Cell based test systems for the antioxidant response pathway

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ABSTRACT: Allergic contact dermatitis (ACD) is the manifestation of an allergic response caused by the skin sensitization of small protein-reactive chemical substances and has turned out to be the most prevalent form of allergic responses. Many chemical substances have skin sensitizing potential, some of them present in our environment in large quantities as plastic additives. Currently, for prediction of skin sensitizing potential animal experiments are necessary, since no validated non-animal assays exist to date. In this work a cell culture test system focusing on the Nrf2-antioxidant response pathway was developed. This assay is based on a cellular defence pathway, which sensitively reacts to chemicals which bind to proteins similar to skin sensitizers. The assay therefore allows a good prediction of skin sensitizing potential. Main emphasis of this work was to improve the sensitivity of the assay to allow measurements of materials directly in contact with the skin.

1 INTRODUCTION

1.1 Allergic contact dermatitis

The skin functions as interface between the environment and the body and therefore is permanently exposed to environmental pathogens and xenobiotics. The repeated exposure to small reactive molecules (haptens) is able to provoke allergic contact dermatitis (ACD) [1]. ACD is the manifestation of an type IV delayed allergic response caused by repeated contact with an allergen with skin sensitization potential and has become a common health issue over the past few decades [1]. Thus, the assessment of the sensitization potential of substances present in formulations of cosmetics or plastics coming in close contact with the skin gained in importance in the same time. Currently, the most commonly used and by the regulatory bodies accepted animal experiment to analyse skin sensitizers is the murine local lymph node assay (LLNA) [2,3]. However, ethical, legislative and monetary demands have actuated intense research to develop alternative, validated non-animal test methods. One of the existing methods for validation is the cell based KeratinoSens assay. It uses the activation state of the transcription factor nuclear factor erythroid 2-related factor 2 (Nrf2) for the identification of skin sensitizers.

1.2 Nrf2-ARE pathway

The Nrf2-antioxidant response pathway is activated by cysteine-reactive compounds [reviewed in 4,5] and induces cytoprotective genes to defend against environmental stresses enforced upon oxidants or xenobiotics. In normal cells the transcription factor Nrf2 is retained in the cytoplasm by being bound to Kelch-like ECH-associated protein 1 (Keap1) (Fig. 1). This tethering further promotes degradation of Nrf2. Inducers of the Nrf2-antioxidant response pathway interact with several critical cysteine residues in Keap1, resulting in conformation changes followed by the release of Nrf2 from the complex. Subsequently, Nrf2 translocates to the nucleus and binds together with a cofactor from the small Maf protein family to antioxidant response elements (AREs) in the promoter region of cytoprotective genes, thus initiating the cellular defence response.

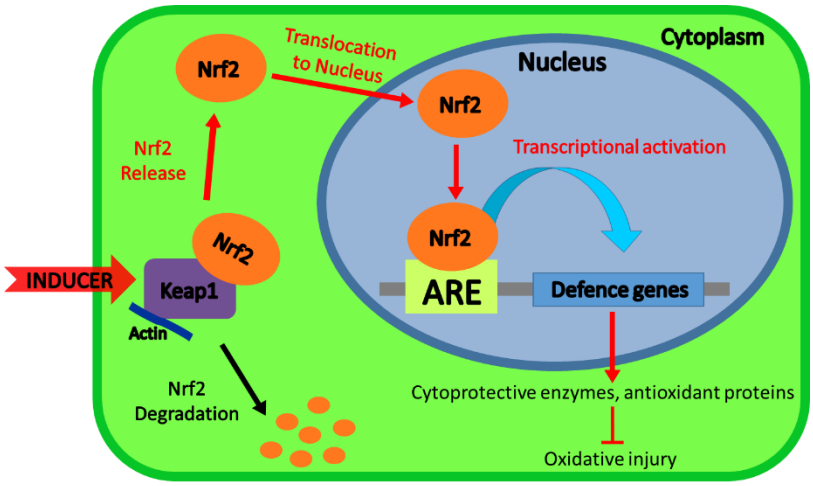

Figure 1. Regulation of Nrf2-antioxidant response pathway. Under normal conditions Nrf2 is bound to Keap1, retained in the cytoplasm and degraded. Inducers of the Nrf2-ARE pathway interact with Keap1 and lead to the release of Nrf2. After translocation to the nucleus Nrf2 binds to antioxidant response elements (AREs) and up-regulates transcription of cytoprotective genes.

