Skin toxicity determined in vitro by three-dimensional, native-state histoculture

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ABSTRACT We describe a gel-supported in vitro system for culturing skin samples in a three-dimensional native state. All the cell types of skin remain viable and maintain their native architecture for at least 10 days. The culture system is used for toxicity measurements by ascertaining cell viability using two fluorescent dyes: 2′,7′-bis(2-carboxyethyl)-5′-(and -6′)carboxyfluorescein acetoxyethyl ester, specific for living cells, and propidium iodide, specific for dead cells. Cell staining with the dye is measured through the tissue block by confocal scanning fluorescence microscopy. The dose-response to three agents—ethanol, doxorubicin, and sodium hypochlorite—is studied, and, in the case of sodium hypochlorite, compared to in vivo skin toxicity with a high correlation. We also demonstrate that the end point of [3H]thymidine incorporation measured by histological autoradiography can be used to measure toxicity. Our results with [3H]thymidine end point demonstrate that the hair follicle cells are the most sensitive to doxorubicin. The native-state model for skin may be an effective replacement for animal systems and superior to the dispersed skin cells systems used previously. It can allow rapid, inexpensive measurements of the effect of manufactured products, drugs, and pollutants on skin.

Determining the toxicity of various substances in animals is expensive, time-consuming, and sometimes misleading because of the complexity of in vitro systems. The need for a suitable in vitro system for the study of skin toxicology has long been recognized (1). In vitro systems have the benefit of allowing the rapid, inexpensive study of a large variety of substances, as well as replacing animals (2).

A number of attempts to develop in vitro systems for toxicology for skin have been reported (3-5). Based on monolayer cell cultures, a toxicity test system has certain advantages. However, the model is oversimplified and distant from the in vivo situation in which total effects are, in part, mediated by complex cell-cell interactions not occurring in monolayers. A three-dimensional reconstructed skin model has been established (6) by seeding dermal fibroblasts onto a nylon mesh to form a dermal equivalent. Melanocytes and keratinocytes are inoculated onto the dermal equivalent and remain above the dermal-epidermal junction. Although this model is closer to the in vivo state than monolayers, it still is markedly dissimilar from true skin. To more closely approximate the in vivo state, skin samples from the mouse, rat, rabbit, guinea pig, marmoset, and human have been cultured but these remain viable for only 24 hr (7).

An adequate in vitro model for toxicology testing requires relatively long-term culture of human and animal skin. The important cellular components must remain viable and grow while maintaining their native tissue architecture. Such a culture system permits study of long-term effects of various agents, such as toxicity, and even malignant transformation. We report here the development of a gel-supported, three-dimensional histoculture system that maintains viability, cell proliferation, and intact tissue architecture of all components of mouse skin for at least 10 days.

Staining with fluorescent dyes allows visualizing all cells within the skin and determines their viability. The staining throughout the skin sample is visualized by using the very large working distances afforded by confocal scanning microscopy. The system described provides an easily quantifiable toxicity assay by using one dye to measure viable cells and another to determine dead cells. Alternatively, we demonstrate here that [3H]thymidine incorporation end points measured by histological autoradiography can determine the effects of toxins on cell proliferation. The in vitro effects of toxins are demonstrated, and those of sodium hypochlorite are compared to the results of the agent in vivo.

MATERIALS AND METHODS

Histoculture of Skin. We have used the method developed earlier by Hoffman et al. (8-12) based on the work of Leighton (13) to culture various tumor and normal tissues. Small pieces of intact athymic nude mouse skin (~2 × 5 mm² and 2.0 mm thick) were cut with scissors under a dissecting microscope and put onto collagen-containing sponges or gels in histoculture in medium as soon as possible. The use of skin from nude mice eliminated the need to shave the animals. Eagle's minimum essential medium supplemented with 10% fetal bovine serum and gentamycin was used and cultures were maintained at 37°C in a gassed incubator with a mixture of 95% air/5% CO₂.

Confocal Microscopy. The confocal microscopy system was with an MRC-600 confocal imaging system (Bio-Rad) mounted on a Nikon Optiphot using a 10× PlanApo objective.

Fluorescence Microscopy. For standard microscopy, a Nikon fluorescence microscope, equipped with fluorescein and rhodamine cubes, was used.

