A New *In Vitro* Method For Identifying Skin Sensitizers and Predicting LLNA EC3 Values.

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ABSTRACT

The purpose of this study was to develop a novel *in vitro* human cell-based model to detect chemicals and finished products that are skin sensitizing agents. *In vitro* alternatives to animal testing are needed to support REACH and the European Cosmetics Directive initiatives. The present study describes the use of human (HaCaT) cells in culture and a 3D human skin model to identify skin sensitizers and predict LLNA EC3 values by utilizing concentration response, exposure time, and expression of genes controlled by the antioxidant response element (ARE). The model was developed using a set of 14 chemicals previously classified as non-, very weak-, weak-, moderate-, strong-, and extreme-sensitizers. A prevalidation study with more than 100 compounds is currently underway. The standard HaCaT cell culture model is efficient, but is limited to chemicals with high solubility. In contrast, the human 3D skin models commercially available from SkinEthic and MatTek provide a test system that can be used to test finished products and chemicals with low solubility. Test chemicals were applied at concentrations that ranged from 0.01 µM to 1 mM. Exposures were for 24 hr at 37°C in a 96-well format. Viability was monitored with MTT or histology. Total RNA was isolated with Trizol and changes in the expression of quinone reductase (NQO1), interleukin 8 (IL-8), and aldoketo reductase (AKR) were measured by RT-PCR. The strong sensitizer 1-chloro-2,4 dinitrobenzene produced significant increases in IL-8 and AKR at an exposure of 10 µM. In comparison, the non-sensitizer benzoic acid had no effect at an exposure concentration of 1 mM. An algorithm based on an analysis of the magnitude of gene expression, concentration response, the number of genes responding, and time provides a single value of response, the *in vitro* toxicity index (IVTI). Comparison of the IVTI to known LLNA EC3 values by exponential regression analysis resulted in an R value of 0.92 in both cell models. This *in vitro* system provides a useful tool for identifying sensitizers and predicting LLNA EC3 values without the use of animals.

INTRODUCTION

The ability to identity chemicals that induce a skin sensitization reaction is of high importance to the cosmetics industry. The proposed ban on the use of animals for the testing of new cosmetic ingredients and finished products requires that a non-animal alternative be developed. Ideally this system should be highly accurate and provide the means to evaluate individual ingredients as well as mixtures and finished products. The mechanisms underlying skin sensitization or allergic contact dermatitis involve two key phases, induction and elicitation. An essential and unifying step in chemical sensitization is the formation of a covalent adduct between the small chemical molecule (hapten) and a cellular proteins. The chemical protein adduct is transported by Langerhan cells to the lymph nodes where a T-cell specific immune response is triggered. It is well known that most chemical sensitizers either possess inherent reactivity (are electrophiles) or can be metabolically modified to reactive metabolites. These chemicals and the reactions discussed are linked to the cytochrome P450 1A (CYP1A) family of enzymes and the reactive products produced by CYP1A metabolism interact with the cytosolic binding protein Nrf2, which with recruitment of other co-factors, binds to the antioxidant response element (ARE) and induces the expression of genes whose protein products protect the cell from oxidative damage. The *in vitro* test system presented here takes advantage of these properties by evaluating chemical reactivity and ARE linked gene expression.

METHODS

Cell Models and Culture: Immortalized human keratinocytes (HaCaT) cells were used as the monolayer system and were cultured based on reported procedures. A human three dimensional skin model (EpiDerm, MatTek and EpiSkin, SkinEthic) were also used in this study.

Description of Experimental Setup and Biochemical Assays

HaCaT cells or 3D human epidermal models were exposed to test articles over a broad (0.1 µM to 2500 µM) concentration range. Maximum concentrations were limited by solubility and cytotoxicity. Dosing solutions were prepared by first preparing stock solutions in DMSO and then final solutions in complete culture medium. A training set of twenty known chemical sensitizers was selected to develop the system (Table 1). Exposures were for 24 hr at 37°C.

Measuring Direct and Indirect Chemical Reactivity.

