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A Novel In Vivo Model for Evaluating Functional Restoration of a Tissue Engineered Salivary Gland

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Abstract

Objectives/Hypothesis: To create a novel model for development of a tissue-engineered salivary gland from human salivary gland cells that retain progenitor cell markers useful for treatment of radiation-induced xerostomia.
Study Design: A 3D hyaluronic acid (HA)-based hydrogel scaffold was used to encapsulate primary human salivary gland cells and to obtain organized acini-like spheroids. Hydrogels were implanted into rat models and cell viability and receptor expression were evaluated.
Methods: A parotid gland surgical resection model for xenografting was developed. Salivary cells loaded in HA hydrogels formed spheroids in vitro and were implanted in the ¾ resected parotid bed of athymic rats. Implants were removed after one week and analyzed for spheroid viability and phenotype retention.
Results: Spheroids in 3D stained positive for HA receptors CD168/RHAMM and CD44, which is also a progenitor cell marker. The parotid gland ¾ resection model was well-tolerated by rodent hosts and the salivary cell/hydrogel scaffolds were adherent to the remaining parotid gland, with no obvious signs of inflammation. A majority of human cells in the extracted hydrogels demonstrated robust expression of CD44.
Conclusions: A 3D HA-based hydrogel scaffold that supported long-term culture of salivary gland cells into organized spheroids was established. An in vivo salivary gland resection mode was developed that allowed for integration of the 3D HA hydrogel scaffold with the existing glandular parenchyma. The expression of CD44 among salivary cultures may partially explain their regenerative potential and the expression of CD168/RHAMM along with CD44 may aid the development of these 3D spheroids into regenerated salivary glands.

Keywords: Bioartificial Organ; 3-D Cell Culture; Salivary Gland; Tissue Engineering; Regeneration; Progenitor cells; Hyaluronic Acid Based-Hydrogels; Hyaluronic acid receptors

Introduction

Treatment for xerostomia and its sequelae post radiation therapy for upper respiratory tract malignancy are presently inadequate. Quality of life can be potentially improved with the generation of a functional, tissue-engineered salivary gland. Tissue engineers continue to accumulate clinical successes through implementation, either in full or in part, of the classic cells-in-scaffolds model. Components of this model often are used separately. For example, textured hip implants and porous cardiac stents represent open “scaffolds” for ingrowth of osteogenic precursors, or overgrowth of endothelial cells, respectively. The clinical successes of combined cell-scaffold therapies represent the closest approximation to native tissue structures. Two recent examples relevant to this study are found in the trachea, where multiple cell types and scaffolds were implemented in clinical trials, and the pioneering work in the tissue engineered urinary bladder.[1,2] These represent some of the limited examples of tissue-engineered constructs using multiple combined cell types, in particular, those of epithelial and mesenchymal lineages.

3D in vitro systems enable extensive testing of biological pathways under a controlled laboratory environment, and provide greater fidelity to the in vivo condition than traditional 2D cell culture. Despite these improvements, mechanisms such as angiogenesis, neurogenesis, and inflammation response can only be effectively observed in an in vivo model. For our goal of restoring salivary function after radiation-induced xerostomia, an appropriate animal model must reflect the observed loss of acinar cell function within the salivary bed. Suitable facilities for replicating radiation damage require appropriately sized machinery and extensive regulatory protocols and training. Such facilities are costly and are not ubiquitous across U.S. research institutions.

We have reported the use of soft hyaluronic acid (HA) hydrogels as biocompatible substrates for salivary gland tissue engineering.[3,4] HA is a ubiquitous extracellular matrix (ECM) polysaccharide found throughout the body. In our current effort toward salivary gland regeneration, we source healthy human parotid gland tissue, and extract acinar-like cells by enzymatic digestion providing salivary acinar-enriched cell cultures.[3–5] HA potentially serves as an optimal material for salivary gland regeneration, as it promotes strong cell-cell junction formation, but can be recognized and potentially remodeled by cells. HA offers utility as a biomaterial, as it can be synthesized as a high molecular weight raw material by bacteria and then chemically functionalized with reactive sites to enable the formation of covalent hydrogel networks.[6] Multiple biocompatible crosslinking chemistries have been

described, including aldehyde-hydrazide systems, and the thiol-acrylate system, which we employ here for encapsulation of cultured human salivary cells in 3D hydrogel scaffolds.

