Development of a Novel Micronucleus Assay in the Human 3-D Skin Model, EpiDerm™.

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Abstract

The rodent in vivo micronucleus assay is an important part of a tiered testing strategy in genetic toxicology. However, this assay, in general, only provides information about materials available systemically, not at the point of contact, e.g. skin. Although in vivo rodent skin micronucleus assays are being developed, the results will still require extrapolation to the human. Furthermore, to fully comply with recent European legislation such as the 7th Amendment to the Cosmetics Directive, non-animal test methods will be needed to assess new chemicals and ingredients. Therefore we have begun development of a micronucleus assay using a commercially available 3-D engineered skin model of human origin, EpiDerm™ (MatTek Corp, Ashland, MA). We first evaluated whether a population of binucleated cells sufficient for a micronucleus assay could be obtained by exposing the tissue to 1-3 ug/ml cytochalasin B (Cyt B). The frequency of binucleated cells increased both with time (to at least 120 h) and with increasing concentration of Cyt B. Three ug/ml Cyt B allowed us to reliably obtain 40-50% binucleated cells at 48h. Mitomycin C (MMC) was then used (in the presence of 3 ug/ml Cyt B) to investigate toxicity and micronuclei formation in EpiDerm™. Exposing the tissue directly through the growth medium for 48h gave a dose response for toxicity between 0.03 and 0.6 ug/ml. Maximum micronuclei induction (~5%) occurred at 0.1-0.3 ug/ml MMC. Experiments conducted with and without Cyt B indicated higher frequencies in the presence of Cyt B as expected. A topical application protocol was then developed using two 10 ul (per 0.64 cm2 tissue) applications of MMC in ethanol 24 and 48h prior to harvest. Maximum micronucleus response (~8%) and toxicity occurred with applications of 6-60 ug/ml MMC. The background frequency of micronuclei was very low (~0.1%). These studies show that micronuclei can be reproducibly induced in a 3-D skin model and are the first steps in developing a routine "in vivo-like" assay for chromosomal damage in human tissue.

Introduction

The in vitro micronucleus assay has been well established for use in detecting potentially clastogenic and aneugenic chemicals (1, 2). Its acceptance has been especially helped by the development of the cytokinesis-block methodology (3) which allows the identification of cells which have undergone one nuclear division, and modulates differences in cell division kinetics due to cytotoxicity, growth conditions or cell origin. However, because there is a need to know whether chemicals which are active in vitro are also bioavailable and active in the whole organism using conventional exposure techniques, in vivo micronucleus techniques have been developed and standardized for several organ/tissue systems, but most often use those of the hematopoietic system (e.g. 4).

Recently an interest has developed in utilizing a tissue, skin, that is often a target organ for human exposure, both from natural substances and consumer products (5-8). However, these methods generally use either the rat or mouse and may not be completely reflective of what would occur in humans. In addition, new regulations in Europe such as the 7th Amendment to the Cosmetic Directive and the REACH program, will make it very difficult, and in some cases impossible, to use animal models in safety assessments.

Therefore we have begun investigations to determine if human three-dimensional skin constructs can be used as a target system for micronucleus studies. Theoretically such models could not only reflect the complexities typical of in vivo exposures, e.g. absorption, tissue specificity, metabolism, etc., but at the same

time reflect human-specific responses in these parameters. We have used a commercially available skin model, EpiDerm™ (MatTek Corp., Ashland, MA), to begin our studies with the model genotoxicants, mitomycin C (MMC) and vinblastine sulfate (VB). We show here that the background frequency of micronuclei is very low and reproducible, and that high frequencies of micronuclei can be induced by both topical exposure and exposure through the medium.

Materials & Methods

Test Chemicals

Mitomycin C, Sigma (St. Louis, MO)
Vinblastine Sulfate, Sigma (St. Louis, MO)

Reagents

Acetone, Aldrich Chemical Co.
Acridine Orange Solution (10 mg/ml), Sigma, (final concentration 40µg/ml)
Calcium/Magnesium-Free Dulbecco's Phosphate Buffered Saline (DPBS) (MatTek, Ashland, MA)
Cytochalasin B, Sigma (St. Louis, MO)
DMSO, Sigma-Aldrich (St. Louis, MO)
EDTA 1g/L, Quality Biological (Gaithersburg, MD)
Ethanol, Pharmco
KCl (0.075M), Sigma
Methanol, Sigma/ Glacial Acetic Acid, Sigma (3:1)
Trypan Blue, Sigma (St. Louis, MO)
Trypsin (0.25%) - EDTA (0.02%), JRH Biosciences (Lenexa, Kansas)
Trypsin Neutralizing Solution: Cell culture media containing 10% serum

Tissue Constructs

EpiDerm™ Tissue (EPI-200), MatTek Corporation (Ashland, MA)

EpiDerm™ tissue is a multilayered, differentiated, tissue consisting of basal, spinous, granular and cornified layers (see 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, 8 mm ID 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 insert sits 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 to the user cold overnight in 24 well plates.

