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Genotoxicity assays with Episkin®, a reconstructed skin model: Towards new tools for *in vitro* risk assessment of dermally applied compounds?

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Abstract

In vitro reconstructed human skin such as Episkin® is a widely used model in safety or efficacy pre-screening tests. It is of growing interest for regulatory purposes as an alternative to animal testing. The reduction and eventually the replacement of In vivo toxicity testing require the development of In vitro models to predict the genotoxic or other endpoint risk. This can be achieved if these new assays take into account the exposure conditions in a more relevant way. To that end, new approaches are proposed using human reconstructed skin model for in vitro toxicology assessment. Reconstructed skin models have not been used for In vitro genotoxicity testing so far, though they present clear advantages over mouse skin for human risk assessment.
This work highlights the development of a specific protocol for performing genotoxicity assays. The skin is indeed a biologically active barrier driving the exposure to compounds and their possible metabolites. A specific co-culture system using Episkin® and target cells to perform a regular micronucleus assay is presented. This first step in using human reconstructed skin for genotoxicity testing has aimed at improving the relevance of exposure conditions in In vitro genotoxicity assays for topically applied compounds.

Keywords: genotoxicity, *in vitro*, reconstructed skin, co-culture, micronucleus assay

Introduction

Genetic toxicity needs to be addressed early in the safety assessment process of chemicals for regulatory purpose. Usually, a tiered strategy including in vitro assays is used (ICH, 1997). Two main endpoints are usually investigated:

- Gene mutation
- Chromosome damage: to detect alterations in chromosome number (polyploidy and aneuploidy) or structure (breaks, deletions, rearrangements)

Although these in vitro assays are routinely used and well accepted by regulators, they do have a poor specificity (tendency to yield a high number of positive results whereas in vivo genotoxicty results are negative). Thus, their relevance to humans is always questioned. Several reasons may account for these limitations:

- Lack of relevant metabolic capabilities in cells lines
- Exposure to "high doses" of chemical and lack of toxicokinetic
- Cell type used may not be relevant for target organ

Reconstructed tissues such as Episkin® are biological models widely used in safety or efficacy pre-screening tests. Moreover, these models have been validated for regulatory purposes as replacement to animal testing for skin corrosivity and skin irritation. Theses assays usually rely on evaluating cytotoxicity and cytokine release.

So far, the published results demonstrate that these models are pretty similar normal human skin. The in-depth characterization of reconstructed skin models is still ongoing. In addition, reconstructed human skin models present clear advantages (structure and biology of the epidermis, commercial availability as an in vitro screening tool) as compared to mouse skin for human risk assessment.

These models are now being used to screen for genotoxicity. Here, we describe how the reconstructed skin was used as a metabolically competent tissue and a biological barrier mimicking realistic condition of use. The tissues were co-cultured with mouse

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lymphoma cells for the micronucleus assay.

In vivo genotoxitcity testing will be phased out starting in March 2009 for cosmetic substances. Thus, relevant in vitro assays are sought to replace the in vivo ones and improve the specificity of the current in vitro ones. Using reconstructed skin models in co-culture with target cells may be a way to tackle this issue.

Material and methods

Episkin®

Episkin® 1.2 cm2 skin equivalents were purchased from Episkin SNC (Lyon, France). Upon receipt, the reconstructed skin inserts were transferred into 12 wells plates containing 5 ml of Episkin® "maintenance" medium and put in a cell incubator at 37°C under 5% CO2 and 90% humidity.

The following day, the medium was replaced by 2 ml of Episkin® "treatment" medium according to manufacturer instructions.

L5178Y cells

L5178Y TK+/- lymphoma cells clone 3.7.2.C were purchased from ECACC (ECACC, Salisbury) and cultured in RPMI1640 medium supplemented with 10% horse heat inactivated serum.

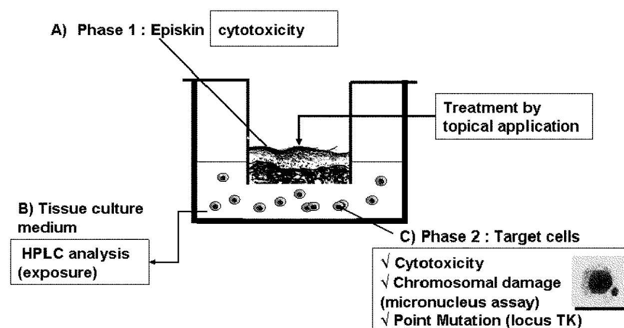

Fig. 1: The skin, a target organ and a physyologic barrier: model and endpoints
Co-culture system made of Episkin in an insert settled in a well containing L5178Y cells. Chemical were applied topically onto the skin surface. Cytotoxicty can be assessed in Episkin as well as in the target cells. The micronucleus assay was performed in the target cells. This system is flexible allowing the use of different types of target cells and different endpoints.

