Monocarboxylate Transporters Mediate Fluorescein Uptake in Corneal Epithelial Cells

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Submitted: October 26, 2016
Accepted: June 21, 2017
Citation: Sun Y-C, Liou H-M, Yeh P-T, Chen W-L, Hu F-R. Monocarboxylate transporters mediate fluorescein uptake in corneal epithelial cells. Invest Ophthalmol Vis Sci. 2017;58:3716–3722. DOI:10.1167/iovs.16-20998

Fluorescein is a marker dye applied to the evaluation of tight-junctional permeability of epithelial cell monolayers and has been used for decades.1–3 The application of fluorescein sodium eye drops during ocular examination was first reported by Pfluger4 for staining epithelial abrasions on rabbit corneas in 1882. It remained an important part of ocular surface assessment for more than 100 years. Whereas corneal fluorescein staining has been observed for years, the interpretation of this clinical observation is not in consensus.5–8 Wilson et al.9 reported that fluorescein uptake could be secondary to ocular cell damage. Tchaow et al.10 using a sodium fluorescein permeability assay, showed that different contact lens multipurpose solutions have differential effects on the integrity of corneal epithelium. Mokhtarzadeh et al.8 suggested that punctate staining (i.e., numerous, discrete dots of fluorescence across the corneal surface) seen in dry eye is the result of fluorescein accumulation in superficial epithelial cells. Bandamwar et al.11 further found that rinsing the corneas with solution-induced corneal staining did not reduce the staining, suggesting that pooling or accumulation of sodium fluorescein solution within intercellular spaces may not be the primary factor contributing to the clinical appearance of micropunctate corneal staining. It was also unlikely that this specific clinical picture could be produced by the rapid stromal diffusion of fluorescein through disturbed cell-cell junctions, which has been suggested by Tabery et al.3 in 1997. Bakkar and his colleagues12 demonstrated that fluorescein was able to enter normal subconfluent cells, and it was present throughout the cell, including the cytoplasm. In 2010, Thinda et al.13 also showed that topically applied fluorescein could penetrate corneal epithelial cells in both normal and dry eye patients.

Although fluorescein uptake intracellularly in corneal epithelial cells has been demonstrated, it remains unknown whether fluorescein trafficking in corneal epithelial cells is mediated by a selective transporter. Interestingly, several studies showed that fluorescein is able to penetrate into intestinal Caco-2 cell in a pH-dependent manner, the process of which is mediated by monocarboxylic acid transporter (MCT).14,15 MCTs are transmembrane proteins capable of transferring lactate and other monocarboxylates across the cell membrane. Chidlow and colleagues16 demonstrated the presence of MCT-1 and MCT-2 in the epithelium and endothelium of rat cornea. Vellonen et al.17 later showed MCT-1 and MCT-4 in human corneal epithelial cells (HCECs) and rabbit cornea. Therefore, in this study we hypothesize that MCT is also involved in fluorescein uptake in corneal epithelial cells. Our findings may provide a better understanding of the pathogenesis of ocular surface diseases for which fluorescein is frequently applied in diagnosis.

MATERIALS AND METHODS

Specimen Collection, RNA Isolation, and RT-PCR

Human and rabbit corneal epithelial cells were obtained from donor cornea and New Zealand albino rabbits following the
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regulations of the committees of Institutional Review Board (201509024RIND) and Animal Research at National Taiwan University Hospital. Total RNA was prepared from human and rabbit corneas by using a kit (RNeasy Mini Kit; Qiagen, Valencia, CA, USA) and reverse transcribed into first-strand cDNA with a kit (High Capacity cDNA Re Reverse Transcriptase Kit; Thermo Fisher Scientific, Waltham, MA, USA) according to the manufacturer’s instructions. Reverse transcription was performed in a final volume of 20 μL containing 10 μL RNA, 2 μL 10× RT buffer, 0.8 μL 25× dNTP, 2 μL 10× RT random primer, 1 μL reverse transcriptase, 1 μL RNase inhibitor, and 3.2 μL RNase-free water, and then these samples were reverse transcribed for 3 hours with a programmed thermal cycle machine. Gene-specific cDNA fragments were amplified and detected with a reagent solution (TaqMan Universal PCR Master Mix; Applied Biosystems, Thermo Fisher Scientific) according to the manufacturer’s instructions. PCR was performed on the resultant cDNA from each sample with specific primers for human MCT-1, MCT-3, MCT-4, and glyceraldehyde-3-phosphate dehydrogenase, which served as an internal control. Amplification was performed with a thermocycler. The 25-μL reaction mixture consists of 2 μL cDNA, 1 μL sense and antisense primer, and 12.5 μL 2× PCR mix. The annealing temperature and cycles for MCT-1, -3, and -4 were all 55°C and 40 cycles. At the end of amplification, the reaction mixture was heated for 10 minutes at 72°C and then cooled to 4°C. A 10-μL sample of each PCR product was separated by performing gel electrophoresis on 2% agarose containing ethidium bromide (Sigma-Aldrich Corp., St. Louis, MO, USA). The 2% agarose gel was analyzed under ultraviolet light against the DNA molecular-length markers.

