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Gene expression changes induced by skin sensitizers in the KeratinoSens™ cell line: Discriminating Nrf2-dependent and Nrf2-independent events

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ABSTRACT

The KeratinoSens™ assay is an *in vitro* screen for the skin sensitization potential of chemicals. It is based on a luciferase reporter gene under the control of the antioxidant response element of the aldoketoreductase gene *AKR1C2*. The transferability, reproducibility, and predictivity of the KeratinoSens™ assay have been investigated in detail and it is currently under assessment at the European Center for Validation of Alternatives to animal testing (ECVAM). Here we investigate the sensitizer-induced gene expression in the KeratinoSens™ cell line at the mRNA level and discriminate Nrf2-dependent and Nrf2-independent events by using siRNA to better characterize this test system at the molecular level. The results show that (i) the sensitizer-induced luciferase signal in KeratinoSens™ cells is completely dependent on Nrf2. The same holds true for the luciferase induction observed for the false positive chemical Tween80, indicating that the false positive result is not due to recruitment of an alternative transcription factor. (ii) Luciferase induction parallels the induction of endogenous Nrf2-dependent genes, indicating that the luciferase signal is representative for the sensitizer-induced Nrf2-response. (iii) The induction by sensitizers of additional genetic markers related to heat shock proteins and cellular stress could be reproduced in the KeratinoSens™ cell line and they were shown to be Nrf2-independent. These results confirm that the KeratinoSens™ cell line is a rapid and adequate screening tool to assess the sensitizer-induced Nrf2-response in keratinocytes.

1. Introduction

The European ban of animal testing for cosmetics in 2013 has triggered large research initiatives with the aim to develop new testing strategies that predict skin sensitization, notably the Cosmetics Europe research program (Aeby et al., 2010), the European 6th framework program Sens-It-Iv (Rovida et al., 2007), and the program of the Netherlands Toxicogenomics Center (www.toxicogenomics.nl). One possibility to identify skin sensitizers is the examination of altered gene expression patterns or the selective induction of signaling pathways in cell cultures challenged with test chemicals.

The aldoketoreductase gene *AKR1C2* gene was one of the first biomarkers that was found to be upregulated by sensitizers in dendritic cells (Gildea et al., 2006; Ryan et al., 2004) and later in keratinocytes (McKim et al., 2010). This gene contains a functional antioxidant response element (ARE) and is therefore dependent on Nrf2 for transcription (Lou et al., 2006). Based on this biomolecular evidence, we developed the KeratinoSens™ cell line which contains the ARE sequence from the human *AKR1C2* gene linked to a luciferase gene (Emter et al., 2010). The KeratinoSens™ assay was studied in detail for its predictivity, transferability and reproducibility (Natsch et al., 2011) and the assay is currently being assessed by ECVAM. As the assay moves forward in this assessment, a more detailed mechanistic understanding will be helpful to compare the reporter-gene approach to other methods, understand the response to false positives and false negatives and to find markers activated by other signaling pathways yielding potential additional information.

Abbreviations: LLNA, local lymph node assay; Nrf2, nuclear factor-erythroid 2-related factor 2; Keap1, Kelch-like ECH-associated protein 1; ARE, antioxidant response element; FCS, fetal calf serum; qRT-PCR, quantitative real time polymerase chain reaction; DMSO, dimethylsulfoxide; DNCB, 2,4-dinitrochlorobenzene; SDS, sodium dodecyl sulfate.

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A wide range of skin sensitizers induce the luciferase gene in this reporter cell line (Ball et al., 2011; Delaine et al., 2011; Emter et al., 2010; Natsch et al., 2013). Nonetheless, some false negative results have also been obtained (Natsch et al., 2013). Among these are several prohaptens, which require P450 mediated enzymatic oxidation to form the reactive species, and chemicals that transfer an acyl moiety especially to lysine-residues (different anhydrides such a phthalic anhydride, trimellitic anhydride and hexahydrophtalic anhydride or phenyl esters such as phenyl benzoate). In addition, some non-electrophilic false positives have been identified, including the non-ionic detergent Tween80. Also, sodium dodecyl sulfate (SDS) induces the luciferase yet only at low cellular viability.

The use of other genetic markers that have previously been identified in keratinocytes might add non-redundant information and thereby aid in correct classification, if they were induced by differentially regulated signaling pathways. A number of recently conducted studies have identified several promising genes and signaling pathways in dendritic cells and keratinocytes. In dendritic cell lines, p38 MAPK activation, induction of Nrf2/ARE-dependent genes, and changes in cell surface thiols are promising candidates for a platform of tests to predict the skin sensitisation hazard (Neves et al., 2011). In keratinocytes, two recent studies (Miyazawa et al., 2011; Vandebriel et al., 2010) showed that the Hsp70 response also has predictive potential. Four recent detailed gene-chip studies examined sensitizer-induced gene expression changes in dendritic cells (Johansson et al., 2011), keratinocyte cell lines (van der Veen et al., 2013; Vandebriel et al., 2010) and mouse skin (Miyazawa and Takashima, 2012). Using RT-PCR, gene expression was studied in HaCaT keratinocytes and epidermal models (McKim et al., 2010, 2012). One can conclude from these studies that the common significantly up-regulated pathway found is the Nrf2-pathway. Nevertheless, a broad range of other markers of interest was also identified.

