Toxicology in Vitro 46 (2018) 155–162

Contents lists available at ScienceDirect

Toxicology in Vitro

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

An alternative biomarker-based approach for the prediction of proteins known to sensitize the respiratory tract

Kathrin S. Zeller[a], Henrik Johansson[b], Trine Østergaard Lund[c], Nanna N. Kristensen[c], Erwin L. Roggen[c], Malin Lindstedt[a,*]

[a] Department of Immunotechnology, Lund, Sweden
[b] SenzaGen AB, Lund, Sweden
[c] Novozymes A/S, Bagsvaerd, Denmark

ARTICLE INFO

Keywords:
Respiratory sensitization
Protein allergens
Cell-based assay
Dendritic cells
Biomarker

ABSTRACT

Many natural and industrial proteins are known to have properties that can result in type I hypersensitivity, however, to date, no validated test system exists that can predict the sensitizing potential of these allergens. Thus, the objective of this study was to develop a protocol based on the myeloid cell-based Genomic Allergen Rapid Detection (GARD) assay that can be used to assess and predict the capacity of protein allergens known to induce sensitization in the respiratory tract. Cellular responses induced by eight selected proteins were assessed using transcriptional profiling, flow cytometry and multiplex cytokine analysis. 391 potential biomarkers were identified as a predictive signature and a series of cross-validations supported the validity of the model. These results together with biological pathway analysis of the transcriptomic data indicate that the investigated cell system is able to capture relevant events linked to type I hypersensitization.

1. Introduction

Allergy is a chronic disease with increasing prevalence and it is of outmost importance for the industry and authorities to identify potential allergens as early as possible to limit the exposure of workers and the general populations. Several hundreds of chemicals are known to be able to cause allergic contact dermatitis (DeGroot, 2008; Luechtefeld et al., 2016), a type IV delayed hypersensitivity reaction, whereas less chemicals are known to sensitize the respiratory tract and to induce type I allergic responses (Boverhof et al., 2008). Most substances causing respiratory allergy are proteins of environmental origin e.g. allergens from house dust mite feces, pollen, or fungi, while others are present in an occupational setting such as enzymes used in flavor, fragrance, detergents and pharmaceutical production (Thomas et al., 2002; Baur, 2005). The risk of developing adverse reactions following occupational exposure exists; thus, a strict focus on occupational safety is mandatory. Sensitization has been observed for workers exposed to certain industrial enzymes such as α-amylase, proteases, pancreatinin, and trypsin (Baur et al., 2013; Budnik et al., 2017). New enzymes are continuously developed for existing as well as for new applications, such as genetically modified enzymes used in food processing and flavor production and may also lead to occupational health risks (Baur, 2005; Budnik et al., 2016).

To date, no validated assay is available for predicting the sensitizing potential of novel proteins, rendering a weight-of-evidence approach to be the most acceptable means of allergy safety assessment. There is, however, a growing consensus that the allergenic potential of compounds, including proteins, should be evaluated with regard to their biochemical characteristics and the protein's potential to induce a specific immune response (European COST Project impARAS (http://imparas.eu/, n.d.)). A combination of physical traits of proteins, the molecular interaction between human cells and proteins, as well as their impact on cell-cell interactions play a role in understanding and eventually predicting protein allergenicity (McClain et al., 2014; North et al., 2016).

The Adverse Outcome Pathway (AOP) concept is a framework for collecting and organizing information relevant to an adverse outcome at different levels of biological organization (OECD et al., 2014). These AOPs are based on available information on substance-response and response-response relationships and allow the development of relevant predictive animal-free test methods and approaches, as well as the contextualization of the results across a diverse range of biological mechanisms and toxicity endpoints. The mechanisms driving respiratory sensitization are not fully disclosed yet, but emerging data suggest that events driving respiratory (chemical- and protein-induced) and skin sensitization (chemical-induced) can be structured in the same

* Corresponding author at: Lund University, Medicon Village (406), 22381 Lund, Sweden.
E-mail address: malin.lindstedt@immun.lth.se (M. Lindstedt).

http://dx.doi.org/10.1016/j.tiv.2017.09.029
Received 3 May 2017; Received in revised form 7 September 2017; Accepted 28 September 2017
Available online 07 October 2017

adverse outcome pathway (AOP). In contrast to skin sensitization, properly evaluated test methods addressing the key events of respiratory sensitization induction are not yet available (Roggen, 2014).

The Genomic Allergen Rapid Detection (GARD) assay was developed to provide information about the capacity of chemicals to induce skin sensitization (accuracy: 89% (Johansson et al., 2014)). This *in vitro* assay utilizes a myeloid cell line resembling dendritic cells (DCs) as a model system. DCs are antigen-presenting cells and central for the induction and regulation of adaptive immune responses (Schuurhuis et al., 2006). This assay was recognized by both the European Reference Laboratory – European Center for Validation of Alternative Methods (EURL-ECVAM) and the OECD as a valuable method for addressing key event 3 (Dendritic cell activation and maturation) of the AOP for skin sensitization (OECD, 2012). Since DCs bridge the innate and adaptive immunity, a requirement of both skin and respiratory sensitization to occur, it can be hypothesized that this cell system may also be suited for the identification of chemicals causing sensitization in the respiratory tract. Forreryd et al. (Forreryd et al., 2015) successfully demonstrated that a modified protocol of the assay is able to predict respiratory chemical sensitizers with an accuracy of 84% based on a biomarker signature consisting of 389 transcripts.

