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Development of an *in vitro* test to identify respiratory sensitizers in bronchial epithelial cells using gene expression profiling

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ABSTRACT

Chemicals that induce asthma at the workplace are substances of concern. At present, there are no widely accepted methods to identify respiratory sensitizers, and classification of these substances is based on human occupational data. Several studies have contributed to understanding the mechanisms involved in respiratory sensitization, although uncertainties remain. One point of interest for respiratory sensitization is the reaction of the epithelial lung barrier to respiratory sensitizers. To elucidate potential molecular effects of exposure of the epithelial lung barrier, a gene expression profile was created based on a DNA microarray experiment using the bronchial epithelial cell line 16HBE14o$^-$. The cells were exposed to 12 respiratory sensitizers and 10 non-sensitizers. For statistical analysis, we used a class prediction approach that combined three machine learning algorithms, leave-one-compound-out cross validation, and majority voting per tested compound. This approach allowed for a prediction accuracy of 95%. Identified predictive genes were mainly associated with the cytoskeleton and barrier function of the epithelial cell. Several of these genes were reported to be associated with asthma as well. Taken together, this indicates that pulmonary barrier function is an important target for respiratory sensitizers and associated genes can be used to predict the respiratory sensitization potential of chemicals.

1. Introduction

Asthma induced by low molecular weight (LMW) chemicals (Sastre et al., 2003) at the workplace is a common type of allergy, as 15–20% of all asthma cases are due to this type of exposure. Occupational asthma is described for several work environments, such as hospitals (Vellore et al., 2006) and hair salons (Moscato et al., 2005), and the socioeconomic- and medical impact is significant (Mapp et al., 2005; Toren and Blanc, 2009). There are currently no widely accepted tests available to identify respiratory sensitizers. This is partly due to the absence of validated predictive *in vitro* and *in vivo* tests. This can be attributed to the lack of mechanistic insight in the pathways involved.

By reviewing all relevant data regarding the mechanism of respiratory sensitization, Kimber et al. (2014) aimed to apply the general principles of modeling an Adverse Outcome Pathway (AOP) for chemical respiratory allergy. The molecular initiating event of LMW respiratory allergy is covalent binding of the chemical to a protein after exposure, forming a hapten that can be recognized by dendritic cells (Kimber et al., 2014). There are two types of methods currently available to determine the chemical reactivity of LMW chemicals to covalently bind proteins. The first are (quantitative) structure activity relationship (QSAR) models using chemical reactivity alerts associated with protein binding and respiratory sensitization. A study implementing QSAR models to predict respiratory sensitization was performed by Dik et al. (2014). The second method is the direct peptide reactivity assay (DPRA) for respiratory sensitizers (Lalko et al., 2012), which correlates peptide depletion by a chemical with the reactivity of the chemical.

The cellular response to a hapten in case of a respiratory sensitizer is similar to skin sensitization (OECD, 2012); dendritic cells recognize the hapten and start to produce inflammatory cytokines (Kimber et al., 2014). In case of respiratory sensitization, this usually leads to the initiation of Th2-type responses (individual references are evaluated by (Kimber et al., 2014)). The Th2-response then causes sensitization through an IgE mediated mechanism. The involvement of IgE in the sensitization process remains unclear however, as some patients have detectable IgE antibodies specific for the chemical, whereas other patients do not (individual references are evaluated by Kimber et al. (2014). Although much research has focused on the process of respiratory sensitization, several biological aspects are still not (fully) understood.

Next to the cellular response of dendritic cells, cellular responses in keratinocytes are identified as a key event in the AOP for skin sensitization (OECD, 2012). Keratinocytes are considered essential for the generation of danger signal, necessary for the adaptive immune response towards skin sensitizers (Natsch, 2010). In case of respiratory sensitization, once a respiratory sensitizer is inhaled, it comes into contact with

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the pulmonary epithelium. Holgate (2008) found that airway epithelium is fundamentally abnormal in asthma and suggested that the barrier function of the epithelium is compromised through defective tight junction formation. Studies that are more recent also show the importance of pulmonary epithelial cells and their barrier function in relation to asthma (Georas and Rezaee, 2014; Rezaee and Georas, 2014). Therefore, lung epithelial cells are considered to be an important target of *in vitro* research. Verstraelen et al. (2008a) studied the applicability of different epithelial cell types as models for the identification of respiratory sensitizers. Among these are bronchial cells (for example BEAS-2B cells) or alveolar cells (for example A549 cells). Both cell lines have already been used to identify biomarkers associated with respiratory sensitizers (Verstraelen et al., 2009a, 2009b). These cells have various benefits compared to other cell lines, as discussed by Verstraelen et al. (2008a). However, these cells do not or only slightly have the ability to form tight junctions. As a decreased barrier function is an important aspect of asthma, we selected a cell line capable of forming proper tight junctions. The 16HBE14o$^-$ cell line was considered appropriate in this regard as it has the ability to form tight junctions and a proper barrier as measured by the trans-epithelial electrical resistance (Cozens et al., 1994).

The aim of this study was to evaluate the predictive performance of a set of gene profiles to identify respiratory sensitizers. Additionally, these gene profiles were evaluated to identify molecular pathways induced by respiratory sensitizers to elucidate the role of bronchial epithelial cells in chemical respiratory sensitization.