So far, the involvement of Nrf2 in the luciferase induction has not been directly demonstrated in the recombinant KeratinoSens™ cell-line. In addition, the reporter gene response has yet to be directly compared to the induction of endogenous genes to evaluate whether the luciferase induction is a true reflection of the endogenous Nrf2-response to sensitizers. To further characterize the response of the KeratinoSens™ cell line, we first tested here whether the luciferase signal is indeed dependent on Nrf2. Next, the response was compared to the response of several endogenous Nrf2-dependent genes. Furthermore, we investigated whether emerging genetic markers in keratinocytes are also Nrf2-regulated in this cell line and whether they may add non-redundant information for correct classification, especially for the false negative chemicals.

2. Materials and methods

2.1. Test chemicals

The test chemicals were obtained from Sigma-Aldrich (Buchs Switzerland) and Givaudan Schweiz AG (Duebendorf, Switzerland). They included chemicals that are typically true positives and chemicals reported previous as either false positive or false negative in the KeratinoSens™ assay. Data on the chemicals and the rationale for their selection is summarized in Table 1. Test chemical concentrations, at which cells maintain >70% viability after 8 h, were selected for all the RT-PCR experiments.

2.2. Cell line and the standard KeratinoSens™ assay

The KeratinoSens™ cell line and method of the KeratinoSens™ assay have been previously described (Emter et al., 2010; Natsch et al., 2011). Unless otherwise indicated, the experiments were performed according to the published procedure (cell density, incubation time, compound addition). Briefly, cells were seeded at a density of 10,000 cells per well in 96-well plates and grown for 24 h. The medium was then replaced with fresh medium containing 1% FCS, containing the test chemical and 1% DMSO. Cells were incubated for 48 h with the test agents, after which luciferase activity and cell viability, using the MTT assay (Mosmann, 1983), were determined.

2.3. RT-PCR experiments

Cells were seeded in 6-well plates at a density of 150,000 cells per well in Dulbecco's modified Eagle's medium containing GlutaMAX™ (Gibco/Invitrogen) and 9% fetal calf serum (FCS). 24 h after seeding, the growth medium was replaced by fresh medium containing 1% FCS, the indicated concentrations of the test chemicals, and 1% DMSO. At the indicated times after compound addition, the cells were harvested by trypsinization. RNA was isolated using the RNeasy® Micro Kit (Qiagen, Hombrechtikon, Switzerland). RNA concentration was measured and 0.4 μg of each sample was used for cDNA synthesis using the QuantiTect® Reverse Transcription Kit (Qiagen). The levels of the specific cDNAs were detemined using the Rotor-Gene Q, the Rotor-Gene™ SYBR® Green PCR Kit (Qiagen), and the primer pairs listed in the supplementary data (Table I). Primers were obtained from Qiagen or synthesized by Microsynth (Balgach, Switzerland). Table 2 lists the rationale for the selection of and the literature reference for the genetic markers investigated in this study.

2.4. siRNA experiments

The transfection mix was prepared by mixing two mL of DMEM w/o FCS, 240 pmol All Stars negative control siRNA (Qiagen) or 240 pmol Nrf2-siRNA (Sense 5′-GUCCCAGUGUGGCAUCACCTT-3′, Antisense 5′-GGUGAUGCCACACUGGGACTT-3′) (Kimura et al., 2009), and 20 μL Lipofectamine RNAiMAX (Invitrogen). This mix was incubated at room temperature for 15 min. Then, 2×10^6 KeratinoSens™ cells were resuspended in 10 ml DMEM containing 9% FCS, added to the transfection mix and transferred to a 10 cm tissue culture dish. Three days later, the cells were harvested by trypsinization and further used for either the RT-PCR experiments as described above or for the luciferase-induction experiments according to the standard protocol (Emter et al., 2010).

3. Results

3.1. The luciferase signal induced by sensitizers in the KeratinoSens™ assay is dependent on Nrf2

In a first set of experiments, cells were pre-treated with Nrf2-siRNA or with control siRNA three days prior to seeding. The response of the siRNA treated cells to the extreme sensitizer 2,4-dinitrochlorobenzene (DNCB) and the two moderate sensitizers cinnamic aldehyde (CA) and (2E)-5,6,7-trimethyl-2,5-octadien-4-one was then measured in the KeratinoSens™ assay performed according to the standard method of the KeratinoSens™ assay. Upregulation of the luciferase signal by all three sensitizers was significantly reduced ($p < 0.005$) in the Nrf2-siRNA-treated cells as compared to the control siRNA-treated cells (Fig. 1). Treatment with Nrf2-siRNA reduced the sensitizer-induced luciferase signal by at least 80% at most test concentrations. The remaining signal can be attributed to the residual presence of Nrf2. At the time of compound addition (24 h after seeding), the knockdown of Nrf2 at the mRNA level was at 87 ± 5% in three independent experiments. At the end of the experiment (i.e. when the luciferase signal