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RESEARCH ARTICLE

An *in vitro* method for detecting chemical sensitization using human reconstructed skin models and its applicability to cosmetic, pharmaceutical, and medical device safety testing

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Abstract

Chemical sensitization is a serious condition caused by small reactive molecules and is characterized by a delayed type hypersensitivity known as allergic contact dermatitis (ACD). Contact with these molecules via dermal exposure represent a significant concern for chemical manufacturers. Recent legislation in the EU has created the need to develop non-animal alternative methods for many routine safety studies including sensitization. Although most of the alternative research has focused on pure chemicals that possess reasonable solubility properties, it is important for any successful *in vitro* method to have the ability to test compounds with low aqueous solubility. This is especially true for the medical device industry where device extracts must be prepared in both polar and non-polar vehicles in order to evaluate chemical sensitization. The aim of this research was to demonstrate the functionality and applicability of the human reconstituted skin models (MatTek Epiderm® and SkinEthic RHE) as a test system for the evaluation of chemical sensitization and its potential use for medical device testing. In addition, the development of the human 3D skin model should allow the *in vitro* sensitization assay to be used for finished product testing in the personal care, cosmetics, and pharmaceutical industries. This approach combines solubility, chemical reactivity, cytotoxicity, and activation of the Nrf2/ARE expression pathway to identify and categorize chemical sensitizers. Known chemical sensitizers representing extreme/strong-, moderate-, weak-, and non-sensitizing potency categories were first evaluated in the skin models at six exposure concentrations ranging from 0.1 to 2500 µM for 24 h. The expression of eight Nrf2/ARE, one AhR/XRE and two Nrf1/MRE controlled gene were measured by qRT-PCR. The fold-induction at each exposure concentration was combined with reactivity and cytotoxicity data to determine the sensitization potential. The results demonstrated that both the MatTek and SkinEthic models performed in a manner consistent with data previously reported with the human keratinocyte (HaCaT) cell line. The system was tested further by evaluating chemicals known to be associated with the manufacture of medical devices. In all cases, the human skin models performed as well or better than the HaCaT cell model previously evaluated. In addition, this study identifies a clear unifying trigger that controls both the Nrf2/ARE pathway and essential biochemical events required for the development of ACD. Finally, this study has demonstrated that by utilizing human reconstructed skin models, it is possible to evaluate non-polar extracts from medical devices and low solubility finished products.

Keywords: Nrf2, Antioxidant response element, allergic contact, dermatitis, reconstructed human skin, *in vitro* skin sensitization, medical devices

Introduction

Recent legislation in the EU has made it necessary for the manufacturers of cosmetics and personal care products, to incorporate alternatives to animal safety testing into their development programs. One impact of this initiative has been an increase in the development of new *in vitro* testing strategies aimed at fully replacing the animal models. To this end, alternative models for skin corrosion

and irritation have been validated by the Interagency Coordinating Committee on the Validation of Alternative Methods (ICCVAM) or by the European Centre for the Validation of Alternative Methods (ECVAM). The developers of alternative methods are now focused on more complex endpoints, such as tests for chemical sensitization, which represents one of the most common types of immunotoxicity observed in humans.

Skin sensitization that manifests as true allergic contact dermatitis (ACD) occurs when a small chemical molecule comes in contact with and penetrates the epidermis of the skin. Once a chemical enters the skin it must bind to a protein forming a hapten. This chemical-protein molecule is large enough to be recognized and taken up by the immune cells of the skin, known as Langerhan's cells. The Langerhan's cells with hapten then migrate to the local lymph node where the hapten (antigen) is presented to T-lymphocytes. The activated T-lymphocytes begin to divide and this division results in clonal expansion of these allergen responsive cells. The now sensitized T-cells are capable of responding to a second exposure to the same chemical with a much more exaggerated response known as ACD (1-6).

The medical device industry is beginning to recognize that *in vitro* testing can not only reduce animal usage, but can significantly reduce the time and cost required to perform standard safety tests. However, before new *in vitro* tests designed to assess the sensitization potential of medical devices will be accepted, manufacturers must be convinced that the test can provide reliable data that is at least as accurate as the data obtained from animal and demonstrate to regulators that the model is sensitive enough to detect biological changes even when there are low levels of a test chemical's effects in device extracts.

Many chemicals can elicit allergic contact dermatitis (ACD) in humans. Current testing guidelines require the manufacturers of medical devices to test for dermal sensitization. Under testing guidelines described in the ISO 10993-10 document, polar and non-polar extracts of medical devices are to be prepared and evaluated for sensitization potential in the mouse local lymph node assay (LLNA) or the guinea pig maximization test (GPMT) (7-13). These animal models have been used successfully for many years to identify chemical allergens. However, with the new focus on alternative methods in the EU, it is clear that the ability to accurately determine the sensitization potential of chemicals, plant extracts, pharmaceuticals, and medical device extracts with *in vitro* models is required.

Several *in vitro* sensitization methods that are currently either under evaluation by the COLIPA (European Cosmetics Association) skin tolerance task force, have completed inter-laboratory ring trials or have been accepted by the European Centre for the Validation of Alternative Methods (ECVAM) for pre-validation (14). These include the Direct Peptide Reactivity Assay (DPRA) (15), the human Cell Line Activation Test (hCLAT), (16-17) the Myeloid U937 Skin Sensitization Test (MUSST) (18-19), KeratinoSens (20-22), and VITASENS (23). Each method has demonstrated usability for identifying whether a chemical is a sensitizer or a non-sensitizer, however, these methods have had varied success in assigning chemicals to potency categories; an important piece of data for risk assessment. Another challenge has been difficulty in testing finished products that have little or no solubility and hence cannot be properly exposed to *in vitro* cell models maintained in aqueous medium.

The concept that genes controlled by the antioxidant response element (ARE) could be used to predict chemical sensitization was first introduced by Natsch and Emter (2008) using cells derived from liver or from human breast cancer (MCF-7) cells. This method was extended by McKim et al., 2010 to include a human keratinocyte cell line (HaCaT), multiple gene expression profiles, dose response, cell viability, and direct reactivity to predict sensitization as well as potency categories (11). This approach has been shown to work well with pure chemicals that have reasonable solubility. However, *in vitro* models that rely on standard culture of cells in monolayer may not be applicable for the evaluation of test compounds with low solubility. This excludes finished products, such as creams and lotions from testing in these models, a major concern for cosmetic companies. Medical device testing requires that a polar and non-polar extract of the device be prepared and tested. As mentioned above, *in vitro* methods that use standard culture techniques in aqueous medium can lose sensitivity with increasing LogP values, making it difficult to test the non-polar preparations. In addition, the ability of an alternative method to evaluate the sensitization potential of insoluble finished products, such as creams and lotions, in the personal care and pharmaceutical industry is not possible with many of the alternative approaches currently being proposed.

The aim of the present study was to evaluate the *in vitro* sensitization assay described by McKim, et al. (2010) (24) in HaCaT cells using two reconstructed human epidermal skin models (MatTek Epiderm® and SkinEthic RHE) which are cultured at an air-liquid interface. This unique tissue model enables chemicals or extracts from medical devices that possess low solubility to be tested. The responses obtained with this study were compared to those observed with the immortalized human keratinocyte (HaCaT) cell line. The three models presented in this study were selected because they have all received considerable review and have either been validated or are in prevalidation phases for chemical sensitization or for other types of skin effects such as irritation. New skin models are being introduced and many may provide advantages over those described here. In addition, these three tissue models possess the mechanisms required for the readouts measured in the assay described. The primary advantage of the HaCaT cell model is ease of handling, low cost, and accuracy for chemicals with adequate solubility. The primary disadvantage is that chemicals with extremely low solubility can not be evaluated in this model due to low exposure. In comparison, the reconstructed human tissue models are cultured at the air-liquid interface and possess the cell layers, and express marker proteins associated with the epidermis. Test chemicals can be applied directly to the tissue surface, which greatly expands the applicability domain for the test system. Although these tissue models do not possess Langerhan's cells, these cells are not required in order to perform the screening test described here because this assay focuses on chemical properties and biochemical changes that occur in the epidermal cells

and not in Langerhan's cells. These biochemical changes observed in the epidermis are essential for recruiting Langerhans cells; an essential step in the development of true ACD. The data presented here shows that both of the human reconstructed models respond in a similar manner and were effective tissue models for performing the sensitization test. Moreover, the ability to directly apply low solubility materials as extracts or finished products to the test system should greatly extend the applicability domain of this alternative method.

Materials and methods

Tissue culture

Three dimensional cell cultures were obtained from two sources SkinEthic (RHE, Leon, France) and MatTek (Epiderm®, Ashland, Massachusetts). Both models are generated using normal human keratinocytes grown in transwell plates. Both models show normal differentiation markers for human skin and show a similar pattern of cell types and cell morphology as compared to human skin including multiple cell layers. Vendor protocols were followed for tissue treatment prior to dosing.

