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Applicability of a keratinocyte gene signature to predict skin sensitizing potential

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ABSTRACT

There is a need to replace animal tests for the identification of skin sensitizers and currently many alternative assays are being developed that have very promising results. In this study a gene signature capable of very accurate identification of sensitizers was established in the HaCaT human keratinocyte cell line. This signature was evaluated in a separate study using six chemicals that are either local lymph node (LLNA) false-positive or false-negative chemicals in addition to nine sensitizers and four non-sensitizers. Similar studies do not apply these more difficult to classify chemicals, which show the true potential for human predictions of an assay. Although the gene signature has improved prediction accuracy compared to the LLNA, the misclassified compounds were comparable between the two assays. Gene profiling also showed a sensitizer specific response of the Nrf2-keap1 and Toll-like receptor signaling pathways. After exposure to non-sensitizing chemicals that induce either of the pathways the signature misclassified all Nrf2-inducers, while the Toll-like receptor ligands were correctly classified. In conclusion, we confirm that keratinocyte based prediction assays may provide essential information on the properties of compounds. Furthermore, chemical selection is critical for assessment of the performance of *in vitro* alternative assays.

1. Introduction

Allergic contact dermatitis is a delayed-type IV hypersensitivity reaction that can be induced after skin contact with chemical haptens. It is a common occupational and consumer health problem which develops through a series of immunological events caused by repeated contact with compounds that have skin sensitizing potential (Kimber et al., 2002). The current methods for assessing the sensitizing potential of chemicals are the Local Lymph Node Assay (LLNA) or guinea pig tests (Guinea Pig Maximization Test (GPMT) or Buehler test) (Gerberick et al., 2007; Kimber et al., 1994). There is great demand for validated non-animal alternatives to replace these animal tests, due to the ban on animal testing described in the 7th amendment to the European Union Cosmetics Directive. In addition, the REACH (Registration, Evaluation, and Authorization of Chemicals) regulation requires that the safety of a large amount of chemicals has to be assessed and it stimulates the use of alternative test methods. These legislative changes, combined with ethical issues and societal acceptance towards animal use in toxicity testing, drive further development of alternative test methods. Although much progress has been made for assessing skin sensitizing potential, no alternative test methods have been validated yet. In recent years, it has become clear that a combination of methods in a testing strategy will be required for correct identification of sensitizers, rather than a single test (Vandebriel and van Loveren, 2010).

Many of current cell based alternative assays for skin sensitization use either keratinocytes or dendritic cells. Read-outs are either changes in gene regulation in these cells (Arkusz et al., 2010; Johansson et al., 2011; Natsch, 2009; Vandebriel et al., 2010), production of cytokines, such as IL-18 in keratinocytes (Corsini et al., 2009) or upregulation of cell surface markers, including CD86 and CD54 on dendritic cells (Aeby et al., 2004; Ashikaga et al., 2006; Sakaguchi et al., 2006; Schreiner et al., 2008) exposed to sensitizers. The prediction accuracy of these assays range between 71% and 99% (Bauch et al., 2011).

In the present study the focus is on the predictive power and the driving pathways involved in the initial response of keratinocytes (KCs). KCs are abundantly present in the skin and play an important role in the initial stages of skin sensitization as they are the first cells to come into contact with chemicals. In addition, KCs are able to secrete several pro-inflammatory mediators and metabolize prohaptens into protein-reactive haptens (Jowsey et al., 2006; Martin et al., 2011; Vandebriel and van Loveren, 2010). More recently, it has been proposed that KCs generate 'danger' signals in response to skin sensitizers that trigger the innate

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immune system through TLR activation (Martin et al., 2011; Natsch, 2009). The signaling cascade following TLR activation leads to MAPK signaling and NF-κβ activation, which induces the release of pro-inflammatory mediators (Kumar et al., 2009). Gene profiling studies in KC indicate a role for the cytoprotective Nrf2-Keap1 pathway in the response to skin sensitizers (Natsch, 2009; Vandebriel et al., 2010). This pathway is involved in antioxidant response signaling and antioxidant response genes such as hemeoxygenase 1 (HMOX1) and NADPH quinone oxidoreductase 1 (NQO1) have been shown to be under Nrf2 control (Martin et al., 2011; Vandebriel et al., 2010; Vandebriel and van Loveren, 2010).

In an earlier array study, we found biologically relevant pathways regulated by skin sensitizers in KCs. In addition, sensitizers could be distinguished from non-sensitizers based on gene regulation patterns with 70% accuracy (Vandebriel et al., 2010). To confirm these findings we have performed a new gene profiling study with more statistical power through an increased number of chemicals. In addition, the accuracy of a gene signature obtained from this study was thoroughly tested in novel approach that included a relatively high number of either false-negative or false-positive chemicals from the LLNA. In addition, as a proof of concept, the performance of the gene signature was challenged by including chemicals that activate the Nrf2-Keap1 or TLR pathways yet are not sensitizers.

