In vitro Differentiation of Skin Sensitizers by Cell Signaling Pathways

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Introduction

Animal testing causes ethical problems and in view of EU regulations (e.g. EU-Guideline (76/768/EEC, Feb. 2003)) and REACH the development of *in vitro* assays has become even more important. In this study, we investigated whether analyses of cell signaling pathways can provide a methodology for the detection of sensitizing compounds *in vitro*. For this purpose a differentiation between unspecific immune reactions (skin irritation) and skin sensitization was of major importance. Pathways were chosen that have a known function in transducing immune responses, which were the MAP-kinases p38, ERK1/2 and JNK1/2 as well as STAT1 and PLCγ. For the induction of a local immune reaction an intact skin barrier plays a key role, since compounds need to be able to penetrate this natural barrier before reaching living immune competent cells. To mimic this situation best, human and murine skin explants were chosen and compared with the reconstituted skin models EST-1000 and AST-2000 (CellSystems, St. Katharinen, Germany) for our investigations.

Materials and Methods

Animals: Hairless mice C3H/TifBom-hr, female, 10-12 weeks old, Taconic M+B, Skensved, Denmark

Chemicals: SDS (sodium dodecyl sulfate), TritonX-100, Oxazolone (4-ethoxy-methylene-2-phenyl-oxazoline-5-one), DNCB (1-chloro-2,4-dinitrobenzene), DNFB (1-fluoro-2,4-dinitro-benzene), Sigma Chemicals (Deisenhofen, Germany). Solvents: PBS (phosphate buffered saline), Gibco (Karlsruhe, Germany) or PG (1,2-propandiol), Sigma Chemicals

***In vitro* reconstituted skin models:** Full-thickness skin model AST-2000 (Advanced Skin Test) and epidermal skin model EST-1000 (Epidermal Skin Test); CellSystems®, St. Katharinen, Germany

Skin explants: Murine skin explants were prepared from the back skin of female hairless C3H/TifBom-hr mice. Human skin explants were obtained from mamma reduction surgeries on female patients younger than 44 years at the Florence-Nightingale-Karankenhaus, Kaiserswerther Diakonie, Düsseldorf, Germany

Culture conditions and skin explantation: AST-2000 and EST-1000 were cultured as described by the manufacturer (CellSystems®, St. Katharinen). Skin explants were cultured on mesh inserts at the air-liquid-interface after removal of fatty and subdermal tissue. All skin models were incubated at 37°C, 5% CO_2 and 95% humidity.

Exposure to irritant and sensitizing compounds: The compounds were titrated on all four skin models using the MTT viability test to determine the concentrations of the lowest observed effect level (LOEL). The LOEL was defined as the concentration resulting in a decrease in viability of about 10% after 24h of exposure. The skin models were exposed to the appropriate concentrations of compounds for 1h or 3h at 37°C, 5% CO_2 and 95% humidity.

Lysis of skin models: Immediately after the end of the incubation period the samples were shock-frozen in liquid nitrogen and stored at -80°C. The human skin explants and and the AST-2000 were cryo-cut, whereas for the EST-1000 and 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 denatured for 5 min at 95°C using 1x denaturation buffer (BD Biosciences, Heidelberg, Germany). Cellular debris was removed by centrifugation and the samples were stored at -80°C for further analysis.

Bradford assay: Total protein concentration of each sample was determined according to the manufacturer‘s instruction using the Coomassie Assay Kit (Pierce, Perbio Science, Bonn, Germany).

Cytometric bead array: Samples were analyzed using the following CBA Cell Signaling Flex Sets (BD Biosciences, Heidelberg, Germany): phospho-p38 (T180/Y182), phospho-ERK1/2 (T202/Y204), phospho-JNK (T183/Y185), phospho-STAT1 (Y701) and phospho-PLCγ (Y783). Samples and standards were prepared according to the manufcturer‘s instructions. Phosphoproteins were measured by flowcytometry (FACSCanto, BD Biosciences). Quantification of samples was performed using the FCAP Array Software (Soft Flow Inc., USA).

Analysis: The measured phosphoprotein concentrations for each sample were normalized to a total protein concentration of 3 mg/ml for the skin explants and the reconstituted skin models. Statistical analysis was performed using the two-tailed student‘s t-test.