Preparing the dosing solutions

Test compounds were dissolved at a concentration of 500 mM in dimethyl sulfoxide (DMSO), Sigma-Aldrich St. Louis, MO. Stock solutions were serially diluted in DMSO to obtain 200x dosing stocks. The dosing stocks were diluted 200-fold in DPBS (SkinEthic RHE or MatTek EpiDerm®, Ashland, MA) and 30 µL applied in triplicate to the apical side of a 96-well tissue plate. The final concentration of DMSO was 0.5%. The final exposure concentrations used for the test chemicals was determined based on previous work in HaCaT cells (24). Exposure concentrations were selected so that cell viability at a minimum of three concentrations was greater than or equal to 50%. In most instances gene changes observed between 100% and 50% viability represent changes caused by chemical interaction with ARE rather than changes related to cell death. Although 50% is the minimum viability used, it is always associated with concentrations that cause less cell death and hence higher viability. Digitonin, at a concentration of 1 mM or Triton-X 100 at a concentration of 1%, were used as controls for maximal cell death. DMSO (0.5% in Dulbecco's Phosphate Buffer Saline (DPBS)) was used as a vehicle control. DMSO was selected as the vehicle to more directly compare responses in the reconstructed skin models to those reported previously in HaCaT cells, and because it is an accepted vehicle for medical device extracts tested in the LLNA method. Tissues were incubated for 24 and 48 h at 37°C with 5% CO₂ and humidity.

Determining solubility of the test chemicals

Test article solubility was evaluated using a Nephaloscan Ascent instrument (LabSystems, Helsinki, Finland). This was done by adding a 200 µL aliquot of the final dosing solutions to a clear, flat bottom microtitre plate in

duplicate. Light scattering in the solutions was measured using a Nephaloscan Ascent instrument. Compounds with scattering values that were greater than or equal to 3-times those recorded for the vehicle control, were considered insoluble (25). The plate was incubated for 24 h at 37°C with 5% CO₂. Following the incubation period, the solubility was reevaluated to verify that the solubility over the 24 h exposure period remained stable.

Determining the effect of vehicles on tissue viability

Tissues received from both MatTek (Epiderm®) and SkinEthic (RHE) were treated with increasing concentrations of DMSO, ethanol (EtOH), olive oil (OO), and sesame oil (SO) to determine the concentration at which tissue viability was affected by each vehicle. Dosing solutions were prepared in DPBS with increasing concentrations of the test vehicles (0.5–100%). These solutions were added to the tissues in quadruplicate according to the dosing protocol described above. After incubation, at 37°C with 5% CO₂ and humidity, viability was assessed using the MTT assay as described below.

Determining the level of background oxidative stress in the EpiDerm® and RHE models

Oxidative stress was assessed in each tissue model using 5-(and-6)-chloromethyl-2',7'-dichlorodihydrofluorescein diacetate, acetyl ester (CM-H2DCFDA) obtained from Invitrogen (Carlsbad, CA). The human reconstructed tissues were received in a 96-well format and cultured according to the manufacturers instructions. Epiderm plates were received and equilibrated in media for 1 h. RHE plates were received and equilibrated overnight (>18 h). Following the equilibration period the tissues were loaded basally with 20 µM CM-H2DCFDA (DCFDA) by incubating for 2 h at 37°C. The loading media was removed by aspiration and replaced with culture medium containing 1% tert-butylhydroperoxide and were incubated for 1 h. Negative controls received DPBS only. Reactive oxygen species were measured by removing the tissue from the insert plates using forceps and placing the tissue into 50 µL of DPBS in a 96-well clear bottom assay plate. DCF-fluorescence was determined with a Packard αHT-Fusion reader at an excitation wavelength of 485 nm and an emission wavelength of 530 nm.

Determining tissue viability (MTT assay)

Viability of dosed cells was assessed using the MTT assay as described previously (26–27). Briefly, 3-(4,5-dimethylthiazo-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) was dissolved in tissue media to a concentration of 0.5 mg/mL. Media was removed from dosed tissues and 250 µL of MTT/media added to the basal side of each tissue. Cells were incubated for an additional 3 h at 37°C under 5% CO₂ with humidity. After 3 h, MTT/media was removed and 250 µL of isopropanol was added to the basal side of the tissues and 50 µL of isopropanol added to the apical side of the tissues. After

shaking for 10 min the insert plate is removed and the absorbance at 570 nm and 650 nm were read on the Packard α HT-Fusion plate reader. Absorbance at 570 nm is subtracted from the 650 nm reading. Dividing the test compound samples by the vehicle controls gives the percent viability. Compound concentrations with less than 50% viability are considered "unusable".

Determining chemical reactivity

Stock solutions of the test compounds (500 mM) were prepared in DMSO. The final reaction mixture contained the following: 20 mM sodium phosphate, (pH 7.4), test compound (20 mM), and GSH (final concentration 100 μ M) in a final volume of 250 μ L was carried out in black 96-well plates. Each reaction was done in triplicate wells. The reactions were incubated for 24 h at room temperature on a plate shaker essentially as described by Natsch, et al. (2007) (28) and Gerberick et al. (2004) (1) and recently reported by McKim, et al. (2010) (24). Following the incubation period, GSH not bound to the test chemical was determined essentially as described by Tietze (1970) (29) with modifications by Griffith (1980) (30). Sample processing was started by adding metaphosphoric acid (MPA) to each well in a volume that yielded a final concentration of 2.8%. The plate was then shaken for 2 min at room temperature. Next, a 50 μ L aliquot of the acidified reaction mix was removed and added to a clear 96-well plate which had been pre-loaded with 10 μ L of a 1.5 M solution of TEAM (triethanolamine). After two additional minutes of shaking, a 150 μ L portion of GSH assay buffer (0.7 mM DTNB, 0.25mM NADPH and 0.6 U/mL glutathione reductase) was added to each well. The plate was shaken for an additional 5 min and the fluorescence change measured with a Packard α HT Fusion plate reader (30). The absorbance at a wave length of 415 nm was directly proportional to the amount of GSH present. The percent depletion of GSH was calculated by taking the mean of the sample triplicates divided by the average of the vehicle controls and multiplying by 100.

Determining the effect of exposure time on tissue viability and gene expression

For time course studies, compounds were dosed as described above, and incubated for 24 or 48 h at 37°C under 5% CO₂ with humidity. After incubation, cell viability was determined with the MTT assay and tissue samples placed in QIAGEN RLT buffer for RNA isolation and stored at -80°C.

Isolating total RNA

RNA was isolated using QIAGEN's RNeasy 96 kit by following the vacuum/centrifugation protocol according to the manufacturer's instructions. Briefly, the tissue inserts were removed from media and placed in a 96-well plate that had been pre-loaded with 200 μ L RLT lysis buffer (containing guanidine thiocyanate). Then an additional 100 μ L of RLT was added apically. Addition of the RLT buffer lysed cells and inactivated RNases. Plates were shaken for 10 min on a plate shaker and stored at -80°C.

Frozen plates were thawed at 37°C and incubated for 10 min to resolubilize all salts in the lysis buffer. The plates were then placed onto a shaker for 10 min at room temperature. After incubation and shaking, apical and basal fractions were combined into the basal side of plate. After pooling RLT, 300 μ L of 70% ethanol was added to the wells, mixed by pipetting and applied to silica columns in an RNeasy 96-well isolation plate. The liquid was pulled through the plate filter by vacuum and 80 μ L DNase in an RDD (DNase digestion) buffer was added to each well. The samples were incubated for 15 min followed by the addition of 1mL of RW1 wash buffer containing guanidine thiocyanate and ethanol. After additional 5 min incubation with RW1 buffer, the liquid is pulled through the wells by vacuum. The samples were washed twice with 1 mL of RPE wash buffer, which was pulled through the wells by vacuum filtration. After a second RPE wash the plate was centrifuged at 5600 rpm in a Sigma 4K15 centrifuge equipped with a QIAGEN/Sigma Nr.09100F rotor for 10 min to remove excess liquid. After this 60 μ L of Nuclease-free water was added to each well of the RNeasy plate. The plate was spun at 5600 rpm for 4 min to elute the RNA. The elution step was repeated with another 60 μ L of Nuclease-free water.

RNA was quantified with a Nanodrop ND-8000 spectrophotometer with 2 μ L of eluted RNA. The concentration of eluted RNA varied from 5 to 20 ng/ μ L with an A₂₆₀/A₂₈₀ ratio greater than 1.6.