Tissue engineering of glandular tissues requires several essential components: 1) primary cells that retain biomarkers typical of the native gland, 2) extracellular matrix proteins that can orchestrate the differentiation of primary/progenitor-like cells into functional structures, and 3) a bio-compatible and bio-degradable scaffold that can hold these components together and re-create the microenvironment found in native glandular tissue. We have shown that isolated human salivary acinar-like cells will self-assemble into spheroids when cultured in 3D HA hydrogels. These cells retain cellular structures such as tight junction proteins ZO-1, CL-1, E-cadherin, and focal adhesion kinase (FAK). They also express the water channel protein, aquaporin-5 and functionally respond with production and secretion of the salivary enzyme α-amylase and intracellular calcium activation in response to the neurotransmitter agonists.[3–5]

This manuscript describes a first of its kind in vivo model with cultured human salivary gland cells that can be potentially adapted to other tissue-engineered exocrine glands including lacrimal and mammary gland, as well as pancreas. We demonstrate resection of three-fourths of the parotid gland in a rat model that enables us to assess incorporation of the hydrogel scaffold into the existing salivary bed. We hypothesize that this in vivo surgical model will promote human salivary spheroid retention and phenotype preservation, and integrate into surrounding tissue with minimal inflammation.

We describe salivary acinar-like cell encapsulation in 3D HA-based hydrogel scaffolds, and characterize the cells' expression of the HA receptors CD44 and RHAMM. These two cell surface markers found on salivary cultured cells bind to HA. HA forms the basic unit of our 3D HA-based hydrogel scaffold that allows cultured salivary gland acinar-like cells to grow in 3D. CD44 expression has previously been reported in acinar, myoepithelial, and basal duct cells of human salivary gland tissue.[7,8] It has also been proposed that CD44 is involved in regulating the growth and renewal of normal salivary gland tissue.[7] CD44 also is posited as a stem cell marker. We hypothesize that primary salivary acinar-like cells that organize into acini-like spheroids in vitro will express CD44 and RHAMM, and retain these morphologic and phenotypic characteristics after in vivo implantation.

Materials and Methods

Tissue and Cell Culture

An Institutional Review Board (IRB-Christiana Care Health Systems) approved tissue procurement protocol is in place for obtaining human salivary tissue from patients undergoing routine salivary gland surgery. Parotid gland tissue was procured from patients between the ages of 40 – 60 years. Human salivary gland

acinar-like secretory cells were obtained via tissue explant outgrowths and tissue dissociation techniques.[3–5] Ductal cell cultures were not used in this study. To dissociate the tissue, the specimen was minced into small pieces and suspended in Hepato-STIM medium (BD Biosciences Discovery Labware). Tissue explants were seeded in six well plates (BD Falcon™) and growth media was not exchanged for 7 days so that they could adhere to the plates and cells could migrate out of the explants. Cells were passaged with 0.05% (w/v) trypsin EDTA (Invitrogen). Trypsin soybean inhibitor (0.05 mg/mL, Sigma) was used to inactivate trypsin.

3D Hydrogel Preparation

An HA-based hydrogel system (Hystem™, BioTime, Inc.) was utilized for the encapsulation of salivary acinar-like cells in 3D. 1×10^5 cells were seeded in every 150μL hydrogel. Hydrogels were prepared according to the kit directions.[3] Hydrogels were prepared in 12mm cell culture inserts (Millipore) placed in 24-well plates (Corning®). Growth medium was added both to the outside chamber of the culture insert and on top of the hydrogel.

Immunofluorescence

Primary antibodies used were polyclonal CD44 (Novus Biologicals) and CD168/RHAMM (Abcam). Secondary antibodies included Alexa Fluor 488 and Alexa Fluor 568 (Life Technologies) against mouse or rabbit IgG. Draq5 (Biostatus) was used for staining nuclei. A Zeiss 510 NLO LSM confocal microscope was used to image all stained samples.

3D HA hydrogel scaffolds (cultured in vitro and in vivo) were stained as follows:[3] Hydrogels were fixed with either 4% (w/v) paraformaldehyde or cold 100% methanol for 15 min at room temperature. Hydrogels were washed, rehydrated with 1 X PBS for 5 min, and permeabilized with 0.2% (v/v) Triton X-100 for 15 min. Hydrogels were washed twice with 1 X PBS and blocked overnight at 4°C in 3% (w/v) BSA in PBS. Primary antibodies were prepared in blocking solution and hydrogels were incubated in the antibody solution for 75 min at 37°C. Samples were washed thrice, 10 min each time, with 1 X PBS. Hydrogels were incubated in secondary antibody for 60 min at 37°C. Draq5 was added immediately after the secondary antibody and was incubated for 10 min at 37°C. Hydrogels were washed again thrice, for 10 min each. Gel Mount (Electron Microscopy Systems) was added on top of the hydrogels prior to imaging.

