Evaluation of Cytotoxicity of Various Ophthalmic Drugs, Eye Drop Excipients and Cyclodextrins in an Immortalized Human Corneal Epithelial Cell Line

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Purpose. An immortalized human corneal epithelial cell line (HCE) was tested as a screening tool for prediction of topical ocular irritation/toxicity by pharmaceuticals.

Methods. Effects of various drugs, excipients and cyclodextrins (CDs) on viability of HCE cells were evaluated using two in vitro cytotoxicity tests, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) dye reduction assay and propidium iodide assay.

Results. Mitochondrion-based MTT test was a more sensitive indicator of cytotoxicity than the plasma membrane-based propidium iodide test. The tests revealed following cytotoxic rankings for ophthalmic drugs: dipivefrin > timolol > pilocarpine > dexamethasone; for excipients: benzalkonium chloride (BAC) > sodium edetate (Na2EDTA) > polyvinyl alcohol (PVA) > methylparaben; and for CDs: α-CD > dimethyl-β-cyclodextrin (DM-β-CD) > sulfobutyl ether β-cyclodextrin (SBE3β-CD) > hydroxypropyl-β-cyclodextrin (HP-β-CD) > γ-CD. In consideration of the in vivo clinical situation, the short exposure time (5 min) is more relevant even though toxic effects of some test substances were seen only after longer exposure times (30 and 60 min).

Conclusions. Immortalized HCE cells are a promising tool for rapid cytotoxicity assays of ocular medications. The cell line is potentially useful in predicting the in vivo corneal toxicity of ocularly applied compounds.

KEY WORDS: cell culture; corneal epithelium; cyclodextrins; cytotoxicity; immortalized cell line; ocular irritation; ophthalmic drugs.

INTRODUCTION

Significant efforts have been directed toward developing in vitro alternatives to replace the animals or to reduce their number in ocular safety assessment. The eye irritation tests in rabbits (i.e. Draize test) are the standard procedures for evaluating topical ocular safety (1). Draize test has been widely criticized for ethical reasons, as well as for the questionable accuracy in predicting the human eye response. An alternative is to use isolated target organs (e.g. the enucleated rabbit or chicken eye, the isolated cornea) for toxicity studies (2,3). In this case, however, animals are needed as a source of the tissues, and the viability of the isolated tissues is questionable. Recently, in vivo confocal microscopy has been shown to be useful in characterizing the rabbit corneal changes occurring after ocular application of irritating substances (4).

The progress in research utilizing cell-based in vitro methods in safety evaluations has been rapid. Many different cell culture systems and endpoints have been proposed for cytotoxicity screening of drugs, surfactants, solvents and various chemicals (5). Primary corneal epithelial cells can be used for rapid screening of acute topical ocular toxicities of large number of compounds (6–8). The disadvantage of primary cultures is their restricted life span, which necessitates seeding of the new primary cells from animals or human eyes frequently. Immortalized cell line can be grown continuously and, therefore, it should be more practical for screening than primary cells.

In the present work, we evaluated the suitability of recently developed immortalized human corneal epithelial cell line (9) for predicting eye irritation/toxicity potential of some commonly used ophthalmic drugs and pharmaceutical excipients.

MATERIALS AND METHODS

Materials

Dipivefrin hydrochloride, pilocarpine hydrochloride, timolol maleate and dexamethasone sodium phosphate were obtained from Leiras Pharmaceuticals (Tampere, Finland). Benzalkonium chloride was purchased from Fluka Chemie AG (Buchs, Switzerland), polyvinyl alcohol (PVA, molecular weight = 124 000–186 000 g mol−1) from Aldrich Chemicals Company, Inc. (Milwaukee, USA), disodium ethylenediaminetetraacetate (Na2EDTA) from Merck (Darmstadt, Germany) and methyl p-hydroxybenzoate from University Pharmacy (Helsinki, Finland). HP-β-CD (Encapsin®, average DS = 0.6; average MW = 1297.4) was purchased from Janssen Biotech (Olen, Belgium) and DM-β-CD (average DS = 14; average MW 1331.5) from Cyclolab R&D Laboratory Ltd. (Budapest, Hungary). (SBE)3β-CD (average DS = 7; average MW = 2160) was kindly supplied by CyDex, Inc. (Kansas City, KS). α-, β- and γ-CD were from Wacker-Chemie GmbH (Munich, Germany).

Propidium iodide was obtained from Molecular Probes (Eugene, OR), digitation from Aldrich Chemical Company, Inc. (Milwaukee, USA) and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) from Sigma Chemicals Co. (St. Louis, MO), N,N-dimethyl formamide (DMF) from Fluka and sodium dodecylsulfate (SDS) from Merck. Dulbecco’s modified Eagles medium (DMEM) with Ham’s nutrient mixture F12 (1:1), L-glutamine, fetal bovine serum (FBS), antibiotic/antimycotic solution, Hanks’ balanced salt solution (HBSS), Dulbecco’s phosphate-buffered saline (PBS) were obtained from Gibco (Paisley, United Kingdom). Epidermal growth factor (EGF) and insulin were from Sigma Chemicals and dimethylsulfoxide (DMSO) from Merck. Cholera toxin was purchased from Calbiochem (La Jolla, CA). All other chemicals were analytical grade and were used as received.

