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Assessment of an *in vitro* model of human cells to evaluate the toxic and irritating potential of chemical compounds

M. Catalano, C. Scanarotti, R. Sanguineti, S. Penco, P. Romano, A. Cadoni, M.A. Pronzato, A.M. Bassi*

Dept. Exp. Medicine, Via L.B. Alberti, 2, 16132, Genova, Italy
* anna.maria.bassi@unige.it

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Abstract

We attempted to induce differentiation in undifferentiated NCTC2544 human keratinocyte line, by exposure to $ZnSO_4$ and $CaCl_2$. Analysis of specific markers, transglutaminase I, involucrin and loricrin, show that basal NCTC2544 (BL) reached spinous- (SL) and granular-like (GL) phenotypes. BL-, SI- and GL-NCTC were exposed to SDS, as irritant stimulus and Neutral red uptake (NRU) and MTT cytoxicity tests evidenced a relatively higher toxicity in SL-and GL cells on lysosomes respect to mitochondria. IL1α cytokine was monitored as early inflammation marker. The complex of data provides evidence for the suitability of our in vitro model to the analysis of cytotoxic/biological effects of topically applied exogenous compounds.

Introduction

To identify non-animal tests for predicting human skin irritation, the European Centre for the Validation of Alternative Methods, ECVAM, promotes prevalidation studies focused on the evaluation of suitable *in vitro* assays.
Epidermal differentiation requires the sequential regulation of specific genes, with their simultaneous activation and inactivation, while the cells move upwards from the basal layer (BL) through the spinous layer (SL) and the granular layer (GL), ending up in the cornified envelope (CE).
A pattern of markers, such as transglutaminases (TG), keratins, involucrin (INV), and loricrin (LOR), permits the discrimination between differentiating and terminally differentiated keratinocytes.
Type I TG (TG1) catalyzes the formation of soluble (e.g. INV) and non-soluble (e.g. LOR) proteins-bonds that form the CE. INV, associated with late SL- and GL-layer, is incorporated into CE, via TG1. LOR, is synthesized in the GL and cross-linked to up to 20 other cytosolic proteins (e.g. INV) by TG1, forming the CE [1-3].
Although several factors can trigger keratinocytes to differentiate, calcium is the most physiological agent. In skin, an increasing Ca^{2+} gradient is established from the basal to the outermost layer of the epithelium, and this regulates the differentiation process [4]. Also Zinc play a critical role in this process.
In response to physical or chemical stress, keratinocytes produce and release inflammatory cytokines. So the analysis of these parameters may be useful to identify the human risk of exposure to potential irritant compound(s).
IL1α, an early inflammation cytokine whose biological active isoform is also stored in keratinocytes, is usually used in the assessment of skin irritation risk [5].
Differentiated and undifferentiated human keratinocyte cell lines (HaCaT and NCTC2544, respectively) result in a different response to irritant stimuli [6], given that a less differentiated phenotype is more susceptible to stressors. In our previous study, NCTC2544 cells showed synthesis and modulation of early cytokine, such as IL1α and LIF, after exposure to selected irritating stimuli [7].
Unfortunately, *in vitro* 3D reconstructed human epithelium show an inter-batch variation for IL1α release, due to different donors. For their phenotype, NCTC2544 cells have many attractive features for an *in vitro* keratinocyte differentiation model. We have just demonstrated that in NCTC2544 cells, Vitamin E increases the expression of differentiation markers, involved in terminal keratinocyte differentiation [8].
The aim of this study was to experimentally modulate the extent of NCTC2544 cell differentiation by exposure to $ZnSO_4$ and $CaCl_2$. To determine the functional outcome of these treatment, we analyse the gene expression of stage specific markers in these keratinocytes, such as TG1, INV and LOR.
To assess the suitability of using NCTC2544, in differentiation stages, for skin toxicity/irritation tests, obtained cultures were exposed to SDS, as an irritant positive control, and it was analyzed cell viability, by neutral red uptake (NRU) and MTT tests and IL1α gene expression levels.

Materials and methods

Cell cultures and treatments

Normal human keratinocyte cell line, NCTC2544, was maintained as previously described [7]. Briefly, cells were seeded in 25-75 cm^2 flasks and 96-well plates at 70-200-

2×10^3 cells, respectively, so as to obtain semi-confluent cultures at the end of culture. $ZnSO_4$, and $CaCl_2$ were added to culture medium in order to achieve final concentrations. The treatment media were changed daily, during experimental times. Semiconfluent cultures were exposed to SDS for 3 h, before performing further analysis. At the end of each treatment, experimental medium was removed and the plates or flasks were washed with saline buffer (PBS) and cytotoxicity tests, or gene expression analysis were assessed.

Cell viability assessment

Cytotoxicity of each treatment was evaluated by two validated methods: MTT and NRU, as previously described [7].