Resection Model

An Institutional Animal Care and Use Committee (IACUC) approved protocol was used for all animal studies. Immunocompetent male, 3 month old, Sprague-Dawley rats (Harlan Laboratories), were used for the implantation of cell-free HA hydrogels while male, 3 month old immunocompromised (hooded T-cell deficient athymic) rats were used for implantation of human salivary cell-loaded HA hydrogels. The animals were anesthetized using a combined ketamine/xylazine intramuscular injection (ketamine: 50–100mg/kg; xylazine: 5–10mg/kg) anesthesia. Once under anesthesia,

the facial area of the rat was shaved. A surgical scrub was used to wipe and disinfect the area. A #11 surgical blade was used to make a 10–15mm incision caudal to the auricle (around 2–3 mm inferior to the ear). Fine scissors were used to dissect through the layers of the skin until the parotid gland was visible. A portion of the parotid gland was elevated using forceps and then resected using fine scissors, leaving ~1/4 of the gland intact. A hydrogel implant was then placed in the parotid bed and the wound was closed using a 4–0 Vicryl™ suture. Sprague-Dawley rats received an HA hydrogel with no encapsulated cells, while athymic rats received an HA hydrogel which had been seeded with human salivary cells, and cultured in vitro for 22 days prior to implantation. Surgical glue was applied. The rats were sacrificed after one-week. The hydrogels were removed, fixed, and stained as described above for further analysis.

Results

Acini-like spheroids express HA receptors in 3D hydrogels
Salivary acinar-like cells seeded in 3D HA hydrogel scaffolds self-assembled into 3D aggregates within 72 hours. These aggregates become organized spheroids within 6 – 8 days of culture. Spheroids growing in 3D HA hydrogels for 22 days expressed CD44 and CD168/RHAMM on their cell membranes (Figure 1 A–C). Salivary spheroids maintained viability in culture for over 150 days (Figure 1 D–I). CD44 and CD168/RHAMM were retained by these larger spheroids over time and were expressed by cells throughout the spheroid as seen in the representative Z-stack slices ranging from 6.55 – 11.22 μm (Figure 1 D–I).

Parotid gland surgical resection and hydrogel implantation in a rat model
A parotid gland surgical resection model was developed to simulate acinar-cell loss in immunocompetent Sprague Dawley rats. No excess bleeding was observed during the ¾ resection of the rat parotid gland (Figure 2). The implant was well tolerated and the wound healed completely in about a week (Figure 2F). No obvious signs of inflammation were seen. Adhesions were observed between the hydrogel implant and the remnant parotid tissue. The acellular HA hydrogels showed vascularization by the host within one-week due to the highly vascularized nature of the parotid bed (Figure 2G). Hydrogels maintained their structure and degraded slowly over 3 weeks (data not shown).

Retention of spheroid structure and biomarker expression after one-week implantation in vivo
Human salivary cell-seeded hydrogels implanted in our athymic rat surgical resection model maintained viability of the encapsulated spheroids. The implanted hydrogel scaffold remained intact in the salivary bed and adhered to the resected gland within a week of implantation (Figure 3A). A clean, intact hydrogel implant is seen in vivo (Figure 3B). Figure 3C shows the site of implantation and remnant parotid tissue after removal of the hydrogel scaffold from Figure 3B. Salivary spheroids encapsulated in the hydrogel scaffold retained expression of CD44 and

RHAMM for a week in vivo (Figure 3D). The same peripheral cell membrane staining observed in the in vitro condition was identified in the explanted in vivo samples, and the cells retained similar spheroid morphology.

Discussion

Our previous work has described the self-assembly of acini-like spheroids and expression of essential salivary biomarkers in 3D HA hydrogel scaffolds including tight junction and adherens junction proteins, salivary enzyme, α-amylase and sympathetic and parasympathetic neurotransmitter receptors.[3] Here we report the expression of HA receptors CD168/RHAMM and CD44 among our salivary spheroid cultures in 3D HA hydrogel scaffolds. Additionally, we report a parotid gland resection model that simulates acinar cell loss and promotes survival of cultured salivary acinar-like cells and, retention of HA receptors in vivo. Our primary human salivary acinar-like cells have demonstrated robust expansion and growth capabilities, with little evidence of senescence. Their near-universal expression of CD44 would support its status as a stem cell marker. We have previously reported that our salivary spheroids survive, retain their morphology and biomarkers, including the salivary enzyme α-amylase, for over three-weeks in vivo when implanted in the back of athymic rats.[3] In this report, we moved to a more relevant site of implantation, the parotid bed.