Fluorescence-Dye Labeling of Live and Dead Cells. Viable cells were selectively labeled with the dye 2′,7′-bis(2-carboxyethyl)-5′-(and -6′)carboxyfluorescein acetoxyethyl ester (BCECF-AM), which is activated to fluorescence by nonspecific esterases present only in living cells (14). Nonviable cells, whose plasma membranes are leaky, were labeled with propidium iodide (PI); a dye that enters only cells with nonintact membranes (14). Since the emission spectra of these two dyes were different they could be used simultaneously on the same specimen. Both dyes were used at a concentration of 5 µM. The double dye-treated cultures were analyzed by fluorescence and confocal microscopy within 30 min of staining.

Abbreviations: BCECF-AM, 2′,7′-bis(2-carboxyethyl)-5′-(and -6′)carboxyfluorescein acetoxyethyl ester; PI, propidium iodide; PIIL, primary irritation index.

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**RESULTS**

Long-Term Intact Skin Cultures. We have used standard and confocal microscopy to analyze the skin histocultures grown for various periods of time. As shown in Figs. 1–3, the mouse skin histocultured on a floating collagen-containing gel in Eagle’s minimum essential medium preserves native-state three-dimensional tissue architecture of all the major cell types in the viable state. The hair follicles (Fig. 1), epidermal cells (Fig. 2), and dermal fibroblasts (Fig. 2) are readily visualized and appear to have normal morphology. The histocultures are viable for at least 10 days.

The cells in the skin histoculture retain their proliferative activity. DNA synthesis and replication are shown by histological autoradiography to occur in hair follicles, epidermal, and dermal cells after 6 days of histoculture (Fig. 3). With a 3-day labeling period with [3H]thymidine, the percentages of labeled cells for the follicle, epidermal, and dermal cells were 38.6%, 75.2%, and 10.2%, respectively.

![Fig. 1](#) (A) Ethanol toxicity dose–response of hair follicles of mouse skin histocultured for 2 days and double-stained with BCCF-AM (green, live cells) and PI (red, dead cells) before (left image) and after (right image) treatment. Top images are 0.5% ethanol treatment. Bottom images are 5% ethanol treatment. Confocal scanning laser microscopy. (B) Ethanol toxicity dose–response of hair follicles of mouse skin histocultured for 2 days and double-stained with BCCF-AM and PI before (left image) and after (right image) treatment. Top images are 30% ethanol treatment. Bottom images are 50% ethanol treatment. Confocal scanning laser microscopy. (×1000.)
Fluorescence Toxicity Test. Two fluorescent dyes, BCECF-AM and PI, staining viable and nonviable cells, respectively, were used to study the in vitro toxic effects of ethanol, doxorubicin, and sodium hypochlorite. The dose–response for sodium hypochlorite was compared to an in vivo animal test (see below).

Fig. 1 demonstrates the viability of hair follicles in cultured mouse skin before and after treatment with ethanol. Increasing concentrations of ethanol cause greater PI staining and less BCECF-AM staining, indicating loss of viability. Fig. 2 shows the toxicity of ethanol on the epidermal and dermal cells of the histocultured mouse skin. Before ethanol treatment most of the cells were viable, but treatment with larger amounts of ethanol caused more epidermal and dermal cells to be nonviable by the dual-fluorescence test. Fig. 4A is the dose–response curve of histocultured mouse skin to ethanol.

The doxorubicin toxicity dose–response curve of histocultured mouse skin demonstrates that increasing amounts of doxorubicin cause increasing percentages of nonviable cells as measured by the dual-fluorescence test shown in Fig. 4B.

**Fig. 2.** (A) Mouse skin histocultured for 2 days and double-stained with BCECF-AM (green, live cells) and PI (red, dead cells) before ethanol treatment. Note that most of the cells are viable (green). Note the distinct epidermal and dermal layers. Confocal scanning laser microscopy. (B) Same specimen as in A. BCECF-AM and PI staining after 70% ethanol treatment for 5 min. Note that all epidermal and dermal cells are dead (red). Confocal scanning laser microscopy. (×1000.)

