

In vitro differentiation of skin sensitizers by cell signaling pathways

L.-M. Koepfer^{a,*}, A. Schulz^b, H.J. Ahr^a, H.-W. Vohr^a

^a Bayer HealthCare AG, Toxicology, Aprather Weg, D-42096 Wuppertal, Germany

^b Florence-Nightingale-Krankenhaus, Kaiserswerther Diakonie, Düsseldorf, Germany

Received 14 August 2007; received in revised form 12 September 2007; accepted 13 September 2007

Available online 22 September 2007

Abstract

Animal testing causes ethical problems and in view of EU regulations (e.g. EU-Guideline (76/768/EEC, February 2003)) or REACH the development of reliable *in vitro* assays has become even more important. Up to now, we use the modified local lymph node assay (IMDS) for toxicological hazard identification of sensitizing and irritant properties of chemicals in accordance with OECD Guideline 429. In this study, we investigated whether analyses of cell signaling pathways can provide a methodology for the detection of sensitizing compounds *in vitro*. Murine and human skin explants as well as reconstituted skin models (epidermal model EST-1000 and full-thickness model AST-2000) were exposed to sensitizing (oxazolone and DNFB) or irritant compounds (SDS and TritonX-100). Phosphorylation of MAP-kinases (p38, ERK1/2 and JNK1/2), STAT1 and PLC γ were determined by cytometric bead array (CBA). In skin explants, all three MAP-kinases were exclusively activated after exposure to sensitizing compounds. For the reconstituted skin models phosphorylations of p38 and JNK1/2 were obtained after stimulation with allergens, whereas treatments with irritant compounds led to ERK1/2 activation. Activation of PLC γ and STAT1 were never detected. In conclusion, MAP-kinase activation provides a promising *in vitro* tool for the discrimination between sensitizers and irritants.

© 2007 Elsevier Ireland Ltd. All rights reserved.

Keywords: Skin explants; Reconstituted skin models; Irritant compounds; Sensitizing compounds; p38; ERK; JNK

1. Introduction

Irritant and allergic skin reactions are undesired side effects in the development of drugs and agrochemicals as well as after contact with environmental or industrial chemicals. Although the clinical symptoms like erythema, oedema, itching and pain are similar, the cellular and molecular events in irritant (ICD) and allergic contact dermatitis (ACD) are different. ICD is defined as a non-immunogenic inflammatory skin reaction, whereas ACD is a specific immunogenic reaction causing the activation of antigen-specific T cells. Up to now, different guinea pig assays (guinea pig maximization test (GPMT), Buehler) or mice tests like the modified lymph node assay (integrated model for the differentiation of skin reactions (IMDS); Vohr et al., 2000; Ehling et al.,

Abbreviations: ACD, allergic contact dermatitis; CBA, cytometric bead array; DI, differentiation index; DNFB, 1-fluoro-2,4-dinitrobenzene; EGFR, epidermal growth factor receptor; ERK, extracellular signal regulated kinase; GPMT, guinea pig maximization test; ICD, irritant contact dermatitis; IMDS, integrated model for the differentiation of skin reactions; IP-10, IFN- γ inducible protein 10; JNK, c-jun NH₂ terminal kinase; LOEL, lowest observed effect level; MAP-kinase, mitogen-activated protein kinase; MCP-1, monocyte chemoattractant protein; PLC, phospholipase C; RANTES, regulated upon activation, normal T cell expressed, and secreted; REACH, registration, evaluation, authorisation and restriction of chemicals; SDS, sodium dodecyl sulfate; STAT, signal transducer and activator of transcription; TGF- α , transforming growth factor α ; TNF- α , tumor necrosis factor α .

* Corresponding author. Fax: +49 202 364137.

E-mail address: lydia-mareen.koepfer@bayerbbs.com (L.-M. Koepfer).

2005a,b) are used as *in vivo* tests for the toxicological hazard identification for ICD and ACD of chemicals and drugs in accordance with OECD Guidelines 406 or 429.

Animal testing causes increasing ethical problems and the predictability of results to the human situation is sometimes questionable. This led to the EU-guideline (76/768/EEC, February 2003), which prescribes a marketing ban for cosmetic ingredients tested for acute toxicity in animals as of 2009. In contrast, the resolution of the REACH-program (registration, evaluation, authorisation and restriction of chemicals) will increase the number of animals needed since approximately 30,000 chemicals have to be evaluated. Although this will be done based on all existing data there will a fair quantity of data gaps be left which require toxicological investigations. To reduce animal testing, several attempts have been made to develop *in vitro* assays. An *in vitro* skin corrosion test based on three-dimensional reconstituted skin models has recently been validated and accepted by regulatory agencies (OECD Guideline 431).

With respect to local immune responses, attempts were mainly based on the expression of certain cytokines, chemokines or surface markers using cultured skin cells, dendritic cells, organotypic skin explants or reconstituted skin models. Nonetheless, a promising protocol for the initiation of validation studies for this end point is still missing. Main problems arose from difficulties in discriminating between sensitizing and irritant compounds *in vitro*, since both result in the generation of a general alarm-signal mediated by cytokines and chemokines (Spiekstra et al., 2005). In addition, other authors did show that surface marker or gene expression pattern were also not able to discriminate both endpoints by *in vitro* experiments (Heisler et al., 2001, 2003). Regarding dendritic cells, it is well known that activation and expression of specific surface markers *in vitro* is not only caused by sensitizing compounds, but also stress, danger signals and skin irritants (Straube et al., 2005; McFadden and Basketter, 2000; McLellan et al., 2000; Sauter et al., 2000).

