Mutation Research 673 (2009) 92–99

Contents lists available at ScienceDirect

Mutation Research/Genetic Toxicology and Environmental Mutagenesis

journal homepage: www.elsevier.com/locate/gentox
Community address: www.elsevier.com/locate/mutres

Further development of the EpiDerm™ 3D reconstructed human skin micronucleus (RSMN) assay

Greg C. Mun[a], Marilyn J. Aardema[b], Ting Hu[b], Brenda Barnett[c], Yulia Kaluzhny[d], Mitchell Klausner[d], Viktor Karetsky[d], Erica L. Dahl[a], Rodger D. Curren[a,*]

[a] Institute for In Vitro Sciences Inc., 30 W. Watkins Mill Road, Suite 100, Gaithersburg, MD 20878, USA
[b] The Procter & Gamble Co., P.O. Box 538707, Cincinnati, OH 45253, USA
[c] Advanced Testing Laboratory, 6954 Cornell Road, Suite 200, Cincinnati, OH 45242, USA
[d] MatTek Corp., 200 Homer Ave., Ashland, MA 01721, USA

ARTICLE INFO

Article history:
Received 10 October 2008
Received in revised form 12 December 2008
Accepted 22 December 2008
Available online 9 January 2009

Keywords:
In vitro skin micronucleus assay
Genotoxicity
3D human skin model

ABSTRACT

The upcoming ban on testing of cosmetics in animals by the European Union's 7th Amendment to the Cosmetics Directive will require genotoxicity safety assessments of cosmetics ingredients and final formulations to be based primarily on *in vitro* genotoxicity tests. The current *in vitro* test battery produces an unacceptably high rate of false positives, and used by itself would effectively prevent the use and development of many ingredients that are actually safe for human use. To address the need for an *in vitro* test that is more predictive of genotoxicity *in vivo*, we have developed an *in vitro* micronucleus assay using a three-dimensional human reconstructed skin model (EpiDerm™) that more closely mimics the normal dermal exposure route of chemicals. We have refined this model and assessed its ability to predict genotoxicity of a battery of chemicals that have been previously classified as genotoxins or non-genotoxins based on *in vivo* rodent skin tests. Our reconstructed skin micronucleus assay correctly identified 7 genotoxins and 5 non-genotoxins, demonstrating its potential to have a higher predictive value than currently available *in vitro* genotoxicity tests, and its utility as part of a comprehensive *in vitro* genotoxicity testing strategy.

1. Introduction

The reliance on animal assays to help determine the relevance of positive *in vitro* genotoxicity results is at odds with the growing emphasis within the scientific and regulatory communities to reduce and eventually eliminate animal use. Recent regulations in Europe, such as the 7th Amendment to the Cosmetics Directive [1], will prohibit the use of *in vivo* genotoxicity tests in safety assessments for cosmetics as of 2009. Further, programs such as Registration, Evaluation, Authorization and Restriction of Chemical Substances (REACH) in Europe will need to rely heavily on *in vitro* methods, which are less costly and time consuming than *in vivo* methods, to evaluate the enormous numbers of chemicals currently projected to require safety testing. This limits the future use of *in vivo* methods to address the relevance of results generated from first tier *in vitro* genotoxicity assays. This is a significant problem since currently used *in vitro* assays produce an unacceptably high rate of false positive results. A number of recent international meetings have addressed this topic, including those sponsored by the International Life Sciences Institute-Health and Environmental Sciences Institute (ILSI-HESI) and International Working Group on Genotoxicity Test Procedures (IWGT) [2,3], the European Center for the Validation of Alternative Methods (ECVAM) [4] and The European Cosmetic, Toiletry and Perfumery Association (COLIPA) [5].

A recent analysis of over 700 chemicals demonstrated that 75–95% of rodent non-carcinogens are positive in one or more *in vitro* genotoxicity tests [6,7]. This analysis was confirmed by a study of over 1000 pharmaceutical compounds [8,9]. Thus, the problem of false positive results in current *in vitro* genotoxicity assays is a broad one that impacts both industry and regulatory agencies that rely on these assays to help evaluate the safety of new products. Currently, chemicals that are genotoxic *in vitro* are typically evaluated further in *in vivo* genotoxicity assays to determine whether the chemical is a potential hazard to humans. Therefore, the high false positive rate has lead to a large number of costly and time consuming animal studies. Many useful chemicals subject to the 7th Amendment to the Cosmetics Directive will likely be discarded solely on the basis of these low-specificity assays. A number of projects are being initiated to address the possible causes of the high rate of false positive results, particularly in mammalian cell assays, which include abnormal DNA repair and cell cycle control, altered xenobiotic metabolism, and the cellular overload associated with testing high, non-physiological concentrations of chemicals and drugs [5].

