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Establishment of a new immortalized human corneal epithelial cell line (iHCE-NY1) for use in evaluating eye irritancy by in vitro test methods

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Abstract In vitro test methods that use human corneal epithelial cells to evaluate the eye irritation potency of chemical substances do not use human corneal epithelium because it has been difficult to maintain more than four passages. In this study, we make a new cell line comprising immortalized human corneal epithelial cells (iHCE-NY1). The IC50 of iHCE-NY1 cells is slightly higher than that of Statens Seruminstitut Rabbit Cornea (SIRC) cells, which are currently used in some in vitro test methods. CDKN1A in iHCE-NY1 cells was used as a marker of gene expression to indicate cell cycle activity. This enabled us to evaluate cell recovery characteristics at concentrations lower than the IC50 of cytotoxic tests.

Keywords Corneal epithelium · SIRC cell · In vitro model · Eye irritation · CDKN1A

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Introduction

A variety of in vivo, ex vivo, and in vitro test methods have been proposed as alternatives to the Draize eye test (Draize et al. 1944; OECD. 2012a). The bovine corneal opacity and permeability (BCOP) test using cow cornea tissue (OECD. 2013) and the isolated chicken eye (ICE) test using a chicken eye (OECD. 2009) are both ex vivo test methods that are suitable for identifying chemical substances with corrosive or medium-to-strong irritation potency, but at present, there are only a limited number of laboratories in Japan capable of performing these tests, which makes studies across multiple facilities problematic.

In contrast, in vitro test methods such as the SIRC-CVS test and the STE (Sakaguchi et al. 2011; Takahashi et al. 2011; ICCVAM. 2013; OECD. 2014) use two-dimensional rabbit corneal epithelial cells cultured from the SIRC cell line. These test methods can be used to classify test substances as either GHS Category 2 (mild irritant) or Non-category (non-irritant) and are useful for initial screening in a bottom-up approach to distinguishing non-irritants from irritants. SIRC cells were immortalized after the rabbit from which the corneal epithelium cells were harvested was infected with a virus (Sato and Kohno 1969). The immortalized SIRC cell line is capable of prolonged culture, making its suitable for prolonged testing at any number of laboratories.

The immortalized human corneal epithelial cell (HCE-T cell) was established in 1995 by Araki-Sasaki (Araki-Sasaki et al. 1995) and is used for culturing three-dimensional models used to test for eye irritation with the Vitrigel-EIT method (Yamaguchi et al. 2015). The immortalization gene (SV40) is introduced to HCE-T cells by infection using a live virus. At present, HCE-T cells supplied from a cell bank do not produce virus particles, even though the use of HCE-T cells

at P2 facilities is desirable. Furthermore, although HCE-T cells appear capable of stratification at the time of establishment, analysis using comparative genomic hybridization (CGH) shows that the genome constitution of HCE-Ts cell differs significantly from that of normal corneal epithelial cells (Yamasaki et al. 2009). This is likely the reason for reports that HCE-T cells either do not stratify or do so only with difficulty (Yamasaki et al. 2009). In addition, since HCE-T cells do not clone at the time of establishment, they form heterogeneous cell groups.

The reconstructed normal human corneal epithelial model (LabCyte CORNEA-MODEL) has a representative corneal epithelium-like structure and is used as a special feeder cell of 3T3-J2 (Katoh et al. 2013). The EpiOcular Eye Irritation is another eye irritation test method that uses human cells reconstructed by culturing normal human epidermal keratinocytes (Kaluzhny et al. 2011; Pfannenbecker et al. 2013).

It is desirable that cell lines used in alternative test methods exhibit minimal fluctuation within repetitive testing as well as a high level of precision for both intra-laboratory and inter-laboratory reproducibility. We also consider in vitro test methods that use human corneal epithelial cells to evaluate the eye irritation potency of chemical substances to be a desirable from the perspective of animal welfare. In actual use, however, it has been difficult to maintain the properties of human corneal epithelium for more than four passages (Yamamoto et al. 2010).

During initial studies of culturing the new immortalized human corneal epithelial cell line (iHCE-NY1), we compared cell growth and reaction to typical substances with that of SIRC cells. We also researched a gene marker that could be used to estimate the effects of exposure to concentrations that are lower than those used in cell viability assays of the iHCE-NY1 cell line.

Materials and Methods

Human corneal tissue Human corneal preparations and primary corneal epithelial cells were harvested from the corneas of a single donor, obtained from the Northwest Lion Eye Bank in Seattle, WA, in accordance with the provisions of the Declaration of Helsinki for research involving human tissue.

