Original Paper

Evaluation of corneal damage caused by the anticancer drug S-1 in human corneal epithelial cells

Kanae Moriya[*1,2], Hisanori Shimizu[3], Daisuke Kamei[4], Satoko Handa[5], Tadanori Sasaki[6] and Yasuhisa Kato[5]

Abstract

The combination drug S-1, which contains tegafur, gimeracil, and oteracil potassium, is a fluoropyrimidine-based oral antineoplastic agent in which the principal drug tegafur is a prodrug of fluorouracil (5-FU). In recent years, many studies have reported eye problems, especially corneal damage, as an adverse effect of S-1 treatment. In this study, we investigated the cytotoxic effects of each of the constituents of S-1 on corneal epithelial cells by measuring viable cell counts and lactate dehydrogenase (LDH) release. Experimental chemosensitivity study for 5-FU and the constituents of S-1 (i.e., tegafur, gimeracil, and oteracil) using a human cell line. We used immortalized human corneal epithelial (HCE-T) cells to estimate viable cell counts (expressed as a percentage of the control cells) and the activity of LDH in a culture medium (expressed as a percentage of the total LDH activity). Decreases in viable cell counts were noted with 5-FU and tegafur, but a significant elevation in LDH activity was noted only with tegafur. The incidence of damage in cells exposed to tegafur significantly decreased on adding tranylcypromine, an inhibitor of CYP2A6 that metabolizes tegafur to 5-FU. In addition, 5-FU did not elevate LDH activity, which is an indicator of cell membrane disruption, and concentration-dependence was not observed when cells were treated with 5-FU doses of up to 1,000 ng/ml. These findings suggest that the disruption of the metabolic activity of the corneal epithelium by 5-FU is involved in the corneal injury mechanism of S-1.

Key words : S-1, human corneal epithelial cell, corneal damage, 5-FU

Introduction

Owing to the economics of medical treatment, as well as changes in patient awareness, anticancer chemotherapy has moved from inpatient to outpatient settings in recent years. This has been accompanied by an increase in the use of oral antineoplastic medications. The combination drug S-1 is a fluoropyrimidine-based oral antineoplastic preparation[1-4] that includes the following agents: tegafur, which is a prodrug of fluorouracil (5-FU); gimeracil, which is an inhibitor of dihydropyrimidine dehydrogenase; and oteracil potassium, which decreases gastrointestinal toxicity at a molar ratio of 1:0.4:1[5]. In 1999, S-1 capsules and granules were released commercially, and in 2013, orally disintegrating tablets[6] were produced for the first time. Since then, S-1 has become a key chemotherapeutic drug in Japan because it is indicated in various cancers, and studies have provided evidence for its effectiveness in combination therapies[7-9].

Myelosuppression, in the form of leukopenia, neutropenia, anemia, loss of appetite, nausea, diarrhea, or stomatitis, is a typical adverse effect of S-1[9]. Eye problems due to S-1 use were first reported in 2005 by Esmaeli et al.[10], and many subsequent studies have reported similar problems, mainly the appearance of

* Corresponding author

✉ Kanae Moriya
  kmoriya@cmed.showa-u.ac.jp

[1] Department of Hospital Pharmaceutics, Showa University School of Pharmacy, 1–5–8 Hatanodai, Shinagawa-ku, Tokyo 142–8555, Japan.
[2] Department of Pharmacy, Showa University Hospital East Branch.
[3] Department of Pharmacy, The Cancer Institute Hospital of Japanese Foundation for Cancer Research.
[4] Department of Healthcare and Regulatory Sciences, Division of Pharmaceutical Regulatory Pharmacy Showa University School of Pharmacy.
[5] Department of Clinical Pharmacy, Division of Cancer Genome and Pharmacotherapy, Showa University School of Pharmacy.
[6] Department of Pharmacy Service, Showa University.

epiphora[11-19]. Although these eye problems are not directly life-threatening adverse reactions, they decrease the quality of life of patients by restricting their visual field or decreasing visual acuity. Moreover, excessive tear secretion may lead to psychological problems in some patients. In severe cases, no improvement is noted in the condition if treatment is continued unchanged, and an extended drug holiday or a change to other medications may be necessary. Therefore, appropriate management of adverse reactions is required to ensure continued medication use by patients.