Immunohistochemical (IHC) Method

To enhance tissue adhesion, 5-μm-thick sections were mounted on pretreated glass slides (Vectabond; Vector Laboratories, Burlingame, CA, USA). After drying, the paraffin was removed with xylene, and the sections were rehydrated by passing them through decreasing concentrations of ethanol. After antigen retrieval in 0.1% trypsin solution under 37°C for 15 minutes and quenching of endogenous peroxidase activity in 3% hydrogen peroxidase, the paraffin sections were blocked with serum for 60 minutes before being incubated with anti-human MCT-1 (sc-14916; Santa Cruz Biotechnology, Dallas, TX, USA) and MCT-4 (AB3316P; EMD Millipore Corp., Temecula, CA, USA) overnight at 4°C. After incubating with biotinylated secondary antibody for 60 minutes at room temperature and washing by PBS three times, the paraffin sections were incubated with reagent (VECTASTAIN ABC Kit; Vector Laboratories, Burlingame, CA, USA) for 30 minutes. Antibody detection was then achieved with 3,3′ diaminobenzidine peroxidase substrate solution for 20 minutes. Sections were counterstained with hematoxylin and eosin (H&E) to facilitate tissue orientation and then mounted. For negative controls, nonimmune serum was used.

HCEC Culture

HCECs were purchased from American Type Culture Collection (CRL-11515; Manassas, VA, USA). The cells were cultured at 37°C with 5% CO2. They were passaged in 75-cm² flasks with culture medium consisting of keratinocyte-serum-free medium (17005-042, Gibco; Thermo Fisher Scientific) with 0.05 mg/mL insulin, 500 ng/mL hydrocortisone, and 0.01μM/mL 100× antibiotic-antimyotic (100 μg/mL penicillin/streptomycin and 1.25 μg/mL amphotericin B) (15240112, Gibco; Thermo Fisher Scientific). After the cells reached approximately 80% confluence, they were dissociated by using a reagent (12605028, Gibco TrypLE; Thermo Fisher Scientific) and seeded at a number of 200,000 cells on 35-mm plastic culture dishes for further experiments.

Cultivated HCEGs Treated by Fluorescein in Culture Medium With Various pH Values and Two Different MCT Inhibitors (Salicylic Acid, 4,4'-diisothiocyanostilbene-2,2'-disulfonate [DIDS])

The fluorescein uptake experiments were measured in HCECs in 35-mm plastic culture dishes. The incubation media with different pH values (pH 6.0, 6.2, 6.6, 6.8, 7.0, and 7.4) and electrolytes were prepared as follows: 1 mM fluorescein mixed with NaCl, KCl, CaCl2, MgCl2, d-glucose, and buffering agents [2-(N-morpholino) ethanesulfonic acid] for pH 6.0, 6.2 to 6.6, and HEPES for pH 7.0 and 7.4. After discarding the original culture medium, HCECs were subsequently incubated in the culture medium consisting of fluorescein and different pH values in the absence or presence of two different MCT inhibitors: salicylic acid (a competitive inhibitor of MCT), 20, 30, and 50 mM, and DIDS (an inhibitor of MCT), 0.2, 1.0, 2.0 mM, for different incubation periods (0, 5, 10, and 15 minutes). Thereafter, the medium was aspirated, and the dish was washed twice with ice-cold original incubation medium (pH 7.4) for 20 seconds. The cells were solubilized by 1 mL 1N HCl and neutralized by 1 mL of 1N NaOH. The solutions were centrifuged at 15,000g for 5 minutes at 4°C. The supernatant was then diluted with 1 M Tris buffer. The fluorescence intensity in the supernatant was detected by a fluorometer (FL-7000 Fluorometer; Hitachi High-Technologies Corp., Tokyo, Japan) with wavelength scans from 350 nm to 550 nm. The supernatant with Tris buffer was used to detect the fluorescence intensity.

