In Vitro Differentiation of Primary Human Submandibular Gland Cells and Immortalized Cell Line

PhD Theses

Szlávik Vanda

Semmelweis University
Molecular Medicines School of PhD Studies

Supervisor: Dr. Varga Gábor, professor, doctor of MTA

Opponents: Dr. Kovács Attila, associate professor, Ph.D.
Dr. Darvas Zsuzsa, associate professor, Ph.D.

Comprehensive exam committee:
Head: Dr. Falus András, professor, member of MTA
Members: Dr. Tóth Sára, associate professor, Ph.D.
Dr. Réz Gábor, associate professor, Ph.D.

Budapest
2008

Introduction

Acinar cells of the salivary glands are responsible for the secretion of electrolytes, water and most salivary proteins, whereas ductal cells largely modify the primary secretion. Acinar cells can be permanently destroyed by radiotherapy or Sjögren's syndrome leading to reduced salivary function and disastrous consequences for oral health and quality of life. Activation of pluripotent progenitor cells could be a potential source for renewal of ablated acini. There is increasing evidence to show that multipotent progenitor cells in salivary glands are localized to the intercalated ducts. These progenitor cells contribute to the renewal of acinar cells via differentiation, though acinar cells may also be renewed by autologous cell division.

Salivary gland morphogenesis requires a complex interaction between cell proliferation, apoptosis and histodifferentiation. Epithelial cell proliferation occurs at all stages of gland development, whereas apoptosis begins with the onset of lumen formation at an early phase of salivary tissue formation. The regulation of apoptosis is critical for developmental homeostasis and normal morphogenesis of embryonic tissues.

In the last two decades, a human salivary gland cell line (HSG) was utilized to study acinar differentiation. Basement Membrane Extract (BME), such as Matrigel, can induce HSG cells to differentiate into acinar structures. The HSG cell line originates from human salivary gland intercalated duct and so it is regarded as a potential model for the repopulation of damaged acinar cells from their progenitors. The advantage of the HSG differentiation model is the uniformity of the cells in the culture, though their neoplastic properties may complicate the interpretation of the experimental results. The occurrence of complete functional differentiation of HSG cells following morphological changes induced by BME is uncertain. Amylase gene expression is frequently unrelated to the acinar morphology and HSG cells are not capable of forming a polarized epithelial layer with tight junctions, which prevents initiation of the vectorial water transport process. It is also questionable whether the tissue renewal capacity in salivary glands can be modeled by a neoplastic cell-line; a human originated, non-neoplastic submandibular gland *in vitro* tissue-culture is required accordingly.

Tran *et al.* have recently developed a method of obtaining primary salivary cell cultures enriched in epithelial cells from human submandibular glands (abbreviated huSMG cells) not exposed to radiation or chemotherapy, and after excluding neoplastic transformation

of the submandibular glands themselves. This cell culture was capable of forming a polarized monolayer with high trans-epithelial resistance and expressing occludin and claudin1. Therefore, huSMG culture is considered to display a ductal phenotype.

The obvious next step in the study of salivary differentiation is to investigate whether primary human salivary gland cells, such as huSMG, can be utilized as a progenitor cell source to develop acinar/ductal structures using BME. The use of primary salivary cells from adult patients may also elucidate whether adult salivary glands are capable of regeneration after application of effective stimuli through their progenitor elements. The subcultivation and differentiation of huSMG cells were not effective, therefore the aim of our work was to modify the huSMG culturing method to obtain differentiated acinar cells using a basement membrane extract.

Aims

The differentiation process of HSG cells in Matrigel® was monitored from engagement to cell death in our work. Primary salivary gland cells were isolated and differentiated into acinotubular structures by extracellular matrix derivates. Our objects were the following:

1. Leading HSG cells to form reproductable acini in Matrigel®
2. Proliferation, survival and apoptosis detection of HSG acini
3. Definition of structural and temporal end of HSG-differentiaton
4. Examination of the reversibility of differentiation process
5. Monitoring the effects of the activated and inhibited signal transduction pathways
6. Foundation of an *in vitro* differentiation model originated from human submandibular gland (PTHSG cell culture)
7. Proliferation studies of differentiated and non-differentiated PTHSG cells
8. Observation of the differentiation dynamics
9. Examination of gene- and protein expression pattern of the differentiated PTHSG cells

