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Development of a method for assessing micronucleus induction in a 3D human skin model (EpiDerm™)

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Abstract

To meet the requirements of the EU 7th Amendment to the Cosmetics Directive, manufacturers of cosmetics products will need to ascertain the safety of ingredients using non-animal methods. Starting in 2009, *in vivo* genotoxicity tests for cosmetics ingredients will not be allowed. Skin is a target area of interest for many cosmetic products because of its relatively high exposure. Therefore, it would be beneficial to have a non-animal, skin-based genotoxicity assay, especially one that utilized human skin *in vitro*. In this paper, we describe the development of a reproducible micronucleus assay that uses EpiDerm™ engineered human skin constructs (MatTek Corp., Ashland, MA). We describe methods for isolating single cells from the 3D skin model and for processing the cells for microscopic analysis of micronuclei (MN). In addition, since little was known about the kinetics of the dividing keratinocytes in the EpiDerm™ model, we evaluated whether cytochalasin B (Cyt-B) could be used to distinguish the population of dividing cells allowing the development of a micronucleus assay in binucleated cells. We found that the frequency of binucleated cells increased both with time and with increasing concentration of Cyt-B. After a 48-h exposure, 30–50% binucleated cells were reproducibly obtained. Finally, we evaluated micronucleus induction using the model genotoxicants mitomycin C (MMC) and vinblastine sulfate (VB). The background frequency of MN is very low and reproducible in this model, and statistically significant increases in the frequency of micronucleated cells were induced by both MMC and VB. These are initial steps in developing a routine "*in vivo*-like" assay for chromosomal damage in human tissue. It is hoped that other investigators utilize these methods to further the understanding of this potentially valuable new non-animal method.

Keywords: Non-animal skin-based micronucleus assay; *In vitro*; Genotoxicity; 3D human skin model

1. Introduction

The *in vitro* micronucleus assay is an established method for detecting potentially clastogenic and aneugenic chemicals [1]. Its acceptance has been furthered by the development of the cytokinesis-block methodology [2], which allows the identification of cells that have undergone one nuclear division, thereby allowing an accurate assessment of the appropriate cell population for quantifying micronuclei (MN) along with an easy assessment of alterations in cell division kinetics due to cytotoxicity. Results from an international validation study have recently been completed [3], a draft OECD guideline for the *in vitro* micronucleus assay has been prepared [4] and the *in vitro* micronucleus

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assay is increasingly being used in the risk-assessment process.

Because current *in vitro* genotoxicity assays induce a high level of false-positive results (75–95% of rodent non-carcinogens are positive in one or more *in vitro* genotoxicity tests) [5] chemicals that are genotoxic *in vitro* are often evaluated further using *in vivo* genotoxicity assays to determine whether the chemical is bioavailable and active in the whole organism. One of the most developed *in vivo* assays is the analysis of MN in bone marrow (see, e.g. [6]). However, the requirements of new regulations in Europe such as the 7th Amendment to the Cosmetics Directive, will make it very difficult, and in some cases impossible, to use animal models in safety assessments in the near future. In addition, large toxicity testing programs, like the REACH program in Europe, will likely not be successful unless *in vitro* methods are utilized for many endpoints previously measured *in vivo*. The inability to use *in vivo* methods to address the relevance of positive results from *in vitro* genotoxicity assays is particularly problematic in light of the high rate of false-positives for current *in vitro* genotoxicity assays [7]. Clearly new methods and approaches are needed.

There has been renewed interest in utilizing skin as a target organ for assessing genotoxicity [8–14] since it is a relevant tissue for many different environmental exposures. Early methods for assessing genotoxicity in rodent skin utilized *in vivo* exposures followed by *in vitro* cell culture to identify MN, e.g. [13,14], while current methods utilize only *in vivo* exposure [8–12], thereby avoiding the complications involved in *in vitro* cell culture. Both of these approaches utilize rat or mouse skin as a surrogate for human skin, and results from such models may not completely reflect what would occur in humans after dermal exposure.

Further, *in vivo* methods such as those described will not be acceptable for use with cosmetics in the future. A task force initiated by the European Commission and led by the European Center for the Validation of Alternative Methods (ECVAM) on which one of us (MJA) participated, recommended a new approach for the safety assessment of dermally applied cosmetics with regards to genotoxicity. This approach would include an *in vitro* assay using skin models [15]. To this end, we have investigated whether human three-dimensional skin constructs can be used as a target system for micronucleus studies. Theoretically such models may better reflect the complexities typical of *in vivo* exposures, e.g. absorption, tissue specificity, metabolism, etc., and at the same time reflect human-specific responses in these parameters. In this paper, we describe the development of methods for isolating and processing cells from the 3D EpiDerm™ human skin model (MatTek Corp., Ashland, MA) and provide initial results with the model genotoxicants, mitomycin C (MMC) and vinblastine sulfate (VB). Preliminary data have been presented previously [16,17].

2. Materials and methods

2.1. Test chemicals and reagents

MMC, VB, cytochalasin B (Cyt-B), DMSO, methanol, acetic acid, KCl (0.075 M; Cat# P9327), and trypan blue were obtained from Sigma (St. Louis, MO). Acetone was obtained from Aldrich Chemical Co., and ethanol (absolute) was obtained from Pharmco (Brookfield, CT). Acridine orange (AO) solution (10 mg/ml) was obtained from Sigma (Cat# A8097) and used at a final concentration of 40 μg/ml in calcium/magnesium-free Dulbecco's phosphate-buffered saline (DPBS) (MatTek, Ashland, MA). EDTA (1 g/l) was obtained from Quality Biological (Gaithersburg, MD) (Cat# 118-090-060). Trypsin (0.25%)–EDTA (0.02%) was obtained from JRH Biosciences (Lenexa, Kansas) (Cat# 59228-100M).

2.2. Cell culture media

New Maintenance Medium (Cat# EPI-100-NMM; NMM) was obtained from MatTek Corporation (Ashland, MA). Its exact composition is proprietary to the manufacturer, but the formulation is based on Dulbecco's Modified Eagle's Medium (DMEM) and contains Keratinocyte Growth Factor (personal communication; MatTek Corporation). DMEM (Quality Biological, Gaithersburg, MD) or equivalent containing 10% FBS and 1% L-glutamine was used as a trypsin neutralization solution.

2.3. Tissue constructs

EpiDerm™ tissue (EPI-200) was obtained from MatTek Corporation. The EpiDerm™ construct is a multilayered, differentiated tissue consisting of basal, spinous, granular and cornified layers (Fig. 1) resembling the normal human epidermis. The tissue is constructed from normal epidermal keratinocytes (foreskin-derived), which are cultured on chemically modified, collagen-coated, 9-mm (i.d.) cell culture inserts (e.g. Millicell CM or Nunc polycarbonate cell-culture inserts). Differentiation is induced by air-lifting the growing cultures so that the cell inserts sit just on the surface of the medium and the apical surface of the tissue is exposed to the atmosphere (Fig. 2). The tissues are shipped cold overnight in 24-well plates.

Cultures were received from MatTek on Tuesdays in 24-well trays packed in insulated shipping containers. Within the containers, the tissue-containing tray was on top of shipping agar and a wet gauze pad covered the top of the tissues. On arrival the gauze pad was removed, the tissue inserts lifted from the agar, and each tissue observed for any gross morphologi-