The GSH Reactivity Assay is prepared in clear polystyrene 96-well plates from Costar (Corning, NY). Each reaction was performed in triplicate and contained 0.5 mg/mL BNF/PB microsomes, 1 mM NADPH, 10 µM freshly prepared GSH, and 100 µM or 1 mM of the test chemical prepared in DPBS, pH 7.4. Briefly, microsomes, NADPH and DPBS were mixed well, followed by addition of the test compound and finally GSH with a final reaction volume of 100 µL. The reactions were incubated for 15 minutes at room temperature. 100 µL of 10% w/v MPA was added and the plate was shaken for 2 minutes at room temperature on a DPC MicroMix 5 plate shaker. 50 µL of each reaction/MPA mixture was transferred to a new clear 96-well plate. 10 µL of 1.5 M TEAM was added followed by another 2 minute shake at room temperature. Finally, 150 µL freshly prepared GSH Assay Buffer (125 mM sodium phosphate, pH 7.4, 6.3 mM EDTA, 0.25 mM NADPH, 0.7 mM DTNB and 0.6 units/mL glutathione reductase) was added. The plate was again shaken at room temperature for 2 minutes followed by 10 minute incubation in the dark. The free GSH was determined by spectroscopy at 415 nM and data expressed as percent of vehicle control (DMSO).

Cytotoxicity

Cell viability was determined by measuring the reduction of 3-[4,5-dimethylthiazol-2-yl] 2,5-diphenyltetrazolium bromide (MTT). The cells in each well were evaluated for their ability to reduce soluble-MTT (yellow) to formazan-MTT (purple). An MTT stock solution was prepared in complete medium just prior to use and warmed to 37°C in a water bath. Once the medium was removed from all wells, MTT solution was added to each well and the plate was allowed to incubate at 37°C for 3-4 hr. Internal method development experiments have demonstrated that color development is linear over this time. After the 3-4 hr incubation, all medium was removed and the purple formazan product was extracted using anhydrous isopropanol. Sample absorbance was read at 570 nm and reference absorbance at 650 nm with a Packard Fusion or equivalent plate reader.

Isolation of Total RNA and qRT-PCR Analysis

Total RNA was isolated from HaCaT cells with Qiagen 96-well isolation kits. Total RNA was isolated from 3D human reconstructed epidermis by the RNAazol method. Induction of target gene mRNA was determined by standard qRT-PCR techniques.

RESULTS

FIGURE 1

Alternatives to Animal Safety Tests Required for Cosmetics

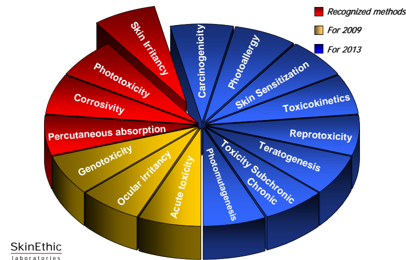


FIGURE 2

Chemical Sensitization Involves Multiple Steps

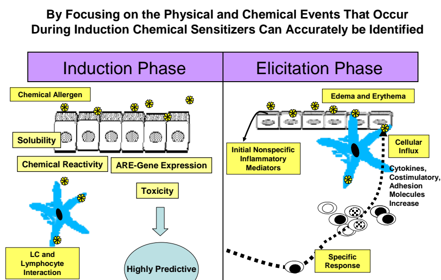


Table 1: List of Known Chemical Sensitizers

Compound	Sensitizer Class
Benzoic Acid	Nonsensitizer
Salicylic acid	Very Weak
Vanillin	Very Weak
Glycerol	Very Weak
Hydroxy Citronellal	Weak
Diethylsulfate	Moderate
Isoeugenol	Moderate
Aminophenol	Strong
Phthalic Anhydride	Strong
Benzoquinone	Extreme
Dinitrobenzene	Extreme

The test compounds above are representative of the more than 40 used to develop this system. The sensitization class is based on *in vivo* LLNA EC3 data.