To surgically simulate acinar cell loss, a parotid gland resection model was developed in which, three-fourths of the left parotid gland of a rat was resected. The right parotid was left intact to minimize detriment to the animal from the reduction in saliva. Additionally, the right parotid can also act as a control. This method allows us to implant our test constructs in a physiologically relevant tissue bed, assess their outcome, and cause minimal harm to the host during the experiments. We observed signs of vascularization within one week in the highly vascularized parotid bed. Integration of the hydrogels adjacent to the remaining one-fourth of the left parotid gland was observed, with minimal inflammation in either Sprague-Dawley or athymic rats. Salivary spheroids remained viable for extended periods of time and continued to express HA receptors. Our HA gels remain stable long enough to support growth and integration of implants with native tissues. These observations, along with the retention of implanted cell morphology and phenotype expression, continue to support the feasibility of our model for salivary gland regeneration. Continued development of this model will include investigations of gel degradation properties, vascularization/innervation of the implant, incorporating complex extracellular matrix proteins, cells, growth factors and eventual comparison with in vivo models of acinar damage from radiation therapy. Studies using human submandibular gland cell cultures to add to our parotid gland cell cultures used in this report have been initiated.

HA is a ubiquitously expressed polysaccharide in the extracellular matrix of vertebrate tissues that, through its interactions with ECM proteins, has been shown

to contribute to the structural and mechanical integrity of the underlying connective tissue.[6] HA has been shown to influence cell behavior through its direct interaction with the proteoglycan CD44 which triggers signal transduction pathways that regulate cell migration, proliferation, and differentiation.[6] The interaction of HA with CD44 has also been associated with activation of Rac1, a small GTP-binding protein, reorientation of cells as well as remodeling of actin cytoskeleton.[9] CD44 and RHAMM signaling may therefore be involved in formation and retention of salivary spheroid structures in HA hydrogels.

During early wound repair processes in adults, excess polymeric HA is produced by stromal cells and functions to slow pathogen invasion and preserve growth factor stability.[10] Subsequently, the HA barrier is gradually degraded and replaced by collagen deposition and initiation of a fibrotic repair response. Conversely, in regeneration (such as observed during early development), fibrosis is delayed in favor of the extended retention of HA.[11] These two mechanisms can be further identified by cell-ECM interactions during each phase through expression of ECM binding receptors on the cell surface. Integrins serve as the primary cell-ECM interface to collagen substrates during a fibrotic repair, and their closest analogues for HA substrates include the CD44 and CD168 (RHAMM) receptors.[12,13] In our model of salivary gland regeneration within HA hydrogels, these two receptors are particularly relevant, as they represent an interface between cells and their surrounding matrix.[7]

Most recently, the investigation of these HA binding proteins in the context of the salivary gland has focused on their observed upregulation around tumor sites.[14–16] These proteins are also observed in cancers of the breast and other tissues, suggesting a role in aberrant, unregulated wound repair. Therefore, the apparent dual nature of CD44 and RHAMM – as markers of healthy regeneration or as markers of unrestricted aberrant neoplasia – reflects the same general function: an interface between the cell and its surrounding matrix.

Conclusions

Here we report a 3D HA-hydrogel culture system capable of supporting long-term growth, structure retention and HA receptor expression. An in vivo parotid gland resection model was developed to mimic acinar-cell loss and to allow for integration of a salivary cell-laden hydrogel scaffold with native glandular tissue. We predict that the expression of the progenitor cell marker CD44 in salivary spheroid cultures may atleast partially explain their regenerative potential. Future studies using this scaffold will allow increased complexity through addition of cytokines and other ECM components to direct the response of both host and donor cells. Our envisioned treatment plan will utilize the patient’s native salivary cells harvested before they are damaged by radiation, then integrated ex vivo with an HA hydrogel scaffold. By supplying the appropriate growth factors and extracellular cues to the harvested cells, as they would present during normal tissue genesis, a fully functional salivary

gland could be formed and implanted in the patient once the radiation treatment is complete.

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