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Increased mucociliary differentiation of human respiratory epithelial cells on hyaluronan-derivative membranes

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ABSTRACT

The selection of a scaffold to facilitate mucociliary differentiation of respiratory epithelial cells (RECs) is crucial in the development of tissue engineering of respiratory epithelium. However, how the differentiation of RECs is influenced by the biomaterials has never been thoroughly explored. Previously, hyaluronan derivatives were considered as unsuitable biomaterials for culture of respiratory epithelium. In contrast, this study demonstrates that the membranous scaffolds made from benzyl esters of hyaluronic acids are capable of providing a more preferential environment for human RECs than conventionally used collagen-based scaffolds. The proliferation and mucociliary differentiation of RECs were examined by MTT assays, scanning electron microscopy, immunofluorescence, immunoblotting and gene expression. The percentage of ciliated cells in cultured RECs increased from 12.4% on collagen to 20.4% on hyaluronan-derivative membranes with a pseudostratified polarized layer that closely resembled the composition of the native epithelium. The expression levels of MUC5AC and MUC5B mRNA were higher on hyaluronan-based scaffolds than those on collagen. The presence of a hyaluronan-binding domain, CD44 and the receptor for hyaluronan-mediated motility of RECs were also demonstrated. Accordingly, the mucociliary differentiation-promoting effect of hyaluronan-derivative membranes indicates that it may be applied to the tissue engineering of respiratory epithelium.

1. Introduction

The respiratory epithelium forms a continuous lining of the airway interacting with the environment, which acts as a physical and functional barrier to external deleterious agents, and subsequently prevents infection via escalation of the mucociliary system. The latter, including ciliated respiratory epithelium and mucous blanket, serves an important defense mechanism in the respiratory system. Clinically, if a patient has a primary tumor in the respiratory tract, after resection the defect should be repaired by flap reconstruction to maintain the defense mechanism. The skin has been widely used to restore the luminal side of the airway, mainly because it is the most convenient and effective graft material [1,2]. Unfortunately, application of squamous instead of mucociliary epithelium causes adverse effects, such as mucus stagnation, graft constriction, desquamation, regrowth of hair and an unpleasant odor [3,4]. In contrast, if respiratory mucociliary epithelium is adopted for the graft material, the above adverse effects may be minimized.

However, native respiratory mucociliary epithelium is hard to obtain, so how to repair defects, especially in those with an extensive tracheal defect after traumatic or iatrogenic lesion, is a constant challenge to plastic and reconstructive surgeons. However, a new therapeutic strategy has been suggested using tissue engineered tracheal replacement, that is, formation of cartilage by cultivated chondrocytes introduced into a biodegradable scaffold framework [5,6]. To maintain the mucociliary function, several studies have attempted to seed the inner surface of tracheal prostheses with respiratory epithelial cells (RECs) [7,8]. A key issue for this strategy is the selection of biomaterials which can facilitate growth and differentiation of both chondrocytes and RECs. However, unlike several biomaterials that have been assessed for cartilage tissue engineering, the optimal scaffold for RECs has yet to be established [9–11].

Hyaluronan, one of the chief components of the extracellular matrix, contributes significantly to cell proliferation, migration and differentiation. Structurally, hyaluronan is a negatively charged, non-sulfated, linear glycosaminoglycan that is characterized as biocompatible, with bioadhesive and non-immunogenic

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properties. These desirable features render hyaluronan widely used in biomedical applications. In the respiratory system, hyaluronan has been proposed to serve a pivotal role in mucosal host defense by stimulating ciliary beating of RECs, which is 13–16 Hz in vivo, thereby increasing the clearance of foreign material [12]. We therefore hypothesized that hyaluronan may serve as the ideal scaffold for tissue engineering of respiratory epithelium.

However, unmodified hyaluronan is found only in gel form and possesses a very short degradation time. For it to be used in tissue engineering, it would need to be chemically modify to improve its structural properties and increase its resistance to degradation. For example, HYAFF, an esterified form of hyaluronan [13], can be spun or woven to form a scaffold for cell growth, and has been shown to be effective as a scaffold for skin and cartilage tissue engineering [14,15]. The aim of this study was to evaluate whether hyaluronan-derivative membranes could be used for culture of RECs with increasing mucociliary differentiation since there is a close association between hyaluronan and mucociliary function [12]. It was found that, by using enzymatically dissociated cells instead of explant outgrowth technique [16], HYAFF is capable of providing a more preferential environment for differentiation of human RECs than conventionally used collagen-based scaffolds. Our final goal was to establish a novel methodology for enhancing the regeneration of respiratory system.

