Adapting Next-Generation Genetic Toxicology Endpoints to the Human *In Vitro* Air-Liquid-Interface Airway Tissue Model

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Abstract

Organotypic *in vitro* tissue models are expected to create unique opportunities to improve predictive toxicology, reduce animal testing, and advance regulatory science. The human air-liquid-interface (ALI) airway tissue model resembles the human large airway epithelium both structurally and functionally and has been used as an *in vitro* alternative for evaluating the toxicity of inhaled substances. In the current study, we have adapted genetic toxicology endpoints to ALI airway cultures: we assayed DNA damage with the high-throughput CometChip assay and quantified patterns of mutagenesis with Duplex Sequencing, an error-corrected next-generation sequencing method capable of detecting a single mutation per million nucleotides. Human ALI airway cultures were treated from the basolateral side with 6.25 to 100 µg/mL ethyl methanesulfonate (EMS) over a period of 28 days. CometChip assays were conducted after 3 and 28 days of treatment, and Duplex Sequencing after 28 days of treatment. Treating the airway cultures with EMS resulted in time- and concentration-dependent increases in DNA damage and a concentration-dependent increase in mutant frequency. The mutations observed in the EMS-treated tissue models were predominantly C→T transitions, which are induced by EMS in other mutation assays. Measurements of physiological endpoints indicated that the EMS treatments had minor effects on the viability of the cultures and no effect on p63-positive basal cell frequency; the treatments however decreased goblet cell and Ki67-positive proliferating cell frequency, reduced cilia beating frequency and mucin secretion, and adversely affected cell morphology. The results from the current study indicate that genotoxicity testing can be conducted in human ALI airway models at dose levels consistent with measuring perturbations to airway function and structure.

Introduction

- The human ALI airway epithelium tissue model is a fully differentiated, organotypic tissue model mimicking many of the structures and functions of *in vivo* human airway epithelium.
- The measurement of genotoxicity in somatic tissues, including the airway, is of importance for the regulatory safety assessment of the carcinogenic potential of inhaled drugs and chemicals.
- Genotoxicity assessment, however, is challenging for many *in vitro* organotypic tissue models because they are predominately composed of highly differentiated, non-dividing cells.
- In this proof-of-principle study, we applied two recently developed tools to measure genotoxicity endpoints in an ALI airway tissue model exposed to the prototypical mutagen, ethyl methanesulfonate (EMS).
- We used the high-throughput CometChip assay for measuring DNA damage.
- Duplex Sequencing, a DNA-based error-corrected next generation sequencing (ecNGS) approach, was used for measuring gene mutations in these cultures. Duplex Sequencing has an error rate below one in ten-million and is capable of directly assessing the mutations distributed across the genome.

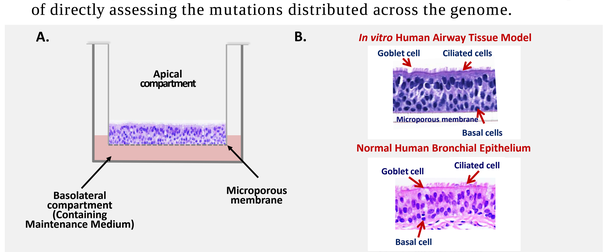

Figure 1. (A). Schematic of air-liquid interface culture of primary human bronchial epithelial cells grown on porous culture inserts. (B). Hematoxylin and eosin (H&E) staining of ALI culture and human bronchial epithelium indicating the presence of multiple epithelial cell types.