Cell Culture

The immortalization of the human corneal epithelial cells (HCE) has been described earlier (9). Mycoplasma free cells

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of passages 42–50 were used. The cells of the HCE cell line were grown at 37°C in a humidified atmosphere of air with 5% CO₂ in a standard medium consisting of DMEM/F12 (1:1), 15% heat-inactivated FBS, 0.3 mg/ml L-glutamine, 5 µg/ml insulin, 0.1 µg/ml cholera toxin, 10 ng/ml EGF, 0.9% DMSO, and 0.1 mg/ml streptomycin, 100 IU/ml benzylpenicillin and 0.25 µg/ml amphotericin B. The cells were plated at 2 × 10⁴ cells/100 µl/well in Nunc 96-well plates (Roskilde, Denmark). Cells were used for cytotoxicity assays when they became confluent on day 3. Cytotoxicity data were obtained from two different experiments by testing three to five concentrations with four wells per concentration.

Methods

Preparation of Test Solutions

The following compounds were used for their cytotoxicity to human corneal epithelial cells: pilocarpine, timolol, dipivefrin, dexamethasone, benzalkonium chloride, EDTA, PVA, methylparaben, α-, β-, γ-, HP-β-CD, DM-β-CD, and (SBE)₃m-β-CD, β-CD was studied at a low concentration (8 mM) due to its poor aqueous solubility. Test substances were dissolved in Hank’s balanced salt solution (HBSS) and pH was adjusted to 7.4. Dilution of the test substances in the experiment to the half was taken into account when preparing test solutions. Osmolarities were measured with an Osmostat OM-6020 osmometer (Daichi Kagaku, Kyoto, Japan). The highest concentrations of pilocarpine, HP-β-CD, DM-β-CD and (SBE)₃m-β-CD were prepared in water. The solutions of HP-β-CD and DM-β-CD (200 mM) were slightly hypertonic (316 and 372 mOsm kg⁻¹), but some solutions of (SBE)₃m-β-CD (100 and 200 mM) and pilocarpine (384 mM, 8%) were strongly hypertonic (>500 mOsm kg⁻¹). Hypertonic control solutions were prepared by adding mannitol to 0.9% sodium chloride solution.

MTT Assay

The MTT assay is a colorimetric method for determining cell viability based on reduction of the yellow tetrazolium salt MTT to a purple formazan dye by mitochondrial succinate dehydrogenase in viable cells. The test was performed according to the procedure of Hansen et al. (10). Cells were incubated with the test solutions (100 µl) for 5, 30, or 60 min. After the exposure period, the reaction medium was removed, and 100 µl of fresh DMEM/F12 medium (without serum) and 25 µl of MTT solution (5 mg/ml in PBS) were added to each well and incubated at 37°C for 2 hours. Formazan was solubilized by adding 100 µl of extraction buffer (20% w/v SDS in DMF/water 50:50, pH 4.7) and incubated overnight at 37°C. The optical densities were measured at 570 nm using a Multiscan Plus multiwell scanning spectrophotometer (Labsystems Oy, Helsinki, Finland). IC₅₀ values (mM), the concentrations of test substances that reduce MTT metabolism to 50% of control levels were determined using nonlinear curve fitting.

Propidium Iodide Assay

Loss of membrane integrity is often a sign of reduced cell viability or direct action of the chemical on plasma membrane. Nonviable cells may be recognized by their uptake of DNA-binding dyes such as propidium iodide. This dye enters the cells only when their plasma membranes have partly lost their integrity. In cells, propidium iodide binds to DNA and becomes fluorescent.

Propidium iodide assay was modified from that of Nieminen et al. (11). Cells were seeded at a density of 2 × 10⁴ cells/well and cultivated on 96-well microtiter plates as described in the MTT assay. Fluorescence measurements were performed in a BioTek® FL500 fluorescence plate reader (Bio-Tek® Instruments, Inc, Vermont, USA) using 530 nm excitation and 590 nm emission filters. Briefly, medium was removed from each well and replaced with HBSS containing propidium iodide (50 µM). After incubation of 20 min at room temperature, 100 µl of the test substances were added. Fluorescence values (F) were measured from the plate after 5, 15, 30, and 60 min incubation at room temperature. At the end of the experiment, digitonin (95 µM) was added to the wells and the plate was shaken at room temperature for 20 min. Fluorescence was measured again to obtain the value of 100% cell death (Fmax). Percentage viability was defined as follows:

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\text{% Viability} = 100 - \left[ \frac{[F - \text{blank}]}{[F_{\text{max}} - \text{blank}]} \right] \times 100
\]

where F is fluorescence at any given time, Fmax is fluorescence after the addition of digitonin, blank1 and blank2 are fluorescence values from the wells lacking cells before and after the addition of digitonin. Two independent experiments were performed using four wells for each concentration of test substances.

RESULTS

MTT Assay

After exposure of 5 min, the dipivefrin was clearly cytotoxic at concentrations of 7 mM (0.25%) or more, while timolol and pilocarpine showed mild cytotoxicity at 63 mM (2%) and 192 mM (4%), respectively. Dexamethasone was not toxic at any tested concentrations (1.3–25 mM). At typical concentrations of eye drops the drugs did not cause significant cellular toxicities (Fig. 1). Cell viability decreased below 50% after

![Graph showing MTT assay results](image-url)