Determining the effects of the test chemicals on gene expression

The eleven target genes chosen code for enzymes that work on a variety of substrates and represent a large number of xenobiotic transformations that can occur in many cell types including keratinocytes. Several of the genes are under the control of the antioxidant response element (ARE), which upregulates the production of cytoprotective proteins. QuantiTect Primer assays for 11 target genes (AKR1C2, NQO1, TXN, IL8, HMOX1, MAFF, GCLC, and ALDH3A1) were obtained from QIAGEN, Inc. (Germantown, Maryland) (Table 1). Metallothionein I (MT1A) and II (MT2A) are under the control of the metal response element (MRE) and cytochrome P4501A1 (CYP1A1) is under the control of the Xenobiotic response element (XRE).

Gene expression was determined by qRT-PCR using QIAGEN's QuantiTect SYBR Green Kit (QIAGEN, Germantown, MD) on a Roche LightCycler 480 (Roche Applied Science, Mannheim, Germany). Reactions were loaded with approximately 50 ng of input RNA. Standard curves were made using serial diluted RNA from non-treated tissues. Reaction components were mixed according to the QIAGEN's QuantiTect SYBR Green Kit Handbook for 20 μ L reactions. Reaction mixture was added to the wells of a 384-well PCR plate (Roche Applied Science, Mannheim, Germany) on ice. RNA was then added to the wells and the plate was sealed and centrifuged at 1500 x g for 3 min. One-step qRT-PCR was run as follows; reverse transcription was 1 cycle of 50°C

Table 1. List of genes monitored by qRT-PCR.

Abbreviation	Gene name
GAPDH	glyceraldehyde-3-phosphate-dehydrogenase
B2M	beta-2-microglobulin
TFRC	transferrin receptor (p90 + CD71)
RRN18S	18S ribosomal RNA
AKRIC2	aldo-keto reductase family 1, member C2
NQO1	NAD(P)H dehydrogenase, quinone 1
TXN	thioredoxin
IL8	interleukin 8
MAFF	v-maf musculoaponeurotic fibrosarcoma oncogene homolog F
HM0X1	heme oxygenase (decycling) 1
ALDH3A1	aldehyde dehydrogenase 3 family, member A1
GCLC	glutamate-cysteine ligase, catalytic subunit
CYP1A1	cytochrome P450, family 1, subfamily A, polypeptide 1
MT2A	metallothionein 2A
MT1A	metallothionein 1A

for 30 min followed by 95°C for 15 min. Amplification and signal acquisition was set for 45 cycles of 94°C for 15 sec, 55°C for 20 sec and 72°C for 20 sec. Melting curve was generated by 1 cycle of 95°C for 5 sec followed by 65°C for 1 min.

Data from the qRT-PCR reactions was first analyzed with LightCycler® 480 software version 1.5.0.39 (Roche Diagnostics Corp., Indianapolis, IN). Initially, standard curve slope, error and efficiency were recorded as well as whether or not the 4 vehicle control replicates had Cp values within 3 cycles of each other. Next, the triplicate samples for each compound at each dose were viewed. A Dixon's Q-test was performed to determine the proximity of each replicate value. Outliers identified by this test were discarded from the analysis. Next the relative abundance of the 4 control genes expressed as fold-change vehicle was plotted in a box and whisker plot. The control gene that showed the lowest variation was selected for each sample. The target gene induction over vehicle was then divided by the control gene to determine a fold induction over background for each target gene.

Calculating the magnitude of gene induction

The fold change in the mRNA for each target gene was determined using qRT-PCR. A standard curve was prepared by adding 0.1, 1, 10, and 100 ng of total RNA isolated from negative control tissues. For each target gene an acceptable range for the slope of the regression line was determined by collecting a minimum of 50 historical slope values for each gene, calculating the mean, standard deviation, and setting the acceptance at two standard deviations. This provided an upper and lower limit of acceptability. In addition, an error value calculated within the light cycler must be less than 0.2. This value is a measure of the accuracy of the quantification results based on the regression line. The fold change in the target genes in

Table 2. Chemicals selected to evaluate the SenCeeTox® method in Epiderm® and RHE reconstructed Human Skin Models.

Compound	Potency category
Glycerol	Non-sensitizer
Benzoic acid	Non-sensitizer
Eugenol	Weak
2,3-Butanedione	Weak
4-Vinylpyridine	Moderate
2-Mercaptobenzothiazol	Moderate
Isoeugenol	Moderate
p-Benzoquinone	Extreme
2-Aminophenol	Strong
DCNB	Extreme
Metol	Strong

To determine if the human skin models (Epiderm and RHE) would respond to chemical sensitizers in a manner similar to the HaCaT cells, 11 chemicals were selected from the set of 112 previously tested in the HaCaT model (11).

treated wells was determined relative to the expression level of the same gene in the negative control wells.

Four house keeping genes (HKG) were monitored for each chemical at each exposure concentration. These included glyceraldehyde-3-phosphate dehydrogenase (GAPDH), beta-2-microglobulin (B2M), transferrin receptor (TFRC), and 18S ribosomal RNA (RPN18S). The mean relative abundance of HKG mRNA was determined. The HKG with the lowest variability in the presence of test compound was determined by plotting the data on a whisker plot. The gene with the lowest variability was selected to normalize the target gene data.

Results

To evaluate whether the human reconstructed skin models produced by MatTek and SkinEthic would respond to chemical sensitizers in a manner similar to the HaCaT cells, eleven chemicals were selected from the original 112 that were tested previously in HaCaT cells (Table 2) (11). Because the mouse local lymphnode assay (LLNA) has been approved for use with several different vehicles, it was necessary to determine how these might affect the viability of human skin tissue models.

Effect of vehicles on tissue viability

Medical device sensitization testing (ISO 10993-10) requires that the device be extracted with a polar (saline or DMSO) and a non-polar (olive oil, sesame oil) vehicle. In addition, many cosmetic companies formulate their finished products in ethanol. In order to use the human reconstructed skin models it is important to understand how potential vehicles might affect tissue viability. Figure 1 shows that when DMSO solutions were applied to the apical surface of the tissues in a volume of 30 µL and incubated for 24 h, the tissues maintained a high level of viability. Ethanol concentrations of 25, 50, 60, 80, and 100% caused significant

toxicity, while Olive oil and sesame oil had little effect on tissue viability up to 100 %.

Importance of measuring background oxidative stress in cell models

The *in vitro* platform described here measures chemical reactivity and chemical activation of the antioxidant response element (ARE), which controls the expression of several cytoprotective genes. This system can lose sensitivity if the constitutive level of reactive oxygen species is high prior to exposing the cell model to the test chemicals. Therefore, each model was evaluated for background levels of oxidative stress (Figure 2). These data indicate that the RHE tissues from SkinEthic had extremely high levels of oxidative stress prior to dosing with test chemicals. This is evident by evaluating tissues treated with DPBS relative to the t-butyl hydrogen peroxide (tBHP) group in EpiDerm® tissues (red bars). In the presence of a positive control for oxidative stress (tBHP) in the DCFDA assay, there was a clear increase relative to negative controls (DPBS) observed in the EpiDerm® tissues (DPBS red bar versus tBHP red bar). In comparison fluorescence was near maximum response in the DPBS treated RHE tissues (blue bar) with no change observed following treatment with tBHP.

Chemical reactivity

The ability of each chemical in the absence of a biological matrix to bind the tripeptide glutathione (GSH) is used as an indicator of direct reactivity and sensitization

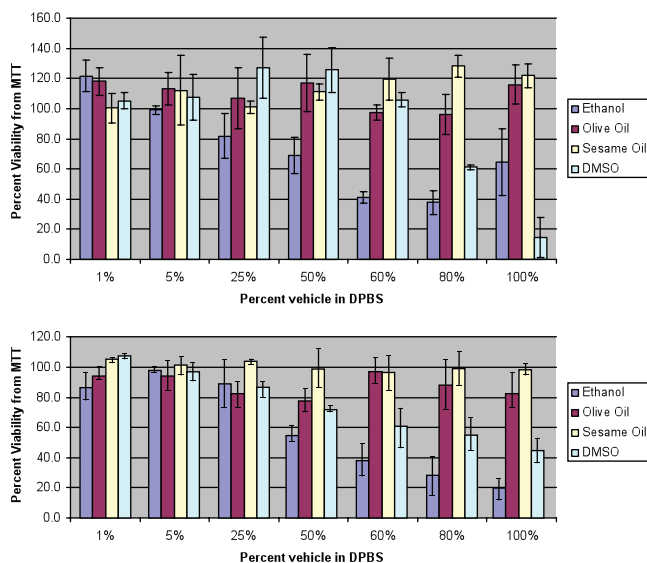


Figure 1. Effects of four commonly used vehicles on tissue viability. RHE tissues from SkinEthic (Top) and EpiDerm® tissues from MatTek (bottom) were treated with varying concentrations of DMSO, olive oil, sesame oil and ethanol and incubated for 24 h at 37°C at 5% CO₂. Tissue viability was assessed by the MTT assay and expressed as a percent viable relative to saline controls. Each value represents the mean of 3 tissues ± SEM.