In this study, this protocol is assessed for its capacity to provide a mechanistic understanding of the protein sensitization at DC level. The objective was to identify a potential gene profile for the classifications of proteinaceous allergens. For this purpose, the effect of eight respiratory protein allergens on the gene expression in the myeloid cell line was investigated using Affymetrix RNA expression array technology. A panel of potential biomarkers distinguishing the allergens from experimental control samples was identified using data-driven approaches including machine learning, and cross-validation exercises indicated that the identified transcripts may indeed be useful for classification. These biomarkers were further investigated with regard to associated biological pathways.

2. Materials and methods

2.1. Respiratory allergens

All allergens contain low levels of endotoxin as summarized in Table 1. LoTox™ recombinant Der p 7 (#LTR-DP7-1) and LoTox™ natural Der p 1 (#LTN-DP1-1) were obtained from Indoor biotechnologies, Charlottesville, USA. The other allergens were provided by Novozymes A/S, Bagsvaerd, Denmark, and tested for endotoxin content by Sahlgrenska Universitetssjukhuset, Bakteriologiska laboratoriet, Göteborg.

2.2. Cell culture and stimulations

The maintenance of the cell line was performed as described in (Johansson et al., 2011). In short, the MUTZ-3 derivative cells were cultured in MEM alpha modification (Nordic Biolabs/GE Healthcare Bio-Sciences, Täby, Sweden) supplemented with 20% fetal bovine serum (Thermo Fisher Scientific/Life Technologies, Stockholm, Sweden) and 40 ng/mL recombinant human GM-CSF (Miltenyi Biotec Norden AB, Lund, Sweden). A dose finding experiment was performed based on earlier protocols optimized for this cell line to identify the highest enzyme concentration resulting in a relative cell viability of ≥ 90% after 24 h of incubation. Longer exposures (48 h) to certain enzymes resulted in substantially decreased cell viability (data not shown). A phenotypic control analysis of cells prior to each experiment was carried out by flow cytometry (see below) in order to assure an immature state. In short, three batches of cells were exposed to allergens and control substances dissolved in complete cell culture medium for 24 h in at least three independent experiments (Table 1, six independent experiments for “unstim”). Proteins present in serum served as vehicle and non-allergen protein controls. Phenotypic controls and viability of the cells were assessed after the stimulation period by analyzing cell surface expression using flow cytometry as described below. Cells aimed for RNA extraction were lysed in TRIzol® (Life Technologies/Thermo Fisher Scentific, Waltham, USA) and stored until further use in minus 20 °C.

Table 1
Identity and concentrations of allergens and vehicle controls.

Substance	Test compound concentration	Endotoxin in well conc. [EU/mL]
Amylase 1	25 µg/mL	0.04
Amylase 2	25 µg/mL	0.059
Protease 1	25 µg/mL	< 0.0012
Protease 2	25 µg/mL	< 0.0006
Glycohydrolase	25 µg/mL	0.033
Lipase	25 µg/mL	0.022
Der p 1	25 µg/mL	< 0.75
Der p 7	25 µg/mL	< 0.25
DMSO	0.1%	NA
Unstimulated		NA

*Unstimulated cells are defined as cells grown in control medium, i.e. not exposed to any potentially stimulating substance.

2.3. Flow cytometry

As a quality control both before and during the experiments, the following monoclonal antibodies were used during phenotypic analysis as described previously (Johansson et al., 2013): CD1a (DakoCytomation, Glostrup, Denmark), CD34, CD86, HLA-DR (BD Biosciences, Franklin Lakes, NJ), all FITC-conjugated; CD14 (DakoCytomation), CD54, CD80 (BD Biosciences), all PE-conjugated. FITC- and PE-conjugated mouse IgG1 (BD Biosciences) were used as isotype controls and Propidium iodide was included as a marker for non-viable cells (BD Biosciences). After 24 h incubation with the allergens and vehicle control samples, viability, CD14, CD1a, HLA-DR and CD86 expression were assessed. FACS samples were analyzed on a FACSCanto II instrument with FACS Diva software for data acquisition. 10,000 events were acquired and further analysis was performed in FCS Express V4 (De Novo Software, Los Angeles, CA). An endotoxin stimulation was included as internal control for cell quality.

2.4. RNA extraction, cDNA and array hybridization

RNA isolation from cells lysed in TRIzol® was performed according to manufacturer's instructions. Labeled sense DNA was synthesized according to Affymetrix' protocols using the recommended kits and controls. The cDNA was hybridized to Human Gene 1.0 ST arrays and further processed and scanned as recommended by the supplier (Affymetrix, Cleveland, USA).

2.5. Data acquisition and analysis

The dataset, comprising 33 samples in total, was quality-controlled and then normalized using Single Channel Array Normalization (Piccolo et al., 2012; Piccolo et al., 2013). Statistical analysis was performed primarily by Analysis of Variation (ANOVA). ANOVAs and PCA visualization of results were performed with Qlucore 3.2 (Qlucore AB, Lund, Sweden). Significance was evaluated with the multiple-hypothesis corrected p-value (q-value, in this article referred to as false discovery rate (FDR) (Benjamini and Hochberg, 1995)). An FDR ≤ 0.05 was considered as statistically significant. Decision values were calculated in a support vector machine (SVM) model (Noble, 2006), constructed in R (R Development Core Team, 2008) and using the package e1071 (R package e1071). The same R package was used to program the backward elimination algorithm (Carlsson et al., 2011). The backward elimination algorithm was applied to a dataset consisting of nine vehicle control and 24 allergen samples and the 1052 most significant