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Performance of the N/TERT epidermal model for skin sensitizer identification via Nrf2-Keap1-ARE pathway activation

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ABSTRACT

Animal testing of chemical ingredients for cosmetic purposes is prohibited. Therefore there is an urgent need for *in vitro* models to identify chemical allergens. In human skin, keratinocytes (KCs) are abundantly present and are key players in initiation of allergic contact dermatitis. One of the pathways that has been shown to be induced by sensitizers is the Keap1-Nrf2-ARE pathway. In this study we compared the response of four keratinocyte-based models including (a) primary human KCs, (b) N/TERT monolayer cultures, (c) the Leiden Epidermal models (LEMs) and (d) the N/TERT epidermal models (NEMs). All keratinocyte-based models were subjected to chemical exposure of the sensitizer 2,4-dinitrochlorobenzene (DNCB) and irritant Sodium dodecyl sulfate (SDS) at nontoxic concentrations. Activation of the Keap1-Nrf2-ARE pathway was evaluated by measuring Nrf2 protein levels as well as nuclear translocation and activation of transcriptional targets of Nrf2. Results show that the Keap1-Nrf2-ARE pathway is activated by the sensitizer DNCB in monolayer keratinocytes and as well as the LEMs and NEMs and not by the irritant SDS. Collectively our data demonstrate that the N/TERT models respond similarly as primary KCs and could therefore serve as an alternative model for skin sensitizer identification, thereby overcoming the need for primary skin tissue.

1. Introduction

Human skin is the first barrier against chemical insults and pathogens and consists for 90% of keratinocytes (KCs). Several lines of evidence support the critical role KCs have as key players in the early initiation of Allergic contact dermatitis (ACD) (Kaplan et al., 2012; Nestle et al., 2009; Nickoloff, 2006; Kollisch et al., 2005). ACD is a common occupational health problem, which results in a skin T-cell mediated inflammation caused by chemical sensitizers that make contact with the skin. The first phase of ACD is referred to as the sensitization phase, which does not lead to clinical manifestations. During this phase chemical sensitizers act as haptens as they have the ability to bind skin-resident carrier proteins. Haptens can efficiently penetrate through the skin and make covalent bindings with carrier proteins. This hapten-protein complex is recognized by the innate immune system leading to production of pro-inflammatory mediators. As a consequence, effector T cells are mediated and enter the blood circulation. The second phase (elicitation) takes place after re-exposure of the skin to the hapten inducing inflammation (Kimber and Dearman, 2002; Cavani, 2008; Gober and Gaspari, 2008; Vocanson et al., 2009; Kaplan et al., 2012).

In order to guarantee the safety of compounds used in the chemical and cosmetic industry, it is essential to identify and predict the sensitizing capacity of these ingredients. At the moment, animal tests such as the local lymph node assay (LLNA) (Kimber et al., 1994; Basketter et al., 2007) are used to identify chemical sensitizers and to classify them according to their potency. Reducing, replacing and refinement of the use of animal experimentation in the cosmetic industry has led to the introduction of the EU 7th Amendment to the cosmetic directive and the implementation of the Registration Evaluation Authorization and Restriction of Chemicals (REACH) regulation. Although there are many promising tests in development, to date there are no *in vitro* test models yet validated by the European Centre for the Validation of Alternative methods (ECVAM). Therefore there is an increasing need for reliable *in vitro* models that are able to identify chemicals causing skin sensitization.

A significant body of data has emerged suggesting that the oxidative stress pathway is a key pathway induced by sensitizers. Sensor protein Keap1 (Kelch-like ECH-associated protein 1) is present as a complex with Nrf2 leading to its constitutive proteasomal degradation. Covalent binding of chemical allergens to Keap1 through activation of the reactive cysteine residues of Keap1, results into

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dissociation of the protein complex and stabilization of Nrf2 levels. Subsequently, Nrf2 translocates to the nucleus and induces transcription of genes containing an antioxidant response element (ARE) (Natsch and Emter, 2008; Natsch 2010; Vandebriel and van Loveren, 2010; Kaplan et al., 2012).

Several human cell lines have been developed for skin sensitizer prediction (e.g. MUTZ-3, THP-1, NCTC2455) either at transcription level or cytokine level (Ade et al., 2009; Saito et al., 2013a,b; Corsini et al., 2009). Here we focus on the KCs as they are the main building blocks of the skin and because they have an important role in initiation of ACD. The KC cell line HaCat has been used for the development of several *in vitro* skin sensitization identification tests (Vandebriel and van Loveren, 2010; Emter et al., 2010; McKim et al., 2010, van der Veen et al., 2013). In addition, several commercial available epidermal models such as EpiCS® (CellSystems, Germany), Epiderm™ (MatTek, USA) and SkinEthic™ RHE (L'Oreal, France), have been used for skin sensitization identification by measuring Il-18 secretion or transcription activation (Gibbs et al., 2013a,b; McKim et al., 2012; Saito et al., 2013a,b). In these models, sensitizers mainly induced transcription activation of the Nrf2 dependent genes.