potential. In Table 3 significant GSH depletion is observed with moderate, strong, and extreme chemicals while little to no GSH binding was observed with non-sensitizers and weak. The relatively low value observed for 2-aminophenol is likely due to need for metabolic activation, suggesting that this compound is a prohapten. 2-mercaptobenzothiazole (MBT) is not classified as an electrophile (31) and as such would not be expected to bind GSH in its parent form. However, this chemical can rearrange to form a thioester tautomer, which can react with nucleophiles (32–33) and therefore can act like an electrophile in the acyl transfer domain (32). Nucleophile-electrophile reactions are controlled by Pearson's hard-soft acid-bases model. Pearson's model dictates that the reaction rate and selectivity of electrophiles and nucleophiles is dependent on their comparable hardness. MBT can bind to the primary amine of lysine, a hard nucleophile (34). A hard nucleophile will more easily bind to a hard electrophile. Based on the unique chemical properties of MBT, binding to GSH would be expected to be low under the experimental conditions used.

Effect of test chemicals on gene expression profiles in three cell models

To determine the relative responses of ARE controlled gene expression to the test chemical training set shown in Table 3 in the EpiDerm, RHE, and HaCaT cell models, each system was treated with the test chemicals at 6 exposure concentrations. Following the standard 24 h exposure period total RNA was isolated and subjected to qRT-PCR analysis. Changes in the expression of the target genes were determined by normalizing the mRNA signal for target to a house keeping gene.

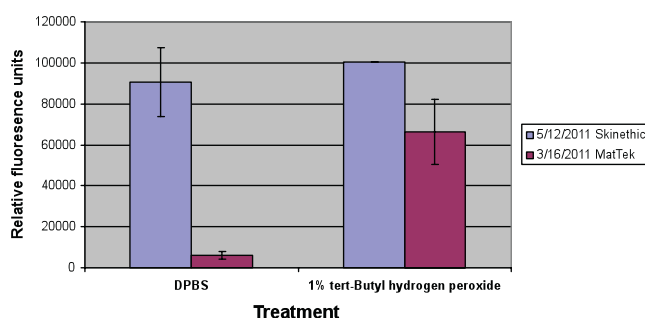


Figure 2. Background levels of oxidative stress in Epiderm and RHE tissue models. Epiderm and RHE skin models were screened for background levels of oxidative stress using the DCFDA assay. The tissues were loaded with 20 µM CM-DCFDA for 2 h at 37°C. Media was removed by aspiration and the tissues treated with either DPBS or tBHP and incubated for 1 h. An increase in FUs indicates oxidative stress. Epiderm tissues had extremely low back ground levels of oxidative stress, and responded fully to exposure to peroxide. In comparison, the RHE tissues had high background levels of oxidative stress (DPBS, blue bar), and showed only a slight response to the peroxide treatment.

A graphical representation of the fold change in target genes is shown in Figures 3–7 for chemicals spanning the Extreme/Strong-, Moderate-, Weak-, and Non-sensitizer potency categories. The graphs allow a direct comparison of gene expression responses between the three cell models.

Genes controlled by the Nrf2/Keap1/ARE pathway included NQO1, AKR1C2, TXN, IL8, ALDH2A1, HMOX1, MAFF, and GCLC. In order to evaluate a potential for metabolic activation, expression of the AhR/Arnt/XRE controlled gene CYP1A1 was also monitored. By scanning the graphs presented in Figures 3–7 it is clear

that AKR1C2 responded with the greatest magnitude of induction. NQO1 was induced above the 2.5-fold threshold, but the magnitude of response was less than AKR1C2. IL-8 responded well in both the Epiderm and HaCaT models, but showed little response in the RHE model. The extreme sensitizer p-benzoquinone did not induce HMOX1 to any significant degree in any of the cell models. In comparison, the strong sensitizer 2-aminophenol produced a significant increase in HMOX1 in HaCaT cells, but not in the other two models (Figure 4). GCLC was induced by both p-benzoquinone and 2-aminophenol in the two human 3-dimensional skin models (h3D), but there was only a small response detected in the HaCaT cell model. Maff, expression was one of the least responsive to the chemicals tested and this was true in all cell models.

Cytochrome P450 (CYP) 1A1 is an important enzyme for the metabolism and activation of many chemicals. Expression of the CYP1A1 gene is controlled by the AhR/Arnt nuclear transcription factor, which when activated binds to the XRE and upregulates CYP1A1. Metabolism of xenobiotics by this enzyme often produces reactive intermediates which can then activate the Nrf2/Keap1 signaling pathway and can also cause chemical sensitization. Therefore, significant increases in the expression of CYP1A1/2 mRNA can identify chemicals susceptible to metabolic activation, such as p-benzoquinone and 2-aminophenol.

Table 3. Direct chemical reactivity of training set chemicals.

Chemical	GSH Depletion (%)	Potency Category
p-Benzoquinone	69	Extreme
1-Chloro-2,4-Dinitrobenzene	76	Extreme
2-Aminophenol	12	Strong
Metol	73	Strong
4-Vinylpyridine	94	Moderate
2-Mercaptobenzthiazol	0	Moderate
Isoeugenol	92	Moderate
Eugenol	0	Weak
2,3-Butanedione	8	Weak
Benzoic acid	0	Non
Glycerol	4	Non

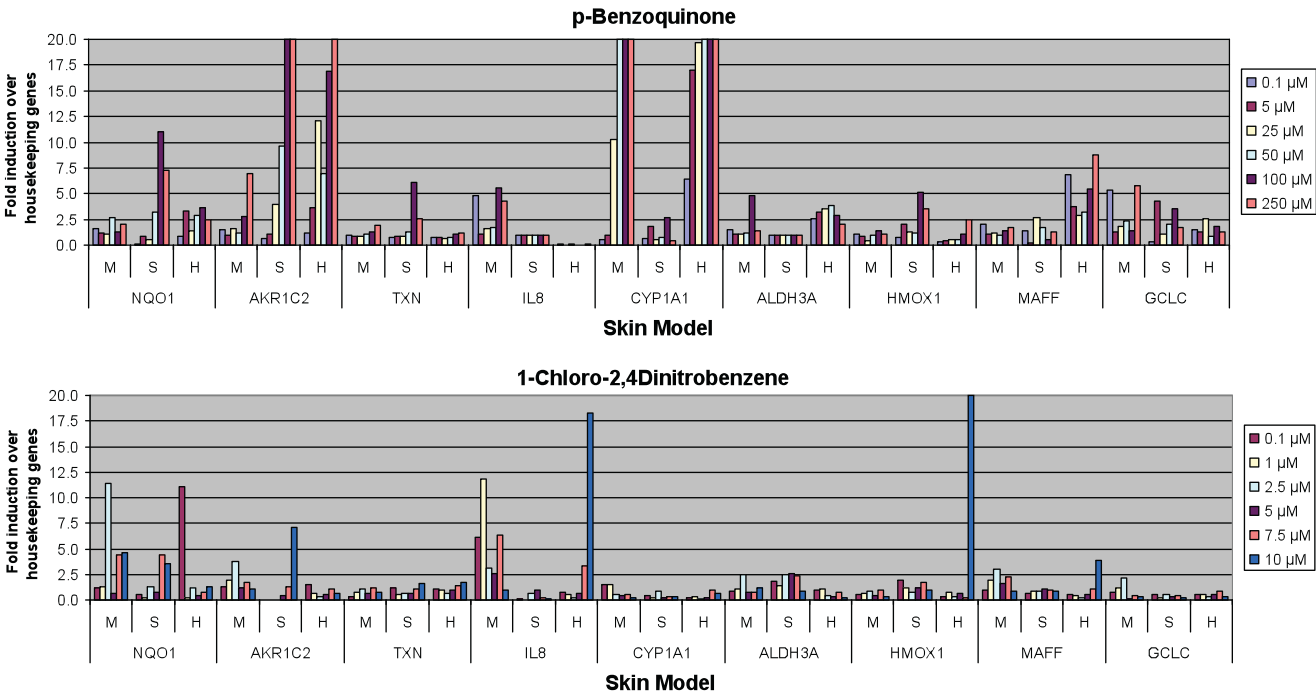


Figure 3. Comparison of Nrf2/ARE gene expression in Epiderm, RHE, and HaCaT skin models following exposure to extreme sensitizers. Representative gene expression profiles are compared between HaCaT (H), SkinEthic RHE (S), and MatTek EpiDerm (M) following exposure to several concentrations of two known sensitizers in the extreme potency category. Values represent the mean of replicate tissue samples or wells. NQO1, AKR1C2, IL8, ALDH3A, HMOX1, and GCLC showed the greatest magnitude of response or potency. MAFF and TXN had very low responses are considered to be the least sensitive of the genes monitored. CYP1A1 expression is controlled by the AhR/Arnt/XRE signaling and is useful for identifying chemicals that may be susceptible to metabolic activation (e.g. prohapten).