Transepithelial Electrical Resistance (TEER)

TEER was measured with a voltohmeter (Millicell-ERS; Millipore, Billerica, MA, USA) to help monitor changes in paracellular permeability. Cells (1 × 104 cells/insert) were seeded on transwell polycarbonate inserts (12-well format, 12-mm insert diameter) with a mean pore size of 0.4 μm (Transwell; Corning, Lowell, MA, USA). After 60 hours, the cells reached confluence (raw resistance is 140 Ω cm²), and the culture medium was replaced as described below. Various incubation media had different pH values: pH 6.0, 6.2, 6.6, 7.0, and 7.4. TEER was measured at 0, 5, 10, and 15 minutes. The background TEER of the blank transwell filter was subtracted from the TEER of the monolayered cells. TEER was calculated from the measured resistance and normalized by the area of the monolayered cells (ohms per square centimeter).

Fluorescein Corneal Staining Test on Rabbit With or Without Acute Dry Eye Treatment

In order to compare the differences of fluorescein uptake between normal and diseased eyes, acute dry eye treatment on the eyes of New Zealand albino rabbits (3.0–3.5 kg, 6 months old) was used in this study (see Fig. 3). The use, care, and treatment of animals followed the regulations of the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. The experimental procedures were approved by the
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Committee for Animal Research of the National Taiwan University Hospital. All procedures were performed on animals under general anesthesia induced by intramuscular injection of ketamine hydrochloride (35 mg/kg) and xylazine hydrochloride (5 mg/kg). The eyes were topically anesthetized with 0.5% proparacaine hydrochloride (Alcaine; Alcon Laboratories, Ft. Worth, TX, USA) before subsequent procedures. The right eye of each animal was used for the experiments, and the left eyes were used as controls.

Acute Dry Eye Treatment. The rabbit eyes were anaesthetized by 0.5% proparacaine hydrochloride according to the manufacturer’s instructions and then opened by eyelid retractor for 4 hours. We used a cotton tip to remove the tears around medial and lateral canthus throughout the entire period.

Fluorescein Application With Acute Dry Eye Treatment. The corneal epithelium was stained by the Fluorescein Ophthalmic Strip (CH3098; Haag Streit, Koniz, Switzerland). After 10-minute incubation, the cornea was photographed under a cobalt blue light source (Carl Zeiss Meditec, Inc., Dublin, CA, USA). Impression cytology was also performed as follows.

Impression Cytology After the Fluorescein Corneal Staining Test on Rabbits

Impression cytology was performed using a mixed cellulose ester membrane filter with a pore size of 0.22 μm (Millipore). Impression cytology after 10-minute fluorescein application was performed on the rabbit eyes with acute dry eye treatment compared with the fellow eye without any treatment. The membrane was applied on the central part of the cornea. The filter membrane strips of impression cytology were all reviewed under fluorescent microscope (TS-100 Olympus; Shinjuku, Tokyo, Japan) and followed by the process of H&E stain. All specimens were fixed with 95% alcohol solution, stained with hematoxylin (Sigma-Aldrich Corp.), and mounted with Permount. Cytology features were observed by microscope equipped with a digital camera and software (Eclipse E800 Nikon Microscope, Spot Digital Camera, Spot version 1.1 CE software; Diagnostic Instruments, Sterling Heights, MI, USA). All the animal experiments were performed in triplicate.

RESULTS

MCT-1 and -4 Are Expressed in Human and Rabbit Corneal Epithelium

To determine the MCTs that are predominantly expressed in human and rabbit corneal, the levels of MCT-1, MCT-3, and MCT-4 were examined using RT-PCR and IHC stain. MCT-1 and MCT-4, but not MCT-3, were detected in HCECs and rabbit cornea (Fig. 1A). Consistently, IHC showed high levels of MCT-1 and MCT-4 immunostaining over the superficial and basal cell layers of human corneal epithelium (Figs. 1B, 1C). Although MCT is a transporter that is primarily located on cytoplasm and cell membrane, certain epithelial cells also showed positivity of staining over the nuclei.