Figure 1. Cross section of an EpiDerm™ tissue on arrival at the laboratory. H&E. Photo courtesy of J. Harbell, IIVS.

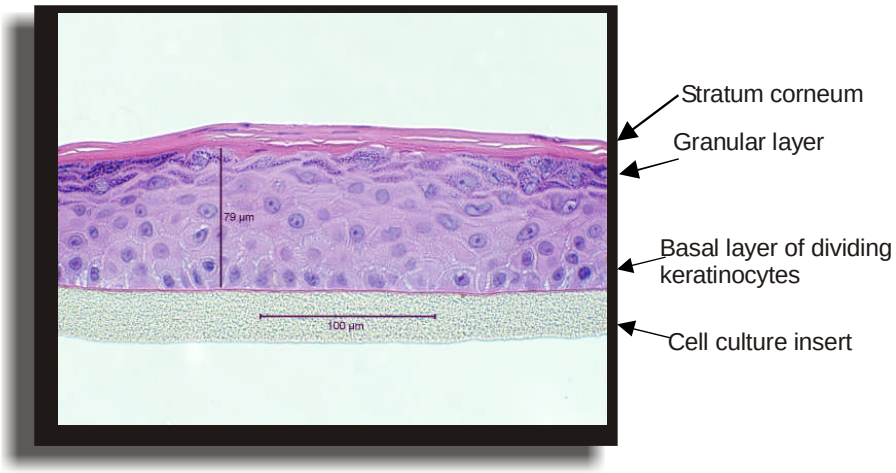
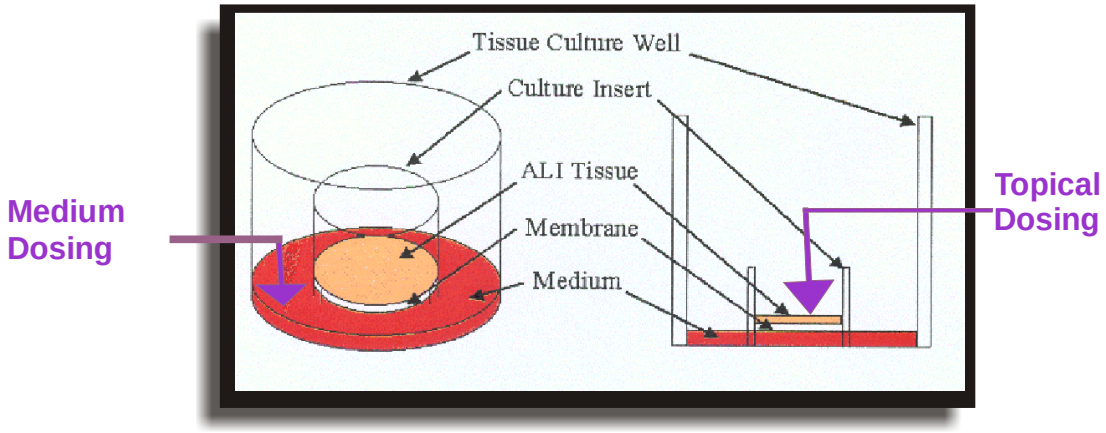


Figure 2. A schematic representation of the EpiDerm™ tissue as used in these experiments. The air-lifted (ALI) tissue sits on a collagen coated membrane with its stratum corneum exposed to the atmosphere. It is fed by medium diffusing through the membrane to the basal cells and above. Diagram courtesy of MatTek Corp.



All experiments described here were conducted with tissue from a single donor. To do this the original monolayer keratinocyte culture was expanded by the manufacturer, and the resulting cells cryopreserved in multiple stock vials. Individual vials from this same freeze lot were then used to establish the three-dimensional cultures before each use.

Cells were grown either in medium EPI-100-NMM or EPI-100-LLMM (MatTek Corp.). Both were designed to maintain acceptable differentiated morphology for one week or longer.

Tissues arriving from the manufacturer were unpacked, observed for any gross morphological defects, placed in 6-well tissue culture plates, fed with 1ml medium NMM (or other medium as noted), and incubated for approximately 24h (unless otherwise noted) at 5% CO_2 and 37C. Treatment was initiated 24h after receipt (Time 0).

Treatment Conditions

Tissues were treated with test chemical either topically or through the medium (Figure 2). For topical dosing, the test material was prepared in the appropriate solvent (generally EtOH), and 10ul was applied directly to the surface of the EpiDerm™ tissue with a micropipette.