Materials and Methods

Human ALI airway tissue models were generated from normal human bronchial epithelial cells (MatTek, Ashland) using the PneumaCult™-ALI medium kit (STEMCELL™ Technologies, Canada). The fully differentiated cultures were treated from the basolateral side by feeding the cultures with Maintenance Medium containing DMSO or various concentrations of EMS for periods of 3-days or 28-days. The cytotoxicity caused by EMS in airway cultures was assessed by measuring LDH release into the basolateral medium using a Lactate Dehydrogenase (LDH) Activity Assay Kit (Roche, Indianapolis, IN) on Days 1, 7, 14, 21, and 28. Cilia beating frequency (CBF) was evaluated using the Sisson-Ammons Video Analysis software (SAVA, Clio, MI). Mucin secretion was examined by an Enzyme Linked Immunosorbent Assay (ELISA). Histological analysis of the ALI cultures was performed by staining tissue sections with H&E and periodic acid-Schiff (PAS) or labeling tissue sections for p63 (a marker of epithelial basal cells) and Ki67 (a marker of proliferating cells) after the 28-day treatment. EMS-induced DNA damage and changes in cell viability were detected using the CometChip assay (Trevigen, Gaithersburg, MD) and the CellTiter® 96 Aqueous Nonradioactive Cell Proliferation MTS Assay (Promega, Madison, WI), respectively, after a 3-day or 28-day treatment. Mutations was measured directly with ecNGS Duplex Sequencing (Twinstrand Biosciences, Seattle, WA) using DNA extracted from cultures treated with EMS for 28 days.

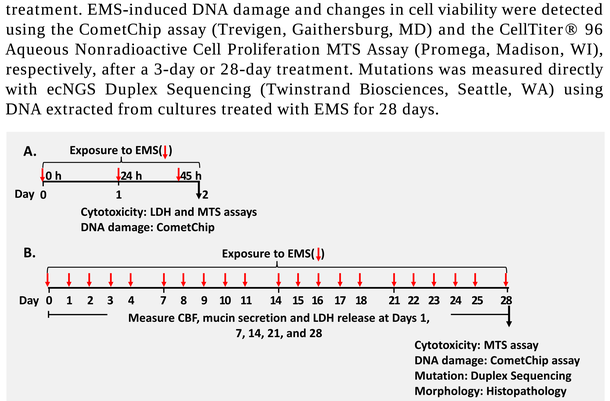

Figure 2. Schematic of the experimental design for the 3 days treatment (A) and 28 days treatment (B). Shorter red arrows: treatment medium added at 0, 24 and 45 hours in (A) and Monday through Friday for 28 days in (B); longer black arrows: assays performed 3 hours after the last treatment (A and B).

Results and Discussion

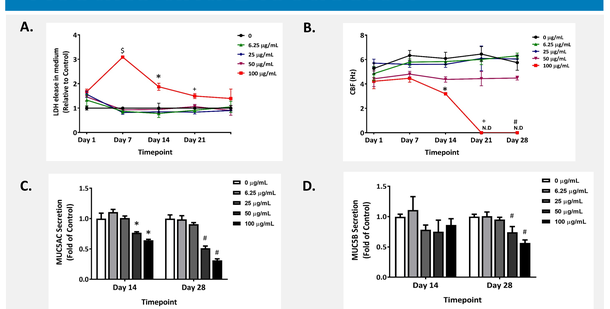

Figure 3. (A). Cytotoxicity in EMS-treated ALI airway cultures. (B) Cilia beating frequency and MUC5AC (C) and MUC5B (D) secretions were measured in cultures used for Duplex Sequencing assays. $, *, +, #p <.05 was considered statistically significant when compared to the concurrent vehicle controls.

Results and Discussion

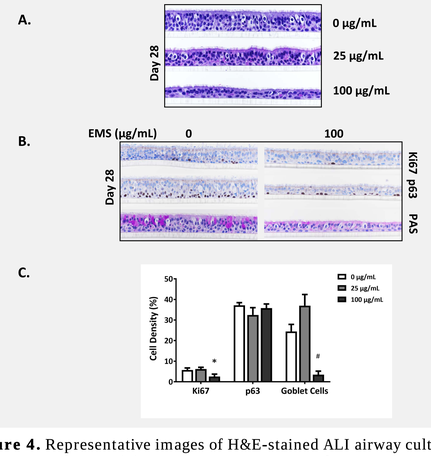

Figure 4. Representative images of H&E-stained ALI airway cultures after a 28-day treatment with EMS (A). Representative images of ALI airway cultures treated with EMS for 28 days and stained for IHC observations (B). Percentages of the periodic acid Schiff-positive goblet cells, p63-positive basal cells, and Ki67-positive proliferating cells (C). *, #p <.05 was considered statistically significant compared to the respective vehicle controls.