2. Materials and methods

2.1. Preparation of membranes

HYAFF (Fidia Advanced Biomaterials, Italy) was dissolved at 180 mg ml^{-1} in dimethyl sulfoxide (DMSO) at room temperature. A thin layer of the HYAFF solution was spread onto a glass plate. Ethanol was added in the proportion of 2 l per 25 ml of HYAFF solution. The polymer sheet was removed gently and ethanol was pipetted to detach it from the coagulation surface. The film was then washed several times with ethanol and water. The resulting sheet was pressed dry under vacuum at 30 °C for 48 h. Before being used for culture, these membranes were sterilized in 70% alcohol overnight and rinsed with phosphate-buffered saline (PBS).

As an alternative, Transwell membrane inserts (Corning Costar Co., Cambridge, USA) were covered with type 1 rat tail collagen (BD Biosciences, Bedford, MA) solution and incubated for 12 h to form a thin coating at a concentration of 5 μg cm^{-2} for culture.

2.2. Isolation and culture of human respiratory epithelial cells

The culture method used for RECs has been published previously [17,18]. Human nasal inferior turbinates were obtained from patients undergoing septomeatoplasty, which was approved by an institutional review board (2008-01-02) and all patients gave informed consent. Tissues were treated with 0.5% Pronase (type XIV protease, Sigma–Aldrich, St. Louis, USA) in a 1:1 mixture of Dulbecco's modified Eagle's medium and Ham's nutrient F12 (DMEM/F12) supplemented with penicillin G sodium (50 IU ml^{-1}) and streptomycin sulfate (50 μg ml^{-1}) for 16–20 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 then suspended in DMEM/F12: bronchial epithelial growth medium (BEGM, Clonetics Corp., San Diego, CA) (1:1) supplemented with antibiotics (penicillin/streptomycin). Cells were preplated on a plastic dish at 37 °C for 1 h in order to eliminate fibroblasts by differential attachment to plastic. The cells in suspension were then collected and resuspended in culture medium at a concentration of 10^5 cells ml^{-1}. Next, 1.5 ml of this cell suspension was seeded on HYAFF membrane or collagen type 1-coated Transwell membrane inserts with 2.6 ml of 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. After confluence, an air–liquid interface (ALI) was created by removing the apical medium and feeding the cultures only from the basolateral compartment. Retinoic acid (10^{-7} M; Sigma–Aldrich) was added to the culture medium during ALI culture and the culture medium was changed every day.

2.3. MTT assay

The proliferation rate of RECs was evaluated by their 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.

2.4. Morphological examination

The living, untreated cultures were observed under light microscope for ciliary beating. Subsequently, 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. The specimens were dehydrated in a graded ethanol series and dried with the critical point technique with liquid CO_2, then sputter coated with gold to a nominal thickness of 25 nm. The samples were examined under a scanning electron microscope (SEM).

2.5. 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 antibodies (Sigma–Aldrich). Species-appropriate secondary antibody conjugated to Alexa 488 (Invitrogen, Carlsbad, CA) was used to visualize tubulin. Additionally, DAPI (Invitrogen) and rhodamine-conjugated phalloidin (Molecular Probes, Carlsbad, CA) were used as nuclear and cytoskeletal markers, respectively. Immunostained cells were visualized by the fluorescent microscope (Zeiss Axiovert 200, Germany) and images were also obtained using confocal microscopy (Zeiss LSM510). To investigate the percentage of ciliated cells, the percentage of pixels occupied by the Alexa 488 was measured in MetaMorph™ image analysis workstation. In order to maintain the fluorescence homogeneity, ProLong™ Gold antifade reagents (Invitrogen) was used to prevent photobleaching. In addition, optical parameters (e.g. shutter speed, aperture and light intensity) were kept constant during image capture to ensure reproducibility. Fluorescence was quantified as the total number of pixels that were above threshold value. This number was divided by the total number of pixels in each image to yield percent fluorescent pixels. Images were captured from 10 random fields with size of 826 × 653 μm. The mean value for these 10 sites was taken as percent area of ciliated cells of the subject.

2.6. Western blot analysis

RECs cultured on day 21 after confluence were lysed with lysis buffer that contained Complete Protease Inhibitor Cocktail Tablets