Expression and Localization of Carnitine/Organic Cation Transporter OCTN1 and OCTN2 in Ocular Epithelium

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PURPOSE. The existence of an organic cation transport process in rabbit cornea and conjunctiva that mediates absorption of carnitine has previously been suggested. This study was conducted to determine the expression and localization of the carnitine/organic cation transporter (OCTN1 and OCTN2) in corneal or conjunctival epithelium.

METHODS. Reverse transcriptase-polymerase chain reaction (RT-PCR) was used for OCTN1 and OCTN2 mRNA expression in cultured human corneal-limbal epithelial (HCLE) or human conjunctival epithelial (HCjE) cells. Immunofluorescence staining with polyclonal antibody against human OCTN1 or OCTN2 was performed to investigate transporter expression in ocular epithelial cells or rabbit corneal and conjunctival epithelium. Polarity of the transporter expression was determined using Western blot analysis of the apical or basal membrane proteins extracted from the cultured cells. Apical or basal uptake of [14C]L-carnitine was determined using the polarized epithelial cells grown onto collagen-coated porous filter support.

RESULTS. OCTN1 and OCTN2 mRNA expression was detected in HCLE and HCjE cells of rabbits and humans. OCTN1 and OCTN2 were predominately localized in the apical membranes of the cells. HCLE and HCjE cells were able to take up L-carnitine; most carnitine uptake occurred through the apical surfaces.

CONCLUSIONS. This report is the first to document OCTN1 and OCTN2 expression in human corneal and conjunctival epithelial cells. These findings suggest potential involvement of OCTN1 and OCTN2 in the transport of carnitine in ocular tissues. (Invest Ophthalmol Vis Sci. 2008;49:4844–4849) DOI: 10.1167/iovs.07-1528

Carnitine (β-hydroxy-γ-trimethylaminobutyrate) is a small, highly polar zwitterionic compound that plays a physiologically important role in the β-oxidation of fatty acids through facilitation in the transport of long-chain fatty acids across the inner mitochondrial membrane and in the modulation of intracellular coenzyme A homeostasis.1,2 Deficiency can cause cardiomyopathy, skeletal muscle myopathy, and hyperglycemia.3 In mammals, carnitine, which is obtained by in situ biosynthesis and from the diet, is maintained at an appropriate level principally by a putative carnitine/organic cation transport system.4 OCTN1 and OCTN2 are members of a solute carrier superfamily of organic cation transporters (OCTs) capable of transporting carnitine.5–7 OCTN1 functions as a pH-dependent proton/cation transporter and is strongly expressed in kidney, trachea, and bone marrow.8 OCTN2 is a unique transporter with a dual mode of transport as both an Na+-dependent OCT and an Na+-dependent, high-affinity carnitine transporter. OCTN2 is expressed in human kidney, skeletal muscle, heart, and placenta.4

Free carnitine and acid-soluble acetylcarnitines are present in various tissues of the rabbit eye.9 Topical administration of carnitine has been shown to increase the concentration of free carnitine in aqueous humor, choroid, and retina and the concentration of free carnitine and acetylcarnitine in the cemal cornea.10 L-carnitine can also protect human retinal pigment epithelium from H2O2-induced oxidative damage.11 Recently, L-carnitine has been shown to act as a compatible solute in protecting corneal cells from hyperosmotic stress in models of dry eye in vitro (Simmons P, et al. IOVS 2007;48:ARVO E-Abstract 428). Artificial tear formulas containing compatible solutes, such as L-carnitine, have demonstrated rapid and consistent improvements in signs and symptoms in patients with dry eye,12 suggesting an intrinsic homeostatic role for carnitine in the eye. In addition, it has been demonstrated that L-carnitine uptakes into rabbit corneal cells, though the underlying mechanisms for this are not yet known.12 Despite functional evidence demonstrating the existence of a carrier-mediated OCT process in the rabbit conjunctiva,13 expression and localization of carnitine/organic cation transporters OCTN1 and OCTN2 in ocular surfaces have not been demonstrated. Therefore, we examined OCTN1 and OCTN2 expression and localization in ocular epithelium using ocular epithelial cell lines and rabbit ocular epithelial tissues.

