Eye tissues grown in 3-dimensional histoculture for toxicological studies

We report here the long-term three-dimensional growth of human and mouse eye tissues, in particular conjunctiva and cornea, and their use in the development of an in vitro ocular safety assay. It has been demonstrated that tissues of the eye can be grown as intact tissue with the maintenance of tissue architecture in a viable state on collagen-gel sponges in vitro for a relatively long period. Human normal conjunctiva can be histocultured for at least 7 days, and mouse conjunctiva can be histocultured for at least 30 days. To develop an in vitro ocular-safety assay, the fluorescent dyes BCECF-AM and propidium iodide (PI) were used to identify living and dead cells in the histocultured eye tissues with analysis of the three-dimensional cultures by confocal scanning laser microscopy. The end-point of incorporation of [3H]thymidine into the cells of the cultured cornea and conjunctiva as measured by histological autoradiography was also utilized. Ethanol was tested as a model toxin for the fluorescent-dye end point. We have demonstrated an ethanol-toxicity dose response on histocultured human conjunctiva. To validate our methodology, ethanol toxicity on histocultured mouse eye tissue in vitro and the ethanol irritation of mouse eye tissue in vivo were compared and a high correlation was found. The long-term culture of conjunctiva that maintains intact tissue architecture such that in vitro toxicity correlates well with in vivo response should be useful for replacement of the controversal in vivo Draize test.

Key words: Eye tissue — Histoculture — in vitro toxicity test

Introduction

It is imperative to develop in vitro testing for ocular safety. In previous attempts to develop in vitro systems, the problem has been the choice of cell types. Cells such as corneal epithelium have been difficult to grow in large quantities, and other cell types have been used as substitutes leading to the question of their relevancy. It is thought there are five areas to consider for the development of in vitro protocols and these include cellular morphology, cytotoxicity, cell metabolism, cellular physiologic function, elicitation of inflammatory/immunologic stimuli, and recovery/repair [1]. It has been difficult to determine a cell type in vitro to measure the above parameters as end points for ocular irritancy. Tissue types that have been used for testing include fibroblasts [2], red blood cells and vaginal mucosa [3-6]. Chorioallantoic membrane preparations have also been utilized for predicting eye toxicity [4].

The need for ocular safety testing became obvious in the 1930's when an...
eyelash dye containing p-phenylene-dimine called Lashlure was marketed in the United States [7]. This and similar products caused corneal ulceration with loss of vision [8]. Based on this high toxicity, the Food, Drug and Cosmetic Act was passed in 1938 and test methods were proposed. There were a number of early publications suggesting the use of rabbits, with the accepted methodology being the publication of Draize et al [9]. The key aspect of Draize was the addition of a summary scoring system. There have been many modifications of the Draize test and a more generic term is now used in eye-irritant testing [8]. There are many problems with the use of animals such as rabbits to test eye irritancy including instilling 0.1 ml of a liquid and 0.1 g of a powder to one eye of each of six rabbits. The responses in the cornea, iris and conjunctiva are relatively subjective and there have been difficulties with standardization. In addition, there are variabilities in how long a time period should pass before the measurements are made, which can vary from 7 to 14 days.

Recently a non-cellular irritancy test has been developed termed Etyex [10]. The Etyex reagent is a lyophilized powder containing proteins, glycoproteins, mucopolysaccharides with buffer salts that are reconstituted with distilled water. A small sample of approximately 100 μl are added to the Etyex mixture with the reaction being measured by optical density units. This system is calibrated with varying amounts of a known chemical irritant. The Etyex test results have correlated well with results obtained in vivo. The original Etyex test was not useful for insoluble compounds. A modification of the Etyex assay called a membrane partition assay (MPA), a two-phase assay system for use with insoluble compounds has been developed. The Etyex MPA test samples are in a membrane cup incubated with Etyex reagents which are biological macromolecules which bind chemicals potentially toxic to the eye. The binding of chemical irritants to components of the Etyex reagent results in aggregation which can be measured optically [10].

Our approach has been to use the collagen-sponge gel native-state histoculture system which was originally developed by Leighton [11] and further developed and exploited by us to culture over 25 types of tumors [12-19] and to culture skin [20]. The histoculture system has shown that skin can be cultured intact for long periods of time and provides an excellent system to study toxicity of all the cell types in skin, including hair follicles which can be measured for cytotoxic responses of various products and reagents. We have taken this approach to study the tissues of the eye, growing them as intact tissue with the maintenance of tissue architecture on collagen gel sponges. We report here the long-term culture of human and mouse conjunctiva with intact tissue architecture and the ability to measure toxicity of an agent that correlates well with in vivo response.