Intracellular Fluorescein Uptake and TEER of HCECs Cultured in the Media of Various pH Values and Different Incubation Periods

To determine the factors that are associated with fluorescein uptake, the level of intracellular fluorescein was examined in HCECs with different time and/or pH value of fluorescein treatment. Fluorescein uptake increased in a time-dependent manner at each tested pH level (Fig. 2A). Meanwhile, fluorescein uptake was also increased at lower pH values of culture media (Fig. 2A). These results suggest that the time and pH value of fluorescein treatment critically affect the fluorescein uptake. Next, to investigate whether the intracellular tight junction can be altered by different time and/or pH values, TEER was examined in cells cultured with various pH values or different incubation periods (Fig. 2B). TEER of HCECs cultured at pH values above 6.2 was initially unchanged, followed by a moderate decrease with 15-minute culture. In contrast, TEER of HCECs cultured at pH values below 6.2 was substantially decreased in a time-dependent manner. Therefore, cell culture at pH value above 6.2 for 10 minutes essentially has no effect on tight junctions.

Inhibition of MCT Decreases Intracellular Fluorescein Uptake in HCECs

To further determine the role of MCTs in fluorescein uptake in HCECs, intracellular fluorescein was measured in cells pretreated with salicylic acid (an MCT inhibitor) cultured at pH 6.6. Because TEER was only minimally changed under these conditions, the intracellular tight junction essentially remained intact, allowing us to better determine the involvement of MCT in trafficking fluorescein in HCECs. As shown in Figure 2C, salicylic acid decreased the HCEC fluorescein uptake in a dose-
dependent manner. Notably, fluorescein uptake was not completely inhibited in the current experiment. Possibly, 50 mM of salicylic acid, the highest dose of salicylic acid used in this study, was nonetheless insufficient to block the transporter function. Or, it is possible that passive diffusion contributes part of the fluorescein ingress in HCECs. Similar results were observed with the use of a separate MCT inhibitor, DIDS (Fig. 2D). Although fluorescein is still possibly trafficking by diffusion and cannot be totally blocked by any of the MCT inhibitors, our results demonstrate that MCT indeed plays a role in fluorescein transportation in HCECs.

The Absorption Spectra of the HCEC Lysates Showed Consistent Results as Intracellular Fluorescein Uptake Detected by Fluorometry in HCECs

To confirm the amount of fluorescein in various pH values and the effect of the MCT inhibitor DIDS, we measured the absorption spectra of the HCEC lysates as well. The absorption spectra of the cell lysates treated with fluorescein in various pH values showed a similar peak around 450 nm (Fig. 2E). Treatment of HCEC with pH-reducing buffer increased its absorbance around the peak wavelength (450 nm). In addition, DIDS caused a dose-dependent inhibition of the absorbance of the cell lysates (Fig. 2F).

Fluorescein Uptake Could Be a Significant Factor Contributing to Fluorescein Staining Observed Clinically

Fluorescein staining frequently observed in patients with dry eye is often interpreted as fluorescein accumulation over detached corneal epithelial cell or penetration through disrupted tight junction. To examine this idea and determine whether fluorescein uptake contributes to fluorescein staining, impression cytology was used over the rabbit cornea with acute dry eye treatment followed by fluorescein treatment. In order to confirm the location of the fluorescent signals under
fluorescent microscope, the filter membrane was processed for H&E stain. The average cell count ratio (fluorescent signal positive cell number/total cell number) under a low-power field (200×) was 0.68 ± 0.19 (average ± standard deviation). We found that the positive stains under fluorescent microscope were matched with the cells in the filter membrane strips after H&E stain. As shown in Figure 3, cytoplasmic fluorescein was observed in a portion of cells over the area of fluorescein staining, suggesting that fluorescein uptake could be a significant factor contributing to fluorescein staining observed clinically.

**MCT Inhibitor Reduces Fluorescein Uptake Over the Rabbit Cornea**

To further demonstrate that MCT is one of the transporters involving fluorescein transportation, MCT inhibitor was applied to rabbit cornea subjected to acute dry eye treatment. DIDS was selected due to its superior in vivo MCT inhibitory effect. DIDS significantly reduced the fluorescein staining over rabbit cornea. The average cell count ratio (fluorescent signal positive cell number/total cell number) with DIDS treatment was 0.30 ± 0.10, which was significantly lower than that without DIDS treatment (P = 0.04, t-test). Importantly, cytoplasmic fluorescein was significantly reduced following DIDS treatment (Fig. 4), suggesting that fluorescein uptake indeed significantly contributes to fluorescein staining.