To create an artificial test system the combination of ARE-sequences with a reporter gene allows detection of pathway activity after exposure to Nrf2-ARE pathway inducers. In our project the in vitro test method using a luciferase assay to detect Nrf2-antioxidant response pathway activity upon induction with established skin sensitizers was optimized to achieve higher sensitivity in comparison to already existing assays [6].The optimization process included the comparison of different ARE sequences, multimerization of the Nrf2 binding sites, testing different luciferase enzymes as reporters and including an internal reference for viability measurement.

2 RESULTS & DISCUSSION

2.1 Reporter construct optimization

First three different variations of an ARE-sequence were tested (Fig. 2A) to find the sequence with the highest binding affinity for Nrf2 and the lowest background activity. The ARE-sequence was taken from the promotor region of the human *AKR1C2* gene [7], which is known to be activated by the Nrf2-antioxidant response pathway [8,9]. One construct contained the ARE core sequence together with 20bp flanking sequences upstream and downstream (AREa). The second only contained the ARE core sequence of *hAKR1C* without flanking regions (AREb) and the third was a mutated form of AREb to destroy the consensus binding site for the AP-1 transcription factor (TGACTCA) within the ARE core sequence of *hAKRC1C* (AREc). All three versions were multimerized up to 12 times to gain higher induction levels and cloned upstream of a minimal promoter and luciferase as reporter gene (Fig. 2B).

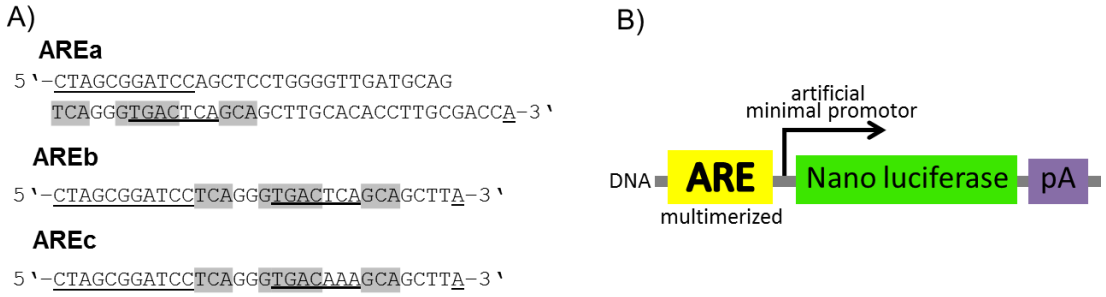

Figure 2. ARE reporter constructs. (A) the three different variations of the ARE sequence of hAKR1C2. ARE core sequences are highlighted in grey. Locations of AP-1 binding sites are underlined (B) Shows the scheme of the optimized reporter constructs with multimerized ARE-sequences upstream of an artificial minimal promotor and the reporter gene Nano luciferase, followed by a polyadenylation signal (pA).

First experiments with transient transfection of reporter constructs containing one to twelve times multimerized ARE-sequences into MCF-7 cells followed by benzylidene acetone treatment for 24 hours revealed that for AREa-sequence two copies, for AREb-sequence four copies and for AREc-sequence twelve copies led to the highest inductions, the highest overall induction could be achieved with multimerized AREa-sequence (Fig. 3A). This result shows that the effect of multimerization on reporter activation is variable between the different ARE reporters. The big differences can be explained by the binding of other transcription factors (e.g. AP-1) close to the Nrf2 binding site. This leads to higher background activation for the longest ARE sequence (AREa) especially at a high multimerization level (data not shown). The poor reactivity of AREc leads to the conclusion, that AP-1 might be involved in the reporter activation upon benzylidene acetone induction. For further reporter construct optimization the reporter gene was exchanged from the well established firefly luciferase (luc) to the more sensitive Nano luciferase (Nluc) [10].

