Acta Biomaterialia 9 (2013) 6783–6789

Contents lists available at SciVerse ScienceDirect

Acta Biomaterialia

journal homepage: www.elsevier.com/locate/actabiomat

Increased mucociliary differentiation and aquaporins formation of respiratory epithelial cells on retinoic acid-loaded hyaluronan-derivative membranes

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ARTICLE INFO

Article history:
Received 28 October 2012
Received in revised form 7 February 2013
Accepted 22 February 2013
Available online 5 March 2013

Keywords:
Retinoic acid
Hyaluronan
HYAFF
Mucociliary differentiation
Aquaporins

ABSTRACT

While playing a major role in maintaining the mucociliary phenotype of respiratory epithelial cells (RECs), retinoids are critical determinants of their normal function. However, despite being a powerful biological agent, retinoic acid (RA) is generally not used in regenerative medicine due to its scarce bioavailability via conventional administration. Therefore, the ability to incorporate RA into biomaterials allows for a combination of the biological effects of RA and biomaterials in influencing cellular behavior. This study attempts to develop RA-loaded hyaluronan-derivative membrane (RA-HAm) and investigates how this membrane affects the mucociliary differentiation and aquaporins (AQP) formation of RECs. In a simulated in vitro culture condition, the RA release from membranes is maintained for 7 days. On the seventh day, the cumulative release rate of RA from supportive biomaterials is ~87% under detect limitation. RECs cultured on RA-HAm reveal numerous mature ciliated cells and microvilli compared to aggregated cilia-like structures on hyaluronan-derivative membrane (HAm). Moreover, the expression levels of MUC5AC and AQP on RA-HAm are higher than those on HAm. The proposed model elucidates the release of hydrophobic RA from hyaluronan-derivative biomaterials. We believe that RA-loaded hyaluronan biomaterials are highly promising biomaterials for use in sinonasal surgery and tissue engineering of the respiratory system.

1. Introduction

While forming a continuous lining of the airway interacting with the environment, the respiratory epithelium acts as a physical and functional barrier to external deleterious agents, subsequently preventing infection via escalation of the mucociliary system. The latter, including ciliated respiratory epithelium, mucous blanket and periciliary fluid, serves as an important defense mechanism in the respiratory system. Therefore, restoration of mucociliary function is important in sinonasal surgery as well as in respiratory tract reconstruction. Clinically, several biomaterials are adopted in the respiratory and sinonasal surgery to control bleeding, facilitate wound healing and prevent synechia [1,2]. As conventional biomaterials damage the nasal mucosa with loss of ciliated mucosal surface [3], developing biomaterials that can facilitate mucociliary restoration of the respiratory epithelium is a priority concern.

As is well known, vitamin A is essential for maintaining the mucociliary epithelium in the conducting airways [4–6]. Chronic vitamin A deficiency leads to replacement of the mucociliary epithelium by a stratified squamous epithelium. Also, systematically administering vitamin A facilitates the regeneration of normal ciliated respiratory epithelium [7]. In vitro studies with organs and primary respiratory epithelial cells (RECs) have established that without retinoic acid (RA), the cultures undergo squamous differentiation and that adding vitamin A to the medium restores mucous differentiation [4,8–10]. However, despite being a powerful biological agent, RA is generally not used in regenerative medicine due to its scarce bioavailability via conventional administration [11]. For instance, providing RA continuously to local sinonasal tissue to promote mucociliary differentiation of RECs is rather difficult. Therefore, if RA is slowly released from biomaterials, RA-loaded biomaterials can serve as a novel biomaterial allowing for continuous influence of cellular behavior.

Earlier studies have demonstrated that hyaluronan-derivative membranes (HAm) can provide a more preferential environment for mucociliary differentiation of RECs than collagen [12,13]. The

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http://dx.doi.org/10.1016/j.actbio.2013.02.038

ability to use HAm loaded with RA for respiratory and sinonasal surgery may facilitate the restoration of mucociliary differentiation of RECs. However, whether RA loaded in HAm can stimulate mucociliary and aquaporins (AQP), differentiation of RECs remains unexplored. In this study, we attempt to develop RA-loaded hyaluronan-derivative membranes (RA-HAm) and investigate their effect in mucociliary and AQP differentiation of RECs.

2. Materials and methods

2.1. Preparation of RA-HAm

180 mg of HYAFF (Fidia Advanced Biomaterials, Italy), which was an esterified form of hyaluronan, was dissolved in 1 ml of dimethyl sulfoxide (DMSO) at room temperature. DMSO was pre-filtered through a 0.22 μm Teflon pre-filter (Millipore, Billerica, USA) and the procedure was conducted in the dark hood. 10 μl of RA at 10^{-3} M in ethanol was added to this solution and mixed well. This solution was then spread on the culture surface at 150 μl cm^{-2}. Ethanol was added in the proportion of 100 times the HYAFF solution to precipitate the HYAFF. Next, the polymer sheet was removed gently and ethanol was pipetted to detach it from the coagulation surface. The resulting sheet was dry under vacuum pressure at room temperature for 12 h in darkness. Before use for culturing, these membranes were rinsed with phosphate-buffered saline (PBS).

