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EVALUATION OF ADVERSE OCULAR EFFECTS OF 5-FLUOROURACIL BY USING HUMAN CORNEAL EPITHELIAL CELL CULTURES

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ABSTRACT

5-Fluorouracil (5-FU) is commonly used in ophthalmology for suppressing fibroblast activity after glaucoma surgery. Adverse effects on corneal epithelial cells have been reported to relate to 5-FU therapy. The effects of 5-FU were evaluated in vitro on SV40-immortalized human corneal epithelial cell (HCE) cultures with two cytotoxicity tests: WST-1 assay as an index of cell proliferation, and lactate dehydrogenase (LDH) assay as an index of plasma membrane integrity. The cells were exposed to 5-FU with various concentrations in serum-free medium and in medium containing 15 % (v/v) fetal bovine serum (FBS) for 1, 24, 48 and 72 hours. One-hour exposure had no effects on HCE cells. Longer exposures caused dose-dependent inhibition of cell proliferation. Exposure to 5 mg/ml 5-FU lowered cell number to 50 % of controls after 24-hour treatment and resulted to complete cell death after 72 hours. Serum protected the cells for 24 hours, but after longer exposure times the protective nature of serum disappeared. 5-FU had only minor effects on LDH release. The LDH leakage was at its peak after 48-hour treatment.

Key Words: 5-fluorouracil; Eye; Corneal epithelial cells; Cytotoxicity

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INTRODUCTION

The use of 5-fluorouracil (5-FU) is relatively common in ophthalmology, especially after glaucoma surgery. Subconjunctival injection of 5-FU for suppressing fibroblast activity after glaucoma filtering surgery was introduced in 1984.[1] Single intraoperative applications of 5-FU are also widely used in patients with a high risk of excess scar formation.[2] Scarring of glaucoma filtering blebs is mainly caused by proliferation of subconjunctival fibroblasts, and their biosynthesis of collagen and other extracellular materials.[3] Fibroblast proliferation from episclera and Tenon's capsule plays an important role in the scarring process. Ideally, an antimetabolite given should enhance the success of filtering surgery by limiting the proliferation of scleral and episcleral fibroblasts, and not by limiting the replication of normal cells in conjunctival and corneal epithelium.[4] However, complications are associated with the 5-FU therapy, especially as corneal epithelial defects.[5–9]

Cytotoxicity of 5-FU in vitro has been studied previously by using rabbit conjunctival fibroblasts[10–13] and rabbit corneal epithelial cells.[4,11,14] In the present study, the direct adverse effects of 5-FU on SV40-immortalized human corneal epithelial (HCE) cells were evaluated. Special attention was paid to differentiate the effects on cell proliferation and viability. The effects of 5-FU on HCE cell cultures were evaluated in serum-free medium, and in medium containing 15% (v/v) fetal bovine serum (FBS), with two cytotoxicity tests: WST-1 assay as an index of cell viability and proliferation, and lactate dehydrogenase (LDH) assay as an index of plasma membrane integrity.

MATERIALS AND METHODS

Human Corneal Epithelial Cell Line

An SV40-immortalized HCE cell line used in this study was established and donated to us by Araki-Sasaki et al.[15] HCE cells were grown in a culture medium containing 1 vol of Dulbecco's modified Eagle's medium and 1 vol of Ham's nutrient mixture F-12 (Gibco, Paisley, UK) supplemented with 15% (v/v) fetal bovine serum (FBS, from Gibco), 1% (v/v) antibiotic–antimycotic solution (penicillin 10,000 U/mL, streptomycin 10,000 μg/mL, and amphotericin B 25 μg/mL, Gibco), 2 m*M* L-glutamine (Gibco), 5 μg/mL insulin (Sigma, St. Louis, MO), and 10 ng/mL human epithelial growth factor (EGF, Sigma).

