Toxicology in Vitro 47 (2018) 94–102

Contents lists available at ScienceDirect

Toxicology in Vitro

journal homepage: www.elsevier.com/locate/toxinvit

Nervonoylceramide (C24:1Cer), a lipid biomarker for ocular irritants released from the 3D reconstructed human cornea-like epithelium, MCTT HCE™

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ARTICLE INFO

Keywords:
Eye irritation
3D reconstructed human cornea-like epithelium
Lipidomics
Nervonoylceramide. C24:1 ceramide

ABSTRACT

Due to invasive and painful procedures during *in vivo* rabbit eye irritation test, *in vitro* alternative methods have been widely investigated. Recently, 3D reconstructed human cornea-like epitheliums (RhCEs) garner a huge attention. RhCEs employ the tissue viability as a primary endpoint to determine ocular irritancy but additional biomarkers may improve its predictive capacity. Here, we explored lipid biomarkers for ocular irritants in MCTT HCE™ RhCE model. Three irritants; sodium lauryl sulfate, benzalkonium chloride and triton X-100 were selected to represent anionic, cationic and non-ionic detergent respectively. After treating MCTT HCE™ with irritants, the alteration of lipids in the treated tissues was examined with Nile Red staining, which revealed the depletion of corneal lipids. We further quantitated the release of ceramides and free fatty acids, major lipid components of cornea, into the medium during the post-treatment incubation, employing a sensitive UPLC-MS/MS method. Among 44 lipid species, nervonoylceramide (C24:1Cer) was found to be released commonly by all three irritants in a concentration-dependent manner. Tests with 10 additional reference substances further supported that C24:1Cer release was significantly correlated with viability. Examination of the genes involved in the biosynthetic pathway for C24:1Cer revealed that stearoylCoA desaturase (SCD) and elongase1 (ELOVL1) were upregulated, suggesting that lipids and related genes may be employed as biomarkers for ocular irritants.

1. Introduction

Cosmetics, contact lenses and personal care products are frequently being placed in direct contacts with human eyes during daily use. Accordingly the evaluation of eye irritation is mandated by regulation (Ng et al., 2012; Ng et al., 2015). Rabbit draize eye test has long been a gold standard method (Draize et al., 1944; OECD, 2002) but with the increasing awareness of animal welfare, demands for the establishment of non-animal-based alternative test methods are escalating. Especially, due to the painful and invasive test procedure, and long restraints of animals during *in vivo* eye irritation test, *in vitro* methods to replace the draize eye irritation test have been explored actively (Wilson et al., 2015; Lee et al., 2017).

There are several alternatives to the draize test: *in vitro* methods using rabbit cornea epithelial cells (Matsuda et al., 2009; OECD, 2015b; Takahashi et al., 2008), and organ culture methods such as bovine corneal opacity permeability (BCOP) and the isolated chicken eye test (ICE) (Gautheron et al., 1992; OECD, 2013a, 2013b; Prinsen, 1996). Another *in vitro* alternative test method using reconstructed human cornea-like epithelium (RhCE), has drawn increasing attention, which may resolve the issues of species difference and enable the identification of weak or moderate irritants. At least four RhCE models have been reported; EpiOcular™ (MatTeck, USA) (Kaluzhny et al., 2015; OECD, 2015c), SkinEthic™ HCE (SkinEthic, France) (Alepee et al., 2010; Pfannenbecker et al., 2013), MCTT HCE™ (Biosolution, Korea) (Jang et al., 2015; Jung et al., 2011) and Labcyte Cornea model (JTE, Japan)

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https://doi.org/10.1016/j.tiv.2017.11.008
Received 25 March 2017; Received in revised form 30 September 2017; Accepted 15 November 2017
Available online 15 November 2017
0887-2333/

Table 1
10 reference substances for correlation test between C24:1Cer release and tissue viability.

Substance	CAS no	Vendor	State	GHS classification
Piperonyl butoxide	1951-03-06	Toronto research chemicals	Liquid	NC
1-Ethyl-3-methylimidazolium ethylsulphate	342573-75-5	Sigma-Aldrich	Liquid	NC
Potassium tetrafluoroborate	14075-53-7	Sigma-Aldrich	Solid	NC
Cellulose, 2-(2-hydroxy-3-(trimethylammonium)propoxy)ethyl ether chloride (91%)	68610-92-4	Sigma-Aldrich	Solid	NC
4-(Methylthio)-benzaldehyde	3446-89-7	Sigma-Aldrich	Liquid	NC
(Ethylenediamine-propyl)-trimethoxysilane	1760-24-3	Sigma-Aldrich	Liquid	Cat 1
Sodium oxalate	62-76-0	Sigma-Aldrich	Solid	Cat 1
1,5-Naphthalenediol	83-56-7	Sigma-Aldrich	Solid	Cat 2A
2,4,11,13-Tetraazatetradecane-diimidamide,*N*,*N*″-bis(4-chlorophenyl)-3,12-diimino-,di-D-gluconate (20%,aqueous)	18472-51-0	Sigma-Aldrich	Liquid	Cat 2A
2,2-Dimethyl-3-methylenebicyclo [2.2.1] heptane	79-92-5	Sigma-Aldrich	Solid	Cat 2B

(Katoh et al., 2013).

Determination of eye irritants in these RhCEs is generally based on the viability decrease elicited by test substances (Stern et al., 1998; Van Goethem et al., 2006). Prediction model is composed of a viability cutoff by which a test substance is determined as an eye irritant or not. However, compounds that affect the viability of corneal tissue may be severely irritating, which is rare in cosmetic ingredients. Indeed, although EpiOcular™ correctly determined most of eye damaging irritants belonging to UN GHS Category 1, some weak or moderate (Category 2A/2B) irritants were falsely determined as non-irritants (OECD, 2015a, 2015c). Against this backdrop, efforts are being directed to discover sensitive biomarkers for ocular irritants (Choi et al., 2015; Meloni et al., 2010).