Epiphora, which is the eye problem with the highest incidence among patients receiving S-1, can be broadly classified into the obstructive and secretory forms. Patients with epiphora can have either form individually or in combination. Obstructive epiphora is produced by stenosis or obstruction of the lacrimal puncta and/ or nasolacrimal duct, which constitute the tear drainage pathway. Secretory epiphora occurs when the cornea is injured, resulting in an increase in lacrimal secretion due to external stimuli.

The mechanism underlying the eye problems associated with S-1 usage is not clearly understood. Currently, it is presumed to involve the obstruction of the lacrimal passage and subsequent corneal epithelial damage caused by 5-FU that has diffused into the tears. The main ocular symptoms reported after intravenous infusion of 5-FU are almost all those seen with obstructive epiphora. Reports of eye problems associated with capecitabine or tegafur/uracil preparations, which like S-1 show an antitumor effect by being metabolized to 5-FU, are rare. However, corneal epithelial damage due to S-1 occurs in approximately 30% of patients with epiphora[17], and although many studies have reported lacrimal passage obstruction accompanied by corneal damage, few have reported this when using 5-FU alone[15-23]. These reports suggest the possibility that the constituents of S-1 (i.e., tegafur, gimeracil, and oteracil) have an effect on corneal cells. Therefore, we conducted this study to investigate the cytotoxic effects of each of these constituents on corneal epithelial cells by assessing viable cell counts and lactate dehydrogenase (LDH) release.

Materials and methods

Preparation of reagents and test solutions

5-FU, tegafur, and gimeracil were dissolved in dimethyl sulfoxide (DMSO). Oteracil potassium and tranylcypromine were dissolved in Dulbecco's modified Eagle's medium (DMEM)/HamF12. Drug concentrations in the culture medium were based on those in the blood and tears. The baseline concentrations were 50 ng/ml for 5-FU, 2,000 ng/ml for tegafur, and 40 ng/ml for gimeracil. As the concentration of oteracil in tears had not been reported, it was set at 40 ng/ml based on its concentration in the blood.

Cell type and culture

SV40-immortalized human corneal epithelial (HCE-T) cells[24-26], donated by RIKEN BCR (Ibaraki, Japan), were cultured in DMEM/HamF12 medium containing 100-IU/ml penicillin, 100-µg/ml streptomycin, 5% fetal calf serum, 5-µg/ml insulin, and 10-ng/ml human epithelial cell growth factor. HCE-T cells exhibit a cobblestone-like appearance similar to normal corneal epithelial cells in culture, with a population doubling time of 24.4 h[26]. The cells were in good condition and could be tested. The inoculated cell count in each experiment was determined in a preliminary experiment. The Ethics Committee has ruled that approval was not required for the study.

Indicators of cytotoxicity

The toxicity of each drug on HCE-T cells was determined by counting the number of viable cells (expressed as a percentage of the control cells) and measuring LDH activity in the culture medium (expressed as a percentage of the total LDH activity). Both indicators are used for studying the corneal epithelium.

Viable cell counts

Viable cell counts were determined using Cell-Counting Kit-8 (Dojindo Molecular Technologies, Kumamoto, Japan) and measured in a fully confluent state to see cell damage. The percentage of viable cells relative to a control group without drug treatment was calculated from the measured absorbance using the following equation: viable cell count (%) = [(As-Ab)/(Ac-Ab)] × 100, where As was the absorbance of the sample, Ac was the absorbance of the negative control, and Ab was the absorbance of the blank.