Episkin® + lymphoma co-culture (Fig. 1)

The Episkin® inserts were placed after their overnight culture in the maintenance medium, in 12 wells plates containing a fresh culture of L5178Y (100 000 cells per ml) in RPMI1640 medium (Fig. 1). The medium was replaced by Episkin® "treatment" medium.

Chemicals and Treatments (Fig. 2)

Mitomycin C (CAS 50-07-7), Cyclophosphamide (CAS 6055-19-2) and Apigenin (CAS 520-36-5), 2,4 Diaminotoluene, 2,6 Diaminotoluene were purchased from Sigma.

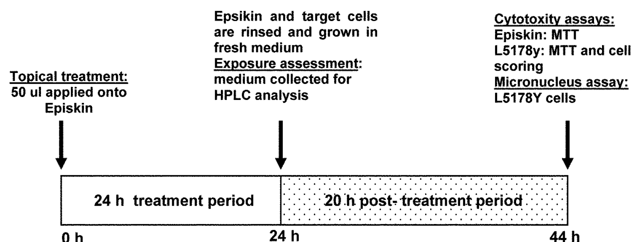

Fig. 2: Summary of treatment and assays performed using the co-culture system

50 microliters of chemical dissolved in PBS or DMSO were applied topically on Episkin® and the system was kept for 24 hours in the incubator at 37°C.

At the end of the treatment period, the epidermises were rinsed three times with PBS. The culture medium was collected by centrifugation for HPLC analysis (*quantification of the exposure*). The L5178Y cells collected from each well of the plate were put back together with "their" respective epidermis in the incubator for an additional 20 hours in fresh maintenance medium.

Cytotoxicity was evaluated in Episkin® as well as in the mouse lymphoma cells grown underneath by an MTT assay. Cell number was also evaluated using a Z1 Coulter counter (Beckmann Coulter) for the L5178Y cells.

Micronucleus assay

The *in vitro* micronucleus assay was performed on the L5178Y cells grown beneath Episkin®.

Briefly, the cells were collected by centrifugation for micronucleus procedure. The cell pellet was rinsed twice with 10% pluronic acid diluted in RPMI1640 culture medium. They were then re-suspended drop by drop in isotonic shock solution (pluronic acid, RPMI1640 medium, PBS (3/13.5/13.5 v/v)), centrifuged and re-suspended twice in the fixative solution (methano / acetic acid 15/1). After another centrifugation, the cell pellets were re-suspended in methanol / acetic acid (3/1 v/v) and spread on microscopic glass slide. The slides were stained with Giemsa (diluted at 5% in mineral water), dried and scored under a microscope.

Bioavailability and HPLC analysis

The culture medium of each well was kept frozen at -20°C until HPLC analysis. The system used is a Waters LC625 equipped with photodiode array detector 996 (Waters). Analysis conditions were optimized for the RPMI serum containing medium and for each chemical. The exposed concentration (that retained in the reconstructed skin) was deducted from the amount of the compound detected in each well.

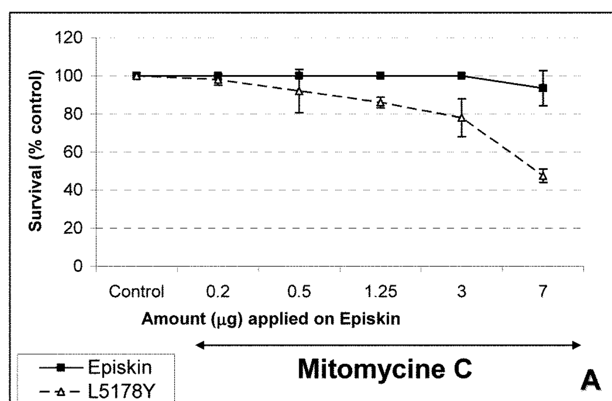

Fig. 3 A: Cytotoxity of Mitomycin C in Episkin and in L5178Y cells: the amount of test agent is hardly toxic to the epidermal cells while a dose-dependent toxicity is evidenced in mouse lymphoma cells

Results

When using the co-culture system, the amount of test agent is hardly toxic the epidermal cells while a dose-dependent toxicity is evidenced in mouse lymphoma cells (Fig. 3 A).