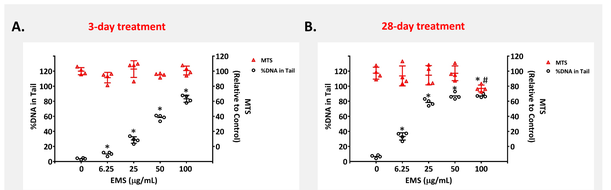

Figure 5. DNA damage was detected using the CometChip assay and the relative cell viability (% of control) was measured using the MTS assay after (A) 3-day and (B) 28-day treatments. *, #p <.05 was considered statistically significant compared to the respective vehicle controls; different symbols refer to comparisons made using different assays.

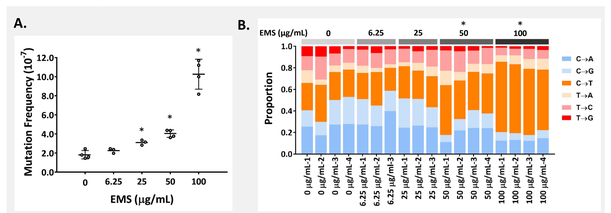

Figure 6. (A). Mutation was measured using Duplex Sequencing after a 28-day treatment with EMS. The individual data are expressed as dot plots, along with the mean ± SD. *p <.05 was considered statistically significant compared to the respective vehicle controls. (B). Proportions of simple base substitutions are plotted for each sample. *p <.05 was considered statistically significant differences in the proportion of C→T transitions, when compared to the concurrent vehicle control.

Table 1. Quantitative mutation analysis in EMS-treated ALI cultures using Duplex Sequencing

Treatment	Replicate	Total Duplex Bases Sequenced (x 10^6)	Mutant Nucleotides Detected	Mutant Frequency (×10^{-7})
Vehicle control	1	481	76	1.58
	2	513	75	1.46
	3	636	156	2.45
	4	727	131	1.80
Mean		**589**	**110**	**1.86**
6.25 µg/mL	1	796	189	2.37
	2	323	64	1.98
	3	709	174	2.45
Mean		**609**	**142**	**2.27**
25 µg/mL	1	699	220	3.15
	2	726	244	3.36
	3	725	205	2.83
Mean		**717**	**223**	**3.11**
50 µg/mL	1	444	161	3.62
	2	456	172	3.77
	3	685	300	4.38
	4	642	280	4.36
Mean		**557**	**228**	**4.03**
100 µg/mL	1	423	423	10.00
	2	398	325	8.17
	3	746	875	11.73
	4	407	458	11.24
Mean		**494**	**520**	**10.28**

Conclusion

- This study integrated the measurement of mutagenesis and DNA damage into the evaluation of toxicity and tissue function in organotypic human *in vitro* airway tissue cultures.
- The high concentration of EMS (100 µg/mL) was mildly cytotoxic, inhibited CBF and mucin secretion, and resulted in a reduced abundance of anti-Ki67-stained proliferating cells. Lower concentrations of EMS had only minor effects on these endpoints.
- The CometChip data indicated that all concentrations of EMS significantly damaged the DNA of the tissue models and that the damage manifested in a time- and concentration-dependent manner.
- The three highest concentrations of EMS (25, 50, and 100 µg/mL) produced significant increases in mutant frequency relative to the negative control. Furthermore, a significant linear relationship between increasing mutant frequency and increasing EMS concentrations was observed.
- Simple base-substitution spectra show that the proportion of pyrimidine-normalized C→T transitions increased with increasing concentrations of EMS, which is consistent with the known mutation signature of EMS identified in several species.
- In summary, we have demonstrated that increases in DNA damage and mutations can be detected in human *in vitro* ALI airway tissue models treated with the known mutagen, EMS.