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Lumen formation in three-dimensional cultures of salivary acinar cells

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Sponsorships or competing interests that may be relevant to content are disclosed at the end of this article.

ABSTRACT

OBJECTIVE: Development of an artificial salivary gland will benefit patients with xerostomia after radiation therapy for upper respiratory cancer. The goal is to devise a three-dimensional (3D) culture system in which salivary cells differentiate into polarized acini that express essential biomarkers and directionally secrete α-amylase. Differentiated acini-like structures in a 3D biomaterial-based scaffold will mimic salivary gland functions.

STUDY DESIGN: Cells were seeded onto HA-based hydrogels containing PlnDIV peptide and allowed to differentiate into acini-like structures. Cell viability and phenotype were examined.

SETTING: Laboratory-based tissue procurement study.

SUBJECTS AND METHODS: Salivary gland tissue was obtained from patients undergoing surgery. Marker expression established the phenotype of salivary gland cells. Perlecan/HSPG2, an important component of the basement membrane, was highly expressed in salivary gland tissue. A culture system consisting of hyaluronic acid (HA) hydrogel and a coupled bioactive peptide derived from domain IV of perlecan (PlnDIV) was used. Prior studies demonstrated differentiation of acinar cells into lobular structures that mimicked intact glands when cultured on PlnDIV peptide-coated surfaces.

RESULTS: Lobular acini-like structures formed on hydrogels and expressed tight junction components such as zona occludens 1. Acini-like structures were stained for the presence of α-amylase. Live/dead staining revealed the presence of apoptotic cells in the center of the acini-like structures, indicative of lumen formation.

CONCLUSION: A novel system supporting acini-like assembly in a 3D culture system was established. Presence of biomarkers and secretion of salivary enzymes confirms functionality in vitro. Future experiments will test the 3D system in an animal model.

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Materials and Methods

Cell Culture

Tissue specimens of the human parotid gland were obtained from patients undergoing head and neck surgery. A protocol...
and consent approved by the institutional review boards of both the University of Delaware (Human Subjects Review Board) and the Christiana Care Health System was utilized for tissue collection. Acinar cells were isolated from the specimen as previously described.4 Briefly, salivary tissue was minced into small pieces that were further dissociated by enzymatic digestion with the Liberase RI enzyme (0.2 U/mL from Roche Diagnostics, Indianapolis, IN) and trypsin (0.1% [w/v]) from Fisher Scientific, Pittsburgh, PA). The resulting slurry was centrifuged (180g for 5 minutes) and pelleted to obtain dissociated acini. The pellet was resuspended in Hepato-STIM medium (BD Biosciences Discovery Labware, Bedford, MA) supplemented with 1 percent (w/v) penicillin-streptomycin and 1 percent (w/v) amphotericin B and seeded into a six-well culture plate. The cells were maintained at 37°C in a humidified atmospheric chamber containing 5 percent (v/v) carbon dioxide.

Photocrosslinkable HA was synthesized according to a previously reported procedure.9 An ultra violet–crosslinkable HA hydrogel that incorporates PlnDIV peptide was generated in cell culture inserts (Millipore, Billerica, MA; diameter 12 mm, pore size 0.4 μm). The hydrogel was allowed to swell in phosphate-buffered saline (PBS; Invitrogen, Carlsbad, CA) for 24 hours. Salivary gland acinar cells were seeded on the hydrogels at a density of 5 × 10^4 cells/gel. Self-assembling structures were allowed to develop in culture for four days before performing experiments.

**Immunohistochemistry**

Primary antibodies used in this study include anti–zona occludens 1 (ZO-1; rabbit; Zymed Laboratories, South San Francisco, CA) and anti–α-amylase (rabbit; Sigma, St Louis, MO). Secondary antibodies such as Alexa 488 and Alexa 568 against mouse or rabbit IgG, were used (Invitrogen). Draq5 (Biostatus, Leicestershire, United Kingdom) was used as a nuclear stain.

Culture medium was removed from cell culture inserts with cell-seeded hydrogels. Hydrogels were gently washed with 1× PBS and then placed in cold methanol for 10 minutes. Hydrogels were then rehydrated with 1× PBS for five minutes and then placed in 0.2 percent (v/v) Triton solution for 10 minutes. After being washed with 1× PBS twice, the hydrogels were blocked in 3 percent (w/v) bovine serum albumin (BSA) in PBS for 14 hours at 4°C. Hydrogels were incubated in primary antibody solutions in a humidified chamber for 45 minutes at 37°C. The primary antibody solution was discarded, and the hydrogels were washed in 1× PBS for 30 minutes. Incubation with secondary antibody solutions was carried out for 40 minutes at 37°C in the humidified chamber. The hydrogels were incubated in Draq5 solution for 10 minutes and then washed again with 1× PBS for 30 minutes. Lastly, the hydrogels were placed in four-well chamber slides (Lab-tek Products, Nalge Nunc International, Naperville, IL), covered with Gel Mount (Biomeda Corporation, Foster City, CA), and stored at 4°C before viewing with the confocal microscope.