2. Materials and methods

2.1. Cell culture

The human keratinocyte cell line HaCaT (Boukamp et al., 1988) was purchased from Cell Lines Service (Eppelheim, Germany). Cells were grown in culture flasks to 80% confluence in Dulbecco's modified Eagle's medium, supplemented with 1% nonessential amino acids, 100 U/ml penicillin, 100 μg/ml streptomycin (all from Gibco, Breda, the Netherlands), and 10% heat-inactivated Fetal Calf Serum (Integro, Zaandam, the Netherlands) (complete medium), at 37 °C in a humidified atmosphere of 5% CO_2 in air. For passaging the cells were washed twice with PBS and then trypsinized (0.05% Trypsin with EDTA 4Na; Gibco). New culture flasks were seeded in complete medium with 1/3rd or 1/6th of the total number of cells of the previous passage.

2.2. Chemical exposure

Before exposure trypsinized cells were resuspended in fresh complete medium to a concentration of 3×10^5 cells/ml. The cell suspension was seeded into 12-well plates (1.5 ml per well; Greiner, Alphen aan den Rijn, the Netherlands). The cells were allowed to adhere and form a monolayer during 24 h, after which the wells were washed with PBS and exposed to the different chemicals in complete medium.

The chemicals that were used in this study are shown in Tables 1 and 2. All compounds were obtained from Sigma–Aldrich (Zwijndrecht, the Netherlands), except for 2-mercaptobenzothiazole, which was obtained from Merck (Schiphol-Rijk, the Netherlands). Chemicals were dissolved in complete medium, absolute ethanol, or dimethylsulfoxide (DMSO). In addition to the compound, the vehicle (DMSO or ethanol) was added to a final concentration of 1% to the cell cultures exposed to compounds dissolved in complete medium. Cells were exposed to ethanol and DMSO to obtain vehicle control samples. For each chemical the concentration resulting in 80% viability (CV80) was determined using colorimetric measurement of WST-1 cleavage. To this end, HaCaT cells were seeded in 12-well plates (4.5×10^5 cells/well) and incubated with a concentration range of the chemicals in duplicate or solvent control in triplicate at 37 °C in a humidified atmosphere of 5% CO_2 in air. After 21 h of exposure, 1.2 mL medium was removed from the wells, leaving 300 μL exposure medium in the wells, and 40 μL/well WST-1 (Roche, Woerden, the Netherlands) was added. After 4 h, 100 μL was transferred to a microtiter plate and WST-1 cleavage was quantified at 450 nm using a microplate reader (Spectramax 190, Molecular devices, Wokingham, UK). After blank correction, WST-1 without cells, the mean optical densities of the replicates were compared to the mean of the corresponding vehicle controls in order to calculate relative viability (data not shown). In a single experiment the HaCaT cells were exposed to each chemical in four replicates for 4 h, which was determined to be the optimal exposure time for classification as this exposure period had higher prediction accuracy compared to 8 h exposure (Vandebriel et al., 2010).

For the Toll-like receptor ligands it proved impossible to determine a CV80 value. The applied concentration induced the same level of IL-8 in the supernatant after 24 h of exposure as did the strong sensitizer benzoquinone, IL-8 was measured using ELISA according to manufacturer's instructions (eBioscience, Vienna, Austria). For the Nrf2 activators the concentration was based on the CV80. Additionally, the ability to induce HMOX1 was assessed by ELISA (R&D systems, Abingdon, UK) for the selected concentration.

2.3. RNA isolation

At the end of the exposure period 400 μL RNAprotect cell reagent (Qiagen, Westburg, the Netherlands) was added to each well. Cells were resuspended and were stored at −80 °C until further analysis. For RNA isolation the cells were lysed after removing the RNAprotect using Qiazol and the lysates were homogenized using Qiashredder columns. RNA was isolated by using miRNeasy Mini Kit in combination with RNeasy MinElute Cleanup Kit (all from Qiagen) according to the manufacturer's instructions. RNA quantity was spectrophotometrically assessed (Nanodrop Technologies, Wilmington, DE), and integrity was determined by automated gel electrophoresis (Bioanalyzer 2000; Agilent technologies, Amstelveen, the Netherlands). For each compound three replicates were selected out of the four RNA samples for DNA microarray analysis, based on concentration and RNA Integrity Number. Control RNA samples from ethanol and DMSO were included in analysis.

2.4. DNA microarray and data analysis

The samples were prepared, hybridized to Affymetrix HT HG-U133 + 2.0 PM arrays and measured by ServiceXS (Leiden, the Netherlands). The quality of the raw data was checked using RMA-Express (Bolstad et al., 2003), which was also used in combination with the BrainArray CustomCDF version 13 for the annotation of 18.040 genes. The expression values were then log2 transformed and corrected for the corresponding vehicle control. The raw data is accessible at Array Express (http://www.ebi.ac.uk/arrayexpress/) under the accession number 943-MTAB-E.

2.5. Identification of significant genes

To detect if genes significantly changed between samples of the sensitizers and non-sensitizers, a *t*-test was done on the control corrected samples of those respective classes. *p*-Values were false discovery rate (FDR) corrected (Benjamini and Hochberg, 1995), an FDR below 0.05 was considered significant. In addition to the comparison between all sensitizing compounds and all irritating compounds, this approach was applied to the potency subsets of sensitizers, as defined by the Globally Harmonized System (GHS).