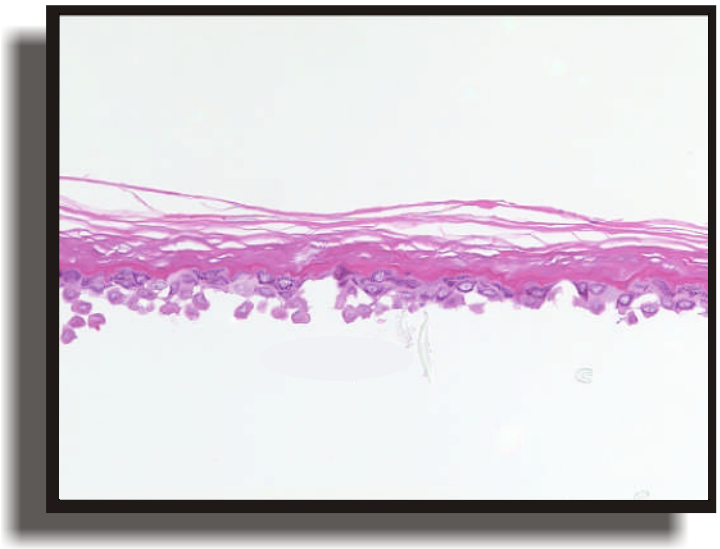
Cell harvesting & slide preparation procedure

Approximately 24 hours following the final dosing, tissues were harvested in the following fashion:

1. Tissues were washed with DPBS, followed by EDTA exposure, and then exposed to trypsin at room temperature.
2. Tissues were then separated from the membrane and again exposed to trypsin.
3. Trypsin neutralizing solution was added to the cell suspension before centrifuging at 100Xg for 5 minutes.
4. After centrifugation, 1 ml of warm (~37^{o}C) KCl was slowly added to the cells followed by 3 ml of cold MeOH/acetic acid (3:1).
5. The cell suspension was recentrifuged, the medium removed and 4 ml of MeOH/acetic acid were added.
6. The cell suspension was again centrifuged and the cells were gently placed on the slide.
7. Slides were stained in AO solution for 2 to 3 minutes, rinsed, and then scored using a fluorescent microscope.

Tissue Remaining After Trypsinization

After trypsinization and cell harvest, a “pad” of material remains which the photomicrograph below shows to be the stratum corneum with some of the granular layer still attached. All other cells, including the basal layer, appear to have been collected in the harvest.



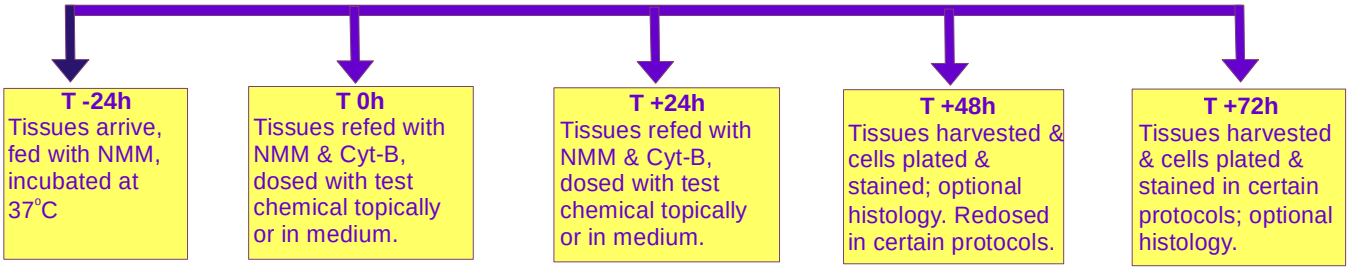
Micronucleus Scoring

In general, 500 total cells were scored per tissue to determine the percentage of binucleate cells, and 1000 cells, when possible, were scored to determine the frequency of micronucleated, binucleate cells.

Histology

In general, tissues to be processed for histology were fixed for at least 24h in 10% buffered formalin by immersing the entire tissue insert in the fluid. Formalin-fixed tissues were then sent to a commercial histology lab for embedding, sectioning and staining. All slides were evaluated at IIVS.

Typical Treatment Protocol



RESULTS

Determination of Cytochalasin B Dose and Exposure Time

EpiDerm™ tissue was treated with various concentrations of Cyt B (Fig 3) in the medium (generally NMM) to determine a) whether the rate of cell division in the tissue was sufficiently high to give a reasonable amount of binucleate cells for counting, and b) what dose of Cyt B was optimal for the production of binucleate cells. In the first trials, cells were harvested at 90 and 144h, and it was clear that treatment with 3 ug/ml Cyt B gave a satisfactory number of binucleate cells. Subsequent experiments at shorter time periods and with 2 and 3 ug/ml Cyt B (Fig 4) gave similar results and indicated that the cell division was sufficient to reproducibly yield 40% - 50% binucleate cells after 48h treatment with 3 ug/ml Cyt B.

Figure 3. Determination of Cyt B dose. EpiDerm™ tissues were refed every 24h with LLMM medium containing the indicated amount of Cyt B. Each bar represents the average of two separate experiments.