RT-PCR

mRNA Isolation and RT-PCR were performed as already described [7]. Gene-specific primers for TG-1, INV, LOR, and GAPDH (housekeeping gene) were designed according to the sequences available at http://www.ncbi.nlm.nih.gov. Digital images of PCR products were analysed by BIORAD Geldoc2000 (Bio-Rad Laboratories), the bands quantified by Quantity One software (Bio-Rad Laboratories) and normalized to their own GAPDH. The results were expressed as percentage of test cells versus control.

Statistics

All experiments were carried out in at least triplicate, using three wells for each sample. The data were analysed using the Instat-Prism software package (Graph-Pad Software, Inc). The effective concentrations, IC50, were determined by using a downhill logistic dose–response curve.

Results

As a first analysis, in order to have a comparative evaluation of the toxicity of differentiation treatments, NRU and MTT viability tests were performed after each experimental exposure time (Tab. 1).

Treatment	Dose	Exposure Time (days)	Viability Index NRU	Viability Index MTT
None	-		100	100
$CaCl_2$	1.2 mM	2	100	100
		4	100	100
	2 mM	2	100	92 ± 3 *
		4	99 ± 1	100
$ZnSO_4$	10 µM		92 ± 8	100
	50 µM		92 ± 5	93 ± 7
	100 µM	6	92 ± 6	92 ± 8 *
	200 µM		14 ± 4 **	2 ± 1 **
	400 µM		11 ± 4 **	0

*Table 1. Cytotoxicity of $CaCl_2$ and $ZnSO_4$ on NCTC2544 cells. Data, expressed as percentage of viability vs untreated cultures and extrapolated by NRU and MTT tests, are the means ± SD of 4 separate experiments run in triplicate. *, ** significantly different ($p<0.05$ and 0.01) vs untreated cultures (see statistics sect.)*

In our experimental conditions, the most toxic treatments were 200µM and 400µM $ZnSO_4$ for 6 days, since in these conditions both viability indexes showed a very significantly dramatic reduction.

NCTC were exposed to non cytotoxic treatments of differentiating agents 1.2 and 2mM $CaCl_2$ (2-4 days) and 10-100 µM $ZnSO_4$ (4 days) and then the gene expression of TG1, INV and LOR was analyzed (Fig. 1).

TG1 mRNA levels increased significantly after 4 days exposure to 1.2 mM $CaCl_2$ and 10 mM $ZnSO_4$. INV and LOR mRNAs showed an up to 1.5 fold increase after exposure to 2 mM $CaCl_2$, in a time-dependent way. Exposure to $ZnSO_4$ decreased LOR and increased INV gene expression. INV mRNA reached a significant level (1.5 fold) but to a lesser extent than after exposure to 2 mM $CaCl_2$. By analysis of INV and LOR gene levels, it can be supposed that NCTC2544 reached SL- and GL-like phenotypes after exposure to 10 mM $ZnSO_4$ and to 2 mM $CaCl_2$, respectively.

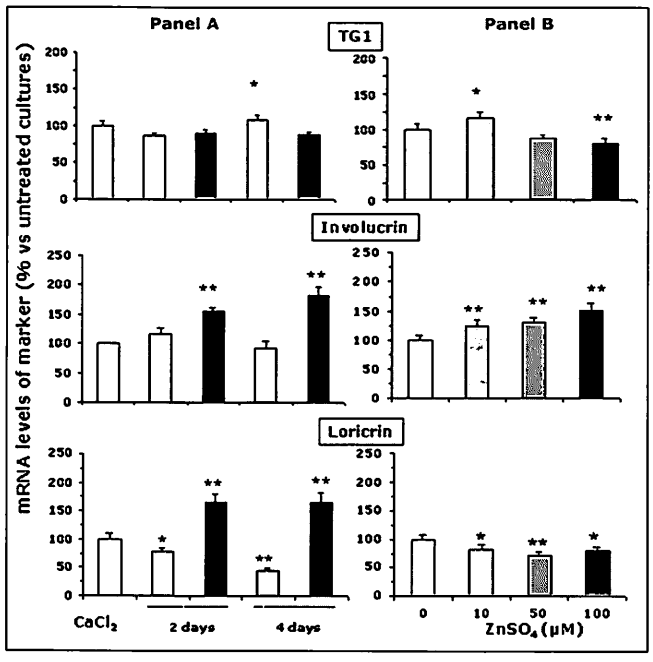

*Figure 1. mRNA expression of TG1, INV and LOR in NCTC 2544 exposed to differentiating treatments. NCTC2544 were exposed to $CaCl_2$ (▨ 1.2-■2 mM), and $ZnSO_4$ (Panel A and B, respectively), as described in Methods. Data are the means ± SD of 3 separate experiments; *,** =p< 0.05 and 0.01 vs respective untreated cultures, (see statistics sect.)*

Phenotype	Viability index NRU	Viability index MTT
BASAL	205 ± 17	271 ± 69
SPINOUS	176 ± 18	234 ± 35
GRANULAR	175 ± 14	267 ± 17

Table 2. Viability of NCTC2544 in different differentiation stages, after 3 hours exposure to SDS. Data are expressed as IC50 (µM), extrapolated by NRU and MTT index, and are referred to 3 separate experiments run in triplicate (see statistics sect.)

