BioResearch

Comparison of Normal and Asthmatic Bronchial Epithelial Cells and Smooth Muscle Cells in Monolayer and RAFT™ 3D Cell Culture System

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Abstract

A hallmark of asthma is remodeling of the lung. To understand the differences between normal and diseased lung cells, we studied human bronchial epithelial and smooth muscle cells from normal (NHBE and BSMC) and asthmatic (DHBE and DBSMC) donors in two-dimensional (2D) culturing surfaces and a three-dimensional (3D) cell culture system. In parallel, co-cultures of BSMC and DBSMC with their bronchial epithelial counterparts were analyzed. The novel 3D cell culture system RAFT™ (Real Architecture for 3D Tissue) allowed us to create tissue-like structures with cells growing within, or on top of, a compressed high-density collagen scaffold. RAFT™ aided the investigation of these co-culture models since there was no overgrowth of one cell type over the other, as observed in 2D. The environmental cues cells experience in 3D culture brings them closer to their *in-vivo* state with respect to cellular structure, interaction with neighboring cells, and overall cell functionality as compared to 2D.

To evaluate differences in normal and asthmatic cells in 2D culture, a bright-field live cell imaging system (CytoSMART™) was used to monitor the different cell types, providing quantitative analysis of their growth properties in real time. Although there were no differences between NHBE and DHBE, the DBSMC grew faster than the BSMC. At initiation of culture, the normal donors averaged 5.8 ± 0.7% (n = 2 donors) cell coverage as compared to the asthmatic donors averaging 4.3 ± 1.1% (n = 2 donors). After 3.8 days of culture time, the coverage means were 59.4 ± 16.4% and 75.1 ± 17.5% respectively and reached statistical significance. These data were corroborated by measuring cellular ATP, indicating proliferation in 2D and 3D, by using a ViaLight™ Plus Assay. After 1 week of culture, DBSMC showed a statistically significant difference in fold change (3.49 ± 0.22 (n = 6 wells)) when compared to BSMC (2.70 ± 0.69 (n = 6 wells)). The growth increase seen in asthmatic smooth muscle compared to normal supports its role in increasing bronchial smooth muscle mass observed in asthma.

2D and 3D cultures were evaluated via immunofluorescent staining of specific epithelial and smooth muscle markers, via ELISA for secreted growth factors and cytokines, and metabolite comparison. Differences were noted in secretion of chemokines in normal compared to asthmatic co-cultures and higher levels of chemokine secretion were observed in 3D. Also, the co-culture 3D environment influenced secretion of some cytokines and growth factors, possibly indicating a portion of the remodeling mechanisms.

These data suggest the ability to perform potentially more relevant modeling studies, and RAFT™ can be a tool for researchers to investigate cellular interactions of different cell types alone or in tissue-mimicking co-culture.

Introduction

Remodeling of the lung is considered as a feature of asthma. To better understand the differences between normal and diseased lung cells, we studied Clonetics™ human Bronchial Epithelial Cells from normal and asthmatic donors (NHBE and DHBE-AS, respectively), and human Bronchial Smooth Muscle Cells (BSMC and DBSMC, respectively) donors in two-dimensional (2D) culturing surfaces and a three-dimensional (3D) cell culture system. Three-dimensional cell culture systems aim to provide cells with a more natural growth environment with the help of methods such as hydrogel matrices or synthetic scaffolds. The environmental cues cells experience in a 3D cell culture environment bring them closer to their *in-vivo* state with respect to cellular structure, interaction with neighboring cells and overall cell functionality as compared to traditional flat two-dimensional (2D) culturing surfaces. Collagen, in particular collagen type I, is one of the most abundant extracellular matrix proteins in the body, and therefore an often-used 3D cell culture material for several cell types. Lonza offers the novel RAFT™ (Real Architecture for 3D Tissue) 3D cell culture system that allows the creation of tissue-like structures with cells growing within or on top of a compressed, high-density collagen scaffold. This novel tool allowed us to gain understanding of the differences between normal and asthmatic cells in single- and co-culture of bronchial epithelial cells with their corresponding smooth muscle counterparts. Real time imaging of 2D cultures were obtained by using the new CytoSMART™ Lux 10X System. The characteristics and functional changes of cells from normal and asthmatic human donor lung tissues were assayed in both 2D and RAFT™ 3D cell cultures. Proliferation was performed with the ViaLight™ Plus assay, which measures total ATP. Immunofluorescence imaging allowed confirmation of cell identity and morphology. Metabolite evaluation was carried out to assess culture characteristics. ELISA analysis was carried out to evaluate the secretions of chemokines, cytokines and growth factors.