* Corresponding author. Tel.: +1 301 947 6527; fax: +1 301 947 6538.
E-mail address: rcurren@iivs.org (R.D. Curren).

1383-5718/$ – see front matter © 2009 Elsevier B.V. All rights reserved.
doi:10.1016/j.mrgentox.2008.12.004

Another approach to solve the problems with the existing *in vitro* genotoxicity assays involves the development of new *in vitro* methods that utilize normal human cells [10,11]. A few years ago we began development of a micronucleus assay in the 3D human EpiDerm™ skin construct [10]. We chose a human skin model because it represents the tissue that has the highest exposure after dermal application of a cosmetic or accidental exposure to many chemicals, and would best reflect the "first pass" absorption and metabolism that occurs following these exposures in humans. Because these artificial tissues are constructed from primary human cells, 3D skin models are expected to more accurately reproduce normal DNA repair and cell cycle control, and to provide a human metabolic capability that is more relevant than the exogenous rodent metabolizing enzymes currently recommended for use with *in vitro* genotoxicity assays. Detailed investigation of the metabolic capability of the EpiDerm™ model is now underway to determine how closely it reproduces the capacity of normal human skin, and whether the model might indeed provide a better estimate of systemic genotoxicity than *in vitro* assays utilizing rodent S-9. In addition, since these tissues have a functional stratum corneum, they will presumably provide more relevant exposure conditions to the target cells, avoiding the non-physiological concentrations of chemicals and drugs that often occur in the currently available *in vitro* genotoxicity tests. We anticipate that these traits of the reconstructed skin models will improve the predictive value of a micronucleus assay relative to existing *in vitro* genotoxicity assays.

In our previous work [10] on the reconstructed skin micronucleus assay, hereafter referred to as the RSMN assay, we described the development of methods for reproducibly isolating and processing large numbers of viable cells from the 3D EpiDerm™ skin model (MatTek Corp., Ashland, MA). We developed methods for incorporating cytochalasin B blockage of cytokinesis, to insure proper analysis of the dividing cell population. The background frequency of micronuclei (MN) was and continues to be very low and reproducible in this model, allowing for the use of robust statistical analyses. We showed that statistically significant increases in the frequency of micronucleated cells were induced by model genotoxins that act via different mechanisms, mitomycin C (MMC) a crosslinking agent that leads to chromosome aberrations, and vinblastine sulfate (VB) a mitotic poison that results in aneuploidy. MN were induced in a dose-dependent fashion either by adding genotoxins directly to the medium that resulted in exposure of the tissue from the basal surface, or by direct application of the genotoxins to the apical surface of the tissue. Based on the success of these initial studies, we have expanded our work to refine the assay procedures and build the database to allow us to begin addressing predictive capability. Since one of the primary objectives was to develop an assay that provided a more relevant assessment of potential genotoxicity, particularly a lower rate of false positive results, we felt that it was important early-on in the development process to evaluate chemicals negative in rodent dermal carcinogenicity studies. In this paper we report negative results for 5 dermal non-carcinogens. In addition, we evaluated 7 other model genotoxins including genotoxic dermal carcinogens. These genotoxins induced statistically significant increases in micronuclei in the RSMN. We also report further investigation of additional experimental parameters such as length of exposure and concentration of cytochalasin B and provide more detail to the procedures described previously.

2. Materials and methods

2.1. Chemicals and reagents

Methyl methane sulfonate (MMS), N-methyl-N'-nitro-N-nitrosoguanidine (MNNG), N-ethyl-N'-nitro-N-nitrosoguanidine (ENNG), 2-phenylphenol (2-PP), 4-nitrophenol, 1,2 epoxydodecane, trichloroethylene, 2-ethyl 1,3 hexanediol, beta-butyrolactone (BBL), dimethylcarbamyl chloride (DCC), n-methyl-nitrosourea (MNU), and n-ethylnitrosourea (ENU), cell culture grade mitomycin C (MMC) with NaCl, cytochalasin B (cytoB), dimethyl sulfoxide (DMSO), methanol, acetic acid, potassium chloride (KCl), trypan blue and acridine orange (AO) were obtained from Sigma–Aldrich Corporation (St. Louis, MO). Trypsin (0.25%)-EDTA (0.02%) was obtained from JRH Biosciences (Lenexa, KS). EPI-100-NMM New Maintenance Medium (NMM) was obtained from MatTek Corporation (Ashland, MA). Dulbecco's phosphate buffered saline (DPBS), Dulbecco's Modified Eagle's Medium (DMEM), Fetal Bovine Serum (FBS) and L-glutamine were obtained from Quality Biological (Gaithersburg, MD).