In this study, a new attempt is presented by analyses of cell signaling. Pathways were chosen that have a known function in transducing immune responses. The mitogen-activated protein kinases (MAPK) p38 and ERK (extracellular signal regulated kinase) are involved in regulating cytokine-expression (Takanami-Ohnishi et al., 2002; Pastore et al., 2005), whereas JNK (c-jun NH₂ terminal kinase) regulates the differentiation of keratinocytes after skin barrier disruption (Gazel et al., 2006). Signal transducers and activa-

tors of transcription (STAT) are the main transducers of cytokine-signaling (Liu et al., 1998) and phospholipase C γ (PLC γ) is known to be activated through certain cytokines in dendritic cells (Kang et al., 2004). For the induction of a local immune reaction an intact skin barrier plays a key role, since compounds need to penetrate this natural barrier before reaching living immune competent cells. To mimic this situation best, human and murine skin explants were chosen as well as the reconstituted skin models EST-1000 and AST-2000 (CellSystems, St. Katharinen, Germany) for our investigations.

2. Materials and methods

2.1. Animals

For the IMDS 8–12-week-old female NMRI mice (Winkelmann, Borken, Germany) were used after at least 5 days of acclimatisation. For the murine skin explants 9–10-week-old female hairless C3H/TifBom hr/hr mice were purchased from Taconic M&B A/S (Skensved, Denmark). The animals were maintained on a freely available standard diet and water provided *ad libitum*. They were used throughout our study in accordance to the German animal protection legislation.

2.2. Chemicals

DNFB (1-fluoro-2,4-dinitrobenzene), oxazolone (4-ethoxymethylene-2-phenyl-2-oxazolin-5-one), SDS (sodium dodecyl sulfate) and TritonX-100 were purchased from Sigma Chemicals (Diesenhofen, Germany). For dermal application performing the IMDS, the test compounds were dissolved in a mixture of dimethylacetamide (40%), acetone (30%) and ethanol (30%) (DAE 433), as described by Maurer et al. (1980). Vehicle compounds were obtained from Merck (Darmstadt, Germany) and Sigma Chemicals. For the cell signaling analysis the chemicals were dissolved in either PBS or 1,2-propandiol (PG), purchased from Invitrogen (Gibco, Karlsruhe, Germany) and Sigma Chemicals.

2.3. The modified lymph node assay, the IMDS

2.3.1. Treatment

Six mice per group were treated on the dorsal sides of both ears with 25 μ l/ear of the test solutions on three consecutive days. The necropsy was performed on day 4, approximately 24 h after the last treatment.

2.3.2. Preparation of lymph nodes and skin

Lateral pairs of lymph nodes (LN) draining the ear tissue were excised, carefully separated from the remaining fatty tissue and weighed on analytical balances as described previously (Vohr et al., 1994; Homey et al., 1998; Vohr et al., 2000). LN were stored on ice in PBS and subjected to the preparation of

single cell suspensions by mechanical tissue disaggregation. Cell suspensions were measured by conductometry (Coulter cell counter Z 1, Coulter Electronics, Krefeld, Germany) gating on a particle diameter above 4.34 μm .

2.3.3. Ear thickness and weight

Ear swelling was determined at the helical edge of the ears using an Oditest micrometer (Dyer, Lancaster, USA). Measurement was carried out before the first treatment and immediately before sacrificing the mice. As an additional parameter for assessing the irritant potential of the chemicals used, an ear punch of 8 mm in diameter was made and weight measured immediately on analytical balances.

2.3.4. Calculation of the IMDS-differentiation-index

The underlying principles of the IMDS are described elsewhere (Homey et al., 1998). For skin inflammation, the increase in ear thickness was measured and for the activation of lymph nodes the increase in LN cell counts index. The maximal increase in ear thickness was defined as $x = 15 \text{ mm} \times 0.01 \text{ mm}$ and the maximal increase in LN cell counts index as $x = 5$. For each experiment the percentages of the defined maximal values were calculated. The differentiation index (DI) is calculated as the relationship between the activation of skin draining lymph nodes (percent of maximal increase in LN cell counts index) and the skin inflammation (percent of maximal increase in ear thickness).

2.3.5. Statistical analysis

The increase in ear thickness is expressed as the mean difference between day 1 and day 4 $\pm$ S.D., and between control and treated animals at day 4. The LN proliferation is expressed as the LN cell count index $\pm$ S.D., calculated as described previously (Homey et al., 1998; Vohr et al., 2000). Statistical significance of differences between the groups was analyzed on the basis of individual data of each mouse using the two-tailed Student's *t*-test. *p*-Values less than 0.05 were considered to be significant.

2.4. Analysis of cell signaling

2.4.1. Human and murine skin explants

Human samples were obtained from skin discarded during cosmetic surgery of the breast. Patients were informed about planned *in vitro* experiments and consented to use their skin explants for these. Murine samples were prepared from the back skin of hairless C3H/TifBom hr/hr mice. Fatty tissue, blood vessels and parts of the dermis were removed. The skin was cut into pieces of approximately 1 cm^2 . The samples were cultured at 37 °C, 5% CO_2 and 95% humidity on 74 μm mesh inserts at the air–liquid interface of keratinocyte basal medium (KBM) supplemented with insulin (5 mg/ml), rhEGF (0.1 U/ml), hydrocortisone (0.5 mg/ml) and GA-1000 (Cambrex, Karlskoga, Sweden). The skin explants were treated after acclimatization overnight. Experiments were performed using five determinations for each concentration and sample.