Results

Determination of lowest observed effect levels

- both irritant and sensitizing compounds have irritant properties leading to a dose-dependent decrease in viability
- to keep irritation of all compounds equal the lowest observed effect levels (LOEL) were determined for each compound and skin model
- the LOEL was defined as the concentration resulting in a decrease in viability of about 10% after 24h of exposure
- skin models were exposed to the appropriate concentrations for the analyses of cell signaling pathways

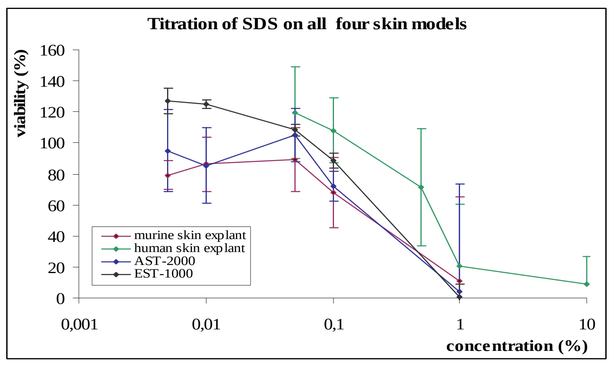

Fig. 1 Titration of SDS to determine the lowest observed effect levels (LOEL). *Results are presented as mean viability in % ± SD referred to the vehicle-treated controls (n = 5 per concentration/vehicle using skin explants and n=3 using the reconstituted skin models AST-2000 and EST-1000). Performed viability test: MTT*

compound	skin explants		reconstituted skin models	
	murine	human	EST-1000	AST-2000
SDS	0,070%	0,070%	0,020%	0,070%
TritonX-100	0,100%	0,001%	0,010%	0,001%
Oxazolon	0,500%	0,070%	0,150%	0,070%
DNFB	0,003%	0,005%	0,010%	0,010%

Table 1 Lowest observed effect levels. *Shown are the LOEL for each compound and skin model. The concentrations were determined by titration of each compound as presented in Fig. 1 and were used for all experiments analyzing cell signaling pathways.*

Analyses of cell signaling pathways

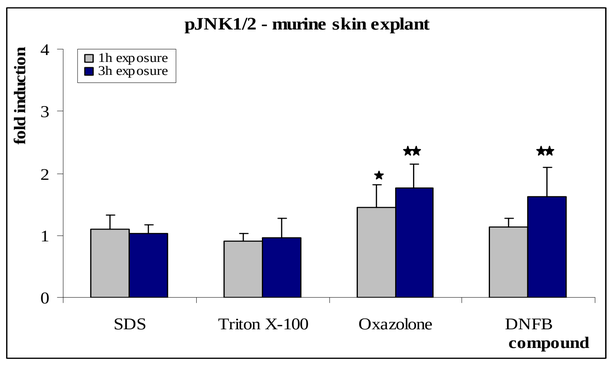

Fig. 2 Induction of phospho-JNK1/2 in murine skin explants after exposure to irritant and sensitizing compounds. *Results are presented as mean fold inductions ± SD referred to the vehicle-treated controls (n = 5 per compound/vehicle). *: p < 0.1 vs. vehicle-treated control; **: p < 0.05 vs. vehicle-treated control*

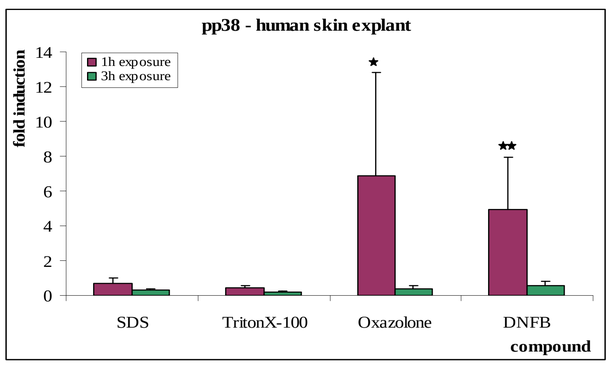

Fig. 3 Induction of phospho-p38 in human skin explants after exposure to irritant and sensitizing compounds. *Results are presented as mean fold inductions ± SD referred to the vehicle-treated controls (n = 5 per compound/vehicle). *: p < 0.1 vs. vehicle-treated control; *: p < 0.05 vs. vehicle-treated control*

MURINE SKIN EXPLANT						
compound		induction of phosphorylation				
		p38	ERK1/2	JNK1/2	PLCγ	STAT1
skin irritant	SDS	-	+/-	-	-	-
	TritonX-100	+/-	-	-	-	-
skin sensitizer	Oxazolone	+++	++	++	-	-
	DNFB	+++	+/-	+	-	-
HUMAN SKIN EXPLANT						
skin irritant	SDS	-	-	-	-	-
	TritonX-100	-	-	-	-	-
skin sensitizer	Oxazolone	+++	+	+	+/-	-
	DNFB	+++	+	+	+/-	-

Table 2 Induction of phosphorylation in murine and human skin explants after exposure to irritant and sensitizing compounds. *Shown is the classification of mean fold inductions of two independent experiments; -: no induction detectable; +: induction of phosphorylation; +/-: no or low inductions*