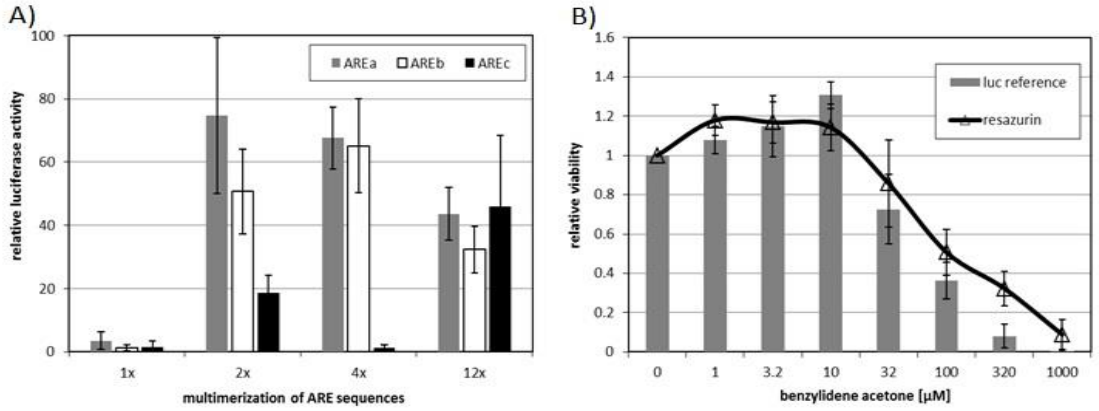

Figure 3. ARE multimerization and internal reference. For comparing ARE reporters with different numbers and variations of the ARE sequence (A) MCF-7 cells were transiently transfected with 10 ng reporter plasmid containing 1,2,4 or 12 times multimerized ARE sequences (AREa [light grey bars]; AREb [white bars] and AREc [black bars]). After 24 hrs transfected cells were induced with increasing concentrations of benzylidene acetone (0-1000 µM) and incubated for 24 hrs before luciferase measurement. Reporter activation is shown relative to untreated cells. Values show mean of 6 replicates. Error bars show standard deviation. For demonstration of the function of the constitutive internal reference luciferase (B) the stable HaCat 2xAREa cell line was treated with increasing concentrations of benzylidene acetone (0 – 1000 µM) for 24 hours and a resazurin assay was performed before luciferase measurement. Y-axis shows viability as relative luciferase or relative resazurin to untreated cells. Error bars show standard deviation. All values are means of 2 independent experiments.

2.2 Stable ARE-reporter cell lines

To generate a cell culture assay with high reproducibility the next step was to create stable cell lines with the optimized ARE reporters. Reporters with 2 AREa sequences or 4 AREb sequences were stably transfected into different cell lines known to have functional Nrf2-ARE pathways [6,11] with the PiggyBac transposase system [12]. A HaCat cell line containing 2 AREa sequences upstream of Nluc as reporter gene and luc as internal reference proofed to be the cell line with the greatest sensitivity and highest induction rates (data not shown). To test optimal assay conditions kinetic studies were performed with two strong inducers of the Nrf2-ARE pathway (benzylidene acetone and cinnammic aldehyde). The inducers were applied for one hour and a dual luciferase assay was performed after different recovery times. The highest level of induction (16.8-fold) was achieved after 6 hours recovery time when 320 µM cinnammic aldehyde were applied (Fig. 4). Even better induction of Nluc activity could be detected after exposure to 1000 µM benzylidene acetone also after a recovery time of 6 hours (99-fold). For both substances a clear decrease in Nluc activity could be observed at later time points. This indicates a fast reaction of the Nrf2-ARE pathway after applying a chemical inducer. A second kinetic study with different incubation times from 1 - 48 hrs demonstrated that if the inducer is left on the cells for longer periods there is still an increase of reporter activation until 24 hrs followed by a decrease at 48 hrs (data not shown). Additionally, cells with a 24 hr incubation reacted with a decreased viability compared to a 1 hr incubation but had their maximum reaction at lower concentrations.

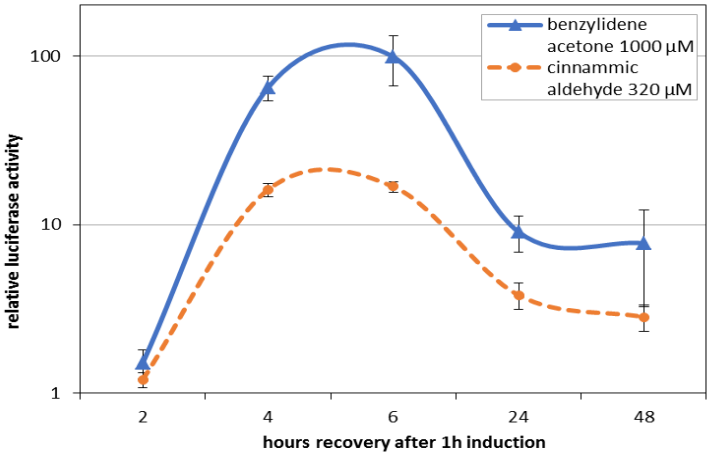

Figure 4. Kinetics of stable cell line. 0.5x104 cells of a stable HaCat cell line containing a 2xAREa reporter were seeded in a 96 well plate. 24 hours after seeding cells were induced with 1000 µM benzylidene acetone and 320 µM cinnammic aldehyde and incubated for 1 hour Afterwards Nluc activity was measured at the indicated time points. Expression of Nluc is shown relative to untreated cells. All values are means of 6 wells, error bars indicate standard deviation.