The evaluation of susceptibility to irritant compound was performed in BL-, SL-, and GL-like NCTC 2544. IC50s (50% effective dose) extrapolated by the two viability tests, NRU and MTT, are quite similar, even though the IC50s derived from NRU were invariably lower than those derived from MTT (Tab. 2).

Only SL-like NCTC cells showed a slight but significant reduction of IL1α, at highest dose of SDS exposure. GL-like phenotype cells demonstrated a dose-dependent increase of IL1α gene expression. Taken together, these results are consistent with the suitability of our model, since IL1α confirmed to be good markers of early inflammation in basal cells and in differentiated cells. Manipulation

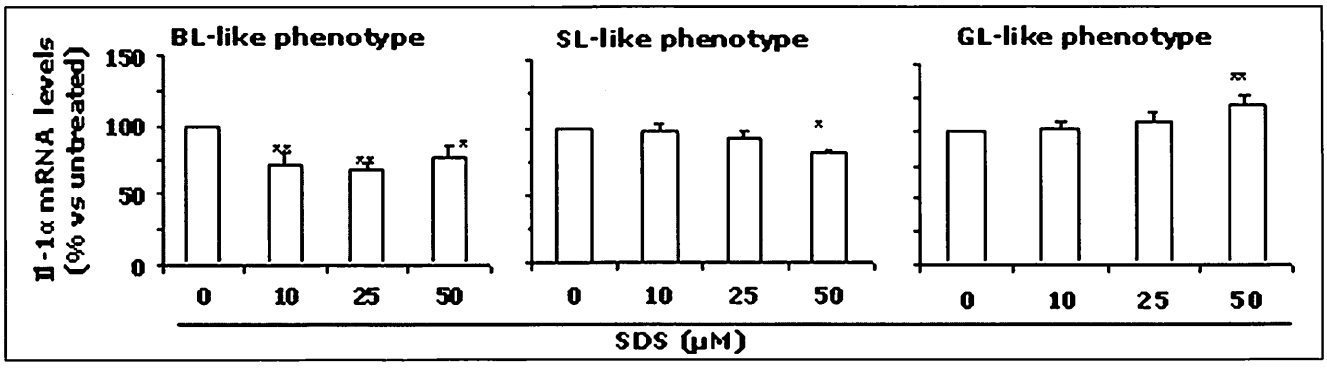

Figure 2. Effect of SDS on IL1α and LIF mRNA expression in NCTC2544 in different differentiation stage. BL-, SL- and GL-NCTC cultures were exposed for 3 h to 10-50 μM SDS. Data, are the means ± SD of 3 separate experiments.,**=p< 0.05 and 0.01 vs respective untreated cultures, (see statistics sect.)*

The effects of SDS on IL-1α gene expression was analyzed in different differentiation stage cultures (Fig. 2). IL1α mRNA was significantly reduced in basal NCTC2544 by SDS. In SL-like cells, only 100μM SDS reduced the cytokine levels. A dose-dependent increase was observed in GL-like cultures (Fig. 2).

Discussion

The aim of this work was to help overcome the problem of the variability of the potential inflammatory responses by 3D models of reconstructed skin, trying to set up alternative models with normal human skin-derived cells, to mimic part of the complex mechanism of the response to stimuli *in vivo* irritation/allergy.

Not excluding the fact that, even in the upper layers (GL and CE) TG1, INV and LOR proteins are expressed in the lower strata, in our study NCTC2544 cells increased the expression of INV mRNA, that is associated with BL and SL-phenotypes after treatment with $ZnSO_4$. After exposure to 2mM $CaCl_2$, NCTC2544 cells acquired a GL-like phenotype, where both INV and LOR are expressed.

TG1 expression follows an inverse trend compared to that of INV, suggesting that, if the target proteins are already produced and linked, the enzyme is no longer necessary.

After exposure to a positive irritant compound, SDS, cytotoxicity tests evidence a perturbation at the lysosomal compartment only in more differentiated cells, being NRU index lower than in the BL-like ones. MTT index did not show differences, suggesting that mitochondria are less influenced by differentiation process.

Some compounds can induce irritation without affect cell viability and, as well-known, cytokines play a key role in initiating and maintaining pathophysiology, as well as in inducing recovery in skin inflammations.

Just for its precocious response in inflammation, IL1α gene expression analysis was carried out after a 3 hours exposure to SDS. It is to keep in mind that in NCTC2544 cells reduced levels of IL1α mRNA were associated with increased protein synthesis [7].

of undifferentiated NCTC2544 cell culture conditions for inducing more differentiate stages, can represent an authentic and convenient model for assessment human risk of skin exposure to potential irritant compounds on keratinocytes with same genotype and different phenotypes.

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