METHODS

Cell Culture

Immortalized human corneal-limbal epithelial (HCLE) and human conjunctival epithelial (HCjE) cell lines derived from primary cultures of HCLE and HCjE cells (a kind gift from Ilene Gipson, Schepens Eye Research Institute, Boston, MA) were used. HCLE and HCjE cells were cultured as described.14,15 Briefly, cells were maintained on plastic at 2 × 10^4/cm² in a keratinocyte serum-free medium (K-SFM; Invitrogen-Gibco, Grand Island, NY), supplemented with 25 μg/mL bovine pituitary extract, 0.2 mg/mL epidermal growth factor (EGF; Invitrogen, Mount Waverley, VIC, Australia), and 0.4 mM CaCl₂ and were grown at 37°C in a 5% carbon dioxide atmosphere. To enhance nutrient composition, cultures were switched at approximately 50% confluence to a 1:1 mixture of K-SFM and low-calcium DMEM/F12 (Invitrogen) to achieve confluence.

For culturing polarized epithelial cells, HCLE or HCjE cells were seeded onto 0.45-μm pore size, collagen-coated, permeable filter sup-

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port (Falcon; Becton Dickinson, Franklin Lakes, NJ) at a density of 1 × 10^6 cells/cm^2. To evaluate the establishment of tight junctions, epithelial cells were grown on a filter membrane, and transepithelial electrical resistance (TER) was assessed using an epithelial volt-ohm meter (EVOM; Word Precision Instruments, SA, Australia) during a 4-day culture period. TER of control filters with no cells was measured as a baseline value. A confluent cell monolayer with peak TER value was used for subsequent experiments.

**Semi-quantitative Reverse Transcriptase-Polymerase Chain Reaction**

Total RNA was extracted from cultured HCLE and HCJE cells (SV Total RNA Isolation System; Promega, Madison, WI). Reverse transcriptase-polymerase chain reaction (RT-PCR) was performed (SuperScript One-Step and Platinum Taq System; Invitrogen, Carlsbad, CA). Purity and integrity of RNA was verified using an ultraviolet spectrophotometer and agarose gel visualization of ribosomal bands, respectively. Transcripts were amplified using the following primers: hOCTN1 sense, CTG GAT GCT CCT AAT TTA CAT GG; hOCTN1 antisense, AGG AGA CIT CTC AGA AAT GGT TGG; hOCTN2 sense, AGT GGG CTA TTT TGG GCT TT; hOCTN2 antisense, GTG CGT AGG CAC CAA GGT AA. This resulted in amplification products hOCTN1 (785 bp) corresponding to the nucleotide position 1227 to 2011 (AB007448) and hOCTN2 (398 bp) spanning nucleotides 1188 to 1585 (AB015050). The control housekeeping gene b-actin was amplified under the same conditions. PCR products were separated by electrophoresis on a 1.2% agarose gel and evaluated by analyzer software (Gel-Pro, version 3.1; Media Cybernetics, Silver Spring, MD). The ratio of integrated density of target genes over b-actin was used to normalize relative mRNA expression. PCR products were purified (Wizard SV Gel and PCR Clean-up System; Promega, Identity of each PCR product was verified by DNA sequencing (Department of Biological Sciences, Macquarie University DNA Analysis Facility, Sydney, NSW, Australia).