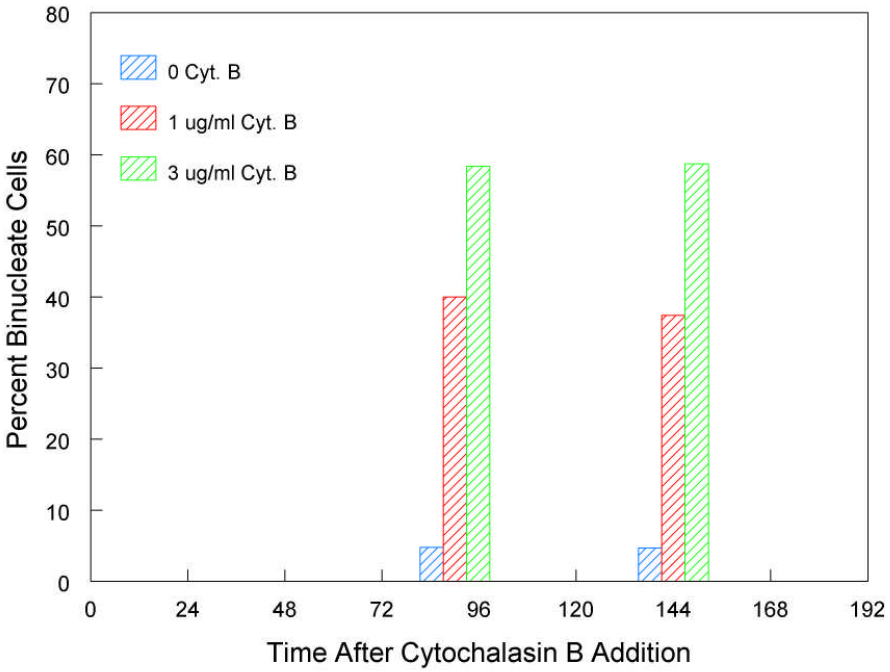
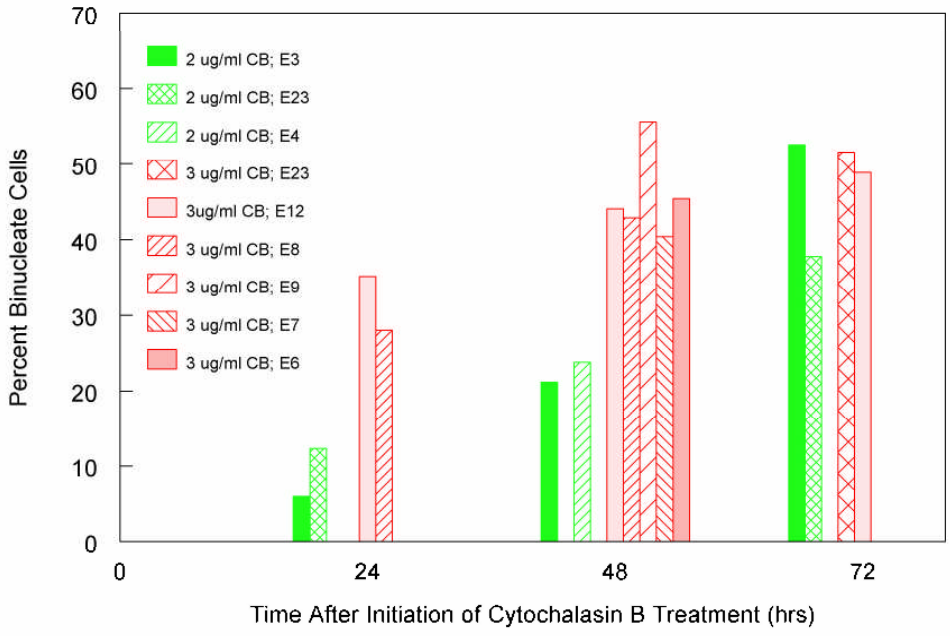