**Fig. 3.** (A) Mouse skin histocultured for 8 days in Eagle's minimum essential medium and labeled with [3H]thymidine for days 3–6. Tissue was fixed and processed for autoradiography and stained with hematoxylin and eosin. Autoradiograms were observed with bright-field and polarizing light. [3H]Thymidine-labeled nuclei appear bright green. Note the high labeling index of hair follicle cells. (B) Same as A. Note the labeled epidermal cells. (A, ×1000; B, ×2000.)

**Autoradiographic Toxicity Test.** Another assay of toxicity uses [3H]thymidine incorporation and subsequent histological autoradiography. A 24-hr exposure of histocultured skin to doxorubicin inhibited DNA synthesis of histocultured skin cells as shown in Fig. 4C. The percentage of cell proliferation relative to control was greatly decreased with increasing concentrations of doxorubicin. The different cell types in skin had different responses to doxorubicin. DNA synthesis in the hair follicle cells was inhibited more than the epidermal or dermal cells and, therefore, they are most sensitive to this toxic agent (17).

**Irritation Test in Vivo Correlated to in Vitro Toxicity.** As shown in Fig. 4D, dose–response to sodium hypochlorite (bleach) was measured in vitro and in vivo. A high in vitro–in vivo correlation was found. In particular, the threshold of toxicity was similar in vitro and in vivo. Both the percentage of killed cells in vitro and the pH in vivo increased with a similar increasing concentration of sodium hypochlorite.

**DISCUSSION**

There are at least three problems in developing adequate in vitro toxicity test systems for the skin:

(i) If cell monolayers are used most of the specific cell–cell contact-dependent effects are lost, which can compromise the accuracy of modeling of tissue.

(ii) The three-dimensional cultures of the skin cells described so far either are constructed in a model not fully
representative of the in vivo state or fail to preserve the cells in the living state in intact skin cultured in vitro for more than a short period.

(iii) If the cells are growing in a three-dimensional system, standard microscopy cannot resolve the individual cells below the outer layer. Histological sectioning remains a rather complex and time-consuming procedure and does not allow for three-dimensional studies or real-time studies. Histological sectioning requires fixation of the sample, thus excluding the prolonged observation of the response of individual cells.

Here we describe a test system to evaluate skin toxicity that overcomes the above-described problems; a three-dimensional gel-supported, long-term histoculture that preserves native tissue architecture. Confocal laser fluorescent microscopy can observe individual fluorescent cells throughout the tissue. Finally, two fluorescent dyes were used to simultaneously evaluate the viability state of individual cells, or $[^3]$Hthymidine incorporation was measured by histological autoradiography to determine cell proliferation.

BCECF, when applied as the acetoxymethyl ester, penetrates the cell membrane and fluoresces only if cellular esterases are active; thus, reporting on cell viability. PI does not penetrate intact plasma membranes and thus stains only membrane-damaged cells (14). In preliminary experiments, we have shown that PI stains at least all the cells stained in the standard dye-exclusion tests with trypan blue (data not shown). After microscopic analysis, the stained specimens can be cultured further and re-stained for analysis. We have determined that analysis with these two dyes is complete; that is, every cell in the culture is stained by one of the dyes. We have never observed cells stained by the two dyes simultaneously.

We have used three substances as models to show that the test is quantitative, dose responsive, and in vivo-like. Two of the substances were ethanol and the chemotherapeutic drug...
doxorubicin, which is used to treat various malignancies such as breast cancer in spite of its high toxicity. In both cases, we observed a dose-dependent toxic effect according to our method. While ethanol kills cells independent of type, doxorubicin is preferentially toxic for hair-follicle cells. This effect of the drug may be related to the alopecia it causes in vivo. Thus, hair follicle cells could probably serve as the most sensitive indicators for in vitro toxicity of skin.

The third toxic agent, sodium hypochlorite, was measurable both in vitro and in vivo. Although the two measurable effects are different, they are compared here only to show that onset and saturation occur at similar concentrations of the toxic agent, suggesting a correlation between the in vitro and in vivo results for sodium hypochlorite.

In experiments using inhibition of \([\text{H}]\text{thymidine as an end point, we have shown that doxorubicin preferentially inhibits follicle cells, which mirrors the in vivo effect of doxorubicin. Inhibition of \([\text{H}]\text{thymidine incorporation may provide an intermediate end point with respect to cell death to measure the effects of substances at high levels of sensitivity.}}\)

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