**Immunocytochemistry and Immunohistochemistry**

For immunocytochemistry, HCLE and HCJE cells were cultured to 70% confluence in KSMF medium in eight-well chamber slides precoated with collagen I (10 µg/cm^2; Auspep, Parkville, VIC, Australia). Medium was removed, cells were washed three times with PBS, and 0.8 mL 3.7% formaldehyde was added to each well. Cells were fixed for 15 minutes at room temperature, rinsed with PBS, and permeabilized using 5% Triton X-100 in PBS for 20 minutes. For immunohistochemistry, rabbit corneal and conjunctival tissues were obtained from New Zealand White rabbits used in our previous study. All procedures were performed in compliance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. Tissues were fixed in situ using 3.7% formaldehyde, and paraffin sections of 2 µm were generated. After the paraffin was removed, rehydrated sections were treated with 0.4% pepsin and incubated at 37°C for 3 hours. Cells and sections were blocked (100 µg/mL rabbit serum, 3% BSA) for 1 hour. Goat anti-human OCTN1 (C-13) polyclonal antibodies (sc-19819; Santa Cruz Biotechnology, Santa Cruz, CA) or goat anti-human OCTN2 (H-13) polyclonal antibodies (sc-19822; Santa Cruz Biotechnology) were applied and incubated at 4°C overnight. Slides were washed with PBS and incubated for 1 hour at room temperature with rabbit anti-goat IgG antibody conjugated with Texas Red (Santa Cruz Biotechnology). Slides were rinsed with PBS, counterstained with DAPI, and mounted using mounting medium (Vectorshield; Vector Laboratories, Burlingame, CA) with 4’,6-diamidino-2-phenylindole (DAPI) nuclear stain (Vector Laboratories). To confirm antibody specificity, blocking peptides to OCTN1 (sc-19819P) or OCTN2 (sc-19822P) (Santa Cruz Biotechnology; 100 µg peptide in 0.5 mL PBS containing 0.2% BSA) were used for competitive binding. Preimmune goat serum served as a negative control.

**Western Blot Analysis of the Polarity of OCTN1 or OCTN2 Expression in HCLE or HCJE Cells**

To further distinguish the polarity of OCTN1 or OCTN2 expression in epithelial cells, Western blot analysis of the extracted apical and basal membrane proteins was performed using anti-human OCTN1 or OCTN2 antibody. Extraction of apical and basal membrane proteins of HCLE or HCJE cells followed the method of Grube et al. Briefly, the cells were resuspended in 5.0 mL of 250 mM sucrose and 10 mM Tris/HCl, pH 7.4 (incubation buffer), supplemented with protease inhibitors (0.1 mM phenylmethylsulfonyl fluoride, 0.3 µM aprotinin, and 1 µM pepstatin). Cells were then lysed with a probe sonicator (Vibra Cell; Sonics & Materials, Newtown, CT) using 10 W power for 30 seconds on ice. Cell debris was further sonicated twice with a 1-minute interval between each round. After each sonication, the supernatants were pooled and centrifuged at 5000g for 10 minutes at 4°C. The remaining cell pellets were resuspended with 2 mL incubation buffer, and the above sonication steps were repeated. Then 15 mL ice-cold buffer was added to the pooled supernatant sample, and the mixtures were stirred slowly at 4°C for 1 hour to precipitate cell membranes. Suspensions were centrifuged at 100,000g for 1 hour in an ultracentrifuge (Discovery 100; Sorvall, Heraeus, Germany).

To separate basal and apical membranes, MgCl_2 was added to a final concentration of 10 mM. After 10 minutes of incubation on ice, the suspensions were centrifuged (2200g, 12 minutes) to separate the basal (pellet) and apical (supernatant) membranes. The pellet containing basal membranes was resuspended in incubation buffer. The apical or basal fraction was then centrifuged at 100,000g for 1 hour, and the pellets of both membrane fractions were resuspended in 0.5 mL buffer and passed through a 27-gauge needle 20 times to facilitate fraction formation. Protein concentrations in each membrane preparation were measured using a peptide quantification kit (LavaPep; Fluorotechnics, Sydney, NSW, Australia) (peptide BSA as the standard). Membrane vesicles were frozen and stored in liquid nitrogen until needed.