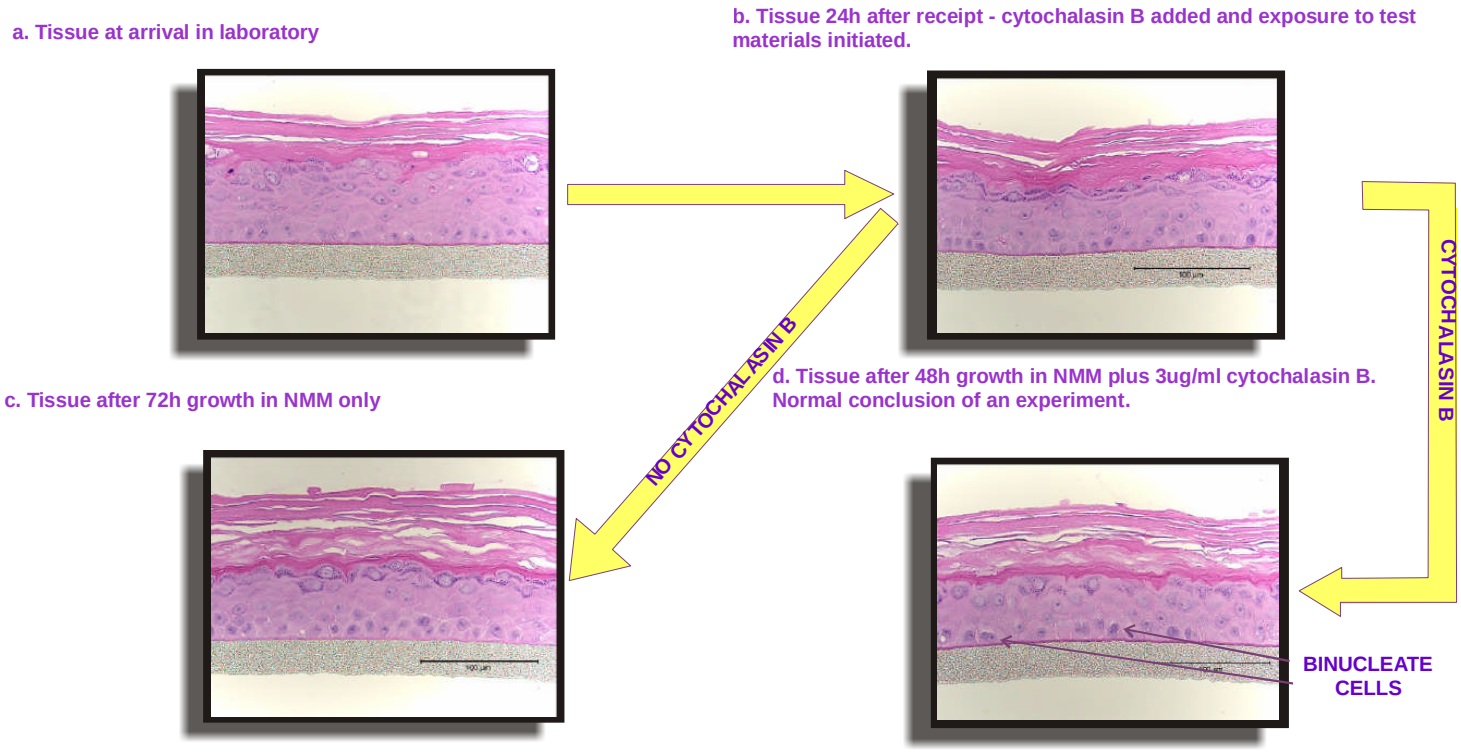
Figure 4. EpiDerm™ tissues were refed every 24h with medium (NMM) containing the indicated amounts of Cyt B. The results for eight different experiments are shown. Each bar represents the results from a single tissue. Bars with the same color and design indicate tissues from the same EpiDerm™ lot treated at the same time.



Normal Tissue Morphology Over Time and Effect of Cytochalasin B

Since EpiDerm™ is a living, continually differentiating tissue, we were concerned how well tissue morphology was maintained over the 2-4 day course of an experiment both in the presence and absence of Cyt B. Single EpiDerm™ tissues were grown in NMM and NMM containing 3ug/ml Cyt B, and the tissues were harvested at 0 through 72h. It can be seen in Fig 5 that some decrease in number of cell layers and an increase in the stratum corneum occurs over 72h in the presence in NMM. Greater changes in morphology can be seen with Cyt B, since far fewer (or no) new cells (due to the action of Cyt B) are being created in the basal layer to replace those that are continuing to terminally differentiate in the upper layers.

Figure 5 a-d. Photomicrographs of the morphology of EpiDerm™ grown (a-c) in medium NMM for 72h (normal duration of a 48h exposure experiment), or (d) in medium NMM for 24h followed by NMM + 3 ug/ml Cyt B for 48h.



Effect of Solvents

A series of solvents were tested to determine if they would affect either cell cycle or tissue morphology. Table 1 shows that ethanol, acetone and a 4:1 mixture of acetone and oil can be used in volumes (2X applications) up to 20 ul. Saline when used at a higher volume of 100 ul also appears to inhibit the cell cycle and has a definite negative effect on morphology causing a tissue with only 1 or 2 viable cell layers.

Table 1. EpiDerm™ cultures received two applications 24h apart of the indicated amount of solvent. Cells were harvested 24h after the final application (48h after initiation of treatment) and percentage of binucleated cells was determined. One EpiDerm™ tissue was sampled for each condition.

Solvents Tested	% Binucleate cells
Untreated	36.9%
10 ul EtOH*	47.3%
20 ul EtOH	44.4%
10 ul acetone	46.6%
20 ul acetone	45.3%
10 ul DMSO	29.2%
10 ul acetone:oil (4:1)	58.2%
20 ul acetone:oil (4:1)	55.1%
100 ul Saline**	17.8%

* = Histology of was equivalent to that of the untreated tissue.
** = Histology indicated extreme differentiation of tissue with only one or two viable cell layers. This response was confirmed in subsequent studies.

Cytotoxicity and Induction of Micronuclei - Dosing in Medium

To determine if micronuclei could be induced in EpiDerm™ tissue, test materials (MMC and VB) were applied directly into the growth medium to expose epidermal cells by diffusion through the membrane. The maximum micronucleus frequency was found between 0.06 and 0.6 ug/ml MMC (Fig 6), and the dose dependent cytotoxicity is shown in Table 2. Dose dependent cytotoxicity and micronucleus induction after VB treatment is shown in Table 3.

Photographic examples of representative micronuclei identified are shown in Fig 7.