**Live/Dead Staining**

Live/dead staining was performed by using a 1:1000 (v/v) dilution of both SYTO-13 (Invitrogen), a green fluorescent nucleic acid stain for the live cells, and propidium iodide (Invitrogen), a red fluorescent nucleic acid stain in 1× PBS. Culture medium was removed from the hydrogels, and 1 mL of the diluted live/dead stain was applied to the hydrogel in the culture insert. After 10 minutes of incubation, the culture insert with the hydrogel was placed in a two-well chamber slide (Lab-tek Products) for viewing with the confocal microscope.

**Results**

**Three-Dimensional Cultures of Salivary Gland Cells**

To mimic in vivo conditions, we devised a 3D system for the culture of salivary gland acinar cells so that they could...

*Figure 1* Formation of acini-like structures in three-dimensional cultures of salivary acinar cells. Light microscopy images show aggregates of salivary acinar cells (A), an immature acinus-like structure (B), and an acinus-like structure rearranging itself to establish polarity (C).
achieve their glandular phenotype. The morphology of cells cultured on hyaluronic acid (HA)-based hydrogels containing PlnDIV peptide was monitored. Although cells were seeded uniformly on hydrogels, the cells migrated toward each other and formed aggregates in certain areas of hydrogel while in culture. Light microscopic images revealed that cells in close proximity to each other were quickly able to self-assemble into acini-like structures (Fig 1B and C). Cells located in distant spots migrated toward each other and to sites of self-assembly (Fig 1A).

**Acini-Like Structures Assemble Tight Junctions and Produce α-Amylase**

Self-assembled acini-like structures were analyzed for tight junction formation. The acini-like structures expressed ZO-1 at their tight junctions (Fig 2A and B). Additionally, the acini-like structures also produced the salivary enzyme α-amylase, as seen by robust staining for the enzyme throughout the self-assembling structures (Fig 3A and B).

**Evidence for Lumen Formation Observed in Self-Assembling Acini-Like Structures**

To determine if the self-assembling salivary units created a lumen, we performed live/dead staining. Red apoptotic cells stained with propidium iodide were observed in the center of developing acini-like structures, indicative of lumen formation (Fig 4B and C).

**Discussion**

Three-dimensional culture systems better mimic in vivo environments compared with the traditional two-dimensional culture systems. Three-dimensional culture systems, therefore, are immensely useful when studying cell morphology and behavior for regenerative medicine applications. In this study, we analyzed the ability of salivary acinar cells to mimic their glandular phenotype and differentiate into functional acini-like structures, when cultured on PlnDIV peptide–containing HA-based hydrogels. Acinar cells cultured on hydrogels were seen to differentiate owing...
to their interactions with each other. Densely populated areas on the hydrogel underwent self-assembly faster than areas with sparse cells, possibly because of an increased accumulation of secreted basement membrane proteins at those locations. Prior studies have demonstrated the secretion and organization of basement membrane proteins by these cells.

To polarize, epithelial tissues require interaction with each other or the ECM, as well as signals from their microenvironment. Formation of tight junctions is essential to the formation of polarized epithelial structures. The presence of tight junction protein ZO-1 in self-assembling salivary structures is indicative of their apical-basal polarity. Additionally, the expression of α-amylase in the lobular salivary units reveals their functionality in the 3D culture system.

Lumen formation is an essential part of organogenesis. In the mammalian salivary gland, the cells at the periphery of the cord polarize and the cells in the center selectively undergo apoptosis. Apoptosis of cells in the center of the salivary units in our culture system is indicative of lumen formation in the acini-like structures. Further studies will be focused on testing the functionality of these salivary units by investigating the secretion of α-amylase and other salivary enzymes in the lumen of these structures. Future studies will involve implantation of hydrogels with salivary units in vivo to determine their functionality in animal models.

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**Author Contributions**

Swati Pradhan, contributed to design of experiments, acquired, analyzed, and interpreted data, designed manuscript figures, drafted article and revised as needed; Chao Liu, contributed to acquisition of data; Chu Zhang, contributed to manuscript design; Xinqiao Jia, contributed to conception and design, critically revised manuscript for intellectual content, approved final version; Mary C. Farach-Carson, contributed to conception and design, interpreted data, critically revised manuscript, approved the final version; Robert L. Witt, contributed to conception and design, critically revised manuscript, approved final version of manuscript.

**Disclosures**

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