The cells were incubated in a humidified atmosphere at 37°C in 5% CO_2/95% air in 80 cm^2 T-flasks, and serially passaged at a split ratio of 1:5 twice a week. The cell cultures were removed by trypsin–EDTA incubation, the cells were counted, and their viability was assessed by the trypan blue exclusion method. Cells with viability >97% were used for cytotoxicity testing. HCE cells were seeded at the density of 15,000 cells/well into 96-well culture plates and grown in a humidified

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atmosphere at 37°C in 5% CO_2/95% air for 24 hr until the cells were exposed to the test solutions.

Treatment of Cells and Cytotoxicity Tests

Fluraplastin® (containing 50 mg 5-FU/mL) was obtained from Pharmacia AB (Stockholm, Sweden). 24 hr after plating, the medium was discarded and replaced with 100 μL of normal growth medium [with 15% (v/v) FBS or 0% FBS as controls] or with a test solution containing 0.0005–5 mg/mL [0.00005–0.5% (w/v)] 5-FU in medium with 15% FBS or 0% FBS. The cells were treated in a humidified atmosphere at 37°C in 5% CO_2/95% air for 1, 24, 48, and 72 hr.

After the treatment period, the test compound-containing medium was removed and used for LDH leakage test. LDH assay as an index of plasma membrane integrity measures the leakage of the cytosolic enzyme LDH into culture medium. LDH test was modified from the "Automated Analysis Boehringer Mannheim LD/LDH assay" (Boehringer Mannheim GmbH, Germany, cat. no. 191353). Test compound-containing sample (25 μL) was pipetted into a 96-well microtiter plate, and 250 μL of coenzyme/buffer containing 56 m*M* Tris buffer (pH 7.4), 0.17 m*M* NADH (disodium salt), and 5.6 m*M* EDTA (tetrasodium salt) was added. The reaction was started by the addition of 25 μL of 14 m*M* pyruvate solution (monosodium salt). The average rate of disappearance of NADH (LDH activity) was monitored at room temperature by measuring the absorbance for 3 min at a wavelength of 340 nm with the Multiskan MS EIA reader (Labsystems Oy, Helsinki, Finland). The background absorbance was measured from the wells containing only culture medium with or without serum. Enzyme leakage into the medium was expressed as percentage of controls (untreated cultures).

After the removal of the test compound-containing medium the cells were rinsed once with the basal medium (Dulbecco's modified Eagle's medium and Ham's F12, 1:1) without serum and the WST-1 cytotoxicity test was initiated. The WST-1 test was based on the ready-to-use cell proliferation reagent WST-1 (Roche Diagnostics GmbH, Germany, cat no. 1644807). WST-1 {4-[3-(4-iodophenyl)-2-(4-nitrophenyl)-2H-5-tetrazolio]-1,3-benzene disulfonate}, a colorimetric assay for nonradioactive quantification of cell proliferation and viability, is based on the cleavage of the tetrazolium salt WST-1 (slightly red) to formazan (dark red), occurring only in the active mitochondria of living cells. Briefly, 100 μL of normal culture medium and 10 μL of WST-1 test reagent were added into each well, and the culture plate was returned to a humidified atmosphere at 37°C in 5% CO_2/95% air for 2 hr. The incubation time with the WST-1 reagent was determined after a series of preliminary experiments. After the incubation, the multititer plate was thoroughly shaken for 1 min, and the absorbances were read at 450 nm with a Victor 1420 Multilabel Counter (Wallac Oy, Turku, Finland). The background absorbance was measured on cells containing only the dye solution and the culture

medium. The results were expressed as percentage of optical density of treated vs. control cultures. The statistical significances of the differences between cultures exposed to 5-FU without FBS and with 15% (v/v) FBS were determined with the Student's two-tailed *t*-test (GraphPad Prism, GraphPad™, San Diego, USA).