The effect of the presence of Cyt B on micronuclei induction by MMC (Fig 8) was investigated by exposing two groups of tissue one continually in the presence of Cyt B, the other in the absence of Cyt B to MMC for 24, 48, or 72 h. We found that micronuclei were induced over a similar dose range under both conditions, but that the absolute frequency was much higher when only the cells known to have divided (binucleate cells) were included in the calculation.

Figure 6. Micronucleus induction in EpiDerm™ tissue after exposure to MMC in the medium. Tissue was refed once after 24h exposure.

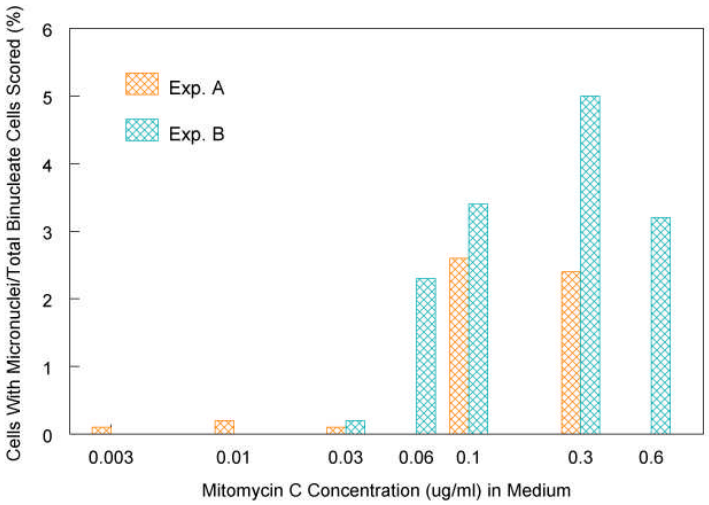


Table 2. Cytotoxicity of MMC as evidenced by reduction in the number of binucleate cells after 48h exposure. Exp. A & B are the same as in Fig 6.

MMC Concentration (ug/ml)	% Binucleate Cells	
	Exp. A	Exp. B
0	50%	44.1%
0.003	57.8%	
0.01	47.4%	
0.03	52.2%	38.9%
0.06		33.0%
0.1	39.1%	33.8%
0.3	9.9%	15.5%
0.6		4.9%

Table 3. Cytotoxicity of VB as evidenced by reduction in the number of binucleate cells after 48h exposure.

VB Concentration (ug/ml)	% Binucleate Cells		Micronuclei Freq.
	Exp. A	Exp. B	Exp. B
0	55.6%	46.8%	<0.1%
0.006	36.8%		
0.01	40.0%	63%	0.1%
0.03	47.6%	61.6%	0.4%
0.06		47.0%	NC
0.1	45.9%	36.7%	0.6%
0.3	36.2%		
0.6	19.3%		

Figure 7 a&b. Examples of micronuclei identified in binuclear human epidermal cells isolated from EpiDerm™ tissues after treatment with MMC