Isolation of human corneal epithelial cells and preparation of immortalized HCE (iHCE) cells The human corneal epithelial (HCE) cells were isolated from corneal limbus and cultured as previously reported by Yamamoto et al. (2010). The cDNA of SV40 Large T antigen was obtained from the Japanese Collection of Research Bioresources (JCRB) cell bank in Osaka, Japan, and was then modified and inserted into the multi-cloning sites of the pAcGFP1-N1 vector (Clontech Laboratories, Inc., in Mountain View, CA). The HCE cells were transfected with the inserted vector by electroporation to prepare immortalized HCE cells. Proliferation was monitored using the fluorescence microscopy (Power IX-71, Olympus, Tokyo, Japan). After passaging, cloning was done by limited dilution as described previously (Umezu et al. 2003). Individual clones were picked and transferred into 96 well plates and frozen at passage 5 following standard protocols. Neutral Red (NR) assay was performed as previously in the present study. The cloning cells were named iHCE-NY1 (clone no. 09–0502) cells. Once the cells were 70 to 80% confluent, they were dispersed with TrypLE™ Select (Invitrogen, Life Technologies Corp., Carlsbad, CA) and were passaged at 1/20 in 60-mm dishes (Blum et al. 2015).

Cell culture medium SIRC cells (JCRB9122, rabbit corneal cell line) were obtained from the JCRB Cell Bank and cultured in Eagle's Minimum Essential Medium (Sigma-Aldrich Co. LLC, St. Louis, MO) containing 10% (*v*/*v*) fetal bovine serum (FBS), 2 mM L-glutamine (Invitrogen, Life Technologies Corp.) and penicillin-streptomycin solution (Sigma-Aldrich). The iHCE-NY1 cells were cultured in Dulbecco's Modified Eagle Medium: Nutrient Mixture F-12 (DMEM/F-12, Sigma-Aldrich) containing 5% (*v*/*v*) FBS, 10 ng/mL epidermal growth factor (EGF, Sigma-Aldrich), Insulin-Transferrin-Selenium (ITS-G, Invitrogen, Life Technologies Corp.), 0.5% (*v*/*v*) dimethyl sulfoxide (DMSO, Sigma-Aldrich), 0.5 μg/mL hydrocortisone (Sigma-Aldrich), and penicillin-streptomycin solution (Sigma-Aldrich). The cells were cultured at 37°C in a 5% CO_2 in a humidified incubator.

Cell growth and density The cells were seeded at a density of 10,000 cells per Falcon® cell culture dish (60 mm diameter, Corning Inc., Amsterdam, Netherlands). The cells were cultured for 7 d, and the medium was replaced every other d. The cells were counted in Burker-Turk at 1:1 dilution with trypan blue solution (Sigma-Aldrich). The cells were seeded at a density of between 3125 and 50,000 cells per well in a 96 well CellBIND™ surface plate (Corning), and proliferation parameters were determined using a neutral red assay.

Neutral red assay The Neutral Red (NR) assay is a means of measuring living cells via the uptake of the vital dye neutral red and was applied using an in vitro toxicology assay kit, neutral red based (Sigma-Aldrich). The ratio of fractional release compared with untreated wells was calculated for each concentration and plotted against the median NR uptake concentration. The half optical density (OD) of untreated wells was calculated from the average concentration-response curves over three runs and was half maximal inhibitory concentration (IC50). One hundred percent cell viability was assumed for the optical density of untreated wells.

Table 1. Test substances

Name	Abbreviation	Classification	CAS Number	GHS Class
Sodium lauryl sulfate	SLS	Surfactant (anionic)	151-21-3	2
Polyoxyethylene octylphenyl ether	Triton X-100	Surfactant (nonionic)	9002-93-1	2
Ethanol	EtOH	Alcohol	64-17-5	2A

Test substances and examination of exposure time The three test substances used are shown in Table 1. They were supplied by the National Institute of Health Science (NIHS). The test chemicals and a negative control of phosphate buffered saline (PBS, Sigma-Aldrich) were each applied to 10 wells. Cells were incubated for periods of 1, 5, and 30 min to determine an optimal exposure time. After exposure, all wells were carefully rinsed five times with PBS, new medium was added, and the cells were incubated for 24 h at 37°C in 5% CO_2 using a humidified incubator. Cell viability was analyzed with the NR assay.