＜ Viable cell count protocol ＞
1. Dispense 100-µl cell suspension (1×10^5 cells/ well) in a 96-well plate. Pre-incubate the plate overnight at 37℃ with 5% CO2.
2. Add 10 µl of various concentrations of substances to be tested to the plate.
3. Incubate the plate for 24 h in the incubator.
4. Add 10-µl CCK-8 solution to each well of the plate.
5. Incubate the plate for 3 h in the incubator.
6. Measure the absorbance at 450 nm using a microplate reader.

LDH activity

A Cytotoxicity LDH Assay Kit-WST (Dojindo Molecular Technologies) was used to measure LDH activity. LDH activity was determined in a sub-confluent state. A sample incubated with the culture medium alone was used as the low control, and a sample containing the cultured cell medium without drugs was used as the high control. The incidence of cell damage (percentage) was calculated from the measured absorbance using the following equation: incidence of cell damage $(\%) = [(A-C)/(B-C)] \times 100$, where A was the absorbance of the test substance, B was the absorbance of the low control, and C was the absorbance of the high control. When measuring LDH activity, the concentrations of 5-FU and tegafur were increased to 2,500 ng/ml and 100,000 ng/ml, respectively, considering the effects of DMSO, which is a solvent for each drug.

< LDH activity protocol >

1) Add 100-μl cell suspension (1×10^4 cells/well) to each well of a flat-bottom 96-well tissue culture plate. Incubate the plate at 37℃ with 5% CO2 overnight to allow the cells, and then, replace the medium with 50-μl fresh medium.

2) Add 50-μl medium containing the test substance adjusted to the desired concentration.

3) Incubate the plate at 37℃ for 24 h.

4) Add 10-μl lysis buffer to each well of the high control. Incubate the plate at 37℃ for 30 min.

5) Add 100-μl working solution to each well. Protect the plate from light, and incubate it at room temperature for 30 min.

6) Add 50-μl stop solution to each well.

7) Measure the absorbance at 490 nm using a microplate reader.

Addition of tranylcypromine

Tegafur, the principal constituent of S-1, shows an antitumor effect when activated by conversion to 5-FU, mainly by CYP2A6, in the liver. The HCE-T cells employed in this study expressed CYP2A6, similar to that in normal human corneal cells[24]. Consequently, the cytotoxicity produced by exposure to tegafur may be due to 5-FU. Therefore, we determined the changes in tegafur cytotoxicity after the addition of tranylcypromine, which inhibits this metabolic process. Accordingly, after culturing cells in a 96-well plate (1×10^4 cells/well), we added 100-μM tranylcypromine to the samples with tegafur and measured the viable cell counts and LDH activity.

Ethical considerations

The Ethics Committee has ruled that approval was not required for the study.

Results

The exposure of HCE-T cells to gimeracil or oteracil did not produce any decrease in viable cell counts. However, a significant decrease in viable cell count was noted after exposure to 500-ng/ml 5-FU. In the case of tegafur, a significant decrease in viable cell count was noted at 20,000 ng/ml (Fig. 1).

Moreover, the HCE-T cells were exposed to high concentrations of 5-FU and tegafur. The viable cell count was 79.9%±2.14% relative to the control group at 500-ng/ml 5-FU and decreased to 70.8%±1.1% at 1,000 ng/ml, 69.4%±1.0% at 2,500 ng/ml, and 69.1%±2.0% at 5,000 ng/ml. All viable cell counts were significantly lower than those of the control ($p < 0.001$), even though concentration dependence was not noted at 1,000 ng/ml and higher concentrations (Fig. 2A). Nevertheless, a significant ($p < 0.001$) concentration-dependent decrease in viable cell counts was noted with increasing tegafur concentrations: 91.4%±0.5% at 20,000 ng/ml, 80.3%±1.5% at 40,000 ng/ml, 69.6%±1.9% at 100,000 ng/ml, and 61.8%±1.9% at 200,000 ng/ml (Fig. 2B).