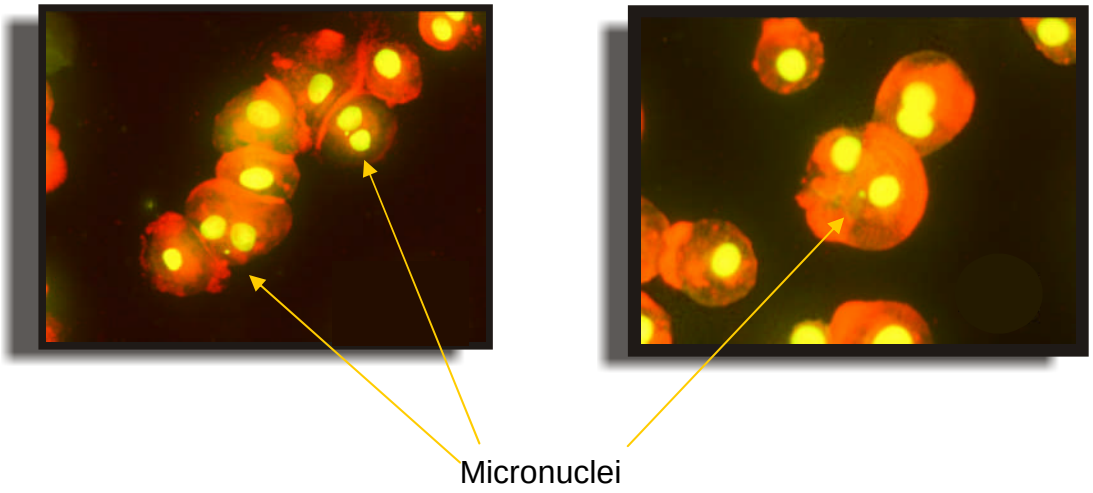
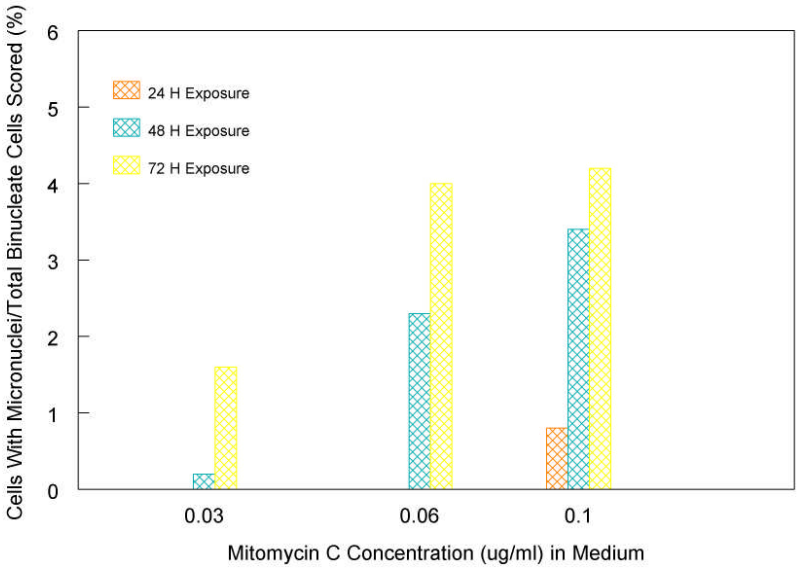
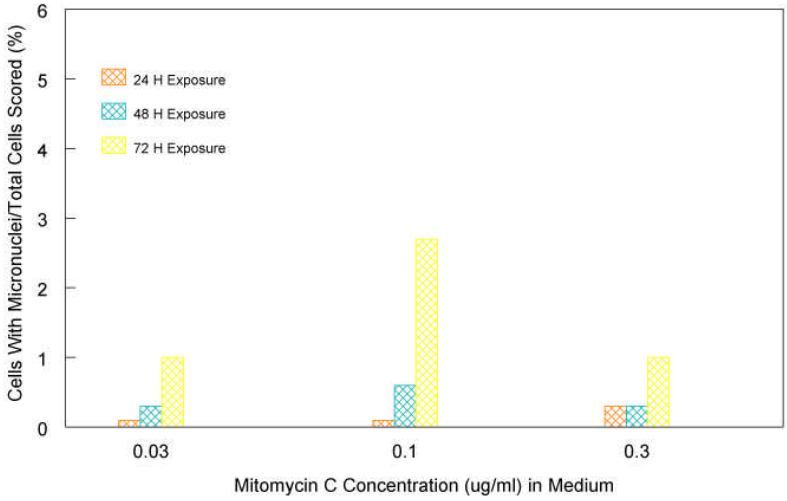


Figure 8 a & b. Effect of Cyt B on micronucleus induction in EpiDerm™ tissue. Tissues were exposed to increasing concentrations of MMC either in a) the presence or b) the absence of Cyt B. Tissue was refed with the appropriate fresh medium and MMC every 24h after the initial exposure.

a. Exposure in the presence of Cytochalasin B.



b. Exposure in the absence of Cytochalasin B.



Cytotoxicity and Induction of Micronuclei - Dosing by Topical Application

To determine if micronuclei could be induced in EpiDerm™ tissue by topical application, test materials (MMC and VB) were applied directly onto the surface of the tissue. Since the tissue seems to form a tight seal with walls of the insert, it was assumed that the epidermal cells were exposed to the test material only after it passed through the stratum corneum barrier. Maximum induction of micronuclei by MMC was found after 2X application of 10-100 ug/ml MMC in 10 ul EtOH (Fig 9). The dose dependent cytotoxicity is shown in Table 4. Maximum induction of micronuclei by VB under the same conditions was found between 0.03 and 0.6 ug/ml (Table 5). The dose dependent cytotoxicity of VB is shown in Table 5.

Background Micronucleus Frequency

In our laboratory, the background level of micronucleated cells per binucleated cell in EpiDerm™ tissue is <0.12% +/- 0.06%; range = <0.1% - 0.3%; N=10. This frequency is considerable lower than that found for many other cell types grown in monolayer or suspension culture.

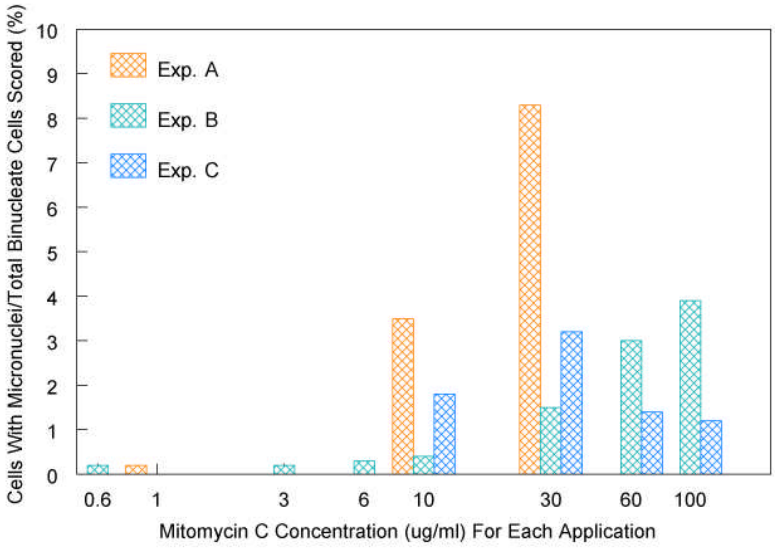
Table 4. Cytotoxicity of MMC dosed topically to the EpiDerm™ tissue as evidenced by reduction in the number of binucleate cells. Exp. A & B are the same as shown in Fig 9. NT = Not Tested; * = Ave. of two tissues.