2.4.2. Reconstituted skin models

The epidermal skin models EST-1000 and AST-2000 were cultured as described by the manufacturer (CellSystems, St. Katharinen, Germany). With respect to the EST-1000, an acclimatization period of 2 h was kept, while the AST-2000 was used after acclimatization overnight. Experiments on reconstituted skin models were performed in triplicates.

2.4.3. Exposure to irritant and sensitizing compounds

The compounds were titrated on all four-skin models to determine the concentrations of the lowest observed effect level (LOEL). The LOEL was defined as the concentration resulting in a viability of about 90% after 24 h of exposure. The final concentrations used for the experiments are shown in Table 1.

Before topical application of test compounds the inserts containing the skin models were placed in fresh medium. Using skin explants a nylon mesh was put on the stratum corneum before application of 20 μl of the compound. With respect to reconstituted skin models, the meshes were placed after application of the test compound. For the EST-1000 50 μl were used and for the AST-2000 12 μl . The skin models were incubated at 37 °C, 5% CO_2 and 95% humidity for 1 h or 3 h. Experiments on skin explants were performed using five determinations for each concentration and sample whereas those on reconstituted skin models were performed in triplicates.

2.4.4. Lysis of skin models

Immediately after the end of the incubation period the meshes were removed and the compound rinsed off the skin using PBS. The reconstituted skin models were unhinged from the inserts. All samples were shock-frozen in liquid nitrogen and stored at -80°C until further preparation. The human skin explants and the AST-2000 were cryo-cut, whereas for the EST-1000 and the murine skin explants no further preparation was necessary. The samples were put on a bead mill (Retsch, Haan, Germany) for 90 s at 30 Hz using 5 mm stainless steel beads. Proteins were then denatured for 5 min at 95 °C using 1 $\times$ denaturation buffer (Cell Signaling Master Buffer Kit, BD Biosciences, Heidelberg, Germany). Cellular debris was removed by centrifugation and the samples were stored at -80°C for further analysis.

Table 1
Determination of lowest observed effect level (LOEL)

Compound	Skin explants		Reconstituted skin models	
	Murine	Human	EST-1000	AST-2000
SDS	2.43 mM	2.43 mM	0.69 mM	2.43 mM
TritonX-100	1.60 mM	16.03 μM	0.16 mM	16.03 μM
Oxazolone	23.02 mM	3.22 mM	6.91 mM	3.22 mM
DNFB	0.16 mM	0.54 mM	0.54 mM	0.54 mM

Shown are the concentrations resulting in a viability of about 90% after 24 h of exposure.

2.4.5. Bradford-assay

The total protein concentration of each sample was determined according to the manufacturer's instruction using the Coomassie Protein Assay Kit (Pierce, Perbio Science, Bonn, Germany). A 1:5 dilution of the samples was necessary because of the high SDS concentration of the denaturation buffer.

2.4.6. Cytometric bead array

The samples were analyzed using CBA Cell Signaling Flex Sets (BD Biosciences, Heidelberg, Germany) for phospho-p38 (T180/Y182), phospho-ERK1/2 (T202/Y204), phospho-JNK1/2 (T183/Y185), phospho-STAT1 (Y701) and phospho-PLC γ (Y783). The samples and standards were prepared according to the manufacturer's instructions. Phosphoproteins were measured by flow cytometry (FACSCanto, BD Biosciences). Quantification of samples was performed using FCAP Array Software (Soft Flow Inc., USA).

2.4.7. Analysis

The obtained phosphoprotein concentrations for each sample were normalized to a total protein concentration of 4 mg/ml for murine skin explants and 3 mg/ml for human skin explants and reconstituted skin models. Fold inductions referred to the vehicle-treated controls were calculated. The statistical analysis was performed using the two-tailed Student's *t*-test.

3. Results

3.1. The modified lymph node assay, the IMDS

The modified local lymph node assay (LLNA), the IMDS (integrated model for the differentiation of skin reactions) is used as an *in vivo* assay for the classification of the skin irritant or sensitizing potential of compounds. As shown in Fig. 1, the two sensitizing compounds oxazolone and DNFB resulted in differentiation indices (DI) of 5.5 and 8.7. $DI > 1$ classify the compound tested as a skin sensitizer, whereas a $DI > 0$ and ≤ 1 classifies a compound as irritant. Thus, oxazolone and DNFB

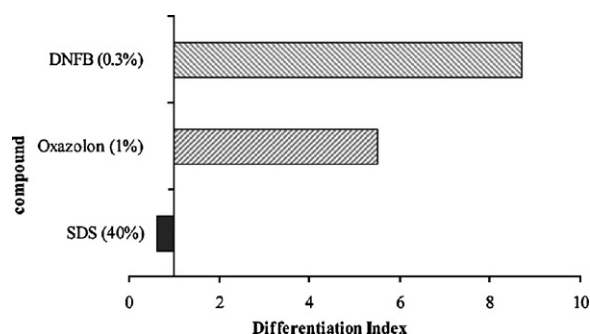


Fig. 1. Differentiation indices (DI) of the IMDS. A $DI > 0$ and ≤ 1 classifies a compound as skin irritant, a $DI > 1$ classifies a compound as skin sensitizer.

were indicated as sensitizers. For SDS a DI of 0.63 was calculated, classifying the compound as irritant. The data shown here originate from earlier experiments. In view of animal welfare we abandoned performing an animal test for TritonX-100 at this stage. As already mentioned before several parameters able to discriminate irritant and sensitizing reactions *in vivo* failed to work *in vitro*. Therefore, the findings by Takanami-Ohnishi et al. (2002) and Pastore et al. (2005) regarding signal transduction opened a new field for *in vitro* analysis.