2.2. Stock chemical solutions

MMC was used as the positive control and was tested concurrently with all test chemicals. Cell culture grade MMC with NaCl (Sigma Cat. No. M 4282) was used, rather than MMC without NaCl as described previously [10], to reduce possible variability in the quality of the MMC stock solutions. A stock solution of MMC (1 mg/ml) was prepared by adding sterile tissue culture grade water at room temperature (2.0 ml) to a vial of MMC (2 mg per vial). The vial was then vortexed to completely dissolve the MMC. Aliquots (100 μl) of stock of MMC were stored at −15 to −25 °C. If a precipitate was observed after thawing, the vial was vortexed and/or sonicated to achieve a uniform suspension. Fresh dosing solutions in acetone were prepared for each assay (generally 10 and 3 μg/ml) and stored at 2–8 °C for the duration of the assay. However in more recent experiments – performed subsequent to those reported in this manuscript – a 0.5 mg/ml stock solution of MMC (2 mg of MMC with NaCl in 4.0 ml water) was used to minimize precipitation during the preparation of the dosing solutions. This modified procedure is currently in use at all laboratories associated with this publication.

Cytochalasin B (cytoB) was prepared by adding either 3.3 ml of dimethylsulfoxide (DMSO) to a vial containing 10 mg of cytoB, then vortexing the vial to completely dissolve the cytoB. Aliquots of 3 mg/ml stock cytoB were stored in cryovials at −15 to −25 °C. For each experiment, an aliquot of stock cytoB was thawed and diluted in NMM to the required concentration (generally 3 μg/ml). NMM supplemented with cytoB was warmed to 37 °C before use and then stored at 2–8 °C for the duration of the assay.

2.3. Tissue constructs

EpiDerm™ tissue (EPI-200-MNA) was obtained from MatTek Corporation. The tissues were supplied on 0.64 cm^2 cell culture inserts. Detailed tissue handling procedures have been described previously [10]. Tissues with signs of detachment from the underlying membrane ("blisters") or with medium pooled on the surface of the tissue (possibly indicating a compromised stratum corneum) were discarded. Upon arrival the tissues were transferred to the wells of six-well plates containing 1 ml of fresh 37 °C NMM and incubated at 37 ± 1 °C and 5 ± 1% CO_2. Culture medium was replaced every 24 h.

2.4. Treatment of tissues with test chemicals

Test chemicals (see Table 1 for more information) were administered topically as described previously [10]. In brief, tissues were equilibrated overnight in 6-well plates containing 1.0 ml NMM per well. The culture medium was then replaced with NMM containing 3 μg/ml cytoB, and 10 μl of the dosing solutions were applied topically to the tissues. Topical dosing and replacement of the culture medium with fresh medium containing cytoB was repeated after another 24 h, and 24 h after that (48 h after the first test chemical treatment) the tissues were collected for analysis. In some cases, the dosing and refeeding was repeated at 48 h and the tissues were collected at 72 h. For these studies, tissues were equilibrated in NMM for 1 h before the first dosing and addition of cytoB. Within each independent experiment, acetone alone was included as a negative or solvent control, and MMC was included as a positive control.

Doses were selected based on toxicity. In a typical assay, a series of concentrations of the test article were evaluated, from concentrations that induced no toxicity, up to concentrations that induced around 70% toxicity (30% survival) as suggested in the draft OECD guidelines [12] that were in place at the time of these studies for the *in vitro* micronucleus assay. Currently, updated guidelines [13] are available, which suggest evaluation only for treatments causing less than 50 ± 5% cytotoxicity.

2.5. Harvesting cells from the basal cell layer

EpiDerm™ tissues were processed for micronucleus analysis as described previously [10], with the following refinements. For the trypsinization step, each tissue insert was placed in 5 ml DPBS at room temperature for 5–15 min, then in 5 ml of EDTA (0.1%) at room temperature for 15 min, then exposed to warm (~37 °C) trypsin-EDTA solution for 15 min. Each tissue was carefully lifted from the supporting membrane with fine forceps. Both the detached tissue and the supporting membrane were transferred to a new well containing 1 ml of 37 °C trypsin-EDTA. The supporting membrane was thoroughly rinsed (4–6 times) with trypsin-EDTA to collect any remaining basal cells, and then discarded. The tissue was agitated to release additional attached cells. Any cell clumps were disrupted by repeatedly drawing the cell suspension into a pipette and gently expelling the