Organotypical epithelial 3D tissues (MatTek®) exposed *in vitro* to whole smoke as a potential alternative to rodent inhalation studies

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Introduction and Objective

The use of reconstituted human airway epithelia exposed *in vitro* to cigarette smoke (CS) closely mimics the situation in human smokers, where lung epithelial cells are exposed to fresh whole smoke at the air-liquid interface (ALI). It is known that CS induces inflammatory processes (Takizawa 2001, Yoshida 2007) that can lead to tissue remodeling, which may eventually result in a diseased lung phenotype. Here we describe an *in vitro* test system to investigate the toxicological effects of single and repeated CS exposure on human organotypical 3D epithelial tissues (EpiAirway™, MatTek). Tissues were exposed at the ALI to either fresh air or to mainstream smoke from the Reference Cigarette 3R4F in the VITROCELL® system (3-well or 24-well exposure chamber).

The objective of the study was to demonstrate whether exposure of human lung tissue to whole smoke *in vitro* can induce similar effects as observed in rodent inhalation studies with regard to selected gene and protein expression.

Materials and methods

Organotypical bronchial epithelial tissue

EpiAirway™ tissues grown on cell culture inserts with a diameter of either 6 mm (24-well culture plate format) or 12 mm (12-well culture plate format) were obtained from MatTek Inc. (Ashland, USA) and shipped to Germany within 2 or 4 days. Each insert was basally attached to an agarose-media preservation matrix and sealed before transport. Tissues were stored at 4-8° C in the original packages until use the following week.

Exposure to whole smoke (Vitrocell system)

Tissues were exposed at the air liquid interface to either fresh air or to mainstream smoke from the Reference Cigarette 3R4F (www.ca.uky.edu/refcig) in the VITROCELL® system. Two experimental designs were used:

1) 20 min exposure per day for 1-3 days to a CS concentration of 20% (12-well format, used in 3-well exposure chamber)
2) 7, 14, 21, and 28 min exposure to a CS concentration of 15% (24-well format, used in 24-well exposure chamber)

After exposure the tissues were transferred to the incubator with fresh culture medium for different post-exposure time points (4h for qRT-PCR, 24h for Viability, 48h for MMP-1 secretion).

Viability test resazurin

The day following CS exposure the tetrazolium salt resazurin (Sigma) was applied to the inserts at the apical and the basolateral compartment simultaneously for 1 hour at 37°C. Both supernatants were combined and the relative fluorescence intensity (FI) was determined in a plate reader (Fluostar Optima, BMG Labtech, Ex 560nm, Em 590nm) and the percent change to the control was calculated.

MMP-1 ELISA

The Quantikine Human Pro-MMP-1 Immunoassay (R&D Systems) was used for the quantitative determination of human Pro-Matrix Metalloproteinase-1 concentrations in cell culture supernatants 48h after exposure. Absorbance was measured at 450nm wavelength using a Fluostar Optima plate reader.

Reverse Transcriptase Real Time quantitative PCR (qRT-PCR)

mRNA was isolated using the Quiazol buffer (Quiagen). The integrity of the RNA samples was determined using the Agilent 2100 Bioanalyzer. qRT-PCR was conducted in duplicate using the high capacity cDNA reverse transcription kit (Applied Biosystems). The mean of duplicate measurements is shown.

Rat Inhalation Studies

The *in vivo* study was performed at Philip Morris Research Laboratories bvba, Leuven, Belgium. Care and use of the animals was in accordance with the American Association for Laboratory Animal Science Policy (1996). All animal experiments were approved by the Institutional Animal Care and Use Committee (IACUC).
In vivo data were obtained from two inhalation studies:

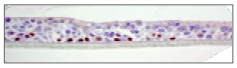
1) Hemoxygenase-1 (HO-1) and Cytochrome P450 1A1 (CYP1A1) gene expression: Sprague-Dawley (SD) rats were whole-body exposed to CS from the Reference Cigarette 2R1 (100 µg total particular matter [TPM/l]) or to fresh air for 3h/day, either once or for 3 wks (7 days a week). CYP1A1 was determined in lung tissue and HO-1 was determined in rat nose epithelial cells.
2) MMP-1 secretion: Spontaneously Hypertensive (SH) rats were nose-only exposed to CS from the Reference Cigarette 3R4F (450 µg TPM/l) or to fresh air for 2h/day, 5 days a week for 4 weeks. MMP-1 was determined in bronchoalveolar lavage fluid (BALF).