According to the kinetics of the stable HaCat cell line further substances, known inducers and non-inducers, were tested with an incubation time of 1 hour followed by a recovery time of 6 and 24 hours incubation. All non-inducing substances led to non-significant induction thereby being consistent with results from literature [6,4,13-17]. Viability was surveyed with the internal luc reference and a resazurin assay (Fig. 3B). Results for the maximal Nluc induction levels are shown in Table 1. For future applications of the cell line a cut off level to distinguish between significant and insignificant reporter activation has to be determined out of a further detailed analysis of inducers and non-inducers. This value can then be used to predict skin sensitizing potential of unknown substances. This fast and easy non-animal test method should be able to provide a sensitive and cost effective alternative to existing methods.

Table 1. Performance of the stable cell line with known inducers and non-inducers. Peak levels of Nluc activity for the stable HaCat cell line measured after short (1 h incubation and 6 h recovery) or long (24 h incubation) exposure to known inducer and non-inducer substances are shown (the concentrations of the substances are given in brackets).

Substance	Fold induction after 1h incubation and 6h recovery		Fold induction after 24h incubation	
Inducers				
benzylidene acetone	99	(1mM)	32.4	(100µM)
cinnammic aldehyde	16.8	(320µM)	34.9	(100µM)
2-mercaptobenzothiazole	33.9	(10mM)	161	(800µM)
p-benzoquinone	6.3	(100µM)	> 40µM cytotoxic	
citral	3.9	(1mM)	3.9	(100µM)
ethylene glycol dimethacrylate	3.8	(10mM)	1.1	(1mM)
glyoxal	3.0	(10mM)	2.7	(32µM)
Non-inducers				
NiCl	1.1	(3.2mM)	1.6	(1mM)
isopropanol	2.0	(3.2mM)	1.4	(100µM)
glycerol	1.9	(1mM)	1.6	(100µM)

3 MATERIALS & METHODS

3.1 Cell line and culture conditions

MCF-7 cells (human breast cancer cell line) were a gift from the Institute for Virology at the University of Veterinary Medicine Vienna. HaCat cells (human keratinocyte cell line) were purchased from CLS Cell Lines Service GmbH. Cells were cultured at 37°C with 5% CO2 in Dulbecco's modified eagle medium (DMEM; HyClone #SH30243.F3) containing 4.5 g/L glucose, Na-pyruvate and L-glutamic acid supplemented with 10% FCS,(HyClone #SV30160.03) 100U/mL Penicillin and 100µg/mL Streptomycin (HyClone #SV30010).

3.2 Transient transfection

A cell density of 1x104 cells in DMEM + 10% FCS were seeded into a PEI coated, white 96 well plates (Greiner #655083) and incubated at 37°C, 5% CO2 until transfection. Transient transfection was done using TurboFect transfection reagent (ThermoScientific #R0531) according to manufacturer's instructions.

3.3 Induction

24 hours after transfection or seeding (for stable cell lines) cells were induced by replacing the medium with fresh DMEM + 10% FCS containing different concentrations of the inducer substances, which were prepared immediately before each experiment. If the reference substance was dissolved in DMSO the DMSO concentration in all dilutions was adjusted to the DMSO concentration in the highest concentrated dilution of the reference substance tested in that experiment.

3.4 Viability measurement

Viability of cells was determined by measuring the metabolic activity of the cells. For that purpose 1/10 of volume of 550µM resazurin (from 5.5mM stock; Santa Cruz #sc-206037A) in 1xPBS per well was added to the medium 2 hours before dual luciferase measurement. Fluorescence was measured with a multimode plate reader (Tecan Infinite F200 pro) with an excitation wavelength at 540nm and an emission wavelength at 590nm. Viability was calculated relative to untreated cells.

3.5 Dual luciferase measurement

For dual luciferase measurement cells were washed once with 40 µL PBS and lysed with 25 µL cell lysis buffer (25 mM Tris-Cl, 0.03% Triton X-100) and dual luciferase measurement of the lysates were done by using a multimode plate reader (Tecan infinite F200 pro). Firefly luciferase substrate buffer (100 µM D-luciferin, 2.5 mM ATP, 6.25 mM Tris pH 7.5, 10 mM MgCl2) is injected followed by luciferase signal measurement. Then Nluc substrate buffer (210 mM DCTA, 6.25 mM Tris pH 7.5, 3.5 µL coelentherazine) was dispensed to the reaction followed by detection of luciferase signal. The relative luciferase activity was calculated by normalising Nluc activity to that of firefly luciferase.relative to untreated cells. Nluc was obtained from Promega GmbH.

4 ACKNOWLEDGMENTS

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