Biomaterials 32 (2011) 7169–7180

Contents lists available at ScienceDirect

Biomaterials

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

The effect of stromal components on the modulation of the phenotype of human bronchial epithelial cells in 3D culture

Steven C. Pageau [a], Olga V. Sazonova [c], Joyce Y. Wong [c], Ana M. Soto [a,b], Carlos Sonnenschein [a,b,*]

[a] Sackler School of Graduate Biomedical Sciences, Tufts University School of Medicine, Boston, MA 02111, USA
[b] Department of Anatomy and Cellular Biology, Tufts University School of Medicine, Boston, MA 02111, USA
[c] Department of Biomedical Engineering, Boston University, Boston, MA 02215, USA

ARTICLE INFO

Article history:
Received 30 March 2011
Accepted 9 June 2011
Available online 2 July 2011

Keywords:
Co-culture
Collagen
ECM (extracellular matrix)
Fibroblast
Lung

ABSTRACT

The stroma plays an important role in the development and progression of human diseases. Pulmonary diseases such as asthma, fibrosis and cancer are thought to be the result of altered communications between the epithelial and stromal tissue compartments. In order to study these epithelial–mesenchymal interactions, we developed a three dimensional (3D) *in vitro* model of the human airway that mimics bronchial morphology and function. This model consists of a type-I collagen matrix, normal human fetal lung fibroblasts (IMR-90) or primary human adult lung cancer-associated fibroblasts (LuCAFs), and a surface epithelium of normal human bronchial epithelial cells (HBECs). When cultured at an air–liquid interface (ALI), the epithelial component generated a well-differentiated pseudo-stratified bronchial epithelium that contained basal, ciliated, and non-ciliated (secretory) epithelial cells. IMR-90 and LuCAFs differentially altered the phenotype of HBECs in distinct ways. While IMR-90 permitted HBECs to form a typical respiratory surface epithelium, LuCAFs promoted HBECs to invade the collagen gel forming both epithelial nodules and cysts, suggesting that LuCAFs may alter the HBEC phenotype by modifying biomechanical signals conveyed through the extracellular matrix (ECM). Furthermore, LuCAFs secreted soluble factors that induced HBECs to express genes associated with immune responses, apoptosis, mitosis, cell survival, differentiation and cancer.

1. Introduction

Physical and biochemical interactions between the epithelium and mesenchyme regulate the development and differentiation of tissues and organs. The pioneering publication by Sakakura et al. demonstrated that embryonic mammary mesenchyme provides sufficient cues to induce the salivary epithelium to morphologically differentiate into a mammary gland-like structure [1]. Similarly, the rat distal embryonic splanchnic mesoderm promotes the proximal rudimentary lung epithelium to develop into a distal alveolar phenotype, while the recombination of proximal mesenchyme with distal lung epithelium induces a proximal tracheal phenotype [2,3].

In addition to its inductive functions, the stroma plays an important role in tissue homeostasis. In recent years, the significance of the stroma in the maintenance of this homeostasis has received renewed appreciation. When using a rodent model of mammary gland carcinogenesis and tissue recombination techniques, carcinogen-exposed rat mammary stroma induced normal unexposed mammary epithelial cells to develop into neoplasias [4]. Conversely, unexposed mammary stroma induced mammary tumor cells to differentiate into normal mammary ducts [5].

In the lung, stromal dysplasia is a distinctive feature of various adult pulmonary diseases, such as asthma, emphysema, chronic obstructive pulmonary disease (COPD), fibrosis and lung cancers. Corresponding with the change from normal to diseased tissue is a concomitant increase in tissue stiffness [6,7]. This may be due to alterations in the cellularity, hydrostatic pressure, and/or composition of the extracellular matrix (EMC). Type-I collagen is the most abundant collagen of the human body, and is the major ECM component of most tissues and organs, including the lung. Under normal homeostatic circumstances, fibroblasts secrete ECM proteins, such as type-I collagen, in a manner that maintains the integrity and functionality of tissues. However, fibroblasts activated under pathologic conditions alter the composition and mechanics of the ECM, and thereby promote disease progression [8,9].

While tissue recombination has been a critical tool for the study of embryonic development and mammary carcinogenesis, the post-natal

* Corresponding author. Sackler School of Graduate Biomedical Sciences, Tufts University School of Medicine, Boston, MA 02111, USA.
E-mail address: carlos.sonnenschein@tufts.edu (C. Sonnenschein).

0142-9612/$ – see front matter © 2011 Elsevier Ltd. All rights reserved.
doi:10.1016/j.biomaterials.2011.06.017

lung is neither accessible nor amenable to this type of experimentation. In order to overcome this shortcoming, our laboratory has developed three-dimensional (3D) tissue culture models by recombining heterotypic cell types and ECM proteins [10–14]. Though others have reported organotypic cultures containing both human lung fibroblast cell lines and either primary or immortalized human bronchial epithelial cells (HBECs) [15–18], the current study differs in that the 3D cultures contained both primary lung fibroblasts and primary bronchial epithelial cells, and examined the effect of collagen concentration and the role of normal and diseased stroma on the phenotype of primary HBECs in a 3D culture environment.