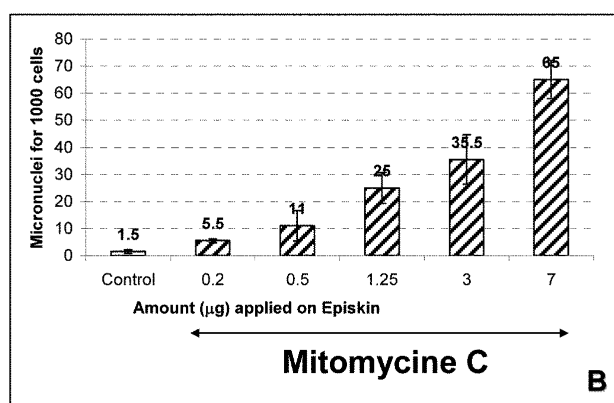

Fig. 3 B: Micronuclei detected in L5178Y cells cultured beneath Episkin and after topical treatment with Mitomycin C: a clear dose-response of micronuclei induction is highlighted

Micronuclei can be detected in L5178Y cells cultured beneath Episkin and after topical treatment with Mitomycin C: a clear dose-response of micronuclei induction is highlighted (Fig. 3 B)

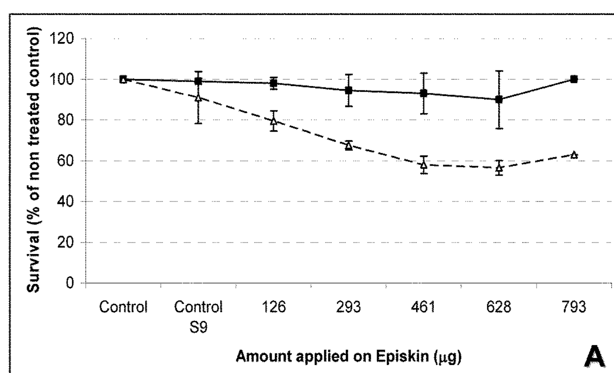

Fig. 4 A: Cytotoxity of Cyclophosphamide in Episkin and in L5178Y cells: similarly to Mitomycin C, Episkin viability is hardly altered by treatment with cyclophosphamide.

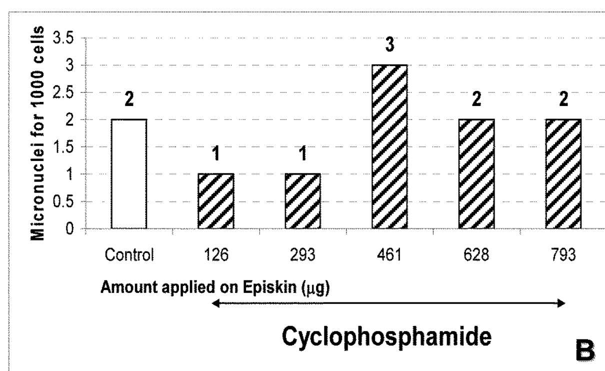

Fig. 4 B: Micronuclei induced in L5178Y cells after topical application of cyclophosphamide were not significantly different from solvent control

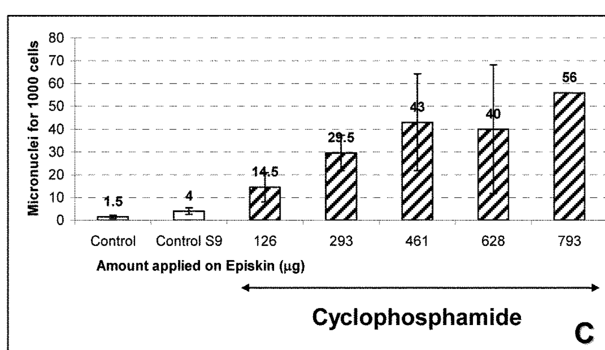

Fig. 4 C: Micronuclei were clearly evidenced in L5178Y cells only when S9 mix was added directly to the medium 3 h before the end of the treatment period

Similarly to Mitomycin C, Episkin viability is hardly altered by treatment with Cyclophosphamide (Fig. 4 A). Micronuclei induced in L5178Y cells after topical application of cyclophosphamide were not significantly different from solvent control (Fig. 4 B).

Micronuclei were clearly evidenced in L5178Y cells only when S9 mix was added directly to the medium 3 h before the end of the treatment period (Fig. 4 C).