3.2. Analysis of cell signaling pathways *in vitro*

To investigate the intracellular signaling pathways of skin models (murine and human skin explants, reconstituted skin models EST-1000 and AST-2000) after exposure to irritant and sensitizing compounds cytometric bead arrays (CBA) were performed. Analyzed were the phosphorylated forms of the MAP-kinases p38, ERK1/2, JNK1/2 as well as PLC γ and STAT1. The irritant compounds SDS and TritonX-100 and the skin sensitizers oxazolone and DNFB were used at the lowest observed effect level (LOEL), which was defined as the concentration resulting in a viability of about 90% after 24 h of exposure. This is a usual procedure to compare *in vitro* effects of compounds with different cytotoxic potential. The rationale for this is that the non-specific effect should be comparable between the test items while specific reactions can easily be separated. To reach the goal of about 90% viability for all models with all compounds tested it is absolutely clear that different concentrations have to be compared in each case.

3.3. Murine and human skin explants

Regarding the murine skin explant, activation of cell signaling proteins was generally detected after exposures of 3 h, while 1-h treatments rarely resulted in pronounced phosphorylation patterns. Fig. 2 shows the analysis of JNK1/2 activation as an example. As can be taken from the figure only oxazolone induced a significant phosphorylation (1.45-fold induction) during a short exposure period of 1 h. Incubation periods of 3 h lead, however, to statistically significant increases ($p < 0.05$) in JNK1/2-activation by both skin sensitizing compounds, i.e. oxazolone and DNFB (1.77- and 1.63-fold). No such effects were determined after application of irritant compounds (SDS and TritonX-100).

Regarding human skin explants, activation of cell signaling proteins was generally detectable after 1 h of exposure, with the exception of PLC γ (3 h exposure). Phospho-p38 (Fig. 3) was clearly induced after 1 h treat-

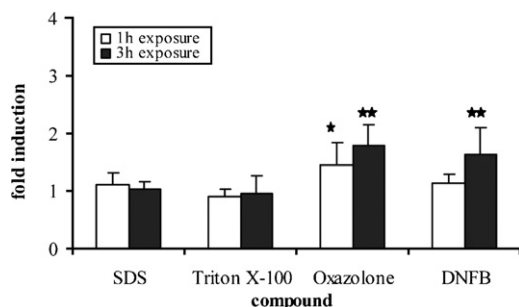


Fig. 2. Induction of phospho-JNK1/2 in murine skin explants after exposure to irritant and sensitizing compounds. Results are presented as mean fold induction $\pm$ S.D. referred to the vehicle-treated controls ($n=5$ per compound/vehicle). * $p<0.1$ vs. vehicle-treated control, ** $p<0.05$ vs. vehicle-treated control.

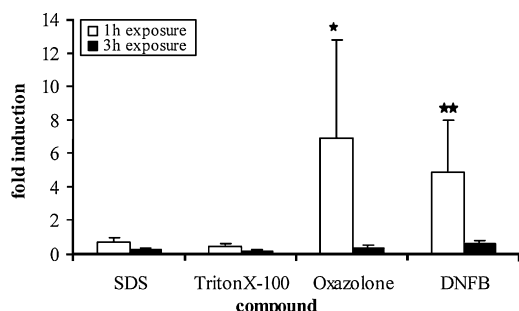


Fig. 3. Induction of phospho-p38 in human skin explants after exposure to irritant and sensitizing compounds. Results are presented as mean fold induction $\pm$ S.D. referred to the vehicle-treated controls ($n=5$ per compound/vehicle). * $p<0.1$ vs. vehicle-treated control, ** $p<0.05$ vs. vehicle-treated control.

ment of human skin explants with the two sensitizing compounds (induction 6.88- and 4.91-fold), while the two irritant compounds did not lead to any activation. After the 3 h exposure period induction level was back at the starting point.

As can be taken from the information in Table 2, the treatment of skin explants from both species lead to similar activation pattern of cell signaling proteins, although maximum increases were detected at different time points. Exposure to the sensitizing compounds oxazolone and DNFB resulted in the activation of the three MAP-kinases tested, whereas the irritant compounds SDS and TritonX-100 did not induce any phosphorylation of these enzymes. For p38 and JNK1/2 significant and reproducible activation were obtained exclusively for the sensitizing compounds. ERK1/2 phosphorylation was also detected after exposure of the explants to sensitizing compounds, although for the murine skin explant results could not be reproduced. Activation of STAT1 has neither been induced by sensitizing nor irritant compounds, and no activation of PLC γ was induced in murine skin explants. In human skin explants a faint phosphorylation of PLC γ was determined after a 3-h treatment period specific for the sensitizing compounds oxazolone and DNFB.

3.4. AST-2000

The AST-2000 is a full-thickness skin model, consisting of a reconstructed epidermis with a supporting dermis equivalent underneath. Regarding p38, treatment of the AST-2000 with the irritant compounds SDS and TritonX-100 did not result in any activation compared to the vehicle-treated control (Fig. 4). Stimulation with the sensitizer oxazolone lead to induction after 1 h and 3 h (1.46- and 2.06-fold), which were, however, of no statistical significance. Exposure to the sensitizer DNFB resulted in 3.86- and 3.54-fold increases in phosphorylation, which became statistically significant after the longer exposure period.