Materials and Methods

Cell Culture
Clonetics™ Normal Human Bronchial Epithelial (NHBE™) and Diseased Human Bronchial Epithelial – Asthma (DHBE™) (Lonza Walkersville, MD) were thawed and maintained in Bronchial Epithelial Cell Growth Medium (BEGM™) according to manufacturer's protocol. Clonetics™ Normal human Bronchial Smooth Muscle (BSMC™) and Diseased human Bronchial Smooth Muscle – Asthma (DBSMC-AS™) (Lonza Walkersville, MD) were thawed and maintained in Smooth muscle Growth Medium (SmGM™). Cells were visualized on the CytoSmart™ Lux 10X System by placing a T-flask on the stage and capturing images every 15 mins for 2 – 8 days. Parallel flasks were harvested at approximately 85% confluence and plated for the various assays. For co-culture in 2D, smooth muscle cells were plated and allowed to attach for no more than 1 hr followed by an overlay of epithelial cells.

RAFT™ 3D Cell Culture
Using the RAFT™ 3D cell culture kit (Lonza Cologne, Germany, Lonza Walkersville, MD USA) 3D cells cultures were produced in 24-well plates and 96-well plates as per the manufacturer's protocol. Briefly, collagen solution was prepared with 10X MEM and cell stock solution was prepared with harvested smooth muscle cells in the appropriate cell culture medium. Cell stock solution was added slowly to collagen solution, gently mixed and plated in the appropriate cell culture plate to form hydrogels. RAFT™ Cultures were made by placing RAFT™ Absorbers on top of the hydrogel to remove the extra liquid and compress the collagen (figure 2). For single or co-cultures, epithelial cells were seeded on the RAFT™ Culture. The appropriate medium was added to the RAFT™ 3D cell cultures.

Cell Proliferation Assay
The cell proliferation assay was performed with a ViaLight™ Plus kit (Lonza Rockland, MD). Cells were seeded in 96-well plates for both the 2D and RAFT™ 3D cell culture. Cultures were allowed to equilibrate for 18 – 20 hours at 37°C. A ViaLight™ Assay read was performed as per manufacturer's protocol except the incubation time for lysis was increased for 20 mins. A second ViaLight™ Assay read was performed on day 7 with a parallel plate. The fold change was calculated with blank adjusted means for the different conditions.

ELISA, Immunofluorescence, and Metabolite Analysis
For the assays, smooth muscle cells were seeded at 4,000 c/cm^2 and epithelial cells at 50,000 c/cm^2 in 2D and RAFT™ 3D cell culture. Cells were fed every other day with appropriate media for 7 days. On day 7, spent media were collected and sent to RayBiotech for ELISA analysis using their Quantibody® Human Angiogenesis Array 3 testing service. The metabolites in spent media were evaluated using the NOVA® Biomedical Flex Metabolite Analyzer. For immunofluorescence, cultures were then fixed with methanol, washed with PBS and stained for human cytokeratin (1/50 dilution, DakoCytomation's #M0821), alpha smooth muscle actin (1/200 dilution, Abcam® #ab119952), β-tubulin (1/250 dilution, Novus Biologicals® #NB600-1514), and visualized with goat-anti-mouse Alexa Fluor® 555 (1/200 dilution, Life Technologies™ #A21425). Diamidino-2-phenyindole, dilactate (DAPI) was used for nuclear staining.

Figure 1. CytoSMART™ 10X Lux Imaging of Normal and Asthmatic Cells

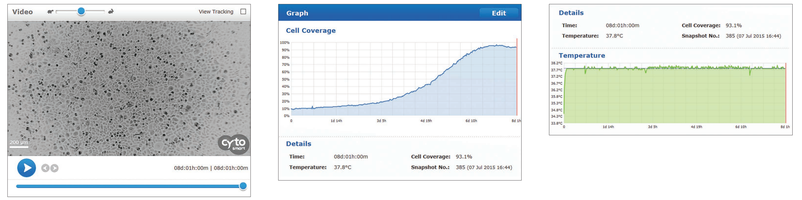

Fig. 1 (A) CytoSMART™ Lux 10X System designed for brightfield live cell imaging allows cell culture monitoring. Human donor lung cells NHBE, DHBE-As, BSMC and DBSMC-As cell culture images were captured by videotaping time-lapse movies that were accessed via a tablet or mobile devices. Representation shown here is of the NHBE images captured every 15 mins over a span of 8 days with the quantitated cell coverage. (B) No significant differences were observed in growth pattern of bronchial epithelial cells, however the Asthmatic bronchial smooth muscle grew at faster rate than the normal.