Quantitative real-time RT-PCR of the SLS exposure cells RNA from cells exposed to SLS was prepared using the TaqMan® Gene Expression Cell-to-Ct™ Kit (Applied Biosystems, Life Technologies Corp., Carlsbad, CA), and quantitative real-time reverse transcriptase-polymerase chain reaction (qRT-PCR) was carried out using a PRISM-7900 HT (Applied Biosystems). This process was performed for three independent runs. TaqMan® Express Endogenous Control Plates use TaqMan® probe-based chemistry and are the standard in quantitative 32 test genes expression (Table 2). The level of half gene expression (GE50) for untreated wells was calculated from average concentration-response curves over three runs. One hundred percent gene expression level was assumed for the cycle threshold (Ct) value of untreated wells.

Results

Morphology and comparison of cell growth The HCE cells prior to gene induction exhibited the normal morphology of corneal epithelial cells. The iHCE-NY1 cells cloned at the time of establishment (clone no. 09–0502) were similar to the HCE cells, except they exhibited slightly less contrast (were somewhat thinner). The SIRC cells exhibited a somewhat fusiform epithelial morphology (Fig. 1). Cell number of the iHCE-NY1 cells began earlier than that of the SIRC cells and was about 3 times as large (3.00 ± 0.69) (Fig. 2*A*).

Determination of cell density The iHCE-NY1 cells were seeded in 96 well plates at different cell densities and cultured for 1 d (day 1). Next, instead of exposure to test substances, the iHCE-NY1 cells were washed with PBS, and a neutral red (NR) assay performed (day 2). After washing in PBS and culturing for 1 d, a NR assay was performed (day 3). When the iHCE-NY1 cells were seeded at a density of 50,000 cells/well and cultured 1 d (day 2), the OD from the NR assay was greater than 0.8. After another day of culturing (day 3), the cells became completely confluent. When the iHCE-NY1 cells were seeded at a density of 25,000 cells/well and cultured 2 d (day 3), the cells were 90% confluent, but there was clearly room for further cell growth. Comparing the OD from day 2 with that from day 3, the absorbance increased 1.5 times.

Table 2. The list of 32 endogenous standard control in gene expression

Gene symbol	Gene name
18S	Eukaryotic 18S rRNA
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
HPRT1	Hypoxanthine phosphoribosyl transferase 1
GUSB	Glucuronidase, beta
ACTB	Beta actin
B2M	Beta-2-microglobulin
HMBS	Hydroxymethylbilane synthase
IPO8	Importin 8
PGK1	Phosphoglycerate kinase 1
RPLP0	Ribosomal protein, large, P0
TBP	TATA box binding protein
TFRC	Transferrin receptor (p90, CD71)
UBC	Ubiquitin C
YWHAZ	Tyrosine 3-monooxygenase / tryptophan 5-Monooxygenase activation protein
PPIA	Peptidylprolyl isomerase A (Cyclophilin A)
POLR2A	Polymerase II polypeptide A
CASC3	Cancer susceptibility candidate 3
CDKN1A	Cyclin-dependent kinase inhibitor 1A (p21, Clip1)
CDKN1B	Cyclin-dependent kinase inhibitor 1B (p27, Kip1)
GADD45A	GROWTH arrest and DNA damase-inducible, alpha
PUM1	Pumilio homolog 1
PSMC4	Proteasome (prosome, macropain) 26S subunit, ATPase 4
EIF2B1	Eukaryotic translation initiation factor 2B
PES1	Pescadilio homolog 1, containing BRCT domain
ABL1	c-abl oncogene 1, non-receptor tyrosine kinase
ELF1	E74-like factor 1 (ets domain transcription factor)
MT-ATP6	Mitochondrially encoded ATP synthase 6
MRPL19	Mitochondrial ribosomal protein L19
POP4	Processing of precursor 4, ribonuclease P/MRP subunit
RPL37A	Ribosomal protein L37a
RPL30	Ribosomal protein L30
RPS17	Ribosomal protein S17