2. Materials and methods

2.1. Cell culture and maintenance

IMR-90 normal lung fibroblasts (CCL-186) were purchased from American Type Culture Collection (ATCC, Manassas, VA, USA). HBECs were generously provided by Dr. Scott Randell (University of North Carolina, Chapel Hill, NC, USA). Approximately 2×10^6 first passage (P1) HBECs were expanded in 100 mm tissue culture dishes (BD Biosciences, San Jose, CA, USA) that had been pre-coated with PureCol® 100 (Advanced BioMatrix, San Diego, CA, USA). HBECs were fed bronchial epithelial growth medium (BEGM), which was prepared in accordance with published protocols [19–21]. IMR-90 and HBECs were cultured in a humidified, 37 °C incubator with 5% carbon dioxide (CO_2).

2.2. LuCAF culture and maintenance

LuCAFs were derived from explanted lung tissues obtained from lung cancer resective surgeries. Briefly, the noncancerous margins of resected lung tissues were washed twice with sterile phosphate buffered saline (PBS) and transferred to sterile 100 mm tissue culture dishes in a biological safety cabinet. Using crossed scalpels, the tissues were minced into pieces measuring approximately 0.4 cm^3. The pieces were transferred to 35 mm tissue culture dishes (BD Biosciences) that had been pre-coated with an aqueous collagen/fibronectin/bovine serum albumin (BSA) solution. This solution contained 6 μg/ml human placental (type-IV) collagen (Sigma-Aldrich, St. Louis, MO, USA), 1 μg/ml bovine fibronectin (Sigma), and 10.0 μg/ml BSA (Sigma). A scalpel was passed through each explanted tissue 1–3 times to promote adhesion to the plastic substrate. The explants were fed Dulbecco's-Modified Eagle Medium/F12 (D-MEM/F12) (Invitrogen, Carlsbad, CA, USA) supplemented with 5% fetal bovine serum (FBS) (Hyclone, Logan, UT, USA) with media changes every other day. Explants were kept in a humidified, 37 °C incubator with 5% CO_2. When cells grew out from an explant, the tissue was removed once the cells reached a distance of approximately 1 cm from the original tissue border. The cells were allowed to continue proliferating until they were approximately 90% confluent, at which point they were split and given the designation passage 1 (P1). LuCAFs were routinely cultured in a humidified, 37 °C incubator with 5% CO_2, and fed DMEM (Invitrogen) supplemented with 5.0% (v/v) FBS.

2.3. 3D cell culture

Rat tail type-I collagen (BD Biosciences) was prepared according to the manufacturer's instructions and was diluted to a final concentration of 1, 2 or 3 mg/ml. Fibroblasts (IMR-90 and LuCAFs) were trypsinized during exponential growth and resuspended in type-I collagen at a concentration of 1.5×10^5 cells/ml. A total volume of 300 μl of the collagen/cell suspension was pipetted into 12 mm diameter Millicell® PICM01250 cell culture inserts (Millipore, Billerica, MA) placed within the wells of 6-well culture plates (≤3 inserts/well). The inserts were placed in a humidified, 37 °C incubator for 15–30 min in order to promote polymerization of the collagen. Once polymerization occurred, 5 μg of human type-IV collagen was added to the apical surface of some of the collagen gels, while others remained uncoated. The type-IV collagen was prepared by diluting a 0.5 mg/ml stock solution in F12 medium. Air–liquid interface (ALI) medium (prepared in accordance with published protocols [19–21]) was added (2 ml/well) and the gels were cultured overnight in a humidified, 37 °C incubator. The following day, semi-confluent plates of HBECs were harvested with the addition of 3 ml of a 0.1 trypsin/1 mM EDTA solution in PBS. Once the cells became freed from the culture vessel surface, the trypsin was inactivated by adding a sterile 2X solution of soybean trypsin inhibitor (STI) (Sigma), which was prepared by dissolving 500 mg STI into 500 ml F12 medium (Invitrogen). 1.5×10^5 viable second passage (P2) HBECs were seeded onto each collagen gel construct surface in a minimal volume of F12 medium (approximately 200–300 μl/insert). The cell-seeded collagen gels were returned to a humidified, 37 °C incubator with 5% CO_2, and the following day, 300 μl PBS was added to each surface and aspirated in order to remove non-adherent cells and cellular debris. ALI medium was replaced every second day, and liquid on the surface of the 3D constructs was gently aspirated in order to maintain an air liquid interface. The cell-seeded constructs were cultured at an ALI for 2 weeks in order to promote functional differentiation of the epithelium. Collagen gels containing either 1.5×10^5 HBECs or 5.0×10^4 fibroblasts served as controls. As a positive control for epithelial cell differentiation, 1.5×10^5 HBECs were seeded on 12 mm diameter Millicell® PICM01250 cell culture inserts that were pre-coated with 5 μg type-IV collagen. All experiments were performed in triplicate.