RESULTS

The treatment of HCE cells with 0.0005–5 mg/mL 5-FU for 1 hr had no effect on cell viability (Figs. 1A and 2A). Treatment with as little as 0.0005 mg/mL 5-FU for 48 hr diminished cell number (Fig. 1). Exposure for a longer time did not kill the cells but hindered cell growth. A hundred times higher concentration decreased cell growth after 24-hr treatment in a similar way, i.e., decreasing cell number. The cell number remained the same for the following 48 hr. Treatment with the highest 5-FU concentration studied (5 mg/mL) for 24 hr decreased cell viability to about 50%, while treatment with the same concentration for 72 hr resulted in complete cell death (Fig. 2). Use of serum clearly promoted the growth of HCE cells (Fig. 1). When the cells were exposed to 5-FU for 24 hr, the protective effect of serum could be observed (Fig. 2). It was statistically significant ($*p = 0.0307$). After longer exposure times, the protective effect of serum was lost ($p > 0.05$). The estimated EC_{50} value after 24-hr serum-free exposure was about 0.5 mg/mL, and in the serum-containing medium about 5 mg/mL.

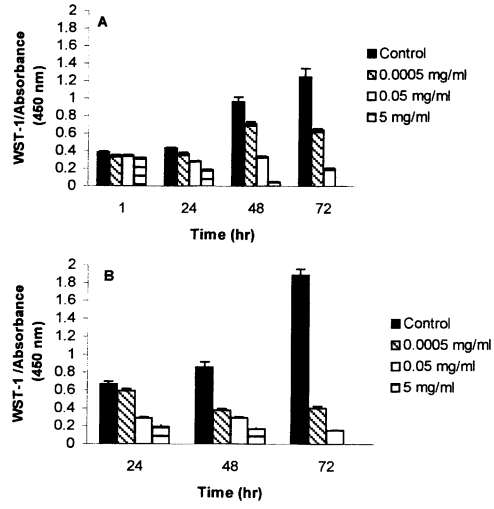

Figure 1. Absorbance of WST-1 formazan at 450 nm in HCE cells exposed to 5-FU in serum-free medium (A) and in serum-containing medium (B) for 1–72 hr.

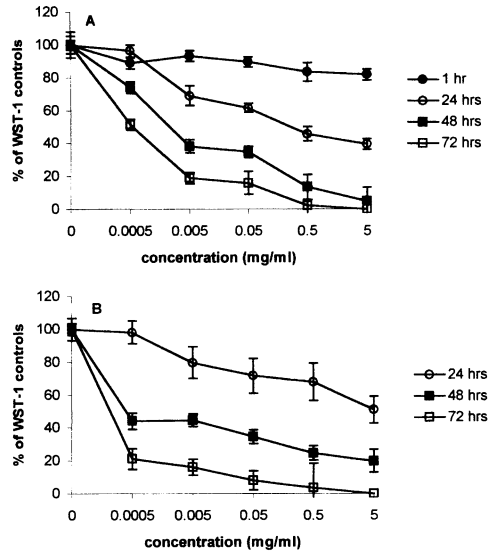

Figure 2. WST-1 formazan reduction with respect to controls in HCE cells exposed to 5-FU for 1–72 hr. Exposure without FBS (A) and with FBS (B).

The effects on LDH leakage after 1-hr treatment were similarly insignificant in all the 5-FU concentrations tested (Figs. 3A and 4A). Exposure to 5-FU for as long as 24 hr did not increase LDH leakage, compared to controls (Fig. 4), whether serum was used or not. The loss of cell membrane integrity and the resulting LDH release was at its highest after 48 hr of treatment with more than 0.05 mg/mL 5-FU in serum-free medium (Fig. 4B). The leakage was smaller in serum-containing medium, but the difference was not statistically significant ($p > 0.05$).

DISCUSSION

5-Fluorouracil [fluorouracilum, 5-fluoropyrimidine-2,4(1H,3H)-dione, 5-FU] synthesized in 1957 is a pyrimidine analog with an antimetabolic activity to uracil.[16,17] After an intracellular conversion to the active deoxynucleotide it interferes with the synthesis of DNA by blocking the conversion of deoxyuridylic acid to thymidylic acid by the cellular thymidylate synthetase. 5-FU primarily inhibits thymidylate synthetase, but it also blocks a variety of other enzymes.[18] It can also interfere with RNA synthesis.[10,17]