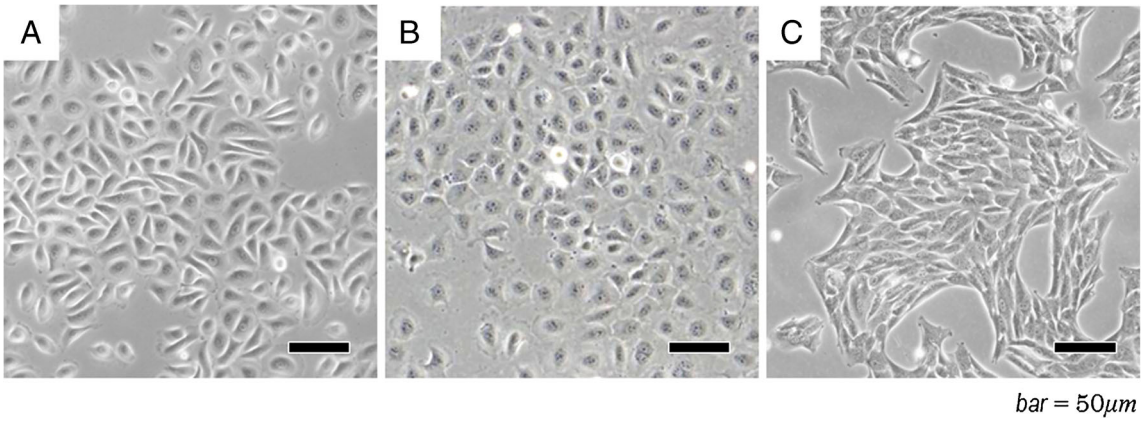

Figure 1. Morphology of the HCE cell, the iHCE-NY1 cell, and the HCE-T cell. HCE cells were observed to have the normal morphology of corneal epithelial cells (*A*), iHCE-NY1 cells were similar to the HCE cells except for a slight thickening (*B*), and SIRC cells were observed to have a somewhat fusiform epithelial morphology (*C*). *Bar* = 50 μm.

Given the above results, we determined that, in order to achieve 100% confluence by day 3, a minimum cell density of 25,000 cells/well was necessary (Fig. 2*B*).

Determination of exposure time using test substance The iHCE-NY1 cells were seeded at the densities of 25,000 and 12,500 cell/well and cultured for 1 d (day 2), after which they were exposed to sodium lauryl sulfate (SLS), a cytotoxic chemical marker, for 1, 5, and 30 min. The exposed cells were washed with PBS and cultured for 1 d, after which a NR assay was performed (Fig. 3). Results of the NR assay showed that wells seeded at 12,500 cells/well had a lower survival rate than those seeded at 25,000 cells/well and also showed significant variation in results across the three runs of the NR assay. A comparison of exposure times shows a stronger effect on cells exposed for 30 min than on those exposed for only 5 min or just 1 min. There was no significant difference in the effects of 5 and 1-min exposure times, but the technicians were able to perform the procedure quite easily using an exposure time of 5 min. Given the above results, we determined that a cell density of 25,000 cells/well and an exposure time of 5 min were the most suitable test parameters.

Comparison of cell viability at the iHCE-NY1 cell and the SIRC cell Viability of the iHCE-NY1 cells and the SIRC cells were estimated in terms of half maximal inhibitory concentration. The IC50 for iHCE-NY1 cells was measured 10 times using 363 ± 72 μg/mL of SLS, 248 ± 34 μg/mL of Polyoxyethylene Octylphenyl Ether (Triton X-100), and 17.7 ± 0.7% ethanol (EtOH). Similarly, IC50 for the SIRC cells was measured 10 times using 239 ± 93 μg/mL of SLS, 242 ± 41 μg/mL of Triton X-100, and 13.1 ± 1.4% EtOH (Fig. 4). Although the IC50 values obtained with this method were slightly higher for the iHCE-NY1 cells than for the SIRC cells, the difference was not considered significant.

Comparison of cell viability and gene expression The mean IC50 for iHCE-NY1 cells seeded at a density of 25,000 cells/well and subjected to a 5-min exposure to a test substance was measured using a NR assay with 363 μg/mL of SLS. Only