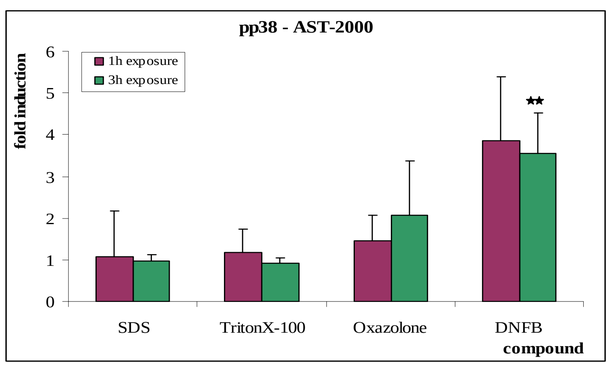

Fig. 4 Induction of phospho-p38 in AST-2000 after exposure to irritant and sensitizing compounds. *Results are presented as mean fold inductions ± SD referred to the vehicle-treated controls (n = 3 per compound/vehicle). *: p < 0.1 vs. vehicle-treated control; **: p < 0.05 vs. vehicle-treated control.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

AST-2000						
compound		induction of phosphorylation				
		p38	ERK1/2	JNK1/2	PLCγ	STAT1
skin irritant	SDS	+/-	+	-	-	-
	TritonX-100	+/-	+	-	-	-
skin sensitizer	Oxazolone	++	-	+	-	-
	DNFB	+++	-	++	-	-

Table 3 Induction of phosphorylation in AST-2000 after exposure to irritant and sensitizing compounds. *Shown is the classification of mean fold inductions of two independent experiments; -: no induction detectable; +: induction of phosphorylation; +/-: no or low inductions*

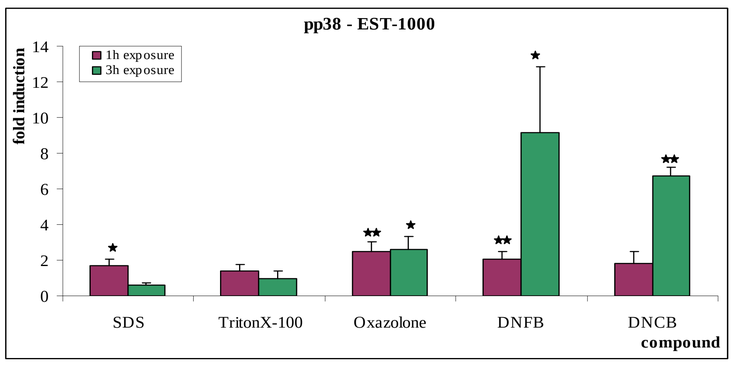

Fig. 5 Induction of phospho-p38 in EST-1000 after exposure to irritant and sensitizing compounds. *Results are presented as mean fold inductions ± SD referred to the vehicle-treated controls (n = 3 per compound/vehicle). DNCB was used at a concentration of 0,015% (LOEL). *: p < 0.1 vs. vehicle-treated control, **: p < 0.05 vs. vehicle-treated control*

EST-1000						
compound		induction of phosphorylation				
		p38	ERK1/2	JNK1/2	PLCγ	STAT1
skin irritant	SDS	+/-	+	-	-	-
	TritonX-100	+/-	+	-	-	-
skin sensitizer	Oxazolone	+++	-	+	-	-
	DNFB	+++	-	++	-	-
	DNCB	+++	-	+++	n.a.	n.a.

Table 4 Induction of phosphorylation in EST-1000 after exposure to irritant and sensitizing compounds. *Shown is the classification of mean fold inductions of two independent experiments, whereas DNCB was used only once. -: no induction detectable; +: induction of phosphorylation; +/-: no or low inductions; n.a.: not analyzed*

Comparison of skin models

compound	phospho-p38		phospho-ERK1/2		phospho-JNK1/2	
	sensitizer	irritant	sensitizer	irritant	sensitizer	irritant
skin explants (murine and human)	+++	-	+	-	+	-
reconstituted skin models (EST-1000 and AST-2000)	+++	+/-	-	+	++	-

Table 5 Comparison of MAP kinase phosphorylation in skin explants and reconstituted skin models. *-: no induction detectable; +: induction of phosphorylation; +/-: no or low inductions*

Conclusion

- analysis of MAP kinase activation provides a promising tool to identify sensitizing compounds *in vitro*
- 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
- disadvantages of skin explants are high inter- and intra-individual variabilities
- especially for human skin explants, the availability is limited and patients need to be of about equal age
- using the AST-2000 a specific activation of p38 and JNK was obtained after exposure to sensitizing compounds
- the EST-1000 showed high induction levels of phospho-p38 specific for exposure to sensitizing compounds and comparable to those found for skin explants
- with respect to availability, variability and simplicity in handling, the EST−1000 turned out to be the model of choice for further analyses of compounds

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