For Western blot analysis, the extracted apical and basal membrane proteins (10 µg) were separated using 4% to 20% SDSPAGE gradient polyacrylamide gel electrophoresis (SDA-PAGE Tris-HCl Ready Gel; Bio-Rad, Sydney, NSW, Australia). Proteins were transferred to nitrocellulose membrane overnight at 50 V and 4°C. The nitrocellulose membrane was blocked with 2% BSA in TST (10 mM Tris and 150 mM NaCl in 0.05% Tween 20) for 18 hours and then incubated with goat anti-human OCTN1 or OCTN2 antibody (1:200 in blocking buffer) overnight at room temperature. Secondary horseradish peroxidase-conjugated donkey anti-goat IgG antibody (Santa Cruz Biotechnology) was used at a 1:1000 dilution.

**Transport of L-Carnitine to Apical or Basal Membrane of Ocular Epithelial Cells**

Culture medium was removed after a peak TER value of a cell monolayer was achieved and was washed twice with a medium consisting of 25 mM Tris/HEPES, 140 mM NaCl, 5.4 mM KCl, 1.8 mM CaCl_2, 0.8 mM MgSO_4, 5 mM glucose, pH 7.4. Cells were then incubated in the medium for 60 minutes at 37°C in humidified 5% CO_2 and 95% air. [3H]-L-carnitine (L-methyl-3H)-carnitine hydrochloride (24 nM; GE Healthcare, Little Chalfont, Buckinghamshire, UK) was subsequently added to the upper or lower chamber, or both, of the filter support in the presence (for nonspecific uptake) or absence (for total uptake) of an excess amount of unlabeled L-carnitine (20 mM) and was incubated for 30 minutes at the end of the incubation, uptake was terminated by removal of the medium and three rapid washes with ice-cold PBS (30 seconds each rinse). Cultures were dissolved in 0.1 M NaOH and 0.1 Triton X-100, and aliquots were removed for liquid scintillation counting and protein concentration measurements using a peptide quantification kit (LavaPep; Fluorotechnics). Specific uptake of [3H]-L-carnitine was calculated as the difference between total [3H]-L-carnitine in the presence and absence of 20 mM unlabeled L-carnitine. Uptake experiments were performed in triplicate and repeated three times.
### Statistical Analysis

All results are expressed as the mean ± SD of at least three experiments. Student’s unpaired t-test was performed with commercial computer software (SPSS; SPSS Inc., Chicago, IL). Post hoc multiple comparisons were analyzed incorporating the Bonferroni correction. Statistical significance was set at $P < 0.05$.

### RESULTS

#### Human OCTN1 and OCTN2 mRNA Expression

Expression of human OCTN1 and OCTN2 was analyzed by semiquantitative RT-PCR in HCLE and HCjE cells (Fig. 1). Amplification products were detected in HCLE and HCjE cells. DNA sequence analysis confirmed the identity of each PCR product. Relative mRNA expression levels of OCTN1 were higher than those of OCTN2 in HCLE cells but much lower than those of OCTN2 in HCjE cells. The difference in relative expression between OCTN1 and OCTN2 in HCLE and HCjE cells was significant ($P = 0.002$ and $P = 0.004$ for HCLE and HCjE, respectively).

#### OCTN1 and OCTN2 Immunolocalization in HCLE and HCjE Cells

Given that mRNA expression of human OCTN1 and OCTN2 was detected in HCLE and HCjE cells, immunocytochemistry was performed to examine the localization of protein expression. OCTN1 expression was observed in HCLE and HCjE cells and appeared to be localized to the cell membrane (Fig. 2). OCTN2 showed similar localization in HCLE and HCjE cells (Fig. 3). Antibody specificity was demonstrated by incubating the cells with preimmune serum or antiserum preincubated with blocking peptide.

**Polarity of OCTN1 or OCTN2 Expression in HCLE or HCjE Cells**

To further distinguish the polarity of OCTN1 or OCTN2 in the cells, Western blot analysis of OCTN1 and OCTN2 was performed with the use of apical or basal membrane fractions. This demonstrated a significantly stronger signal for the apical fraction compared with the basal fraction for both transporters and for HCLE and HCjE cells (Fig. 4). These results indicated that OCTN1 and OCTN2 were localized primarily to the apical membrane of the epithelial cells.