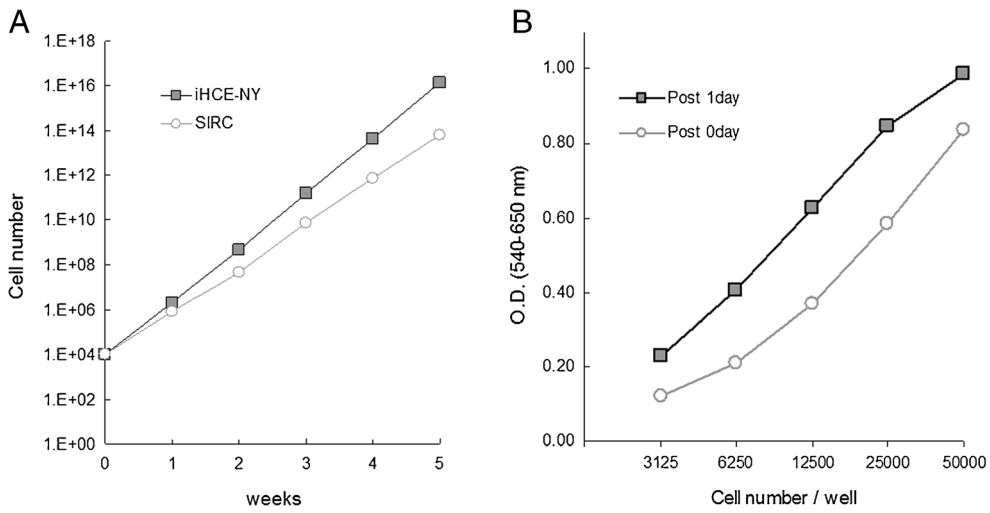

Figure 2. Cell growth curve and effect of day 3 culture. The iHCE-NY1 cells appear to grow about three times more quickly than the SIRC cells (*A*), and the minimum cell density needed to achieve 100% confluency by day 3 was 25,000 cells/well (*B*).

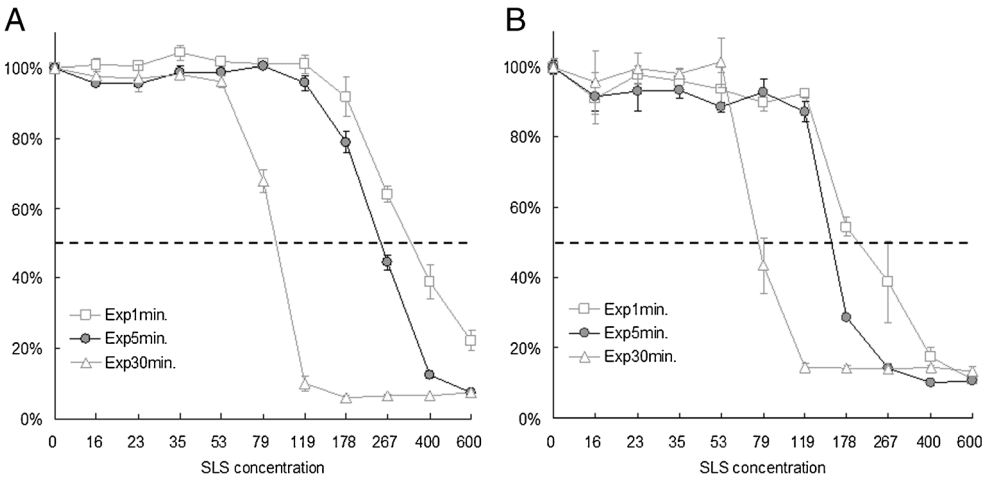

Figure 3. Exposure at the cytotoxic chemical marker of SLS by several condition. The iHCE-NY1 cells were seeded at a density of 25,000 (*A*) and 12,500 (*B*) cells/well cultured for 1 d (day 2) and then exposed to a cytotoxic chemical marker of SLS for either 1, 5, or 30 min. A cell density of 25,000 cells/well and an exposure time of 5 min were determined to be the most suitable test parameters.

CDKN1A exhibited decreased gene expression at concentrations lower than the IC50 calculated using a NR assay, and the GE50 was 178 μg/mL (Fig. 5).

Discussion

As has also been reported from similar studies of immortalized human corneal epithelial cell lines in the EU (Robertson et al. 2005), it is generally not possible to achieve gene introduction using conventional techniques such as electroporation and lipofection, so a live virus is used to introduce an immortalization gene (SV40) into corneal epithelial cells (e.g., HCE-T cells) (Araki-Sasaki et al. 1995).

The iHCE-NY1 cells used in this study comprise a new line of immortalized corneal epithelium cells derived by the researchers from a normal human corneal limbus with the following features:

1. The iHCE-NY1 cells were not derived from rabbit corneal epithelium (SIRC cells), but from normal human corneal epithelial cells.
2. The iHCE-NY1 cells were not immortalized per the introduction of a live virus and were made cloning at the time of establishment (e.g., HCE-T cells).
3. The iHCE-NY1 cells do not require special feeder cells (LabCyte CORNEA-MODEL).