Materials and Methods

- Primary culture preparing (PTHSG)
- Cell cultures, subcultivation) and differentiation of HSG and PTHSG cells by extracellular matrix derivates (Matrigel®, Basal Membrane Extract (BME))
- Cell proliferation /viability assays (MTT, XTT, ^{3}H Thymidin incorporation)
- Apoptosis detection (Annexin-V-FITC flow cytometry, caspase-3 activity assay)
- Real Time PCR
- Immunohistochemistry
- Videomicroscopy
- Pharmacological inhibition of signal transduction pathways
- Statistics

Results

I. Results of HSG Cell Experiments

Morphological changes of HSG cells cultured in Matrigel

As expected, HSG cells grown on a plastic surface formed monolayers, first making epithelial fields and then proliferating continuously until confluency. In growth factor reduced Matrigel (4.55 mg/ml final concentration), HSG cells formed spherical structures within 24 h and developed microvilli on the cell surfaces. Increasing numbers of cells formed acinar structures by the next day. Dense granules appeared in the bodies of the acinar-like cells at Day 4 and lumen formation was detectable in the centre of the acinar structures, which also exhibited strong amylase expression. Most 4 day acini in Matrigel included 10-12 cells. After Day 5 the acinar structures disintegrated and most of the cells lost contact with each other. The small size and shrunken appearance of these detached cells suggested apoptotic cell death.

In consecutive experiments HSG cells were cultured for three days in Matrigel, then isolated carefully from the Matrigel with dispase (so as not to destroy the acini) and then remixed with fresh Matrigel. The integrity and growth of replated acini were followed by phase-contrast microscopy. These replated acini disintegrated after 2-3 days in the fresh

Matrigel in a very similar manner to the acini grown in Matrigel for 5-6 days without replating. In other experiments, HSG cells cultured in Matrigel for 3 or 6 days were recovered, and single cell suspensions were replated onto plastic culture plates. HSG cells originating from 3-day Matrigel acini attached to the plastic surface, while HSG cells originating from 6-day Matrigel acini failed to attach. Cells originating from 3 days in Matrigel, replated on plastic, formed again undifferentiated, cobblestone patches, suggesting the reversibility of the differentiation after 3 days of Matrigel culture.

Effect of Matrigel on cell proliferation, viability and DNA synthesis of HSG cells

Cell proliferation and viability of HSG cultures grown either on plastic surface or embedded in Matrigel were evaluated first by XTT assay. When cells were grown on plastic, a growth curve was observed that is characteristic of immortal cells: a rapid increase in cell number until confluency was reached, followed by a plateau and/or a modest decline. This decline, observed at Day 6, is primarily due to the loss of firm contact of cells with the plastic surface; these cells are subsequently removed when the culture dish is rinsed. HSG cell numbers in Matrigel exhibited a very different curve during the 6-day course of the experiment. During the first 3 days in culture, cell growth was similar—though slightly lower—on Matrigel compared to plastic, suggesting that cell proliferation is still considerable during this period. At Day 4, however, HSG cell proliferation in Matrigel slowed noticeably, followed by a dramatic decline of cell numbers at Days 5 and 6. Together with the morphological changes described above, our data suggest that in Matrigel HSG cells first proliferate and differentiate, but soon after that extensive cell death occurs in the culture.

In line with the cell viability data, DNA synthesis did not differ during the first cell cycle (i.e. within 20 h) between cells grown on plastic or Matrigel. Thymidine incorporation significantly decreased at 26 h, 2 and 4 days, in Matrigel cultures compared to cells grown on plastic, suggesting an inhibition of proliferation during this period.

In a separate set of experiments, HSG cells cultured in Matrigel for 3 or 6 days were recovered by enzymatic digestion of Matrigel. The recovered single cell suspensions were replated onto plastic culture plates. Following a further 2 days of cultivation on plastic, ^{3}H-thymidine incorporation into the replated cells was measured and compared to those of 2-day HSG cells cultured only on plastic (control group). The level of DNA synthesis in the 3-day Matrigel replated cells (98.4 ± 4.3 % of the control group) did not differ from the control cells

grown on plastic. HSG cells replated from 6-day-Matrigel acini failed to survive, and the DNA synthesis of these cells was practically undetectable.