FIGURE 3

Strong Chemical Sensitizers Can be Highly Reactive

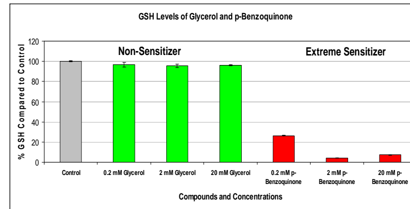
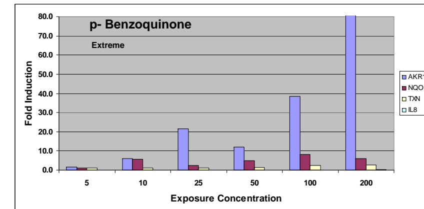


FIGURE 4

The Potent Sensitizer Benzoquione Induces Key ARE-linked Genes in a Concentration-Dependent Manner

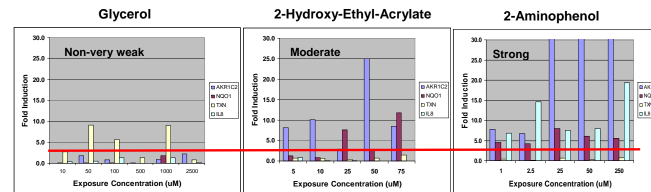


More than 10 ARE-linked genes are monitored in this system. The four shown in this study are NADPH-quinone oxidoreductase, aldoketo reductase, thioredoxin, and interleukin 8.

FIGURE 5

Chemicals with Varying Degrees of Potency Induce Different Gene Expression Profiles

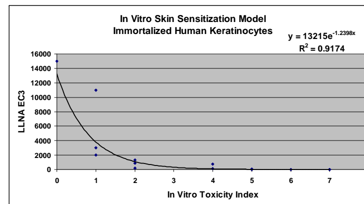
HaCaT cells used as model



Detailed concentration response data expressed as mass per unit area may allow the NOEL to be estimated for human exposure.

FIGURE 6

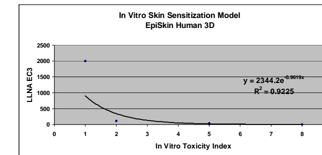
Chemical Reactivity and ARE-Linked Gene Expression Provide Good Correlation with LLNA EC3 Data



A CeeTox proprietary algorithm evaluates multiple gene expression responses, cytotoxicity, and chemical reactivity to develop an *in vitro* toxicity index or IVTI. When the IVTI was compared to LLNA EC3 values by regression analysis the correlation (r^2) was >90%.

FIGURE 7

ARE-Linked Gene Expression in Human 3D Skin Model Also Predict Sensitization and Correlate with LLNA

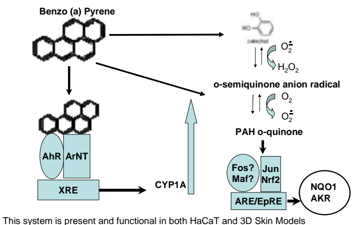


Compound	Sensitizer Class
Salicylic acid	Very Weak
Isoeugenol	Moderate
Propyl Gallate	Strong
Aminophenol	Strong
Benzoquinone	Extreme

A representative group of chemicals from Table 1 were also tested in a human 3D skin model. The results were nearly identical to those obtained in the HaCaT cell line.

FIGURE 8

An Intrinsic Sensor System Capable of Responding to Reactive Chemicals and Metabolites



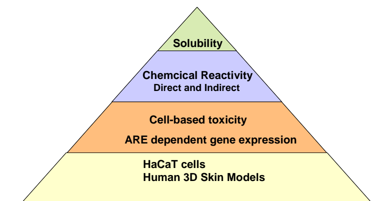
This system is present and functional in both HaCaT and 3D Skin Models

SUMMARY

- Chemicals that are known to be sensitizers react with GSH based on their potency.
- Genes controlled through Nrf2 and the antioxidant response element (ARE) responded to chemical sensitizers in a concentration related manner.
- The magnitude of gene expression and the number of genes responding correlated with *in vivo* potency.
- Several human cell systems responded in a similar manner.

CONCLUSIONS

The ability to correctly identify and correctly classify chemical sensitizers can be accomplished by combining chemical reactivity, gene expression profiling, and cytotoxicity. These data can also be used to estimate *in vivo* parameters, such as the mouse LLNA EC3 and human HRIPT. It may also be possible to estimate human NOELs from this type of information. Large collaborative validation studies are currently underway with corporate partners.



A tiered approach to *in vitro* testing for skin sensitizers increases accuracy and reduces false positive and false negative results.

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