LDH activity measurements showed considerable variations. However, no significant elevation in LDH release was noted after exposure to 5-FU at the concentrations used (Fig. 3A). With tegafur, a significant elevation in LDH release to 30.9%±20.9% ($p < 0.001$) was noted at 100,000 ng/ml (Fig. 3B).
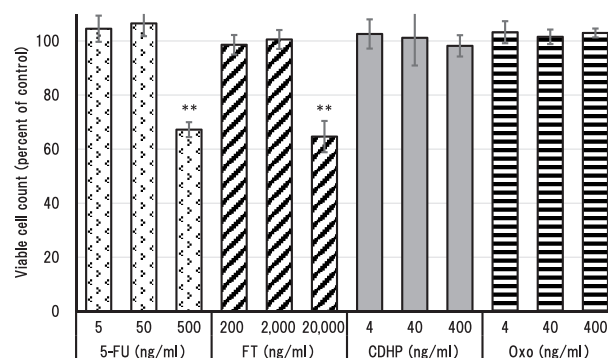

Fig. 1. Counts of viable immortalized human corneal epithelial cells treated with 5-fluorouracil (5-FU), tegafur (FT), gimeracil (CDHP), and oteracil potassium (Oxo). Data are expressed as the mean percentages of control ± standard deviation (n = 4) (*p < 0.05, **p < 0.01).

Changes in viable cell counts in the absence and presence of added tranylcypromine are shown in Figure 4A. Changes in LDH release are shown in Figure 4B. In the tranylcypromine-added group, no significant cytotoxicity was observed compared with the control group. In the samples exposed to 200,000-ng/ml tegafur, the viable cell count decreased to 41.2%±26.4% in the absence of tranylcypromine and significantly improved to 91.4%±4.8% ($p<0.001$) after exposure to this inhibitor. Moreover, a significant decrease was noted in LDH release: 4.7%±6.4% in

samples with tranylcypromine compared with 30.9%± 20.9% in samples without tranylcypromine ($p=0.007$).

In LDH measurements, the values of the results varied; however, the reproducibility was confirmed in three experiments.

Discussion

In this study, we examined whether each of the three drug constituents of S-1 (tegafur, gimeracil, and oteracil) and the active metabolite of tegafur (i.e., 5-FU) were cytotoxic to HCE-T cells. Two indicators of cytotoxicity were used: viable cell count and LDH release. The two components that caused a decrease in viable cell counts were 5-FU and tegafur. No indication of cytotoxicity was noted after corneal epithelial cells were exposed to gimeracil or oteracil. Tegafur is metabolized to 5-FU by CYP2A6, mainly in the liver[27, 28]. By adding tranylcypromine, a competitive CYP2A6 inhibitor, the decrease in viable cell counts and elevation in the incidence of cell damage (i.e., LDH release) observed in HCE-T cells exposed to tegafur were significantly improved. This suggests that the cytotoxicity in the samples exposed to tegafur was due to the metabolism of tegafur to 5-FU by these cells.