2.2. In vitro RA release study

The in vitro culture condition was simulated by placing RA-HAm on Transwell insert with 1.12 cm^2 surface area; the culture medium was changed daily. At each time point, the RA-HAm was dissolved in 500 μl DMSO and the amount of remaining RA in membranes was quantified by measuring the OD value at 350 nm with an ELISA reader. The concentration of RA evaluated using a series dilution of RA in DMSO was used as the standard curve. All procedures were performed in a dark room at 37 °C. The RA cumulative concentration in a medium was calculated as well.

2.3. Isolation and culture of human RECs

The culture method has been described elsewhere [12,14]. Human nasal inferior turbinates were obtained from patients undergoing septomeatoplasty. Tissues were treated with 0.5% Pronase (type XIV protease, Sigma–Aldrich, St Louis, MO, USA) in a 1:1 mixture of Dulbecco's modified Eagle's medium and Ham's nutrient F12 (DMEM/F12) supplemented with antibiotics (i.e. penicillin/streptomycin) for 16 to 20 h at 4 °C. The cell suspension was filtered through a 40 μm cell strainer to remove cell aggregates and debris. After centrifugation, the cells were suspended in DMEM/F12: bronchial epithelial growth medium (BEGM, Clonetics Corp., San Diego, CA, USA) (1:1) supplemented with antibiotics. Manufacturer-provided supplements except RA were added in the medium. Cells were then pre-plated on a plastic dish at 37 °C for 1 h to eliminate fibroblasts by differential attachment to plastic [15]. The cells in suspension were collected and resuspended in a culture medium at a concentration of 10^5 cells ml^{-1}. Next, 1.5 ml cell suspension was seeded on RA-HAm or HAm that was placed on Transwell membrane inserts, respectively, with 2.6 ml of the medium deposited on the basolateral side. Cultures were maintained at 37 °C in an atmosphere of 5% carbon dioxide in air. Cells were grown submerged before confluence and the culture medium was changed after 48 h first and every other day thereafter. Following confluence, an air–liquid interface (ALI) was created by removing the apical medium and feeding the cultures only from the basolateral compartment.

2.4. Morphological examination

Living, untreated cultures were observed under a light microscope. Furthermore, cultures were rinsed in PBS and in 0.1 M cacodylate buffer, fixed for 1 h in 2.5% glutaraldehyde in 0.05 M cacodylate buffer, rinsed for 1 h in 0.1 M cacodylate buffer and then post-fixed in 1% OsO_4 in 0.05 M cacodylate buffer for 1 h. Next, the specimens were dehydrated in a graded ethanol series and dried with the critical point technique with liquid CO_2 and then sputter coated with gold to a nominal thickness of 25 nm. Finally, the samples were examined under a scanning electron microscope.

2.5. MTT assay

The viability of RECs was evaluated by cellular ability to reduce 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT; Sigma–Aldrich). The culture medium was removed at the indicated time points (4 h, 12 h, 1 day, 3 days, 5 days and 7 days), and the cells were incubated with 0.1 ml of MTT (2 mg ml^{-1} in PBS) for 3 h at 37 °C. After incubation, the MTT solution was aspirated and the formazan reaction products were dissolved in 200 μl DMSO and shaken for 20 min. The optical density of the formazan was read on an ELISA plate reader at 570 nm.

2.6. Immunocytochemistry

The Transwell inserts were fixed in 4% paraformaldehyde for 20 min and permeabilized with 0.1% triton X-100. The cells were blocked in 2% bovine serum albumin overnight and stained by incubation with anti-acetylated tubulin (Sigma–Aldrich) antibodies, anti-MUC5AC antibody (Abcam, Cambridge, MA, USA), or anti-AQP5 antibody (Millipore). Tubulin or MUC5AC was then visualized using species-appropriate secondary antibody conjugated to Alexa 488 (Invitrogen, Carlsbad, CA, USA). Next, DAPI (Invitrogen) was used as nuclear marker. Rhodamin conjugated phalloidin (Molecular Probes, Carlsbad, CA, USA) were used as cytoskeletal or AQP5 markers, respectively. Images were further taken with a confocal microscope (LSM510, Zeiss, Germany).

2.7. Reverse transcription polymerase chain reaction (RT–PCR) for MUC5AC and AQP5

Total RNA was extracted from cultured cells on day 21 after confluence using Trizol® reagent (Invitrogen) according to the manufacture's instruction. 4 μg of total RNA were reverscribed with Transcriptor High Fidelity cDNA Synthesis Kit (Roche Diagnostics GmbH), and PCR was carried out by using the primers. Standard PCR conditions were used and the annealing temperature of MUC5AC and AQP5 were 55 °C. The band intensities were quantified using AlphaEaseFC™ software to compare the levels of gene expression.

2.8. Western blot analysis

RECs cultured on day 21 after confluence were lysed with a lysis buffer that contained Complete Protease Inhibitor Cocktail Tablets (Roche Diagnostics GmbH, Germany) for 30 min on ice. After centrifugation, the supernatant was transferred to a pre-cooled fresh Eppendorf tube, and 3 μl of each protein sample solutions was used for quantification by Quant-iT™ Assay System (Invitrogen). The protein samples were denatured and separated by sodium dodecyl sulfate–polyacrylamide gel electroresis and then transferred onto the Immobilon-P membrane (Millipore) by a semidry system. After they were blocked in 1% bovine serum albumin at 4 °C overnight, the membranes were probed with anti-MUC5AC antibodies or anti-AQP5 antibodies at room temperature for 2 h.