Results: *In vitro*

Tissue Staining showing intact 3D epithelium

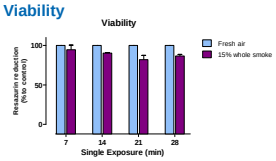
H&E staining (Hematoxylin Eosin)

AB-PAS staining (Alcian Blue – Periodic Acid Schiff)

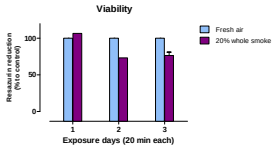


P63 immunostaining

Cell Viability

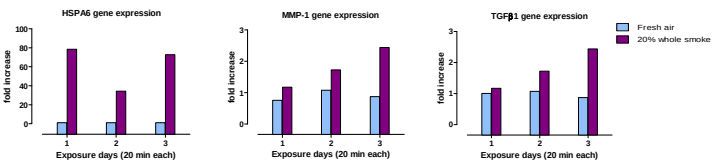


Single exposure to 15% CS; mean ± SEM, N=3



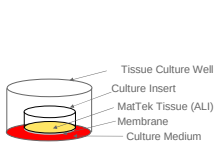
Repeated exposure to 20% CS (20 min duration/day); mean ± SEM, N=2-4

Gene expression (MatTek)



- Heat shock 70kDa protein A6 (HSPA6), involved in (oxidative) cell stress regulation, was strongly up-regulated (qRT-PCR) under all conditions.
- Matrix metalloproteinase-1 (MMP-1) and transforming growth factor ß1 (TGF ß1), which may play a role in tissue remodeling, showed moderate but dose-dependent induction after repeated exposure.

Experimental Setup

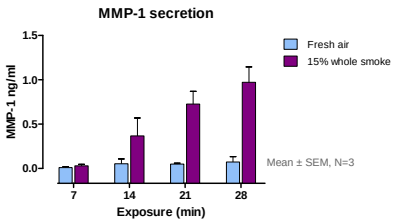


Tissue Culture Insert

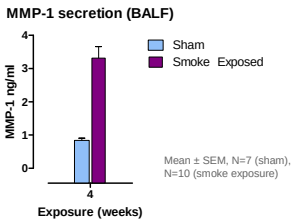
Exposure Chamber

VITROCELL® System

Results: *In vitro – in vivo* comparison

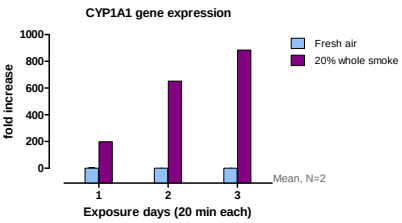


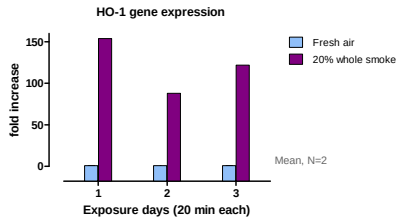
- MMP-1, an enzyme possibly involved in the pathogenesis of COPD, was dose-dependently increased.



- MMP-1 was strongly increased in BALF after exposure to CS for 4 weeks (2h/d, 450 ug TPM/l).

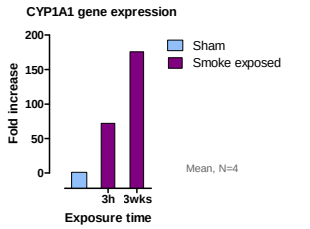
Gene Expression (MatTek®)

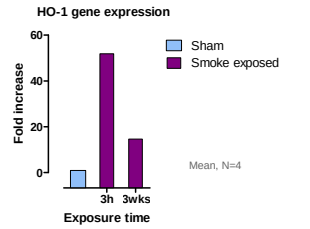




- CYP1A1 and HO-1, markers for xenobiotic drug detoxification and oxidative stress, respectively, were strongly up-regulated (qRT-PCR).

Gene Expression (SD Rat)





- CYP1A1 and HO-1 were strongly up-regulated (qRT-PCR) in rats exposed to 100 ug TPM/l either once or for 3 weeks (3h/day).

Summary and Conclusion

- The study design for single or repeated *in vitro* exposure of human lung tissue to whole smoke was successfully established.
- Markers involved in (oxidative) cell stress regulation (HSPA6, HO-1), xenobiotic drug detoxification (CYP1A1) and tissue remodeling (MMP-1, TGFß1) were seen *in vitro*.

Results for HO-1 and CYP1A1 and MMP-1 are comparable to *in vivo* data from previous rodent inhalation studies; thus, this *in vitro* study design may serve as a potential alternative to *in vivo* inhalation studies for the investigation of the toxicology of aerosols, and supports the 3R strategy of refinement, reduction, and replacement of animal experimentation.

References

Takizawa H., Tanaka M., Takami K., Ohtoshi T., Ito K., Satoh M. et al. (2001) Increased expression of transforming growth factor beta 1 in small airway epithelium from tobacco smokers and patients with chronic obstructive pulmonary disease (COPD). *Am J Respir Crit Care Med*. **163**, 1476-1483.

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