The metabolic capacity in Episkin may differ (quantitatively and/or qualitatively) from that of normal human skin. This may justify why cyclophosphamide was unable to produce a significant number of micronuclei in the mouse lymphoma cells cultured beneath the skin after topical application. An unsuccessful attempt to solve this issue was made by setting a sandwich culture where the mouse lymphoma cells were grown between the attached HepG2 cells and the skin insert (data not shown). Micronuclei were observed only when Aroclor induced rat S9 mix was added to the target cell medium (systemically) three hours before the end of the treatment period. Extensive studies are ongoing to characterize the metabolic capabilities of Episkin.

Table 1 summarizes data of different agents tested in the co-culture system after topical application and

Table 1: summary data of different agents tested in the co-culture system after topical application and in a "simple" L5178Y cell culture.

Test agent	Micronucleus assay in L5178Y cells		Micronucleus assay in Episkin/ L5178Y co-culture (topical route)	
	Without S9 mix	With S9 mix	Without S9 mix	WithS9 mix
Apigenin	Positive	Negative	Negative	Negative
2, 4 Diaminotoluene	Negative	Equivocal	Negative	Positive
2, 6 Diaminotoluene	Positive	Positive	Positive	Positive

in a "simple" L5178Y cell culture.

Results from the dyes (2, 4 diaminotoluene and 2, 6 diaminotoluene) were pretty consistent in the two systems except for the equivocal call in the L5178Y cells. 2, 4 diaminotoluene is a rat and mouse hepatocarcinogen while its isomer 2, 6 diaminotoluene is not. Both are genotoxic in vitro and in vivo. From the literature positive results in the In vitro micronucleus assay have been obtained treating HepG2 cells. With the In vivo rat micronucleus assay, weakly positive (positive at results were obtained at fairly toxic doses) with both dyes. Similarly to these in vivo results from bone marrow cells, L5178Y cells may not be the appropriate target cells for 2, 4 diaminotoluene (liver and bladder carcinogen, George et al., 1997).

Interestingly and as shown with apigenin, some substances able to induce micronucleus formation when incubated directly with target cells turned out negative in the co culture system. This means that:

- Either the chemical could not cross the physiological barrier
- And/or was metabolized to non-genotoxic compounds
- The amount reaching the target cells was not sufficient to elicit the genotoxic insult

Discussion

To comply with the seventh amendment to the cosmetic directive, cosmetic industry will rely on alternative to in vivo genotoxic toxicity assays starting in March 2009.

The current in vitro assays, especially the mammalian cell-based ones suffer from their poor specificity (tendency to yield positive results that are not confirmed by in vivo assays). Several reasons may account for this tendency to produce the so called "false positive results":

- Lack of toxicokinetics data
- Lack of exposure (bioavailability) data
- Lack of appropriate metabolic system
- The cell type used may not be representative of the target organ for a given chemical
- Chemical concentrations used may be by far "higher" than those reached in In vivo conditions.

Usually, in vitro genotoxicity tests are performed by applying **a single application** at "high" doses

Using reconstructed skin models can help to tackle (part of) these issues. The skin is (together with hair) the main target organ for cosmetics (skin irritation, eye irritation, phototoxicity, genotoxicity). Using these reconstructed skin models in combination with target cells is very helpful:

- The exposure of the target cells to a given substance can be assessed after topical application as was the case here. Episkin was used as a metabolically active tissue and a physiologic barrier. The test compound can be metabolized by the skin and/or by the target cells. Metabolism is an important event to consider in genotoxicity and skin sensitization evaluation.
- Compared to cell models, a broad variety of chemicals with different physico-chemical features can be evaluated in this system: compounds with different pH, physical state (liquids, gels, solids, formulations).

This co-culture system is much closer to physiological conditions than a monolayer cell culture. These skin models are valuable tools since they are commercially available and produced with high quality standards.

In order to address the poor specificity of the current cell-based genotoxicity assays, a co-culture system made of reconstructed skin and suspension cells was developed.

The preliminary results obtained are promising. A thorough characterization of the skin equivalents (that is ongoing), especially their metabolism is needed to better understand some of the results. The step forward is to evaluate a broader set of chemicals using this system to get an inside into its performances.

Also, different target cells can be used as surrogate target organs that may be affected after topical application of a substance.

Other endpoints like mutation (tK locus assay in heterozygous tK cells like the L5178Y described here), gene expression changes can be assessed as well.

To address the need for more relevant in vitro assays, there are number of ongoing projects. One of them is a Colipa funded collaborative project lead by the genotoxicity Project Team. Different engineered skin models are used to evaluate the genotoxic potential by performing the comet and/or the micronucleus assay in epidermal cells after topical application.

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