When HCE-T cells were treated directly with 5-FU at concentrations of up to 1,000 ng/ml, the percentage of cells surviving after 24 h was lower than that in the control group. However, no significant elevation
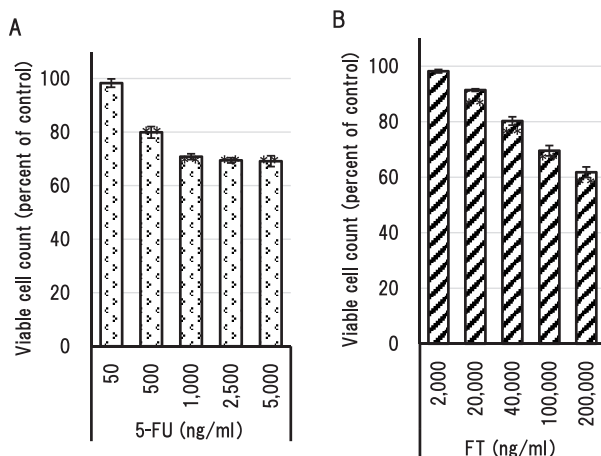
Fig. 2. Counts of viable immortalized human corneal epithelial cells treated with (A) 5-fluorouracil (5-FU) and (B) tegafur (FT). Data are expressed as the mean percentages of control ± standard deviation (n=4) (*p < 0.05, **p < 0.01).
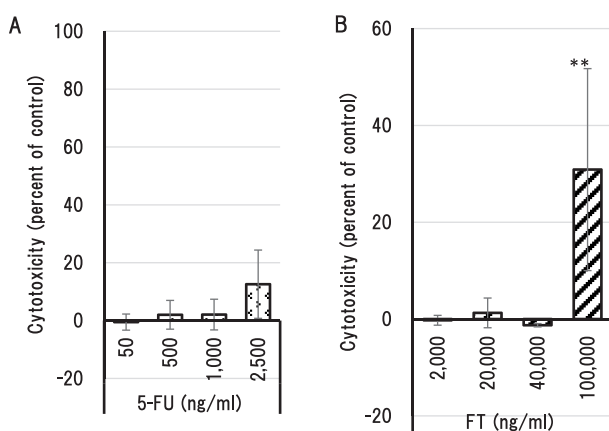
Fig. 3. Cytotoxicity assay of immortalized human corneal epithelial cells treated with (A) 5-fluorouracil (5-FU) or (B) tegafur (FT). Data are expressed as the mean percentages of total lactate dehydrogenase released into the medium ± standard deviation (n=4) (*p < 0.05, **p < 0.01).
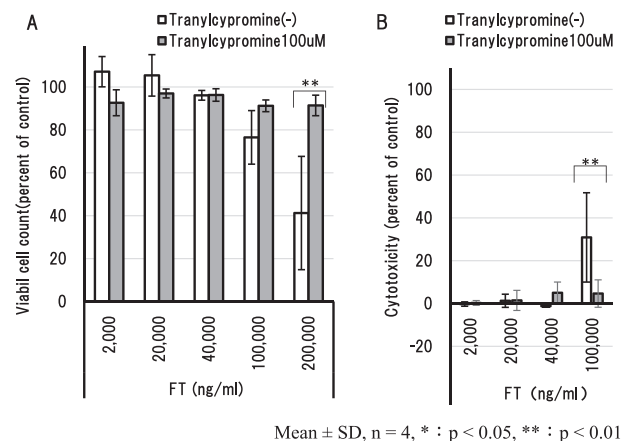
Mean ± SD, n = 4, * : p < 0.05, ** : p < 0.01

Fig. 4. (A) Viable cell counts and (B) cytotoxicity of immortalized human corneal epithelial cells treated with tegafur (FT) in the presence or absence of 100-μM tranylcypromine. Viability data are expressed as the mean percentages of control ± standard deviation (SD) (n=4). Cytotoxicity data are expressed as the mean percentages of total lactate dehydrogenase released into the culture medium ± SD (n=4) (*p < 0.05, **p < 0.01).

in the incidence of cell damage calculated based on LDH release within the range of 5-FU concentrations used was observed. Nevertheless, irregularities in cell arrangement and detachment were observed under a microscope. Thus, it may contribute to the development of SPK（superficial punctate keratopathy） in patients.

This study was designed to examine the cytotoxicity of each constituent of S-1 and 5-FU; thus, another study on growth inhibition is needed. 5-FU is a fluoropyrimidine antimetabolite antineoplastic drug. Its toxicity depends on exposure time rather than concentration. Thus, when the concentration of 5-FU was increased beyond 1,000 ng/ml, the decrease in cell survival did not show any concentration dependence. When derived from the metabolism of tegafur in S-1, the concentration of 5-FU in the blood is maintained at a high level because the metabolism of 5-FU is inhibited by the inclusion of gimeracil. Therefore, conditions conducive to the development of metabolic disturbances may occur in limbal basal epithelial cells, which include corneal stem cells, because of exposure to 5-FU for a longer time than that following the intravenous injection of 5-FU.