#### OCTN1 and OCTN2 Immunolocalization in Rabbit Ocular Epithelium

In addition to human ocular epithelial cells, localization of OCTN1 and OCTN2 was determined in rabbit ocular epithelium using immunohistochemistry (Fig. 5). OCTN1 and OCTN2

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**Figure 1.** RT-PCR detection of OCTN1 and OCTN2 expression in human ocular epithelial cells. (A) Representative image of PCR-amplified human OCTN1, OCTN2, and β-actin products. Lane 1, MW marker; lanes 2, 4, 6, HCLE; lanes 3, 5, 7, HCjE. (B) Relative expression of human OCTN1 and OCTN2 mRNA in ocular epithelial cell lines. Data represent the mean ± SD (n = 3) of the ratio of integrated density to β-actin. *Significant difference from OCTN2 (bracketed). $P = 0.002$ for HCLE and $P = 0.004$ for HCjE.

**Figure 2.** Immunocytochemical detection of OCTN1 expression (red) on HCLE (A, C, E, G) and HCjE (B, D, F, H) cells. (A, B) 100× objective. (C-H) 40× objective. (A-D) Anti-human OCTN1 polyclonal antibody (dilution, 1:50). (E, F) Anti-human OCTN1 polyclonal antibody preincubated with OCTN1 blocking peptide (dilution, 1:50). (G, H) Preimmune goat serum (dilution, 1:500). Cell nuclei were stained with DAPI (blue).
proteins were strongly expressed in rabbit corneal and conjunctival epithelia, and expression appeared to occur predominantly on the apical surface of the epithelium. Furthermore, for OCTN1, little difference in immunoreactivity between corneal and conjunctival epithelia was found, but OCTN2 immunoreactivity in rabbit conjunctival epithelium appeared much higher than that of corneal epithelium, which is consistent with mRNA expression.

Carnitine Uptake by HCLE and HCjE Cells

To demonstrate the functional activity of OCTN1 and OCTN2 in human ocular epithelial cells, transport of \(^{3}H\)-L-carnitine into apical and basal surfaces of the cells was measured. When cells were cultured onto a permeable filter support, TER increased as cells became confluent. Peak TER values were achieved on the third day after cell seeding and decreased thereafter for HCLE and HCjE monolayers (Fig. 6), indicating that tight junctions were formed and that epithelial cells were polarized.\(^{16,17}\) Transport of L-carnitine by apical or basal surface revealed that 73% ± 4% and 72% ± 6% of \(^{3}H\)-L-carnitine was taken up across the apical surfaces of HCLE and HCjE cells, respectively, whereas 23% ± 2% and 13% ± 2% was taken up by the basal surfaces of the HCLE and HCjE cells, respectively (Fig. 7).
DICUSSION

L-carnitine is expressed in significant quantities in the eye and is capable of protecting corneal cells from hyperosmotic stress in dry eye and of protecting the human retinal pigment epithelium from H$_2$O$_2$-induced oxidative damage (Simmons P, et al. IOVS 2007;48:ARVO E-Abstract 428). $^{11,12}$ suggesting this micronutrient plays an important role during oxidation-induced ocular disorders. L-carnitine is transported by the organic cation transporter system, specifically by OCTN1 and OCTN2, to maintain a steady state concentration in many tissues, including skeletal and heart muscles. $^{18-20}$ Functional evidence has demonstrated the existence of a carrier-mediated organic cation transport process in the cornea (Simmons P, et al. IOVS 2007;48:ARVO E-Abstract 428)$^{12}$ and conjunctiva. $^{15}$ Protein(s) involved in this transfer, however, have not been determined. Our results provide the first direct evidence that ocular epithelial cells express carnitine/organic cation transporters OCTN1 and OCTN2, which are strong candidate proteins for mediating carnitine transport.