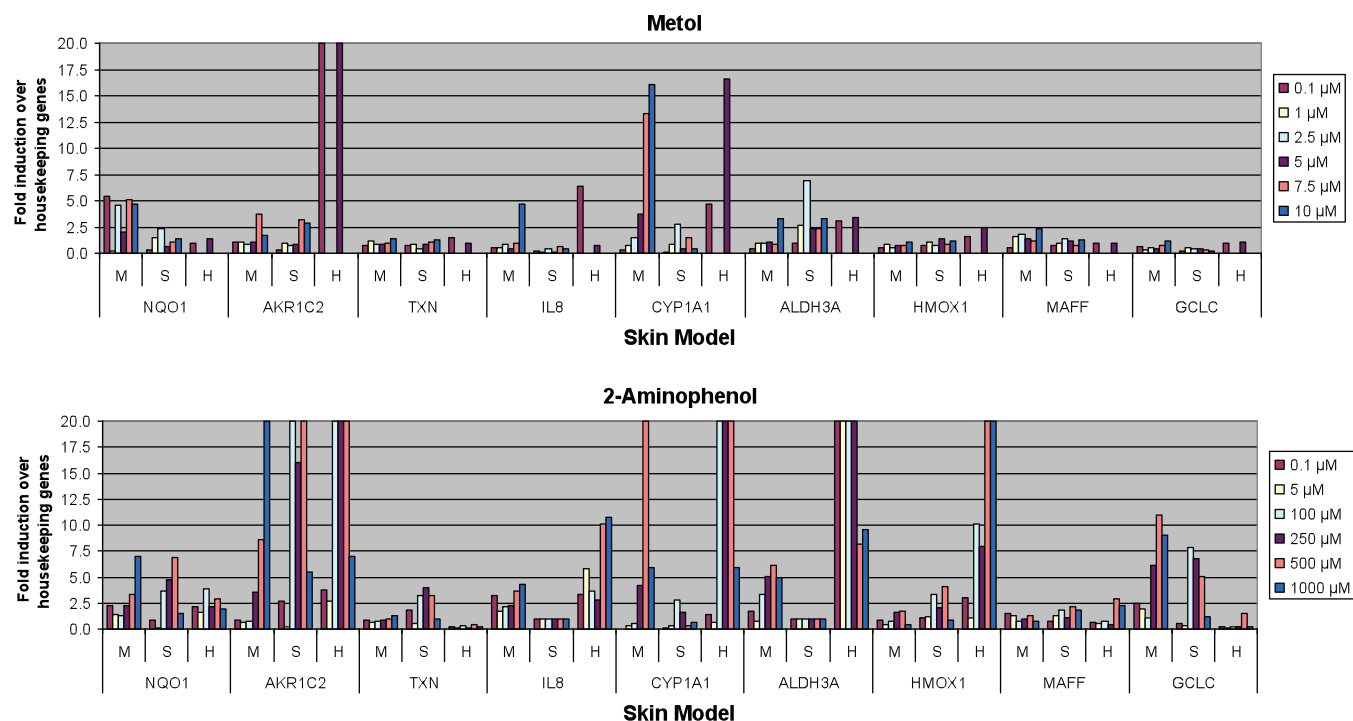


Figure 4. Comparison of ARE and XRE mediated gene expression in Epiderm, RHE, and HaCaT skin models following exposure to strong sensitizers. Representative gene expression profiles are compared between HaCaT (H), SkinEthic RHE (S), and MatTek EpiDerm (M) following exposure to several concentrations of two known sensitizers in the strong potency categories. Values represent the mean of replicate tissue samples or wells. NQO1, AKR1C2, IL8, ALDH3A, HMOX1, and GCLC showed the greatest magnitude of response. MAFF and TXN had very low responses and are considered to be the least sensitive of the genes monitored. CYP1A1 expression is controlled by the AhR/Arnt/XRE signaling and is useful for identifying chemicals that may be susceptible to metabolic activation (e.g. prohaptens).

Compounds considered to be moderate sensitizers [4-vinylpyridine (4VP), 2-mercaptobenzothiazol, (2MBT) and isoeugenol, (IE)] are compared in Figure 5. 4-VP produced an increase in NQO1 in EpiDerm and RHE, but not in HaCaT. AKR1C2 was significantly increased in all three cell models. TXN was only increased in the RHE model, while IL-8 was increased solely in the EpiDerm model. ALDH3A was induced in EpiDerm and RHE, by 4VP, and 2MBT, but not by IE. In comparison, the HaCaT cells responded to all three moderate sensitizers. IE induced CYP1A1 in HaCaT cells only, while 4VP had no detectable effect on CYP1A1 expression in any of the three models. In comparison, 2MBT dramatically increased CYP1A1 mRNA in all cell models. The expression of the genes evaluated is controlled by ARE and XRE and they respond to varying degrees in different tissue models. However, because multiple genes are monitored the net result of the expression analysis provides the correct answer. These findings underscore the importance of multiple endpoints, and the evaluation of multiple genes.

Figure 6 shows the gene expression data for 2 weak (2,3-butanedione and Eugenol) and Figure 7 depicts data from two non-sensitizing (benzoic acid and glycerol) compounds. For most of the weak and non-sensitizers there was little or no response. One exception was eugenol in the HaCaT cells, where several genes were upregulated.

Evaluating chemicals used in medical devices

The training set of eleven compounds (Table 2) was used to establish the sensitivity of the human skin models (Epiderm and RHE) to chemical sensitizers covering a range of potencies. Once the response of these chemicals in the test tissues was confirmed a smaller set of seven chemicals used in the manufacture of medical devices were tested (Table 4). Analysis of these data showed a 100% sensitivity and specificity when moderate, strong and extreme were grouped as positives and when weak and non-sensitizers were grouped as negatives. The ability of the assay to accurately place compounds into one of the four potency categories (non-, weak, moderate, and strong/extreme) was 71%.

Linking upregulation of ARE controlled genes with allergic contact dermatitis

Induction of an allergic response and activation of the Nrf2/Keap1/ARE signaling pathways can be activated through phosphatidylinositol 3 kinase (PI3K) and the subsequent activation of the kinases (extracellular signal regulated kinases (ERK) and Akt also known as protein kinase B (Figure 8). There was upregulation of the cytokine TNF- α , and the chemokine RANTES (regulation on activation, normal T cell expressed and secreted) also known as CCL5 following exposure to known sensitizers (Figure 9). PI3K-ERK1/2 pathways and the Nrf2/ARE pathways are present and activated by sensitizers. The