Apoptosis detection inHSG cultures

Annexin-V-FITC flow cytometry

Cells grown on plastic showed a very low level of annexin binding, even after 5 or 6 days in culture. In contrast, when grown in Matrigel, the percentage of Annexin-V-FITC positive cells in the investigated HSG cell population increased with the length of incubation. The proportion of cells with a high level of annexin binding (HA fraction) gradually increased over the fraction having low annexin binding (LA fraction), suggesting not only a qualitative but also a quantitative relationship between annexin binding and the progress of apoptosis.

Survivin mRNA and protein expression of HSG cells in Matrigel

Survivin is a member of the antiapoptotic protein-family. Survivin mRNA expression in the HSG cultures, evaluated by quantitative PCR, was similar for the first 3 days of incubation in Matrigel and on plastic. At Day 5, survivin mRNA expression decreased markedly in Matrigel compared to the cells on plastic**.** As the last column shows, survivin mRNA expression was much lower in normal human submandibular tissue than in HSG cells either on plastic or in Matrigel.

Both the nuclei and the cytoplasm of undifferentated HSG cells grown on the plastic surface showed survivin immunopositivity. Therefore, in these cells the nucleus appears to be yellow, exhibiting the overlap of the red propidium iodide dye and the green survivin staining. Survivin could still be detected in the cytoplasm, but not in the nuclei, of acinar-like HSG cells at Day 4, cultured in Matrigel. The cytoplasm of the few compact acini at Day 6 was still survivin immunoreactive, whereas the survivin protein could not be detected in the disintegrating acini. The nuclei appear more granulated - suggesting nuclear condensation - in disintegrated acini compared to the homogenous propidium iodide staining in intact acini.

Caspase-3 activity of HSG cells in Matrigel®

Caspase-3 is an apoptotic effector-enzyme. Caspase-3 activity changed in an inverse manner compared to survivin expression. When compared to the activity of cells grown on plastic (100 ± 49 %), caspase-3 activity did not change in culture at Day 1 and Day 2 (79 ± 5 and 176 ± 72 %, respectively), but a significant increase was observed at Day 3 in Matrigel

(446 ± 11 %, $p<0.001$, n=3-3), suggesting the activation of caspase-3-dependent apoptotic processes.

Effect of major signaling pathway inhibitors on cell viability and morphology of HSG cells

This part of the study was performed to gain some insight as to the involvement of major intracellular signaling pathways involved in the Matrigel-induced proliferative and apoptotic processes. The application of various inhibitors caused similar changes, regardless of the number of days (3 versus 5) of incubation. PKC and NFκB inhibitors decreased the proliferation of cells both on plastic and in Matrigel, compared to the cultures where no inhibitors were used. The inhibitory effect of NFκB on growth rate was much more pronounced in Matrigel (interaction: $p<0.001$). In the presence of PI3K inhibitor the proliferation significantly decreased on plastic, but was unchanged in Matrigel. Contrary to these observations, when MMP inhibitor was applied the proliferation was not affected on plastic, but was slightly increased in Matrigel. Finally, tyrosine kinase inhibition slightly decreased the number of the cells on plastic, but significantly increased numbers on Matrigel, thus attenuating the Matrigel-induced effects.

When observing morphological changes in Matrigel cultures in response to the inhibitors at Day 3, the most pronounced change was the appearance of small, shrunken, undifferentiated cells with stretched cytoplasm when staurosporine or the NFκB inhibitor was used. On Day 5, all of the Matrigel cultures disintegrated regardless of whether any inhibitor or none was applied.