Thus, these cells have significant advantages as an alternative to the cells conventionally used in in vitro test methods for evaluating ocular irritation potency in humans. On the other hand, the iHCE-NY1 cells that are easily cultured and maintained (similar to SIRC cells, HCE-T cells), making it suitable for verifying intralaboratory reproducibility or simultaneous study of interlaboratory reproducibility at multiple facilities.

Morphology of the iHCE-NY1 cells seems similar to the HCE cells, and the iHCE-NY1 cells grow earlier than SIRC cells. The IC50 of the iHCE-NY1 cells is slightly but not significantly higher than that of SIRC cells in SLS, Triton X-100, and EtOH. The use of CDKN1A as a marker enabled detection of changes in gene expression throughout a range of cell densities and exposure times as well as at concentrations lower than that of IC50 of cytotoxicity tests. IC50 and GE50 of 31 test genes except CDKN1A are the same values. CDKN1A, which is also known as p21, is a stress-induced

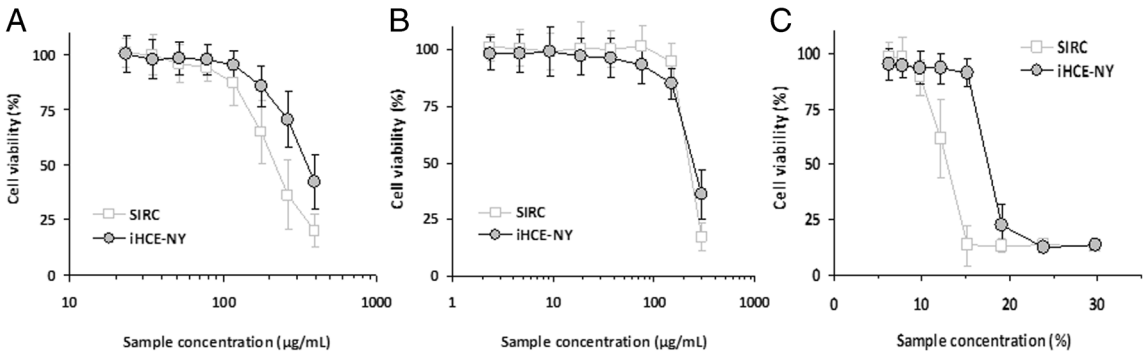

Figure 4. Comparison of cell viability at the iHCE-NY1 cell and the SIRC cell at SLS, Triton X-100, and EtOH. The viability of the iHCE-NY1 cells and the SIRC cells were estimated in terms of the half maximal inhibitory concentration per exposure to SLS (*A*), Triton X-100 (*B*), and EtOH (*C*). The IC50 of the iHCE-NY1 cells was slightly but not significantly higher than that of the SIRC cells.

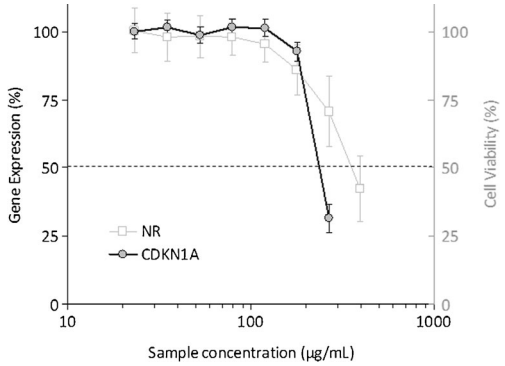

Figure 5. Comparison of cell viability and gene expression. CDKN1A exhibited decreased gene expression at concentrations lower than the IC50 calculated using a NR assay.

antiproliferative gene (Abbas and Dutta 2009; Benson et al. 2014). Because the expression of CDKN1A genes at concentrations lower than IC50 of the NR assay is lower than that of the control (non-exposure), comparison with the control can be used as an indicator of cell cycle activation, which can be used to evaluate proliferation and recovery characteristics of the cells.

The iHCE-NY1 cell is an immortalized cell line for which a differentiation marker is expressed in outer layer corneal epithelial cells when cultured in a suitable differentiation medium (data not shown). Although the iHCE-NY1 cell is an immortalization cell line, it has been shown that iHCE-NY1 cells differentiate per changes in constitution of the culture medium.

To summarize, we compared iHCE-NY1, a newly developed line of immortalized human corneal epithelial cells, with SIRC cells conventionally used in vitro test methods for evaluating of eye irritation potency. Future research will look at the manufacture of the three-dimensional reconstructed cornea models using iHCE-NY1 cells with cell culture inserts and the air lift method.

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