The use of human based reconstructed skin models for immune-toxic screening has major advantages compared to conventional monolayer cell lines since it consist of a competent barrier. This allows topical application of chemicals with low aqueous solubility and finished (cosmetic) products. In order to apply and evaluate the penetration of a drug, compound or chemical through the skin, such *in vitro* models should recapitulate a competent skin barrier (Ponec et al., 1997; El Ghalbzouri et al., 2008; Thakoersing et al., 2010; Thakoersing et al. 2012). More important is the xenobiotic metabolizing capacity of these *in vitro* skin models as skin penetration is considered to be the key route for inducing sensitization (Hu et al., 2010; Neis et al., 2010; Gotz et al., 2012; Hewitt et al., 2013). Currently, most epidermal models are engineered using primary KCs in order to mimic *in vivo* characteristics (Ponec et al., 1997; El Ghalbzouri et al., 2008; Thakoersing et al., 2010). However, the use of a KC cell line that generates a reconstructed epidermis is desired and considered as an added value since the bio-variability (donor-to-donor variation) is drastically reduced and offers a constant supply of skin tissue. Here we demonstrate that the N/TERT epidermal model (NEM) show similar activation of the Keap1-Nrf2-ARE pathway as the Leiden epidermal model (LEM) that is generated with primary KCs. This indicates that the NEM is a promising screening tool to predict the sensitizing potential of chemicals and may contribute to the reduction of animal experimentation.

2. Materials and methods

2.1. Cell culture

2.1.1. Primary human KCs

Primary human KCs were isolated from human mammary skin after informed consent according to the principles and guidelines of the Declaration of Helsinki, as described earlier (Ponec et al., 1997; El Ghalbzouri et al., 2008). In short, fresh skin tissue was obtained from surgical mamma reductions. First the epidermis is enzymatically separated from the dermis overnight using dispase II (Roche Diagnostics, Mannheim, Germany). The next day KCs were isolated from the epidermis using trypsin for 15 min at 37 °C followed by trypsin inactivation. Cells were filtered using a 70 μm cell strainer (BD Biosciences, Breda, The Netherlands) and cultured in keratinocyte medium at 37 °C and 7.3% CO_2. Primary KCs were cultured in DMEM/Ham's F12 medium (ratio 3:1) supplemented with 5% HyClone calf serum (Greiner, Nürtingen, Germany), 0.5 μM hydrocortisone, 1 μM isoproterenol, 0.1 μM insulin (Sigma), 100 U/ml penicillin and 100 μg/ml streptomycin (Invitrogen, Breda, the Netherlands). In all experiments secondary cultures were used. Cultures were refreshed every 2 days.

2.1.2. N/TERT keratinocyte cell line

Human N/TERT cell line (J. Rheinwald laboratory, Harvard Medical School, Boston, USA) is an hTERT-immortalized human keratinocyte cell line (Dickson et al., 2000). Cells were cultured at 37 °C and 7.3% CO_2 in keratinocyte-serum free medium (K-SFM) supplemented with the following final concentrations; 25 μg/ml Bovine pituitary extract (BPE), 0.4 mM $CaCl_2$, 0.2 ng/ml hEGF, 100 U/ml penicillin and 100 μg/ml streptomycin (Invitrogen, Breda, the Netherlands). Cultures were refreshed every 2 days.

2.1.3. Reconstructed epidermis

The LEMs and the NEMs were constructed by seeding 200.000 cells on insert filters (12 wells plate, polyester membrane, Costar) in Dermalife K medium including life-factors (Lifeline Cell Technology, Walkersville, MD) until full density was reached. Thereafter cells were cultured in CnT-BM.3-500 medium (basal medium plus supplement kit (CnT-02-3DP.S), CellnTec, Bern, Switzerland). This medium was supplemented with 2.4×10^{-5} M bovine serum albumin, 25 μM palmitic acid, 15 μM linoleic acid and 7 μM arachidonic acid (Sigma). Next, cultures were lifted to air/liquid interface and after 1 day the linoleic acid concentration was increased to 30 μM. Medium was refreshed every 2 days. LEMs and NEMs were air-exposed for 12 days at 37 °C and 7.3% CO_2 prior analysis.

2.2. Morphology and immunohistochemistry

LEMs and NEMs sections were washed in phosphate-buffered saline (PBS), fixed with 4% formaldehyde and paraffin-embedded. Sections were cut at 5 μm thickness, deparaffinized, rehydrated, and stained with haematoxylin and eosin (HE). Immunohistochemical analysis was performed for Keratins 10, 16 and Ki67. Heat-mediated antigen retrieval was performed in citrate buffer pH-6 followed by blocking non-specific binding using PBS containing 1% bovine serum albumin (BSA, Sigma) and 2% normal human serum (NHS, Sanquin, Leiden, the Netherlands). Primary antibodies were diluted in PBS + 1%BSA + 2%NHS and incubated overnight at 4 °C). After washing with PBS sections were incubated with streptavidin–biotin–peroxidase complex system (GE Healthcare, Buckinghamshire, UK) according to the manufacturer's protocol. After washing in PBS, the signal was developed with 3,3′-diaminobenzidine (DAB) solution, and all slides were counterstained with haematoxylin. As negative controls, tissue sections were processed under the same experimental conditions as described above, except that they were incubated overnight at 4 °C in PBS + 1%BSA + 2%NHS without primary antibody.

2.3. Immunofluorescence

Cells were grown on glass coverslips and exposed to chemicals at CV 80% concentrations. After 24 h cells were fixed with 4% formaldehyde for 15 min at room temperature (all of the following steps were done at room temperature) and permeabilized in 0.2% Triton X-100-phosphate-buffered saline for 5 min. Cells were washed with phosphate-buffered saline and blocked with 5% goat serum for 1 h, incubated with primary antibody (anti-Nrf2 rabbit polyclonal, 1:500, Santa Cruz, CA, USA) for 1 h, washed, and incubated with secondary antibodies for 30 min, following extensive washing. All sections were counterstained with 4′,6-diamidino-2-phenylindole, dihydrochloride (DAPI; Molecular Probes, Invitrogen) for 2 min. Sections were mounted with Vectashield