II. Results of PTHSG Cell Experiments

Primary culture and subculture

Portions of human submandibular glands were obtained from the Department of Oral and Maxillofacial Surgery of Semmelweis University. The samples were collected from patients undergoing head and neck tumor operations, with permission from the Regional Human Research Ethical Committee (permission number: 67/2005). The mechanically disrupted and enzymatically digested submandibular gland tissue mainly consisted of floating aggregates of cells. After single cells attached clonal expansion of discrete epithelial fields was observed. On day 4 of culture the PTHSG cells grew in discrete patches with a cobblestone-like epithelial appearance; elongated fibroblast-like cells were also observed sporadically. Seven days after seeding, PTHSG cultures reached 70% confluence, mainly

growing in monolayer, although sporadic 3D clumps were also found. PTHSG cells from passage 1 had a very similar appearance to passage 0, but the 3D clumps diminished. Cells formed a monolayer at passages 2 and 3 and the abundance of fibroblast-like cells increased relative to epithelial cells. At all passages of PTHSG cell cultures cellular senescence was observed after 25-30 days of cultivation; eventually the cells lost their contact with the plastic substrate.

Morphological changes on the surface of BME

On the surface of growth factor-reduced BME, PTHSG cells from passage 1 formed spherical, acinotubular structures within 24 hours. Time-lapse microscopy revealed that cells migrated rapidly after plating, forming small aggregates that developed into acino-tubular structures over the course of a day or less. Additional movement of the cells and some reorganization of the aggregates were observed in the following days. The cell aggregates appeared very similar regardless of the type of BME used.

Sections of paraffin-embedded BME cultures showed ductal networks with lumens and acinus-like structures (Fig 4B and C) whereas the cells on plastic formed monolayer.

Immunocytochemical analysis of amylase, vimentin, C-kit, CD34 and occludin expression

PTHSGs from passage 0 contained epithelial fields with strong amylase immunoreactivity but also a notable number of weakly amylase-immunoreactive cells, on the plastic substrate. First passage cells grown on plastic showed very weak and sporadic amylase immunoreactivity, while cells having an acinar structure on BME were strongly positive on average. Confocal microscopy revealed that the acinar structures consisted of both amylase-positive and -negative cells. The amylase immunoreactivity of cells on BME was less intense if the cells had first undergone seven passages on plastic.

First passage PTHSG cells displayed occludin immunoreactivity on both plastic and BME. First passage cultures grown on plastic also contained vimentin positive cells (data not shown), while globular acinar structures on BME were vimentin negative. Primary (p0) PTHSG cell cultures contained occasional 3D clumps, which resembled stem cell-niches. Some cells within the niche-like 3D clumps had small, dense nuclei and showed C-kit and CD34 immunoreactivity.

RT-PCR analysis of gene expression in PTHSG

In five patients the quantity of salivary gland tissue samples was enough for harvesting RNA directly from the tissue and culturing the remaining cells. AMY mRNA expression in first passage PTHSG cultured on plastic was compared with expression in the tissue. In one case, there was little difference in expression between the primary tissue and the first passage plastic culture. However, in the other four cases AMY expression decreased dramatically between harvest and first passage culture. Expression in the first passage cultures varied over 2 orders of magnitude between different patients.

The effects of BME, and of the protein concentration of the BME preparation used, on the expression of salivary gland-associated AMY mRNA was examined in first passage PTHSG cells cultured from 12 different patients, with statistical analysis by 2-way ANOVA. Protein concentration of the BME had no effect on the outcome within the range examined (9.1-17.1 mg/ml; $p=0.9935$), whereas cells from different patients displayed a wide range of AMY induction ($p<0.001$), and great variability in the expression of other genes. To estimate the trend in change of gene expression, the data from replicate cultures, from various BMEs and from the different patients were grouped together and the average change in expression for each gene was calculated. The expression of AMY increased significantly on BME, but no significant changes were found in the mean expression values of the other genes (CLN1, CLN3, KLK1 and VIM). Virtually half of the samples (cells from 6 patients) showed no substantial increase (fold change was less then 1.5) on BME and the other half (n=6) had higher than 2.5 fold increase. Therefore, BME-treated samples were divided into two groups: one with AMY expression considered to be upregulated (at least 2.5 fold increase, Group A), and the other with no change in AMY expression (below 1.5 fold, Group B). In Group A the CLN3 gene was also upregulated, and the expression levels of other genes were unchanged. In Group B CLN1 was downregulated, whereas expression of the other genes was unchanged.