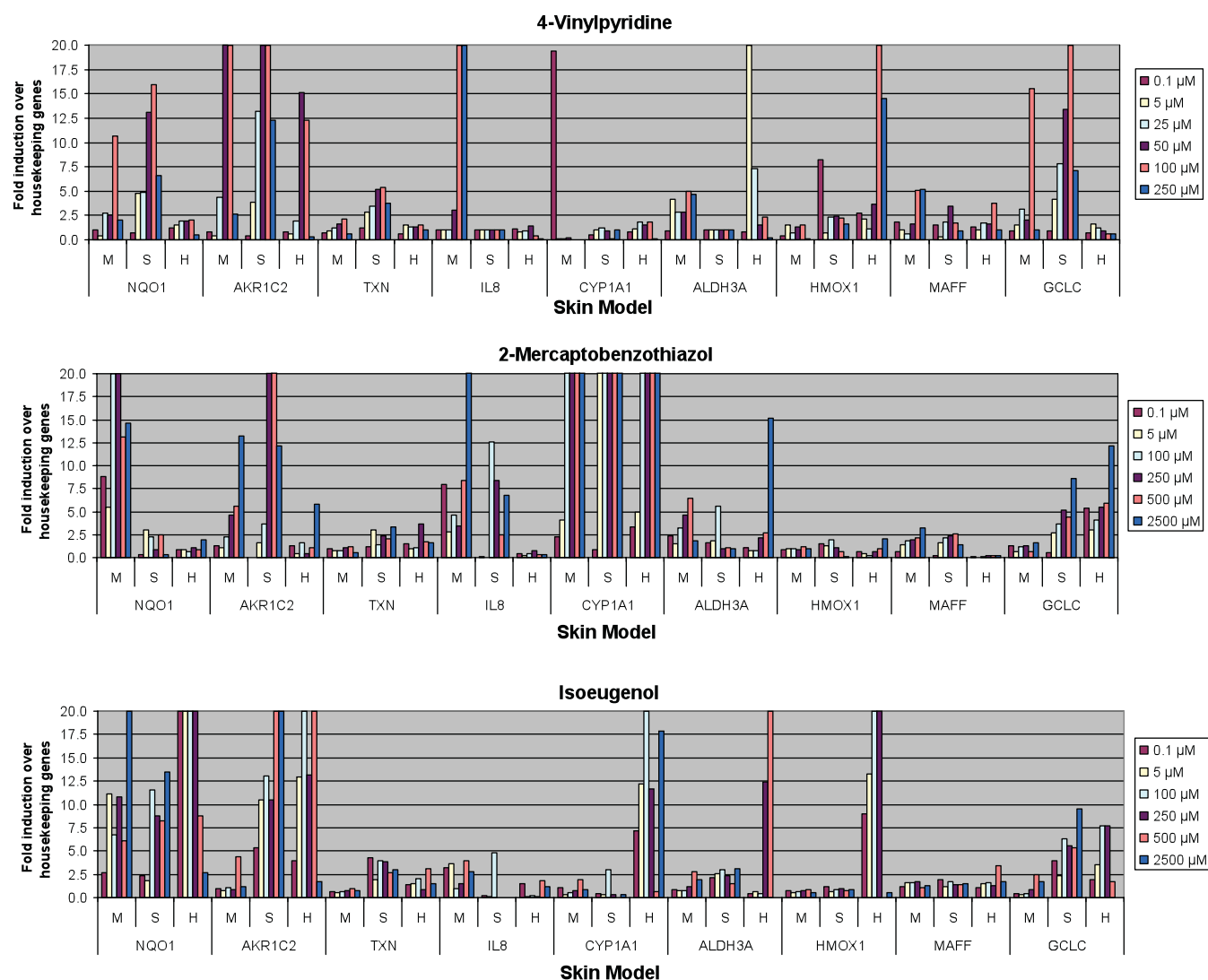


Figure 5. Comparison of Nrf2/ARE gene expression in Epiderm, RHE, and HaCaT skin models following exposure to moderate sensitizers. Representative gene expression profiles are compared between HaCaT (H), SkinEthic RHE (S), and MatTek EpiDerm (M) following exposure to several concentrations of three known sensitizers in the moderate potency category. Values represent the mean of replicate tissue samples or wells. NQO1, AKR1C2, IL8, ALDH3A, HMOX1, and GCLC showed the greatest magnitude of response or potency. MAFF and TXN had very low responses and are considered to be the least sensitive of the genes monitored. CYP1A1 expression is controlled by the AhR/Arnt/XRE signaling and is useful for identifying chemicals that may be susceptible to metabolic activation (e.g. prohapten).

constitutive levels of several of these key signal molecules was quite high and relative comparisons could be made by comparing the cross over point C_p of the RNA curves obtained during RT-PCR analysis. The higher relative abundance of these genes could indicate signal control mechanisms that occur post-translationally.

Discussion

The correlation between activation of the Nrf2/Keap1/ARE signaling pathway by sensitizers was first proposed by Natch and Emter (2008) (35). Later, McKim, et al. (2010) (24) demonstrated that upregulation of ARE controlled genes in combination with a direct reactivity assay, cytotoxicity, and concentration response data could be highly predictive of chemical sensitization in a human keratinocyte cell line (HaCaT). This method was able to

assign chemicals to three potency categories (extreme/strong, moderate, and weak-non). In order for the screening method to have practical value to the medical device industry, the method must allow for the testing of non-polar extracts. Therefore, this method was evaluated in reconstructed human three dimensional tissue cultures obtained from SkinEthic and MatTek Corporation. These human skin cultures are grown at an air/liquid interface, which allows for the direct application of the test material to the apical surface. Exposure can be expressed either as a concentration, or as a mass per unit area of skin.

The data presented in this study clearly demonstrates that the Nrf2/Keap1/ARE pathway is intact and responsive to chemical sensitizers in the reconstructed human skin models. Although both models responded in a similar fashion, skin cultures obtained from SkinEthic had an unusually high background level of oxidative stress

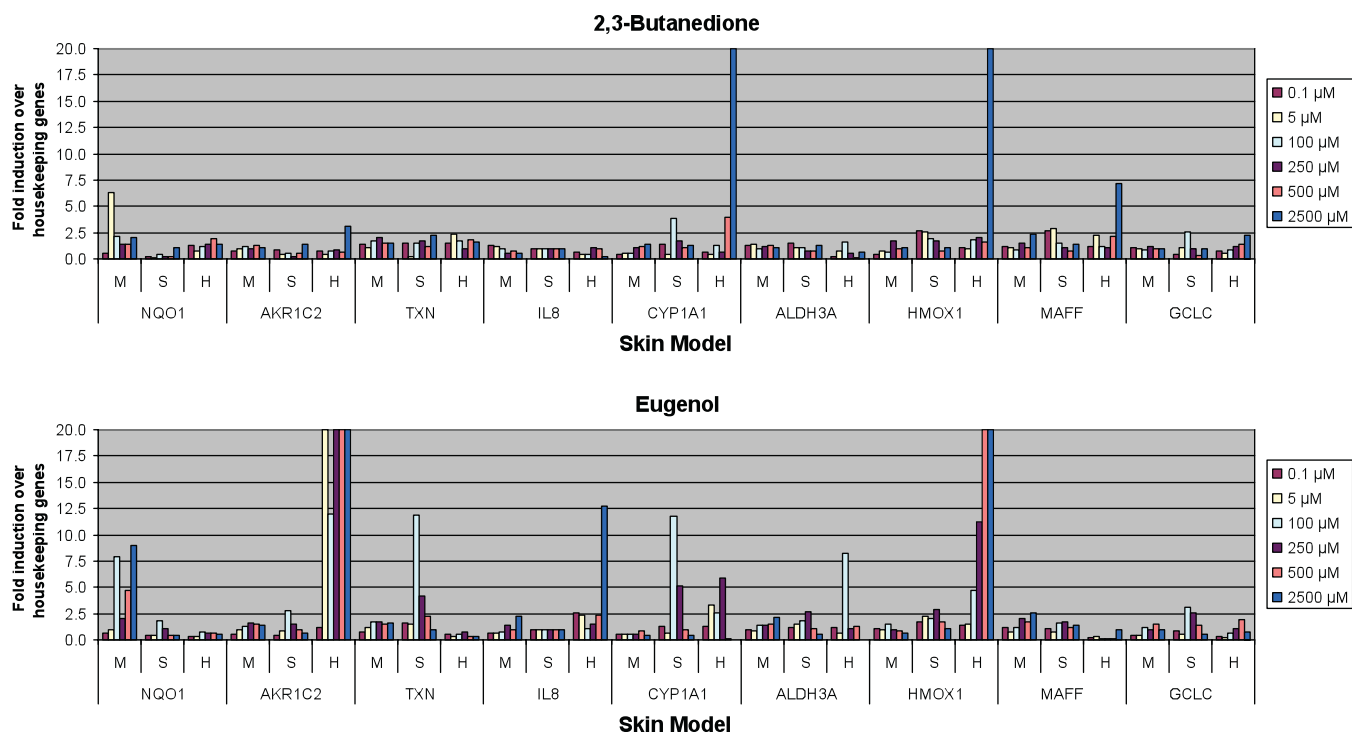


Figure 6. Comparison of Nrf2/ARE and AhR/Arnt/XRE mediated gene expression in Epiderm, RHE, and HaCaT skin models following exposure to weak sensitizers. Representative gene expression profiles are compared between HaCaT (H), SkinEthic RHE (S), and MatTek EpiDerm (M) following exposure to several concentrations of two known sensitizers in the weak potency category. Values represent the mean of replicate tissue samples or wells. NQO1, AKR1C2, IL8, ALDH3A, HMOX1, and GCLC showed the greatest magnitude of response or potency. MaFF and TXN had very low responses and are considered to be the least sensitive of the genes monitored. CYP1A1 expression is controlled by the AhR/Arnt/XRE signaling and is useful for identifying chemicals that may be susceptible to metabolic activation (e.g. prohaptens).