Table 2

Induction of phosphorylation in murine and human skin explants after exposure to irritant and sensitizing compounds

Compound		Induction of phosphorylation				
		p38	ERK1/2	JNK1/2	PLC γ	STAT1
Murine skin explant						
Skin irritant	SDS	0.87 (–)	1.24 (±)	1.10 (–)	1.13 (–)	1.06 (–)
	TritonX-100	1.08 (±)	0.99 (–)	0.99 (–)	0.95 (–)	1.02 (–)
Skin sensitizer	Oxazolone	6.01 (+++)	1.94 (++)	1.87 (++)	1.04 (–)	0.86 (–)
	DNFB	2.55 (+++)	1.16 (±)	1.40 (+)	1.12 (–)	0.97 (–)
Human skin explant						
Skin irritant	SDS	0.64 (–)	0.91 (–)	0.81 (–)	0.91 (–)	0.74 (–)
	TritonX-100	0.40 (–)	0.85 (–)	0.89 (–)	0.88 (–)	0.89 (–)
Skin sensitizer	Oxazolone	4.02 (+++)	1.44 (+)	1.45 (+)	1.14 (±)	0.56 (–)
	DNFB	3.36 (+++)	1.40 (+)	1.62 (+)	1.23 (±)	0.66 (–)

Results are presented as mean fold induction from two independent experiments. Shown in brackets is the classification of mean fold induction; –: no induction detectable; +: induction of phosphorylation; ±: no or low induction.

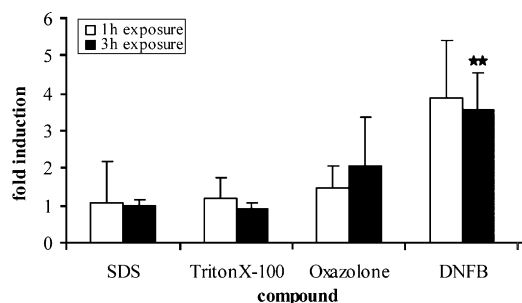


Fig. 4. Induction of phospho-p38 in AST-2000 after exposure to irritant and sensitizing compounds. Results are presented as mean fold induction $\pm$ S.D. referred to the vehicle-treated controls ($n=3$ per compound/vehicle). * $p < 0.1$ vs. vehicle-treated control, ** $p < 0.05$ vs. vehicle-treated control. Measurements of samples treated for 1 h and 3 h were performed using different CBA batches.

An overview showing all phosphoproteins analyzed is given in Table 3. Although not always statistically significant, p38 phosphorylation after treatment with oxazolone was reproducibly induced, as well as the activation of other proteins analyzed. In comparison to skin explants, the results obtained for p38 and JNK were similar, indicating an activation specific for the exposure to the sensitizing compounds. In contrast to this, a phosphorylation of ERK1/2 was detected after 1 h exposure to the irritant compounds.

3.5. EST-1000

Treatment of the EST-1000 resulted in significant inductions of p38 phosphorylation (Fig. 5), which were more pronounced after application of skin sensitizing compounds. After 1 h exposure, statistically significant induction by oxazolone and DNFB (2.48- and 2.05-fold; $p < 0.05$) were measured. A slight induction was also detected by the irritant compound SDS (1.70-fold; $p < 0.1$). However, a longer exposure period of 3 h resulted in an exclusive p38-activation specific for the sensitizing compounds oxazolone and DNFB (2.63- and 9.16-fold).

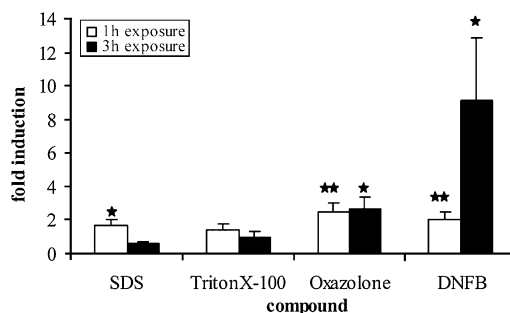


Fig. 5. Induction of phospho-p38 in EST-1000 after exposure to irritant and sensitizing compounds. Results are presented as mean fold induction $\pm$ S.D. referred to the vehicle-treated controls ($n=3$ per compound/vehicle). * $p < 0.1$ vs. vehicle-treated control, ** $p < 0.05$ vs. vehicle-treated control.

The overview given in Table 4 shows that reproducible results were obtained for p38 activation specific for the sensitizing compounds oxazolone and DNFB. Treatment of EST-1000 for 1 h induced phosphorylation of ERK1/2 only when using the irritant compounds SDS or TritonX-100. JNK activation was obtained for the sensitizing compounds in one experiment after 3 h exposure, but the results were difficult to reproduce since JNK-concentrations in EST-1000 were mainly at the detection threshold. Like the results for the AST-2000, STAT1 and PLC γ were not phosphorylated using either class of compounds.