Conclusions

It has been shown that a human salivary gland cell line (HSG) is capable of differentiation into gland-like structures, though little is known of how morphological features are formed or controlled. We investigated the changes in cell proliferation and apoptosis upon terminal differentiation of HSG cells in Matrigel, an extracellular matrix derivative. Changes in the expression of survivin, a prominent anti-apoptotic factor, and caspase-3, a key apoptotic

factor were also measured. Results of these studies demonstrate that cytodifferentiation of HSG cells to an acinar phenotype is accompanied first by a decrease of cell proliferation and then by a massive programmed cell death, affected by multiple signal transduction pathways. The reversibility of apoptosis is depended on the culturing length; the cell death is irreversible after 3 days in Matrigel®. Thus, the validity of HSG cells as a model for salivary differentiation is limited. Matrigel alone is insufficient for the full maturation and long-term survival of the newly formed acini: the presence of other factors is necessary to complete the acinar differentiation of HSG cells. The feedback communication of differently specialized cells has the key role in real organogenesis and has not been realized by monoclonal HSG cells with neoplastic origin.

Improved cell culture techniques are important to generate sufficient amounts of cells with the capacity to differentiate for replacing damaged salivary tissues. Primary submandibular gland cells (PTHSG) were isolated from human tissue and cultured *in vitro* by a new method, in which single cells form an expanding epithelial monolayer on plastic substrates. Differentiation, morphology, number and organization of these cells were then followed on basement membrane extract (BME). On the surface of BME, PTHSG cells formed acinotubular structures within 24 hours and did not proliferate. The morphology of the acinotubular structures has developed for 6 days. The PTHSG cultures consisted of progenitor cells, specialized epithelial and mesenchymal cells. The acinotubular structures were of epithelial origin. The level of organization of the acinotubular structures and the changes in cell-type–specific markers (amylase, claudin1, claudin3, kallikrein and vimentin) on BME varied between cultures derived from different patients. The culture conditions supported by BME probably induce the beginning of tissue regeneration and basic structure formation. The PTHSG culture model suggests that human salivary gland may be capable of regeneration via reorganization and differentiation, and that basement membrane components play a crucial role in the morphological and functional differentiation of salivary cells.

The PTHSG model system, with its capacity to generate acinotubular structures on BME, might have applications in regenerative medicine and progenitor-cell-based tissue engineering as it maintains a complex cell population-mixture, which is suitable for generating differentiated cells. Furthermore, a large-scale experiment would be useful to determine the effect of clinical factors such as age, disease, symptoms and gender on regeneration capacity.

Publications

Related References

Szlavik, V., J. Vag, K. Marko, K. Demeter, E. Madarasz, I. Olah, T. Zelles, B.C. O'Connell, and G. Varga, *Matrigel-induced acinar differentiation is followed by apoptosis in HSG cells.* J Cell Biochem, 2008. 103(1): p. 284-95. **IF: 3,075**

Szlávik, V., Szabó, B.,Vicsek, T., Barabás, J., Bogdán, S., Gresz, V., Varga, G., O, Connell B.C. and Vág, J. *Differentiation of primary human submandibular gland cells cultured on basement membrane extract.* Tissue Eng., 2008 –accepted **IF: 3,725**

Unrelated References

Kornyei, Z., V. Szlavik, B. Szabo, E. Gocza, A. Czirok, and E. Madarasz, *Humoral and contact interactions in astroglia/stem cell co-cultures in the course of glia-induced neurogenesis.* Glia, 2005. 49(3): p. 430-44. **IF: 4,809**

Racz, G.Z., A. Szucs, V. Szlavik, J. Vag, B. Burghardt, A.C. Elliott, and G. Varga, *Possible role of duration of PKC-induced ERK activation in the effects of agonists and phorbol esters on DNA synthesis in Panc-1 cells.* J Cell Biochem, 2006. 98(6): p. **IF: 3,075**

Nagy, K., V. Szlavik, G. Racz, G. Ovari, J. Vag, and G. Varga, *Human submandibular gland (HSG) cell line as a model for studying salivary gland Ca2+ signalling mechanisms.* Acta Physiol Hung, 2007. 94(4): p. 301-13.