5-FU is used as an antineoplastic in the adjuvant treatment of breast and gastro-intestinal malignancies.[17] It is also used in the treatment of solar keratoses and superficial neoplasms of the skin. The main adverse effects of 5-

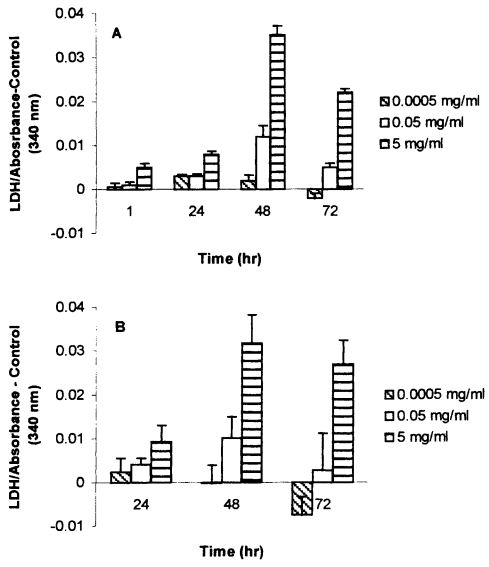

Figure 3. Mean absorbance change of LDH per min minus control at 340 nm in HCE cells exposed to 5-FU in serum-free medium (A) and in serum-containing medium (B) for 1–72 hr.

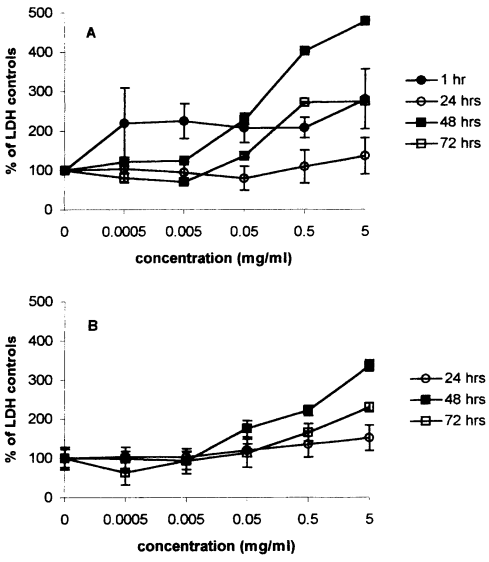

Figure 4. LDH leakage compared to controls in HCE cells exposed to 5-FU for 1–72 hr. Exposure without FBS (A) and with FBS (B).

FU are on the bone marrow and the gastro-intestinal tract. Administration of 5-FU by serial subconjunctival injections or by single intraoperative applications is used for adjunctive treatment in filtration surgery in glaucomatous eyes, i.e., those with poor surgical prognosis. Systemic 5-FU treatment has been associated with various types of ocular toxicity, including corneal epithelial defects.[5–9,19]

5-FU has been reported to cause antiproliferative effects in vitro in rabbit conjunctival fibroblasts,[10–13] human scleral fibroblasts,[4] and human Tenon's capsule fibroblasts.[20] 5-FU has also been shown to induce antiproliferative effects in other ocular cells, such as rabbit corneal epithelial cells,[4,11,14] human retinal pigment epithelial cells,[21] rabbit lens epithelial cells,[22] and bovine corneal endothelial cells.[23] The EC_{50} values and the method used for evaluation of the antiproliferative effect of 5-FU are presented in Table 1. In most of the studies, the cells were grown in complete serum-containing medium to confluency in the presence of 5-FU, after which cell numbers were calculated by using a hemocytometer or a Coulter counter. It should be noted that the estimated inhibitory doses for both fibroblasts and epithelial cells are in the same concentration range, around 0.0005 mg/mL.