2.4. Analysis of collagen gel contraction

To determine whether increasing collagen concentrations would prevent fibroblasts and epithelial cells from contracting the collagen gels, we cast 2 mg/ml or 3 mg/ml type-I collagen gels (4-mm thickness) that were acellular or that contained either IMR-90 or LuCAFs (50,000/gel) into 12-mm Millicell™ inserts. After 7 days HBECs were added (150,000, P2) and cultured under ALI conditions for an additional 2 weeks. The inserts were observed daily using a Zeiss stereo microscope, and once contraction was observed, photographs were taken every other day using a Zeiss AxioCam HRc color CCD camera. Measurements of collagen gel contraction were made using Zeiss Axiovision version 4.4 software and calculated as the average of the longest diameter from three replicates divided by the original diameter of an uncontracted gel.

2.5. Histology and immunohistochemistry

At harvest, the inserts were placed in 10% formalin in PBS, processed with an automatic tissue processor containing a xylene alternative (SlideBright™; American Bio-Safety, Rocklin, CA, USA), and embedded in paraffin for subsequent histological and immunohistochemical analyses. Histology stains included hematoxylin and eosin (H&E) and periodic acid Schiff with alcian blue (PAB). Primary antibodies, suppliers and working dilutions are listed in Table 1. Antibodies requiring antigen-retrieval are denoted with an asterisk. The antigen-retrieval technique consisted of a microwave pretreatment in 0.01 M sodium citrate buffer (pH 6). All antigen–antibody reactions were visualized using the streptavidin–peroxidase complex with diaminobenzidine tetrahydrochloride (DAB) (Sigma). Tissues were counterstained with Mayer's hematoxylin. Tissues were visualized and images were captured with a Zeiss Axioscope 2 Plus microscope fitted with an AxioCam HRc color CCD camera (Carl Zeiss). Epithelial thickness was determined using AxioVision software from H&E stained sections and analyzed using GraphPad Prism statistical analysis software.

2.6. Scanning electron microscopy

Harvested inserts were fixed overnight in a neutral aqueous buffer containing 2.5% glutaraldehyde and 0.1 M sodium cacodylate. The fixed gels were post-fixed in 1% osmium tetroxide followed by dehydration to 100% ethanol. The samples were critical point dried under CO_2, mounted, and sputter-coated for viewing in a scanning electron microscope.

2.7. Biomechanical characterization of fibroblast-secreted matrices

IMR-90 and primary fibroblasts were seeded onto gelatin-coated 24 mm round glass cover slips according to published protocols [22]. Briefly, fibroblasts were seeded at a density of 7.5×10^4 cells/cover slip and cultured in DMEM supplemented with 5% FBS in a humidified, 37 °C incubator with 5% CO_2. When the cells on the cover slips reached 100% confluence, the growth medium was replaced with matrix medium consisting of DMEM supplemented with 5% FBS + 50 μg/ml ascorbic acid, in order to promote the deposition of a stable ECM. The cell-seeded cover slips were fed with freshly-prepared matrix medium every second day for a period of 8 days. On day 8, the cell-seeded cover slips were harvested and analyzed with atomic force microscopy (AFM), fixed in a solution of 4% paraformaldehyde + 5% sucrose in PBS for subsequent analysis by confocal microscopy, or used for gene expression analysis.

Mechanical properties of fibroblast matrices were determined using an MFP-3D atomic force microscope (Asylum Research, Santa Barbara, CA, USA). Samples were indented with a silicone nitride cantilever (manufacturer's reported spring constant = 0.06 N/m) which was functionalized with a borosilicate bead ($r = 10$ um). The cantilever was calibrated with the thermal method prior to each experiment to determine actual spring constant. Indentation was performed in PBS at room

Table 1
Primary antibodies for immunohistochemistry.

Antigen*	Supplier	Cat. No./Clone	Specie	Dilution
Pan-keratin*	Sigma	C2562	Mouse	1:1000
Keratin 18*	Sigma	C8541	Mouse	1:100
P63*	Santa Cruz	SC-8431	Mouse	1:100
E-cadherin*	Novocastra	NCL-E-Cad	Mouse	1:75
Type I collagen*	Novus	AF5610	Mouse	1:500
Laminin V	Millipore	MAB19562	Mouse	1:200
Ki-67*	Novus	NB600-1252	Rabbit	1:100