When the external epithelial layer is exposed to irritants, lipid bilayer of cells gets perturbed, leading to the release of constituting lipids. Loss of lipids may also lead to the transcriptional activation of lipid-synthesizing enzymes for their recuperation. Indeed, it has been shown that some skin irritants increase the expression of lipid synthesizing enzymes in keratinocytes to restore skin barrier lipids (Proksch et al., 1992; Törmä et al., 2006). Several detergents are known to affect lipid-synthesizing enzymes in cultured keratinocytes, suggesting that lipids can be potential biomarker for eye irritants (Wei et al., 2006). Based on these studies, we investigated whether ocular irritants may lead to the depletion of lipids in the RhCE tissue and concomitant release into media, and the alteration of lipid-related-genes.

Three reference ocular irritants, sodium lauryl sulfate, benzalkonium and triton X-100 that are widely used in cosmetics and pharmaceutical formulations (Steiling et al., 1999) were employed to investigate the effects on lipids of MCTT HCE™ RhCE. After MCTT HCE™ was treated with the irritants, cytotoxicity and the number of remaining lipid granules in MCTT HCE™ were examined through special staining. In addition, we quantitated the amount of ceramides and free fatty acids in the post-incubated medium employing a sensitive UPLC-MS/MS technique to identify lipid biomarkers specific to ocular irritants. We also examined the expression level of lipid-synthesizing enzymes in MCTT HCE™ and explored the applicability of the lipid biomarker with 10 reference substances with known irritancy to investigate its potential utility as a secondary marker for ocular irritancy in *in vitro* eye irritation test method using RhCEs.

2. Materials and methods

2.1. Chemicals and reagents

Reference irritants and chemicals for lipid extraction and staining were purchased from Sigma-Aldrich (St. Louis, MO, USA); sodium lauryl sulfate (SLS), benzalkonium chloride, triton X-100, methyl tert-butyl ether (MTBE), methanol glycerol and Nile Red. For reverse transcription-PCR, power SYBR® Green PCR master mix was purchased from Applied Biosystems (Warrington, UK). 4-[3-(4-Iodophenyl)-2-(4-nitrophenyl)-2H-5-tetrazolio]-1,3-benzenedisulphonate (WST-1) was from Roche (Mannheim, Germany). Negative (phosphate-buffered saline (PBS)) and positive controls (1% w/v SLS in PBS) was run in each assay to monitor the integrity of tissue. For analysis of lipids, C16:0, C18:0, C20:0, C24:0, C24:1 Cer[NS] (nonhydroxy fatty acid conjugated to sphingosine), C16:0 SM (*N*-palmitoyl-D-*erythro*-sphingosylphosphorylcholine) and C18:0 SM (*N*-stearoyl-D-*erythro*-sphingosylphosphorylcholine) were purchased from Avanti Polar Lipids (Alabaster, AL). Free fatty acids (C12:0, C14:0, C16:0, C16:1, C18:0, C18:1, C18:2, C20:0, C22:0, C22:1, C24:0, C24:1, C26:0 and C28:0) were obtained from Sigma-Aldrich (St. Louis, MO). 2,2-dithiodipyridin (DTDP), 2-picolylamine (PA), triphenylphosphine (TPP), ammonium formate and formic acid were also purchased from Sigma-Aldrich. HPLC-grade methanol, isopropyl alcohol, acetonitrile and chloroform were purchased from Burdick & Jackson (Muskegon, MI). The water used were ultrapure deionized water (18.2 MΩ·cm) produced from Millipore Milli-Q Gradient system (Millipore, Bedford, MA). Other materials were of the highest quality available. Ten reference materials to examine the correlation of lipid markers with viability were selected among the proficiency substances of OECD TG492 such that equal number of irritants and non-irritants were included as listed in Table 1.

2.2. Manufacture of MCTT HCE™ model

The human corneal epithelial tissue model, MCTT HCE™ model was manufactured in Biosolution Co. (Seoul, Korea) from cultured primary human corneal cells, of which details were well described previously (Jung et al., 2011). Briefly, in accordance with the tenets of the Declaration of Helsinki and proper informed consent, human limbal epithelial cells were obtained from limbal tissues remaining after corneal transplantation. Limbal epithelium was detached by incubating with dispase II (Roche, Germany, 1.2 U/mL at 4 °C for 16 h) and gentle scraping. The limbal epithelial cells were isolated by treating the detached epithelium with 0.05% trypsin–EDTA (Invitrogen, Carlsbad, CA, USA) at 37 °C for 10 min. Isolated cells were co-cultured with feeder 3T3 fibroblasts (1×10^4 cells/cm^2) inactivated by mitomycin C treatment to facilitate the expansion of human limbal epithelial cells according to a previous report (Meller et al., 2002). The expanded human limbal epithelial cells were seeded onto an insert (0.6 cm^2 polycarbonate Millicell™ cell culture insert, Millipore, Billerica, MA, USA), and further cultivated with chemically defined medium at the air–liquid interface for 7 days. The model was shipped to the laboratory at 5 °C with testing media and the batch control information was available on request to the vendor (Biosolution Co., Seoul, Korea).

2.3. In vitro eye irritation test with MCTT HCE™ RhCE

Eye irritation test (EIT) with MCTT HCE™ was conducted as described previously (Jang et al., 2015; Jung et al., 2011) and recently (Lee et al., 2017; Yang et al., 2017). Briefly, after the shipment, 900 μL