Table 1. Toxicity of 5-FU In Vitro

Cell Type	Inhibitory Dose (EC_{50}, mg/mL)	Exposure Time	Analysis Method	References
Fibroblasts				
Rabbit conjunctival fibroblasts	0.0002	2–3 days	Hemocytometer	[10]
Rabbit conjunctival fibroblasts	0.0005	5 days	Coulter counter	[11]
Rabbit conjunctival fibroblasts	0.0006	6 days	Hemocytometer	[12]
Rabbit conjunctival fibroblasts	0.0014 0.0018	3 days 5 days	Coulter counter	[13]
Human scleral fibroblasts	0.00043	5 days	Hemocytometer/ cell counter	[4]
Human Tenon's capsule fibroblasts	10	5 min 24 hr recovery	[^{3}H]-thymidine uptake	[20]
Epithelial cells				
Rabbit corneal epithelial cells	0.0006	5 days	Coulter counter	[11]
Rabbit corneal epithelial cells	0.00042	5 days	Hemocytometer/ cell counter	[4]
Rabbit corneal epithelial cells	Significant inhibition 0.00013	30 hr	[^{3}H]-thymidine uptake	[14]
Human retinal pigment epithelial cells	0.00039	3 days	Coulter counter	[21]
Rabbit lens epithelial cells	0.0006	13–14 days	Hemocytometer	[22]
Endothelial cells				
Bovine corneal endothelial cells	Cytotoxicity threshold 50	30/60 min	MTT	[23]

In the present study, the effects of 5-FU were assessed on preconfluent HCE cells by the WST-1 assay as an index of the metabolic activity of various mitochondrial dehydrogenases and cell proliferation, and by the LDH leakage test as an index of cell membrane integrity. The 5-FU concentrations tested after 1-hr exposure had no acute toxic effect on corneal epithelial cells. 5-FU affected cell proliferation, as has also been shown previously in other studies with various ocular cell types. After 24-hr treatment, the estimated EC_{50} values were 5 mg/mL in the presence of 15% (v/v) serum and 0.5 mg/mL in the absence of serum. Serum also protected cells against the loss of membrane integrity assessed by the LDH release assay. In all, 5-FU had minor effects on LDH leakage.

For corneal epithelial cells, 0.05 mg/mL 5-FU appears to be the threshold concentration of cytotoxicity. When HCE cells are exposed to less than 0.05 mg/mL 5-FU, cell death is not induced, only cell growth is prohibited. Higher 5-FU concentrations are cytotoxic and induce cell death when exposure time is extended. Considerably low concentrations of 5-FU can induce adverse corneal effects in vitro compared to the clinically used doses. During the single intraoperative 5-FU exposure of glaucoma filtration surgery, 5-FU is mostly delivered at the concentration of 50 mg/mL to the inner surface of the sclera and subconjunctiva for a couple of minutes, and then it is rapidly washed away. In the postoperative subconjunctival injection, 5 mg (0.1 mL of 50 mg/mL) is typically used. In the postoperative injection, 5-FU may leak out into the tear film and can be in contact with the corneal epithelium for much longer periods and thus induce more corneal epithelial side effects. In the repeated subconjunctival injections of 5-FU, leakage into the tear film and to the corneal surface must be prevented. The authors also suggest careful rinsing after injections with artificial tear substitutes. To minimize any corneal side effects, it is important to make sure that the wash-out is also thorough after intraoperative 5-FU exposure.

From the technical point of view, it should be noted that with the WST-1 and LDH assays, long exposure times are needed for the cytotoxicity testing of nonirritating test agents. The acute toxicity of highly irritating substances such as benzalkonium chloride can be assessed with the WST-1 and LDH tests after short 5–60 min exposure times.[24] When the effects of antiproliferative agents such as 5-FU and related substances are tested with the WST-1 and LDH tests, it is essential to use long exposure times to induce detectable influence. In the clinical use, the repeated postoperative injections of 5-FU may cause similar prolonged exposures to 5-FU in the lacrimal fluid and the corneal surface, especially if conjunctival leakage occurs. However, some caution is necessary as always when extending the in vitro cytotoxicity data to in vivo. The use of 5-FU for adjunctive treatment in filtration surgery in glaucomatous eyes appears to be the case of complex balancing between the optimal dose to achieve maximal fibroblast growth inhibition and minimal corneal side effects.

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