In the present study, significant levels of OCTN1 and OCTN2 mRNA expression in human HCLE and HCjE cells were detected, and the specificity of each PCR product was confirmed by DNA sequencing. In HCLE cells, the relative level of expression of OCTN1 was higher than that of OCTN2, but in HCjE cells, the expression of OCTN2 was much higher than that of OCTN1. These observations are consistent with the expression of the respective proteins in epithelial cells and even more so in rabbit ocular epithelium, suggesting that OCTN1 and OCTN2 are differentially expressed in the anterior segment of the eye. Using antibodies against acetylated tubulin for colocalization, other studies have shown OCTN2, but not OCTN1, to be expressed in cilia. $^{21}$ The implication of the differential distribution of OCTN1 and OCTN2 in various cell types remains unclear.

Immunofluorescence staining using polyclonal goat antibody against human OCTN1 and OCTN2 showed significant expression of OCTN1 and OCTN2 in human ocular epithelial cells and rabbit ocular epithelium. We further identified in human ocular epithelial cells that both transporters were localized primarily to apical domains of the cells. Apically polarized expression of OCTN1 and OCTN2 in airway epithelium has been reported. $^{21}$ Using human placenta, OCTN2 has also been found localized in the apical membrane of the syncytiotrophoblast. $^{6}$

In addition, we investigated the function of OCTN1 and OCTN2 in carnitine uptake by apical or basal surface of the HCLE or HCjE cells. A polarized monolayer of cells grown on porous filters is known to have the characteristics of epithelial cells in tissue. $^{16}$ Our experiments using a polarized monolayer of HCLE or HCjE cells showed most carnitine uptake was on the apical surfaces of the cells. Elucidation of the expression, localization, and function of the carnitine transporters in ocular cells is important. In ocular tissues carnitine is capable of protecting retinal pigment epithelial cells from H$_2$O$_2$-induced oxidative damage, whereas esters of carnitine have been shown to enhance optic nerve growth and to increase visual function. $^{11,22}$ Carnitine and its esters are also capable of protecting the chaperone activity of α-lens crystalline, decreasing post translational modifications induced by oxidative stress, and preventing cataract formation. $^{23,24}$ Further, dry eye is usually experienced as a consequence of aging $^{25}$ and is characterized by hyperosmotic tear film and proinflammatory changes in the ocular surface. It has been suggested that L-carnitine acts as a compatible solute in protecting corneal cells from hyperosmotic stress (Simmons P, et al. IOVS 2007;48:ARVO E-Abstract 428), suggesting an intrinsic homeostatic role for carnitine in the eye that may be harnessed as a potential therapeutic in dry eye treatment. This study provides a structural and functional basis for the concept that transport processes for carnitine exist in corneal and conjunctival epithelia. It remains to be elucidated how OCTN1 and OCTN2 mediate the transport of carnitine. Further research is needed on the functional identification of these ocular organic cation transporters for carnitine.

In conclusion, this is the first report of OCTN1 and OCTN2 expression in human corneal and conjunctival epithelial cells and in rabbit ocular epithelium. These findings suggest OCTN1 and OCTN2 may play a significant role in the transport of carnitine in ocular tissues.

![Figure 6. TER measurements of HCLE or HCjE cells cultured onto filter support on days 1, 2, 3, and 4 after seeding (mean ± SD; n = 3). Cell seeding density was 1 × 10⁶ cells/cm².](image-url)

![Figure 7. Specific uptake of [³H]-L-carnitine by apical or basal surface of the filter-grown HCLE and HCjE cells. After preincubating cells with uptake buffer at 37°C in humidified 5% CO₂ and 95% air for 60 minutes, 24 nM [³H]-L-carnitine was added to the apical or basal side in the presence (for nonspecific uptake) and absence (for total uptake) of 20 mM unlabeled L-carnitine. Specific uptake was defined as total uptake minus nonspecific uptake. Values are mean ± SD (n = 4). *P < 0.01 versus total. †P < 0.001 versus basal domain.](image-url)
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