Figure 2. RAFT™ 3D Cell Culture Process Overview

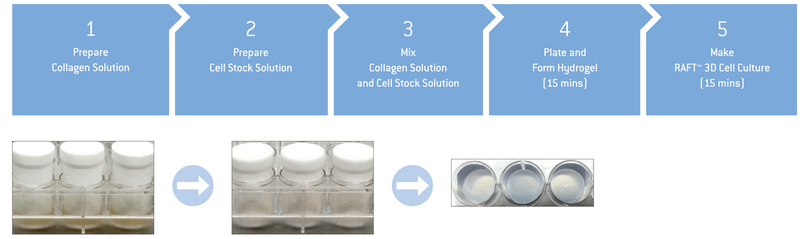

Figure 3. Proliferation-Vialight™ Plus Assay

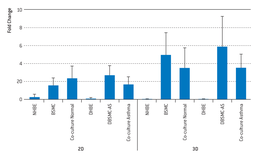

Fig.3 ViaLight™ Plus Assay to measure intracellular ATP. Bioluminescent detection of intracellular ATP was evaluated at baseline and again at day 8 of culture by reading on a luminescent plate reader. Each condition was measured in triplicate, blank well readings were subtracted and fold change was calculated by dividing day 8 values by day 1 values.

Figure 4. Metabolite Analysis of 2D and RAFT™ 3D Cell Cultures

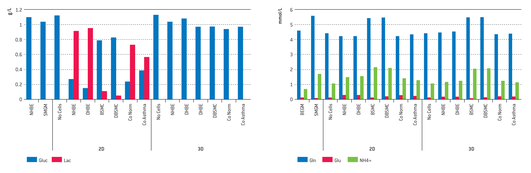

Fig. 4 Metabolite Analysis of spent media after 8 days of culture using the NOVA® Biomedical Flex Metabolite Analyzer (A) Comparison of glucose consumption and lactate production of 2D and 3D cultures. (B) Comparison of glutamine, glutamate, and ammonium levels.

Figure 5. Immunofluorescence 2D Vs 3D

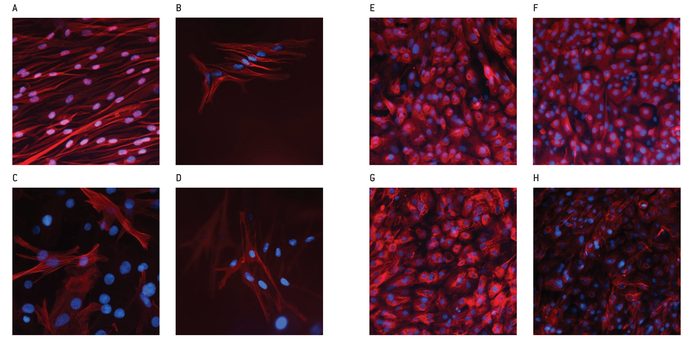

Fig. 5 (A, B) BSMC stained with anti-β-tubulin in 2D and 3D. (C ,D) Asthmatic co-culture stained with anti-α-smooth muscle actin in 2D and 3D.
(E ,F) Anti-pan cytokeratin staining of 2D co-cultures of Normal and Asthma. (G ,H) Anti-pan cytokeratin staining of 3D co-cultures of Normal and Asthma.

Figure 6. ELISA of Normal and Asthmatic Cells in 2D and RAFT™ 3D

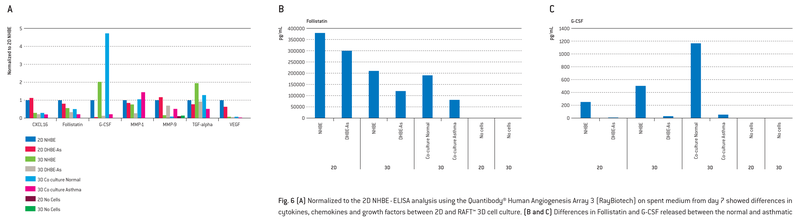

Fig. 6 (A) Normalized to the 2D NHBE - ELISA analysis using the Quantibody® Human Angiogenesis Array 3 (RayBiotech) on spent medium from day 7 showed differences in cytokines, chemokines and growth factors between 2D and RAFT™ 3D cell culture. (B and C) Differences in Follistatin and G-CSF released between the normal and asthmatic bronchial epithelial cells with the later more pronounced when grown in RAFT™ 3D cell culture and in co-culture.

Summary

- The asthmatic bronchial smooth muscle cells growth is different compared to the normal cells, however, no differences were observed in the growth of normal and asthmatic bronchial epithelial cells. Furthermore, compared to 2D the proliferation of smooth muscle cells was increased in RAFT™ 3D cell culture.
- The consumption of glucose is more prominent in 2D compared to the RAFT™ 3D cell culture. No differences were observed between normal and asthmatic.
- The morphology of the smooth muscle cells appeared to be influenced by the RAFT™ 3D cell culture system.
- There were differences detected in secretion of several proteins by normal and asthmatic bronchial epithelial cells. The RAFT™ 3D cell culture system influenced some of the protein expression compared to the 2D.

Conclusion

The RAFT™ 3D cell culture system provides a valuable tool to investigate different cell types singularly or in co-cultures in an *in-vivo* like collagen based microenvironment. Future studies would include more cell types and configurations to create a lung model using the RAFT™ 3D cell culture system.