2. Methods

2.1. Cell culture

The human bronchial epithelial cell line 16HBE14o− was obtained as a kind gift from Dr. Gruenert (National Institutes of Health [NIH], San Francisco, CA). Cells were grown to 80–90% confluence in DMEM/F12 cell culture media supplemented with 100 U/ml Penicillin/Streptomycin solution (Gibco) and 10% Fetal Bovine Serum (FBS; Greiner)(complete medium). Cells were cultured in plastic culture flasks (Greiner) and were maintained at 37 °C, in a humidified atmosphere of 5% CO2 in air.

2.2. Chemical exposure

The chemicals used in this study were twelve respiratory sensitizers (belonging to five different chemical subclasses) and ten respiratory non-sensitizers that are not skin sensitizers. The chemicals tested in this study can be found in Table 1, which shows the identity of the chemical, whether the chemical is a respiratory sensitizer (RS), respiratory irritant (RI) or non-sensitizer (NS) (based on our previous work (Dik et al., 2014)), the vehicle in which the test chemicals were dissolved and the final exposure concentration. The respiratory sensitizers consisted of five chemical classes: Isocyanates, acid anhydrides, amines, salts and aldehydes. All chemicals were obtained from Sigma Aldrich (Zwijndrecht, The Netherlands). The final exposure concentrations were determined by calculating the concentration that results in a decrease of cell viability to 80% (CV80), based on a colorimetric assay using the tetrazolium dye MTT (Morgan, 1998) (data not shown). The highest concentration tested was 4 mM. If a chemical did not show toxicity at this concentration, experiments were conducted using 4 mM.

Before exposure, cells were diluted to a concentration of $6 * 10^5$ cells/ml fresh complete medium, seeded into 6-wells plates (4 ml per well; Costar, VWR) and incubated at 37 °C in a humidified atmosphere of 5% CO2 in air for 24 h to form a monolayer. The cells were subsequently washed with HBSS and exposed to chemicals in medium without FBS or solvent controls without FBS for 4 h. The DMSO or HBSS content in all samples was 1%. At the end of the exposure, 700 µl RNAprotect cell reagent (Qiagen, Westburg, The Netherlands) was added to each well. Cells were resuspended and stored at −20 °C until further analysis within the same week.

Table 1
Overview of all chemicals tested in this study, identified by name and CAS number. The vehicle in which each chemical was dissolved (dimethylsulfoxide (DMSO) or Hanks Balanced Salt Solution (HBSS)) as well as the exposure concentration (CV80) is also provided.

Chemical	Abbreviation	CAS number	Vehicle	CV80 (mM)[a]
Respiratory sensitizers				
Hexamethylene diisocyanate	HDI	822–06-0	DMSO	1.10
Toluene diisocyanate	TDI	584–84-9	DMSO	0.15
Maleic anhydride	MA	108–31-6	DMSO	2.65
Phthalic anhydride	PA	85–44-9	DMSO	1.33
Trimellitic anhydride	TMA	552–30-7	DMSO	2.73
Ethylene diamine	ED	107–15-3	HBSS	4.00
Triethylenetetramine	TETA	112–24-3	HBSS	4.00
Ethanolamine	Etha	141–43-5	HBSS	4.00
Sodium hexachloroplatinate	HcPt	16,929–58-7	HBSS	0.42
Chloramine-T	Chlor-T	127–65-1	HBSS	1.83
Glutaraldehyde	Glut	111–30-8	HBSS	0.19
Formaldehyde	Form	50–00-0	HBSS	0.11
Respiratory irritants				
Acrolein	Acro	107–02-8	HBSS	0.04
Methyl salicylate	MS	119–36-8	DMSO	1.17
Epichlorohydrin	EPI	106–89-8	HBSS	0.08
Sodium dodecyl sulfate	SDS	151–21-3	HBSS	0.11
Respiratory non-sensitizers				
Glycerol	GLY	56–81-5	HBSS	4.00
Lactose	LAC	63–42-3	HBSS	4.00
Mannitol	MAN	69–65-8	HBSS	4.00
Vanillin	VAN	121–33-5	DMSO	4.00
Saccharin	SAC	81–07-2	DMSO	4.00
Lactic acid	LAA	50–21-5	HBSS	4.00

[a] In all cases where the CV80 is 4.00, the chemical was not found to be toxic at this concentration.

2.3. RNA isolation

RNA was isolated by using an RNeasy Plus Mini Kit (cat.no. 74134, Qiagen) according to the manufacturer's instructions. RNA concentrations and qualities were determined using a NanoDrop Spectrophotometer (Isogen-Life Sciences) and an Agilent 2100 Bioanalyzer (Agilent), respectively. All RNA samples were diluted in RNase-free water to a concentration of 200 ng/µl in 15 µl and stored at −80 °C. Control RNA samples from HBSS and DMSO exposed cells were included in the analysis as well.

2.4. DNA microarray analysis

RNA samples were further processed for hybridization to Affymetrix HT HG-U133+ PM plates at the Microarray department (MAD) of the University of Amsterdam, The Netherlands. Amplification, labeling and hybridization were performed according to Affymetrix protocols, using an automated Affymetrix Genechip console. Samples were randomized prior to processing and hybridization. Array plates were scanned with a Genechip HT array plate scanner and analyzed with the Affymetrix HT software suite. Quality control and normalization of Affymetrix CEL files were performed using the ArrayAnalysis website (www.arrayanalysis.org/) (Eijssen et al., 2013), using the Robust Multichip Average (RMA) method (Bolstad et al., 2003) and the MBNI custom CDF version 14 (Dai et al., 2005). Normalized data consisted of log2 transformed signal values for 18,909 genes.

2.5. Statistical analysis

Further statistical analysis including classification was performed in R (www.r-project.org) (version 3.0.3) (R Development Core Team,