3.6. Comparison

Since similar results were obtained for the murine and human skin explants as well as for the reconstituted skin models AST-2000 and EST-1000, an overview combining the results of the two classes of skin models is given in Table 5. A p38 activation was specifically detected after exposure to sensitizing compounds in both the skin explants and the reconstituted skin models. In addition phosphorylation of JNK1/2 was only induced by sensitizers, but not as strong as p38, and in case of the reconstituted skin models the values were

Table 3
Induction of phosphorylation in AST-2000 after exposure to irritant and sensitizing compounds

	Compound	Induction of phosphorylation				
		p38	ERK1/2	JNK1/2	PLC γ	STAT1
Skin irritant	SDS	$\pm$	+	—	—	—
	TritonX-100	$\pm$	+	—	—	—
Skin sensitizer	Oxazolone	++	—	+	—	—
	DNFB	+++	—	++	—	—

Classification bases on mean fold induction as shown in Table 2; —: no induction detectable; +: induction of phosphorylation; $\pm$: no or low induction.

Table 4

Induction of phosphorylation in EST-1000 after exposure to irritant and sensitizing compounds

Compound		Induction of phosphorylation				
		p38	ERK1/2	JNK1/2	PLC γ	STAT1
Skin irritant	SDS	±	++	–	–	–
	TritonX-100	±	++	–	–	–
Skin sensitizer	Oxazolone	+++	–	+	–	–
	DNFB	+++	–	+	–	–

Classification bases on mean fold induction as shown in Table 2; –: no induction detectable; +: induction of phosphorylation; ±: no or low induction.

Table 5

Comparison of protein phosphorylation in skin explants and reconstituted skin models

Compound	Phospho-p38		Phospho-ERK1/2		Phospho-JNK1/2	
	Sensitizing	Irritant	Sensitizing	Irritant	Sensitizing	Irritant
Skin explants (murine and human)	+++	–	+	–	+	–
Reconstituted skin models (EST-1000 and AST-2000)	+++	±	–	+	+	–

–: no induction detectable; +: induction of phosphorylation; ±: no or low induction.

just above the detection thresholds. For the activation of ERK1/2 different results were obtained using skin explants or reconstituted skin models. Using the latter, exposures to irritant compounds lead to phosphorylation, while in skin explants sensitizers caused ERK1/2 activation.

4. Discussion

Data shown here reveal that the integrated model for the differentiation of skin reactions (IMDS) correctly predicts the sensitizing and irritant properties of the model compounds SDS, oxazolone and DNFB. Furthermore, they confirm previous results proving that the IMDS is able to discriminate between irritant and sensitizing compounds (Homey et al., 1998; Vohr et al., 2000; Ehling et al., 2005a,b; Vohr and Ahr, 2005). For the toxicological hazard identification of local immune reactions to chemicals or drugs it is very important that irritant compounds do not lead to false-positive results (Montelius et al., 1994; Scholes et al., 1992; Homey et al., 1995; Gerberick et al., 2002). This is of special importance for test systems based on the induction phase (LLNA) without analyzing substance-specific activation by memory cells (CP assays). Therefore, like in the *in vivo* situation, a reliable *in vitro* assay has to discriminate between the sensitizing and irritant properties of compounds, too. Based on the data presented here by analyzing cell signaling pathways this differentiation seems to be possible.

In murine and human skin explants as well as the reconstituted skin models p38 activation was induced specifically after exposure to sensitizing, but not irritant compounds. This result correlates with the findings of a two-phase *in vivo* experiment, where p38 phosphorylation in skin was obtained through treatment with DNFB (Takanami-Ohnishi et al., 2002). Furthermore, the group demonstrated that p38 up-regulates the expression of chemokines and cytokines like IL-1 β , tumor necrosis factor α (TNF- α), IL-18, monocyte chemoattractant protein 1 (MCP-1) and interferon γ inducible protein 10 (IP-10). Investigations using dendritic cells showed that the expression of activation-state specific markers (CD80, CD86 and CD83) depends on p38 activation, which occurred only after treatment with sensitizing compounds (Arrighi et al., 2001). Nevertheless, the main source for phospho-p38 in the skin models used for the experiments presented here seems to be the keratinocytes, since this is the only cell type present in all four skin tissues analyzed here.

The analyses of ERK1/2 phosphorylation led to opposite results for skin explants and reconstituted skin models. In skin explants, phosphorylation was induced by treatment with sensitizing compounds, whereas in reconstituted skin models irritants caused activation. Activated ERK1/2 suppresses inflammatory chemokines like MCP-1, RANTES (regulated upon activation, normal T cell expressed, and secreted) and IP-10 *in vivo* and in cultured keratinocytes after stimulation of epidermal growth factor receptor (EGFR) (Pastore et al.,

2005). Transforming growth factor α (TGF- α) is a ligand for EGFR which is released from keratinocytes after stimulation with pro-inflammatory TNF- α and IFN- γ (Mascia et al., 2003). Since TNF- α is also known to stimulate p38 MAP-kinase (Pearson et al., 2001; Wajant et al., 2003), this confirms the obtained activation of ERK1/2 in skin explants at the same time points as the p38 phosphorylation. One could speculate that complex interactions like the regulation of chemokine expression by ERK1/2 and p38 do not work in reconstituted skin models. The phosphorylation of ERK1/2 in reconstituted skin models after 1 h exposure to irritant compounds was reproducible, but not statistically significant. Nonetheless, the same result might be obtained for skin explants at a different time point due to another kinetic.

For the experiments presented here, JNK1/2 activation was specific for exposure of skin models to sensitizing compounds. In contrast to p38 and ERK, JNK is not active in healthy skin (Takahashi et al., 2002). This explains the low phospho-JNK1/2 concentrations measured in reconstituted skin models since they were not affected by a preparation procedure like the skin explants. Inhibition of JNK leads to differentiation of keratinocytes, i.e. its function is regulatory, too (Gazel et al., 2006). JNK can also be activated through TNF- α (Pearson et al., 2001; Wajant et al., 2003), which again confirms the activation of all three analyzed MAP-kinases in skin explants at the same time points.