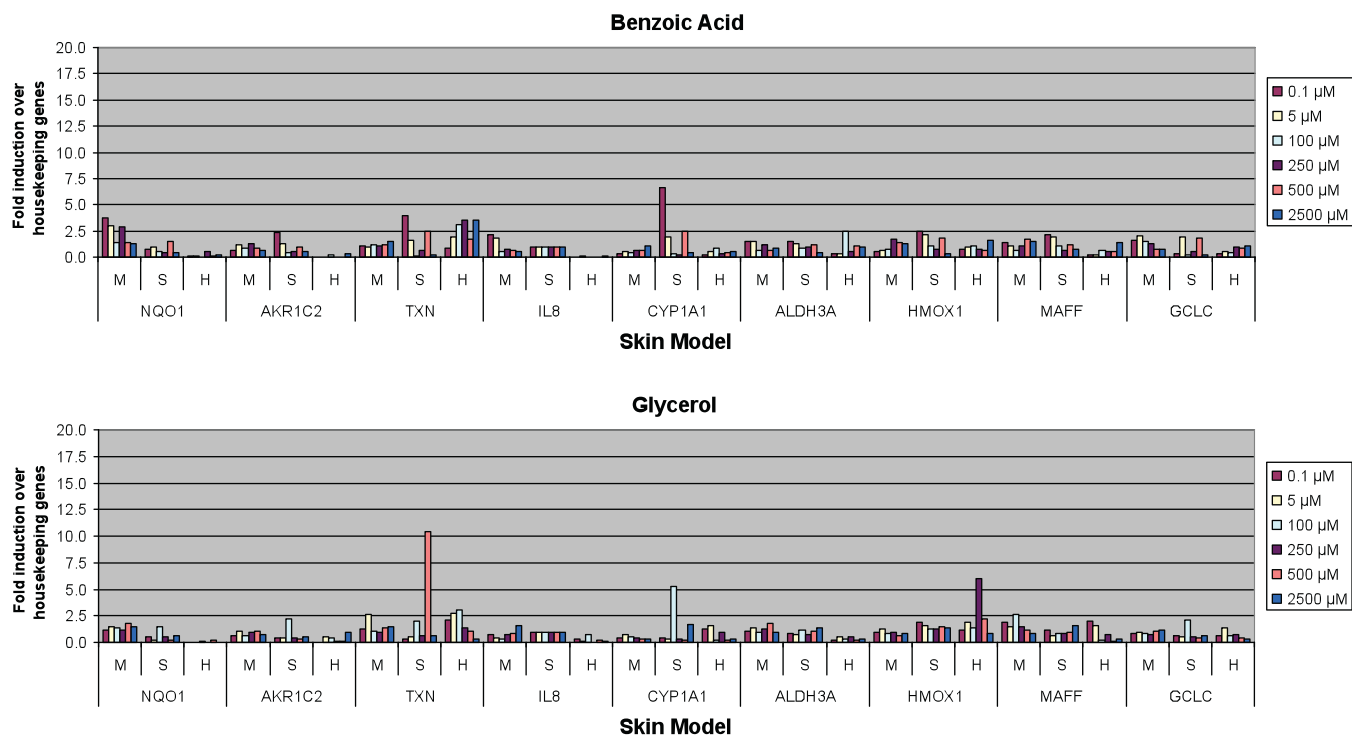


Figure 7. Comparison of Nrf2/ARE and AhR/Arnt/XRE mediated gene expression in Epiderm, RHE, and HaCaT skin models following exposure to non-sensitizers. Representative gene expression profiles are compared between HaCaT (H), SkinEthic RHE (S), and MatTek EpiDerm (M) following exposure to several concentrations of two non sensitizers. Values represent the mean of replicate tissue samples or wells. NQO1, AKR1C2, IL8, ALDH3A, HMOX1, and GCLC showed the greatest magnitude of response or potency. MaFF and TXN had very low responses are considered to be the least sensitive of the genes monitored. CYP1A1 expression is controlled by the AhR/Arnt/XRE signaling and is useful for identifying chemicals that may be susceptible to metabolic activation (e.g. prohaptens).

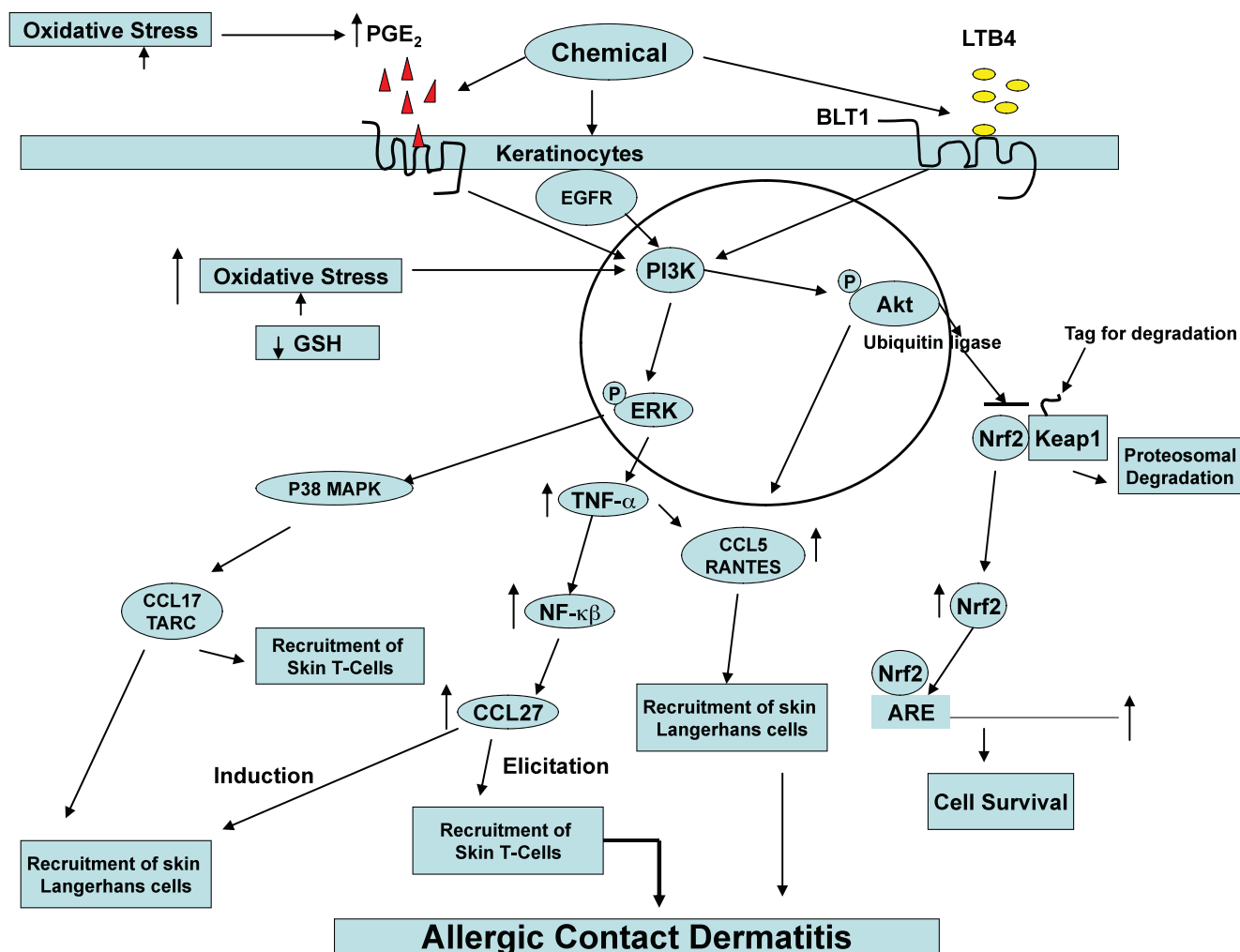


Figure 8. Proposed signaling pathways linking chemical reactivity and oxidative stress, to activation of ACD and Nrf2/Keap1/ARE signaling pathways. A diagrammatic representation depicting a putative link between PI3K activation and activation of signaling pathways that lead to allergic contact dermatitis (ACD) and to activation of the Nrf2/Keap1/ARE pathway. This links the cellular defense mechanism with development of ACD in a mechanistic manner and explains in part why changes in genes controlled by Nrf2/Keap1/ARE are good predictors of chemical sensitization.

as measured by the DCFDA assay. We hypothesize that this could be due to the shipments being exposed to high levels of X-rays during transit (border inspections). X-ray exposure was confirmed by placing detection films in the shipping cartons (data not shown). The *in vitro* sensitization assay presented in this work keys off of signaling pathways that are controlled by oxidative stress; therefore, a high level of oxidative stress could alter the dynamic range and sensitivity of these tissues when attempting to use this method. These findings underscore the importance of characterizing the cellular and biochemical properties of any cellular system that is to be used for toxicity testing.