**Results and discussion**

**Long-term eye histoculture**

Standard and confocal microscopy were used to analyze the eye tissues grown for various periods of time. As shown in Fig. 1, the mouse conjunctiva histocultured on a floating collagen containing gel in Eagle’s Minimum Essential Medium maintained its native-state three-dimensional tissue architecture. With regard to structure, one can see the histocultured mouse conjunctiva were viable for at least 30 days and the histocultured human conjunctiva were viable for at least 7 days.

**Fluorescence toxicity test**

Two fluorescent dyes, BCECF-AM and PI, staining viable and non-viable cells respectively, were used to study the in vitro toxic effects of ethanol. Figure 2 demonstrates by confocal microscopy the viability of human conjunctiva before and after treatment with increasing concentrations of ethanol. Increasing concentrations of ethanol caused greater PI staining and less BCECF-AM staining indicating loss of viability.
Fig. 1. Mouse conjunctiva tissue histocultured for 30 days and double-stained with BCECF-AM (green) and PI (red). Note most of the cells are viable (green). Confocal scanning laser microscopy. Stereo image. Magnification 1000x.

Fig. 2 a-f. Human normal conjunctiva tissue histocultured for 24 h and double-stained with BCECF-AM (green, staining living cells) and PI (red, staining dead cells). a, b and c are the histocultures before 5%, 30% and 70% ethanol treatment, respectively. Note most of the cells are viable (green). d, e and f are the same histocultures as a, b and c but after 5%, 30% and 70% ethanol treatment for 5 min, respectively. Note only small fraction of cells (red) were killed after 5% ethanol treatment (d), but after 30% ethanol treatment, the majority of cells were killed (red, e) and after 70% ethanol treatment, all of the cells were killed (red, f). Confocal scanning laser microscopy. Stereo images. Magnification 625x.
[¹H] thymidine incorporation in conjunctiva and cornea

Four-day cultures of mouse cornea revealed incorporation of [¹H]thymidine after three-days labeling in the majority of the corneal epithelial cells and in a minority of the associated stromal cells as seen in the histological autoradiogram in Fig. 4.

Four-day cultures of mouse conjunctiva revealed extensive incorporation of [¹H]thymidine in the conjunctival epithelium after three-days labeling and [¹H]thymidine incorporation in the associated stromal cells, as seen in Fig. 5.

Irritation test in vivo correlated to in vitro toxicity

To validate toxicity testing of eye tissues in vitro, primary eye irritation tests in vivo were performed on the mouse with ethanol. As shown in Fig. 3, there is a high correlation between eye tissue toxicity in vitro and eye irritation in vivo. The percentages of killed cells (PI-positive cells) increased along with the increasing concentration of ethanol both in vivo and in vitro.

The cultured tissue was killed by a model toxin, in this case ethanol, in a dose-dependent manner which matches in vivo response to the same toxin, thereby validating the in vitro system to replace in vivo eye toxicity tests such as the Draize test in many instances. The results suggest that conjunctiva and cornea cultured from one or two mice can replace many rabbits to study ocular toxicity.

It can be seen that cornea and conjunctiva from mouse and human can be cultured for long periods of time with maintenance of tissue architecture. We have demonstrated two end points that can be used to test toxicity, fluorescent dye-exclusion and inclusion, measured by scanning laser confocal microscopy as well as [¹H]thymidine incorporation measured by histological autoradiography utilizing a combination of bright-field and polarizing microscopy. The histocultured toxicity system described here should have many uses not only to measure toxins but to measure the physical effects of drugs effecting conjunctiva and cornea.

Materials and methods

Histoculture of eye tissues

Human conjunctiva from surgical biopsy, and fresh dissected mouse conjunctiva and cornea were
**Fig. 4.** Mouse cornea histocultured for 4 days and labeled with $[^3]H$thymidine for days 2-4 as described in the text. Histological autoradiograms were prepared and observed as described in the text. An IGS cube for polarization of light was used to observe the autoradiograms. The exposed silver grains over the radionabeled nuclei reflect the polarized light as bright green. It can be seen that the majority of the corneal epithelial cells have incorporated $[^3]H$thymidine during the 3-day labeling period. The stromal cells have also incorporated label but to a lesser degree. Magnification 540x.