Recently, epiphora following S-1 therapy has been increasingly observed in patients. However, eye problems may be underreported. Corneal epithelial damage due to S-1 is probably caused by a collection of contributing factors. The results of this study suggest that one of these factors is the inhibition of 5-FU metabolism in corneal epithelial cells. Unfortunately, no established methods are currently available for treating and preventing these eye problems. The findings of this study should aid future research aimed at establishing effective and safe methods for treating and preventing eye problems associated with S-1 treatment of cancer.

Conflict of interest disclosure

The authors have no conflicts of interest to declare.

References

1. Shirasaka T, Taguchi T. Timeline from discovery of 5-FU to development of an oral anticancer agent S-1 and its drug concept. *Gan To Kagaku Ryoho*. 2006;**33 Suppl 1**:4–18. （in Japanese）.

2. Fukushima M. Antitumor activity and function of S-1, a new oral tegafur-based formulation. *Gan To Kagaku Ryoho*. 2006;**33 Suppl 1**:19–26. （in Japanese）.

3. Shirasaka T, Tsukuda M, Inuyama Y, *et al*. New oral anticancer drug, TS-1 （S-1）--from bench to clinic. *Gan To Kagaku Ryoho*. 2001;**28**:855–864. （in Japanese）.

4. Shirasaka T, Shimamato Y, Ohshimo H, *et al*. Development of a novel form of an oral 5-fluorouracil derivative （S-1） directed to the potentiation of the tumor selective cytotoxicity of 5-fluorouracil by two biochemical modulators. *Anticancer Drugs*. 1996;**7**:548–557.

5. Shirasaka T, Shimamoto Y, Kato T, *et al*. Invention of a tumor-selective 5-fluorouracil derivative named S-1 by biochemical modulation of 5-fluorouracil. *Gan To Kagaku Ryoho*. 1998;**25**:371–384. （in Japanese）.

6. Ohnishi Y. Development and formulation design of TS-1 combination OD tablet. *Pharm Tech Jpn*. 2014;**30**:617–621. （in Japanese）.

7. Ichinose Y, Yoshimori K, Sakai H, *et al*. S-1 plus cisplatin combination chemotherapy in patients with advanced non-small cell lung cancer: a multi-institutional phase Ⅱ trial. *Clin Cancer Res*. 2004;**10**:7860–7864.

8. Yamada Y, Takahari D, Matsumoto H, *et al*. Leucovorin, fluorouracil, and oxaliplatin plus bevacizumab versus S-1 and oxaliplatin plus bevacizumab in patients with metastatic colorectal cancer （SOFT）: an open-label, non-inferiority, randomised phase 3 trial. *Lancet Oncol*. 2013;**14**:1278–1286.

9. Koizumi W, Narahara H, Hara T, *et al*. S-1 plus cisplatin versus S-1 alone for first-line treatment of advanced gastric cancer （SPIRITS trial）: a phase Ⅲ trial. *Lancet Oncol*. 2008;**9**:215–221.

10. Esmaeli B, Golio D, Lubecki L, *et al*. Canalicular and nasolacrimal duct blockage: an ocular side effect associated with the antineoplastic drug S-1. *Am J Ophthalmol*. 2005;**140**:325–327.

11. Shioda K, Tanabe K, Kimura S, *et al*. Treatment of severe canalicular blockage secondary to peroral TS-1, an antineoplastic agent. Jpn J Clin Ophthalmol. 2009;**63**:1499–1502. （in Japanese）.

12. Kitamura H, Miyanaga T, Shin H, *et al*. Investigation of epiphora following S-1 therapy. *Gan To Kagaku Ryoho*. 2011;**38**:259–262. （in Japanese）.