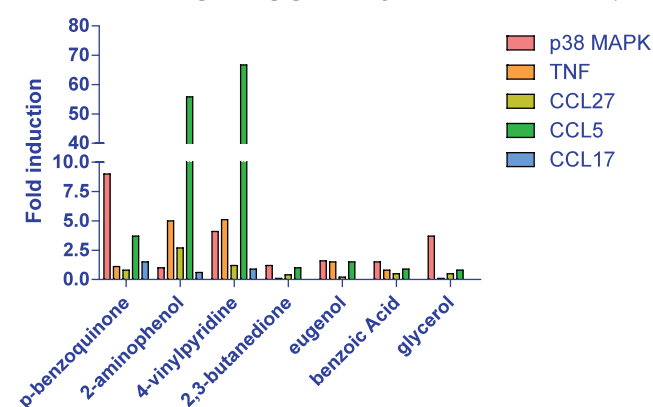
It is possible that the Nrf2/Keap1/ARE signaling pathway used in this study to determine whether or not a chemical is a sensitizer may simply be a measure of reactivity and cellular injury. The upregulation of the ARE controlled genes is considered to be a protective mechanism for cells in response to oxidative stress and to cellular injury. A unifying trigger has not been described

for both induction of ACD and activation of the Nrf2/Keap1 signaling pathway. In Figure 8, a pathway is proposed in which a single cellular event can activate two signaling pathways, one that involves PI3K-Akt (36) with downstream activation of Nrf2 mediated ARE controlled gene expression, and one that involves PI3K-ERK1/2 and initiation of key events required for a full ACD reaction to develop. PI3K is important for the activation of inflammatory cells and for mast cell activation associated with allergens that cause airway hyperresponsiveness and asthma (37–40).

It is known that keratinocytes, when activated by sensitizers, release the chemokines CCL5 (RANTES), CCL17 (TARC), and CCL27 (also known as CTACK). Receptors for these chemokines are present on dendritic cells of the skin and on T-memory cells (41–44). These chemokines are produced by activated keratinocytes and function in the recruitment of Langerhans cells to skin keratinocytes where they pick up and shuttle the haptenized chemical to the local lymph node where it can

to activate T-lymphocytes (induction phase of ACD) making memory cells. Upon reexposure to the chemical allergen, the keratinocytes again produce CCL27 which now recruits the skin homing memory T-cells formed

Induction of signaling genes by sensitizers at 100 μ M



Induction of signaling genes by sensitizers at 250 μ M

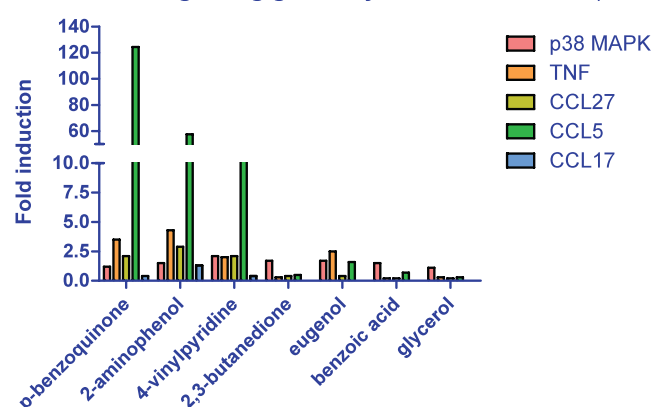


Figure 9. Chemical sensitizers cause changes in the expression of downstream effectors of allergic contact dermatitis. Identifying primary signaling pathways functioning in the EpiDerm® skin model following exposure to known sensitizers. Under these experimental conditions there was a marked increase in the expression of the chemokine CCL5 (RANTES) and TNF-, but not CCL17 (TARC) or CCL27 (CTACK). p-benzoquinone and 4-vinylpyridine showed a modest increase in p38 MAPK. The 2 weak sensitizers (2, 3-butanedione and eugenol) had no measurable effect on gene expression as did the two non-sensitizers Benzoic acid and glycerol. Values represent the mean of three tissues following a 24 h exposure at 100 μ M (Top) and 250 μ M (bottom) concentrations with DMSO (0.5%) as the vehicle.

during the induction phase of skin sensitization. These cells, along with attracted dendritic cells can now produce a magnified response through the release of many inflammatory cytokines (44–46). The signaling pathway proposed in the present study begins with the activation of PI3K followed by activation of Erk1/2 and downstream upregulation of TNF- α , upregulation of CCL5 and/or an increase NF- κ B, which in turn controls CCL27 expression (47). Thus, in addition to CCL27, PI3K-ERK activation can also increase cellular levels of CCL5 (RANTES) via TNF- α and CCL17 (TARC) via p38 MAPK (48–50). All three chemokines (CCL27, CCL17, and CCL5) are known to recruit Langerhans cells and T-cells in skin.

Activation of PI3K can also activate Akt, which through interaction with a Ubiquitin ligase blocks the binding of Nrf2 to Keap1 allowing it to accumulate in the cytosol and translocate to the nucleus, where it binds to ARE and induces cytoprotective genes (36,51–52). Akt can also increase the expression of CCL5 (RANTES) which can lead to ACD. These signaling pathways are known to be activated by oxidative stress, which can occur outside the cell via release of prostaglandins and inside the cell by reduction in cellular levels of glutathione. Evidence for this combined signaling pathway scheme is supported in this study by the observation that TNF- α , CCL5, and Nrf2/Keap1/ARE controlled genes are upregulated by sensitizers, but not by non-sensitizers (Figure 9).

Moreover, previous work has demonstrated that p38 MAPK and ERK1/2 in human dendritic cells are differentially activated by sensitizers, but not by non-sensitizers (53), however, this response was not evaluated in keratinocytes and a unifying mechanism was not proposed.

In this study, the effects of three sensitizers (p-benzoquinone, 2-aminophenol, and 4-vinylpyridine) on the expression of down-stream targets of ERK indicate that the primary pathway in the EpiDerm® human skin model involves TNF- α , CCL5, and to a lesser degree CCL27 (Figures 8 and 9). The activation of these signaling pathways provides a clear link between the development of ACD and upregulation of ARE controlled genes. The important point is that both pathways can be initiated by the same triggering event, namely the activation of PI3K. Induction of the CCL5 pathway can be stimulated by both ERK and Akt mediated events. The relative contribution of the signaling pathways shown in Figure 8,

Table 4. Chemicals used in medical devices.

Compound	Recognized potency category	Predicted potency category	Accuracy
p-Benzoquinone	Extreme	Extreme	C
Tolulene diisocyanate	Strong	Moderate	W
Phthalic anhydride	Strong	Strong	C
2-hydroxyethylacrylate	Moderate	Strong	W
2-Mercaptobenzothiazol	Moderate	Moderate	C
L-Lactic acid	Non-sensitizer	Non-sensitizer	C
Glycerol	Non-sensitizer	Non-sensitizer	C

A group of seven chemicals used in the manufacturing of medical devices were tested in the developed assay. The sensitivity and specificity were 100% when positives included moderate, strong and extreme and negatives included non-sensitizers and weak. The accuracy of the method, or the ability to correctly predict the potency category was 71%. C = correct potency category, W = wrong potency category.

may change based on the chemical class, the time point selected to measure changes and the tissue model. It is possible that the activation of these upstream kinases may provide an improved method for differentiating sensitizers when using the *in vitro* sensitization method described here. Based on previous work discussed above and the experimental data presented here, it is proposed that the activation of PI3K can be linked to both ACD and expression of ARE controlled genes. Ongoing work in our laboratories is focused on closer evaluation of PI3K, Akt, ERK1/2, and cellular levels of CCL5 and CCL27.

It is likely that the most accurate method for the detection and categorization of chemical sensitizers will include an integrated set of test systems that mechanistically link chemical properties, dermal absorption, keratinocyte signaling, and activation of dendritic cells in a panel of assays run in parallel. To this end a skin model encompassing both epidermis and dermis with dendritic cells could provide an even more robust model (54). This integrated approach can also be combined with historical animal data to develop a weight-of-evidence analysis for assessing risk associated with chemical sensitization (55).

The data presented here shows that the *in vitro* sensitization method originally described using HaCaT cells (24) can be improved by using human reconstructed skin grown at an air/liquid interface. The ability to use these models allows the evaluation of test compounds or finished products with low aqueous solubility. The performance of both Epiderm® and RHE skin models related to the detection of sensitizers versus non-sensitizers was high and the ability to place chemicals into potency categories provides added value for risk assessment. In addition, this work has put forth evidence for a unifying cellular event that links Nrf2/ARE gene expression and ACD. In conclusion, the method described should provide a rapid and accurate means to evaluate polar and non-polar medical device extracts as well as chemicals and finished cosmetic products with low solubility for their sensitization potential.

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Declaration of interest

CeeTox is a contract research laboratory that offers SenCeeTox® as a service.

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