**DISCUSSION**

In this study, we determined the basis of which fluorescein staining is applied to ocular disease diagnosis. We provided in vitro and in vivo evidence showing that fluorescein uptake is primarily mediated by MCT-1 and MCT-4, which, in combination with disrupted tight junction, contributes to fluorescein staining. Since fluorescein is frequently used for ocular surface disease diagnosis, our study focusing on fluorescein transportation may provide a better understanding of the pathogenesis of these diseases.

While the loss of tight junctions, pooling in areas of shed cells, and cellular desquamation are often considered as the basis of fluorescein staining, growing evidence suggests that transcellular transportation of fluorescein may also contribute to the staining. This notion is based on the finding that fluorescein dye predominantly accumulates within the cells over a corneal button and that corneal punctate spots disappear following polytetrafluoroethylene membrane im-

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**Figure 3.** Pictures of the membrane strips taken for impression cytology after 10-minute fluorescein dye application on New Zealand white rabbit’s cornea with acute dry eye treatment. (A, B) Intracellular fluorescein staining noted from impression cytology under fluorescent microscopy. (C, D) H&E stain of the same membrane strips in A and B. (A, C) 200×. (B, D) 400×.
Consistently, we showed that fluorescein staining spots on the rabbit cornea could be removed by impression cytology and that the cells on the membrane strip demonstrate intracellular fluorescent staining. These findings indeed support the idea that fluorescein staining is at least partly mediated via transcellular transportation.

Since fluorescein staining can be mediated by transcellular transportation, it is important to determine the transporter(s) responsible for this process. MCT has been demonstrated to play a role in the ocular drug absorption in both human and rabbit corneal epithelial cells.\textsuperscript{17} The ability of MCT to transport fluorescein is revealed in intestinal Caco-2 cells, with the demonstration that fluorescein uptake is significantly reduced by an MCT inhibitor.\textsuperscript{14} In support, we demonstrated the existence of MCT-1 and -4 in both human and rabbit corneas. Importantly, we found that corneal fluorescein intracellular uptake is reduced by different MCT blockers and can be increased by reduced pH levels, likely due to the fact that MCT is a proton-linked transporter. Taken together, these findings support a role of MCT in ocular fluorescein transportation.

In addition to MCT, the quenching effect between fluorescein and intracellular protein may possibly have an effect on fluorescein ingress in the corneal epithelial cell. In 2008, Barbero et al.\textsuperscript{19} noted the fluorophore could quench the intrinsic fluorescence emission of bovine serum albumin. Glasgow\textsuperscript{20} also demonstrated intracellular quenching of fluorescein in corneal epithelial cells in 2016. Fluorescein bound to intracellular proteins, including albumin, could reveal fluorescence quenching, both static and dynamic. In addition, Doughty\textsuperscript{21} reported that the fluorescence of sodium fluorescein was clearly influenced by both pH and concentration. Both the absorption and resultant fluorescence declined as the pH is reduced from 8.5 to lower pH values.

Although current findings support the involvement of MCT in fluorescein staining, our in vitro data are nonetheless limited to the use of a single HCEC line. This cell line was established via SV40-mediated transformation to gain constant replicating potential. However, a major concern is that cell transformation may possibly increase the expression of a certain protein, including membrane transporters, resulting in an overestimation of their functional contribution. Nonetheless, the involvement of MCTs in fluorescein uptake is also demonstrated with a rabbit in vivo model in this study, suggesting that our finding is not restricted to a specific cell type. Notably, although our results suggest a possible role of MCT in fluorescein uptake, our findings could not negate that other mechanisms, such as passive diffusion, may contribute to the intracellular movement of fluorescein as transcellular fluorescein transportation.

**Figure 4.** Pictures of the membrane strips taken for impression cytology after 10-minute fluorescein dye application with 10-minute DIDS preincubation on rabbit’s cornea with acute dry eye treatment. (A, B) Only few cells with fluorescein staining noted from impression cytology under fluorescent microscopy. (C, D) H&E stain of the same membrane strips in A and B. (A, C) 100×. (B, D) 200×.
is incompletely blocked, even with a high dose of MCT inhibitor.

In conclusion, we found that the penetration of fluorescein in corneal epithelial cells could be an active process mediated by MCT family. Translationally, this finding may provide molecular pathogenesis of certain clinical ocular presentations that are commonly presented with fluorescein staining. We believe further study of the MCT-mediated transportation in corneal cells will benefit ocular differential diagnosis and, possibly, contribute to a better treatment modality.

Acknowledgments

Disclosure: Y.-C. Sun, None; H.-M. Liou, None; P.-T. Yeh, None; W.-L. Chen, None; F.-R. Hu, None

References