13. Kim N, Park C, Park DJ, *et al*. Lacrimal drainage obstruction in gastric cancer patients receiving S-1 chemotherapy. *Ann Oncol*. 2012;**23**:2065–2071.

14. Moriya K, Shimizu H, Handa S, *et al*. Incidence of ophthalmic disorders in patients treated with the antineoplastic agent S-1. *Gan To Kagaku Ryoho*. 2017;**44**:501–506. （in Japanese）.

15. Kanazawa T, Tanakaya K, Takeuchi H, *et al*. Two cases of lacrimal duct occlusion caused by S-1 therapy in patients with gastrointestinal cancer. *J Jpn Coll Surg*. 2008;**33**:150–154. （in Japanese）.

16. Shibahara H, Kuze S, Kyokane T, *et al*. Optic lesions in patients with epiphora during S-1 therapy. *Gan To Kagaku Ryoho*. 2010;**37**:1735–1739. （in Japanese）.

17. Sasaki T, Miyashita H, Miyanaga T, *et al*. Dacryoendoscopic observation and incidence of canalicular obstruction/stenosis associated with S-1, an oral anticancer drug. *Jpn J Ophthalmol*. 2012;**56**:214–218.

18. Sakai J, Inoue Y, Kashiwagi H, *et al*. Multi-institutional

survey on the lacrimal side effects of TS-1（R）. *Jpn J Clin Ophthalmol*. 2012;**66**:271–274.（in Japanese）.

19. Sato K, Nishimura S. Adverse effects of the oral anticancer drug s-1: lacrimal passage impairment and specific features of corneal epitheliopathy. *Open Ophthalmol J*. 2013;**7**:85–86.

20. Hosotani Y, Sotozono C, Inatomi T, *et al*. Corneal epithelial lesion presumably due to anticancer drug TS-1（R）. *Jpn J Clin Ophthalmol*. 2007;**61**:969–973.（in Japanese）.

21. Sakamoto H, Sakamoto M, Hamada T, *et al*. A case of corneal lesion following treatment by peroral TS-1（R）. *Jpn J Clin Ophthalmol*. 2008;**62**:393–398.（in Japanese）.

22. Kobashi H, Kamiya K, Shimizu K. A case of corneal epithelial lesion and keratoconjunctival pigmentation due to anticancer drug S-1. *Jpn J Ophthalmol*. 2011;**55**:163–165.

23. Yamada R, Sotozono C, Nakamura T, *et al*. Predictive factors for ocular complications caused by anticancer drug S-1. *Jpn J Ophthalmol*. 2016;**60**:63–71.

24. Kolln C, Reichl S. mRNA expression of metabolic enzymes in human cornea, corneal cell lines, and hemi- cornea constructs. *J Ocul Pharmacol Ther*. 2012;**28**:271–277.

25. Nagai N, Ito Y, Okamoto N, *et al*. An *in vitro* screening test for corneal damages by various anti-glaucoma eye drops: comparison of suppression to cell growth of corneal epithelial cell line SV40（HCE-T）by them. *J Eye*. 2008;**25**:553–556.（in Japanese）.

26. Araki-Sasaki K, Ohashi Y, Sasabe T, *et al*. An SV40-immortalized human corneal epithelial cell line and its characterization. *Invest Ophthalmol Vis Sci*. 1995;**36**:614–621.

27. Ikeda K, Yoshisue K, Matsushima E, *et al*. Bioactivation of tegafur to 5-fluorouracil is catalyzed by cytochrome P-450 2A6 in human liver microsomes *in vitro*. *Clin Cancer Res*. 2000;**6**:4409–4415.

28. Komatsu T, Yamazaki H, Shimada N, *et al*. Roles of cytochromes P450 1A2, 2A6, and 2C8 in 5-fluorouracil formation from tegafur, an anticancer prodrug, in human liver microsomes. *Drug Metab Dispos*. 2000;**28**:1457–1463.