**Fig. 5 a, b.** Mouse conjunctiva tissue cultured for 4 days and labeled with $[^3]H$thymidine for days 2-4 as described in the text. Histological autoradiograms were prepared and observed as described in the text. The bright green grains indicate radionabeled nuclei as described in Fig. 4. Note the extensive labeling of the conjunctival epithelial and relatively lesser labeling of the stromal cells. a magnification 270x; b magnification 540x.
used for histoculture and ocular toxicity assay experiments. Small pieces of each type of eye tissues (about 2 x 4 mm²) were put onto collagen-containing gels in histoculture for medium as soon as possible. The gels are derived from pigskin (Spongostan, Health Design, Rochester, NY). The dry gels were cut into 1-cm pieces and hydrated for at least 4 h in cell culture medium before use. The medium used was Eagle's Minimum Essential Medium supplemented with 10% fetal bovine serum, and gentamycin (0.1 mg/ml). Cultures were maintained at 37 °C in a gassed incubator with a mixture of 95% atmosphere: 5% CO₂.

Confocal microscopy

The confocal microscopy system used was an MRC-600 Confocal Imaging System (BioRad) mounted on a Nikon Optiphoto using a 10X PlanApo Objective.

Fluorescence microscopy

For standard microscopy, a Nikon fluorescent microscope, equipped with fluorescein and rhodamine cubes, was utilized.

Fluorescent-dye labeling of live and dead cells

Viable cells are selectively labeled with the dye BCECF-AM, which is activated to fluorescence by non-specific esterases present only in living cells [20]. Non-viable cells, whose plasma membranes are leaky, are labeled with propidium iodide (PI), a dye which enters only cells with non-intact membranes [20]. Since the emission spectra of these two dyes are different they can be used simultaneously on the same specimen. Both of PI and BCECF-AM were used at a concentration of 15 μM. The cultures were stained by BCECF-AM and PI for 30 min, then rinsed with Eagle's medium 3 times after staining. The double-dye-treated cultures were analyzed by fluorescence and confocal microscopy within 30 min of staining.

[^3]H]thymidine labeling of proliferating cells

Briefly, cells within the three-dimensional cultures capable of proliferation were labeled by administration of [^3]Hthymidine at 4 μCi/ml for 3 days. Cellular DNA is labeled in any cells undergoing replication within the tissues. After 3 days of labeling, the cultures were washed with phosphate-buffered saline, placed in histology capsules and fixed in 10% formalin. The cultures were then dehydrated, embedded in paraffin, and sectioned by standard methods. After the slides were deparaffinized, they were prepared for autoradiography by coating with Kodak NTB-2 emulsion and exposed for 5 days, after which they were developed [12-20]. After rinsing, the slides were stained with hematoxylin and eosin. The slides were then analyzed by determining the percentage of cells undergoing DNA synthesis in cultures, using a Nikon or Olympus photomicroscope fitted with epi-illumination polarization. Replicating cells were identified by the presence of silver grains, visualized as bright green in the epi-polarization system, over their nuclei due to exposure of the NTB-2 emulsion by radioactive DNA [16].

Toxicity testing

Ethanol was used for toxicity testing in dose-response measurements on histocultured eye tissues. After 24 h of incubation, the histocultured conjunctiva was stained with BCECF AM and PI before treatment as an untreated self-control. After the initial observation, the conjunctiva preparations were exposed to various concentrations of ethanol for 10 min. After treatment, the culture medium was removed and replaced with fresh medium and the culture re-stained with BCECF-AM and PI. The same area examined before treatment was observed post treatment. The toxicity of ethanol was measured by confocal scanning laser microscopy. The percentage of killed cells was calculated from the following formula: % of killed cells = (Ra-Rb)/(total cells(Ga + Ra)), where Ra is the number of red cells after treatment, Rb is the number of red cells before treatment, and Ga is the number of green cells after treatment.

Eye irritation test in vivo

For comparison to the toxicity test in vitro, primary eye irritation tests in vivo were performed on the mouse with ethanol. The concentration of ethanol used was the same as that used in the in vitro test. For treatment, two drops of each concentration of ethanol were put into the eyes. After treatment of 10 min, the treated and untreated eye conjunctiva were cut with a scissors under a dissecting microscope while the mouse was under anesthesia and stained with BCECF-AM and PI immediately. The toxicity of ethanol treatment in vivo was measured by confocal scanning laser microscopy. The percentage of killed cells (PI-positive cells) compared to total cells was calculated.

Acknowledgement. The manuscript was expertly typed by Polly Jayne Pomeroy, whom we greatly appreciate. We thank Dr. Sandy Feldman for human conjunctiva tissue. We thank George Himel for the expert photographs from the confocal microscope.