Analyses of PLC γ and STAT1 activation did not show differential results for sensitizing or irritant compounds. Regarding dendritic cells, it was previously shown that sensitizing compounds do not activate any of the known STATs (Valk et al., 2002).

Why the three analyzed MAP-kinases are differentially activated by sensitizing but not by irritant compounds remains unclear. One can speculate that either different chemokines and cytokines are released after exposure to sensitizing or irritant compounds or that the release occurs at different time points, leading to a differential kinetic of activation of MAP-kinases at the specific time point of measurement.

It can be stated that the analysis of MAP-kinase activation provides a promising tool for the identification of sensitizing compounds *in vitro*. A differentiation between two irritant and two sensitizing model compounds was possible, but further investigations with more compounds are, of course, necessary for verification. Skin explants, either murine or human, seem to have the best capability for identifying sensitizing compounds

since complex interactions leading to an activation of all three MAP-kinases can be measured. But there are disadvantages using skin explants as well. First, there is a high inter-individual variability in penetration rates of compounds by factor 4 (Williams, 2004) due to variations in stratum corneum thickness (de Jongh et al., 2006) by factor 2–3 (Anderson and Cassidy, 1973). An intra-individual variability between different skin areas was also described by Cua et al. (1990). Second, especially for human skin explants, the availability is limited and patients need to be of equal age, since younger patients were shown to be more susceptible to skin irritation (Cua et al., 1990). With respect to availability and variability, reconstituted skin models would provide the preferred alternative. Using the full-thickness skin model AST-2000, a specific activation of p38 and JNK was obtained after exposure to sensitizing compounds. The epidermal skin model EST-1000 on the other hand showed high induction levels of phospho-p38 specific for exposure to sensitizing compounds, comparable to those found for skin explants.

Acknowledgement

Thanks to Prof. Dr. Jutta Liebau, Florence-Nightingale-Krankenhaus, Kaiserswerther Diakonie, Düsseldorf, Germany, for providing human skin explants from mamma reduction surgeries.

References

- Anderson, R.L., Cassidy, J.M., 1973. Variation in physical dimensions and chemical composition of human stratum corneum. *J. Invest. Dermatol.* 61, 30–32.
- Arrighi, J.F., Rebsamen, M., Rousset, F., Kindler, V., Hauser, C., 2001. A critical role for p38 mitogen-activated protein kinase in the maturation of human blood-derived dendritic cells induced by lipopolysaccharide, TNF- α , and contact sensitizers. *Immunology* 166, 3837–3845.
- Cua, A.B., Wilhelm, K.P., Maibach, H.I., 1990. Cutaneous sodium lauryl sulfate irritation potential: age and regional variability. *Br. J. Dermatol.* 123, 607–613.
- de Jongh, C.M., Jakasa, I., Verberk, M.M., Kezic, S., 2006. Variation in barrier impairment and inflammation of human skin as determined by sodium lauryl sulphate penetration rate. *Br. J. Dermatol.* 154, 651–657.
- Ehling, G., Hecht, M., Heusener, A., Huesler, J., Gamer, A.O., van Loveren, H., Maurer, T., Riecke, K., Ullmann, L., Ulrich, P., Vandebriel, R., Vohr, H.W., 2005a. An European inter-laboratory validation of alternative endpoints of the murine local lymph node assay: first round. *Toxicology* 212, 60–68.
- Ehling, G., Hecht, M., Heusener, A., Huesler, J., Gamer, A.O., van Loveren, H., Maurer, T., Riecke, K., Ullmann, L., Ulrich, P., Vandebriel, R., Vohr, H.W., 2005b. An European inter-laboratory validation of alternative endpoints of the murine local lymph node assay: second round. *Toxicology* 212, 69–79.