MMC Concentration (ug/ml in EtOH); 2 10 ul applications	% Binucleate Cells		
	Exp. A	Exp. B	Exp. C
0	42.3%	42.2%	35.9%*
0.6	NT	38.4%	NT
1.0	55.4%	28.1%	NT
3.0	48.1%	37.4%	NT
6.0	NT	38.6%	NT
10.0	32.2%	40.7%	14.3%
30.0	19.9%	21.8%	11.6%
60.0	9.9%	7.9%	8.4%*
100.0	NT	8.7%	9.8%*

Table 5. Cytotoxicity and induction of micronuclei after topical application of VB to EpiDerm™ tissue. NC = Not Counted.

VB Concentration (ug/ml in EtOH); 2 10 ul applications	% Binucleate Cells		Cells with micronuclei per total binucleate cells scored (%)	
	Exp. A	Exp. B	Exp. A	Exp. B
0	42.3%	34.2%	<0.1%	<0.1%
0.01	49.5%	45.1%	0.1%	<0.1%
0.03	44.1%	27.3%	<0.1%	0.3%
0.1	51.7%	37.3%	0.5%	0.5%
0.3	20.5%		<0.1%	
0.6		38.8%		0.4%
1.0	18.7%		NC	
3.0	7.2%		NC	

Figure 9. Micronuclei response in EpiDerm™ tissue after exposure to MMC by topical application. Tissue was dosed twice, 48h and 24h prior to harvest of the tissue.



CONCLUSIONS

- Epidermal cells in three-dimensional reconstructed human skin models e.g. EpiDerm™, are susceptible to cytokinesis blockage by cytochalsin B.
- Binucleate cells can be reproducibly obtained from EpiDerm™ cultures at satisfactory levels (40 - 50%) after 48h treatment with 3 ug/ml cytochalasin B.
- A cytotoxicity dose response to MMC and VB can be demonstrated in EpiDerm™ cultures either exposed topically or via the medium.
- There is a low, reproducible background frequency of micronucleated cells in the EpiDerm™ cultures.
- Increases in micronucleated cells can be found after treatment (topically and through the medium) with MMC & VB. There is a clear dose-dependent response for micronuclei induction with both chemicals.
- There is variability in individual tissue response that occurs within experiments which will have to be addressed in future studies.

REFERENCES

1. Kirsch-Volders M, Sofuni T, Aardema M, Albertini S, Eastmond D, et.al. (2003) Report from the in vitro micronucleus assay working group. Mutat. Res. 540(2):153-163.

2. Garriott, M.L., Phelps, J.B., and Hoffman, W.P. (2002) A protocol for the in vitro micronucleus test I. Contributions to the development of a protocol suitable for regulatory submissions from an examination of 16 chemicals with different mechanisms of action and different levels of activity. Mutat. Res. 517: 123-134.

3. Fenech, M., and Morley, A.A. (1986) Cytokinesis-block micronucleus method in human lymphocytes: effect of in vivo aging and lowdose X-irradiation. Mutat. Res. 161:193-198.

4. MacGregor, J.T., Heddle, J.A.., Hite, M., Margolin, B.H., Ramel, C., Salamone, M.F., Tice, R.R., and Wide, D. (1987) Guidelines for the conduct of micronucleus assays in mammalian bone marrow erythrocytes. Mutat. Res. 189: 103-112.

5. Nishikawa, T., Haresaku, M., Adachi, K., Masuda, M., and Hayashi, M. (1999) Study of a rat skin in vivo micronucleus test: data generated by mitomycin C and methyl methanesulfonate. Mutat. Res. 444, 159-166.

6. Nishikawa, T., Haresaku, M., Fukushima, A., Nakamura, T., Adachi, K., Masuda, M., and Hayashi, M. (2001) Further evaluation of an in vivo micronucleus test on rat and mouse skin: results with five carcinogens. Mutat. Res. 513, 93-102.

7. Weiner, S.K., Schuler, M.J., Ku, W.W. and Guzzie, P.J. (2001) Evaluating the use of the in vivo rat skin micronucleus assay for the detection of genotoxic agents following topical application. EMS Poster Presentation 2001.

8. Gibson, D.P. and Aardema, M.J. (2003) The in vivo mouse skin micronucleus assay for detecting site of contact genotoxicity induced after dermal exposure. EMS Poster Presentation 2003.

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