- Gazel, A., Banno, T., Walsh, R., Blumenberg, M., 2006. Inhibition of JNK promotes differentiation of epidermal keratinocytes. *J. Biol. Chem.* 281, 20530–20541.
- Gerberick, F.G., Cruse, L.W., Ryan, C.A., Hulette, B.C., Chaney, J.G., Skinner, R.A., Dearman, R.J., Kimber, I., 2002. Use of a B cell marker (B220) to discriminate between allergens and irritants in the local lymph node assay. *Toxicol. Sci.* 68, 420–428.
- Heisler, E., Ahr, H.J., Vohr, H.W., 2001. Induced immune reactions in *in vitro* models for irritation and sensitization. *Immunobiology* 205, 67.
- Heisler, E., Ahr, H.J., Vohr, H.W., 2003. A high sensitive DNA-array method for the analysis of differential gene-expression profiles during early phase of local immune reactions. *Immunobiology* 208, 186.
- Homey, B., Vohr, H.W., Schuppe, H.C., Kind, P., 1995. UV-dependent local lymph node reactions: photoallergy and phototoxicity testing. *Curr. Probl. Dermatol.* 22, 44–50.
- Homey, B., von Schilling, C., Blümel, J., Schuppe, H.C., Ruzicka, T., Ahr, H.J., Lehmann, P., Vohr, H.W., 1998. An integrated model for the differentiation of chemical-induced allergic and irritant skin reactions. *Toxicol. Appl. Pharmacol.* 153, 83–94.
- Kang, H.K., Lee, H.Y., Lee, Y.N., Jo, E.J., Kim, J.I., Kim, G.Y., Park, Y.M., Min, D.S., Yano, A., Kwak, J.Y., Bae, Y.S., 2004. Up-regulation of phospholipase C γ and phospholipase D during the differentiation of human monocytes to dendritic cells. *Int. Immunopharmacol.* 4, 911–920.
- Liu, K.D., Gaffen, S.L., Goldsmith, M.A., 1998. JAK/STAT signaling by cytokine receptors. *Curr. Opin. Immunol.* 10, 271–278.
- Mascia, F., Mariani, V., Girolomoni, G., Pastore, S., 2003. Blockade of the EGF receptor induces a deranged chemokine expression in keratinocytes leading to enhanced skin inflammation. *Am. J. Pathol.* 163, 303–312.
- Maurer, T., Weirich, E.G., Hess, R., 1980. The optimization test in guinea pig in relation to other predictive sensitization methods. *Toxicology* 15, 163–171.
- McFadden, J.P., Basketter, D.A., 2000. Contact allergy, irritancy and ‘danger’. *Contact Dermatitis* 42, 123–127.
- McLellan, A.D., Bocker, E.B., Kampgen, E., 2000. Dendritic cell activation by danger and antigen-specific T-cell signaling. *Exp. Dermatol.* 9, 313–322.
- Montelius, J., Wahlkvist, H., Boman, A., 1994. Experience with the murine local lymph node assay: inability to discriminate between allergens and irritants. *Acta Derm.-Venereol.* 74, 22–27.
- Pastore, S., Mascia, F., Mariotti, F., Dattilo, C., Mariani, V., Girolomoni, G., 2005. ERK1/2 regulates epidermal chemokine expression and skin inflammation. *J. Immunol.* 174, 5047–5056.
- Pearson, G., Robinson, F., Beers Gibson, T., Xu, B.E., Karandikar, M., Berman, K., Cobb, M.H., 2001. Mitogen-activated protein (MAP) kinase pathways: regulation and physiological functions. *Endocr. Rev.* 22, 153–183.
- Sauter, B., Albert, M.L., Francisco, L., Larsson, M., Somersan, S., Bhardwaj, N., 2000. Consequences of cell death: exposure to necrotic tumor cells, but not primary tissue cells or apoptotic cells, induces the maturation of immunostimulatory dendritic cells. *J. Exp. Med.* 191, 423–434.
- Scholes, E.W., Basketter, D.A., Lovell, W.W., Sarll, A.E., Pendlington, R.U., 1992. The identification of photoallergic potential in the local lymph node assay. *Photodermatol. Photoimmunol. Photomed.* 8, 249–254.
- Spiekstra, S.W., Toebak, M.J., Sampat-Sardjoepersad, S., van Beek, P.J., Boorsma, D.M., Stoof, T.J., von Blomberg, B.M.E., Scheper, R.J., Bruynzeel, D.P., Rustemeyer, T., Gibbs, S., 2005. Induction of cytokine (interleukin-1 α and tumor necrosis factor- α and chemokine (CCL20, CCL27, and CXCL8) alarm signals after allergen and irritant exposure. *Exp. Dermatol.* 14, 109–116.
- Straube, F., Grenet, O., Bruegger, P., Ulrich, P., 2005. Contact allergens and irritants show discrete differences in the activation of human monocyte-derived dendritic cells: consequences for *in vitro* detection of allergens. *Arch. Toxicol.* 79, 37–46.
- Takahashi, H., Ibe, M., Nakamura, S., Ishida-Yamamoto, A., Hashimoto, Y., Iizuka, H., 2002. Extracellular regulated kinase and c-Jun N-terminal kinase are activated in psoriatic involved epidermis. *J. Dermatol. Sci.* 30, 94–99.
- Takanami-Ohnishi, Y., Amano, S., Kimura, S., Asada, S., Utani, A., Maruyama, M., Osada, H., Tsunoda, H., Irukayama-Tomobe, Y., Goto, K., Karin, M., Sudo, T., Kasuya, Y., 2002. Essential role of p38 mitogen-activated protein kinase in contact hypersensitivity. *J. Biol. Chem.* 277, 37896–37903.
- Valk, E., Zahn, S., Knop, J., Becker, D., 2002. JAK/STAT pathways are not involved in the direct activation of antigen-presenting cells by contact sensitizers. *Arch. Dermatol. Res.* 294, 163–167.
- Vohr, H.W., Homey, B., Schuppe, H.C., Kind, P., 1994. Detection of photoreactivity demonstrated in a modified local lymph node assay in mice. *Photodermatol. Photoimmunol. Photomed.* 10, 57–64.
- Vohr, H.W., Ahr, H.J., 2005. The local lymph node assay being too sensitive? *Arch. Toxicol.* 79, 721–728.
- Vohr, H.W., Blümel, J., Blotz, A., Homey, B., Ahr, H.J., 2000. An intra-laboratory validation of the integrated model for the differentiation of skin reactions (IMDS): discrimination between (photo) allergic and (photo) irritant skin reactions in mice. *Arch. Toxicol.* 73, 501–509.
- Wajant, H., Pfizenmaier, K., Scheurich, P., 2003. Tumor necrosis factor signaling. *Cell Death Differ.* 10, 45–65.
- Williams, F.M., 2004. Evaluations and predictions of dermal absorption of toxic chemicals. *Int. Arch. Occup. Environ. Health* 77, 150–151.