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Ubiquitin Carboxyl-Terminal Esterase L1 Promotes Proliferation of Human Choroidal and Retinal Endothelial Cells

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Abstract

Purpose—We aimed: (1) to establish endothelial expression of ubiquitin carboxyl-terminal esterase L1 (UCHL1) in human choroid and retina and; (2) to investigate a role for UCHL1 in basic processes involved in intraocular neovascularization.

Design—Controlled translational experimental study.

Methods—Ethanol-fixed human choroid and retina (n = 3 eyes) were indirectly immunostained with rabbit anti-human UCHL1 antibody. Endothelial proliferation and migration assays were performed using cultured human choroidal and retinal endothelial cells (n = 6 isolates/assay). Cells were transfected with UCHL1-targeted or non-targeted small interfering (si)RNA and a commercially available transfection system, and used 48 hours later in experiments. Cell proliferation was evaluated using an assay in which cellular DNA was fluorescently tagged for quantification by microplate reader. Cell migration was examined in an assay that involved counting the number of endothelial cells moving across a perforated membrane. Transcript silencing was verified by Western blot for all assays.

Results—Immunohistochemistry confirmed expression of UCHL1 by endothelium in human choroid and retina *in vivo*. UCHL1-specific knockdown resulted in significantly less proliferation ($p < 0.0001$) for 3 human choroidal endothelial isolates and 3 human retinal endothelial isolates, and significantly less migration ($p = 0.016$) for 2 of 3 human choroidal endothelial isolates and 1 of 3 human retinal endothelial isolates.

Conclusions—Our results suggest that UCHL1 may be involved in choroidal and retinal endothelial proliferation in most persons, and endothelial migration in some persons. UCHL1 may be a suitable target for a new treatment of intraocular neovascularisation.

Keywords

Choroid; Retina; Endothelial Cell; UCHL1

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Conflicts of Interest: None are declared.

Introduction

Intraocular neovascularisation is directly responsible for loss of vision in age-related macular degeneration and diabetic retinopathy. These diseases are causes of substantial vision impairment and loss worldwide, particularly in high income regions.¹ A major advance in the treatment of eye diseases characterized by ocular neovascularisation has come in the last decade in the form of pharmacological inhibition of vascular endothelial growth factor (VEGF-A), which is a master regulator of vascularisation. VEGF-A blockade, achieved by RNA aptamer, soluble receptor, specific antibody or antibody fragment, and delivered directly to the eye by intraocular injection, regresses intraocular neovascularisation and significantly improves vision in patients with age-related macular degeneration in particular.^{2,3} However, the potential for toxicity to choroid and retina has stimulated interest in therapeutic targeting of alternative regulators of ocular neovascularisation.⁴

Ubiquitin carboxyl-terminal esterase L1 (UCHL1) – also known as protein gene product (PGP) 9.5 – is a deubiquitinating enzyme that was identified 30 years ago as a “brain-specific protein”.⁵ Indeed, UCHL1 is estimated to account for up to 2% of human brain soluble protein.⁶ UCHL1 is present in peripheral and CNS neurons, including retinal ganglion and horizontal cells, where it is essential for axonal transport.^{7,8} Expression is repressed in non-neuronal cells by repressor element 1 silencing transcript factor, which binds neuron restrictive silencer element.⁷ UCHL1 has had much attention in the pathology literature as an immunohistochemical neuronal marker, and separately in the neurological literature as a mediator of neurodegenerative disease.^{7,9} A handful of reports, including our own,^{10–13} have identified UCHL1 in endothelial subpopulations, but functional studies are yet to be published.

Our previous profiling studies identified UCHL1 transcript and protein in choroidal and retinal endothelial cells isolated from human eyes.^{12,13} Work from the field of oncological science indicated UCHL1 might prevent HIF-1 α degradation.¹⁴ Hypoxia inducible factor (HIF)-1 is a transcription factor that is activated when oxygen is scarce. The HIF-1 heterodimer consists of oxygen-sensitive HIF-1 α and constitutively expressed HIF-1 β subunits. In normoxia, HIF1- α is targeted for degradation via E3 von Hippel-Lindau tumor suppressor protein (VHL)-chaperoned ubiquitination. Hypoxia permits HIF1- α and HIF1- β dimerization, generating active HIF-1, which is a principal regulator of VEGF-A gene expression. This suggested to us the possibility that endothelial UCHL1 might influence intraocular neovascularisation. After verifying endothelial expression of UCHL1 in whole human choroid and retina, we investigated the involvement of UCHL1 in human choroidal and retinal endothelial proliferation and migration, which are key processes in blood vessel growth.¹⁵

Methods

Immunohistochemistry

Choroid and retina were dissected from human cadaveric eyes (Lions VisionGift, Portland, OR) (n=3), fixed in graded ethanol solutions and embedded in paraffin. Tissue cross-sections were cut 5 microns in thickness, deparaffinized in xylene, and rehydrated through

graded ethanol solutions. Sections were boiled in a microwave for 10 minutes in 10 mM citrate buffer at pH 6.0. After cooling, they were washed in a buffer of 50 mM Tris and 0.15 M sodium chloride at pH 7.5 (TBS). Non-specific binding was blocked for 1 hour with a solution of TBS, 0.1% bovine serum albumin, 0.3% Triton-X 100 and 2% v/v normal goat serum. Sections were incubated overnight at 4 °C with rabbit polyclonal anti-human UCHL1 antibody (Cedarlane Laboratories, Burlington, NC) or as a negative control, rabbit serum (Vector Laboratories, Burlingame, CA), both diluted 1:5000 in blocking solution, and subsequently they were washed with TBS. They were incubated with biotinylated goat anti-rabbit immunoglobulin (Vector Laboratories), diluted 1:200 in blocking solution for 45 minutes, washed with TBS, and finally treated with streptavidin-biotin complex conjugated to alkaline phosphatase (Vector Laboratories). To visualize antibody complexes, the sections were incubated with Fast Red (Biogenex, San Ramon, CA), as described by the supplier.

Human ocular endothelial cells

Choroidal or retinal endothelial cells were obtained from non-diseased eye pairs of 8 human cadaveric donors (Lions VisionGift), adhering closely to our previously published method.¹⁶ In brief, choroid or retina were dissected from posterior eye cups and digested with graded solutions of Dispase (Life Technologies-Gibco, Carlsbad, CA) and type II collagenase (Sigma-Aldrich, St. Louis, MO) in MCDB-131 medium (Sigma-Aldrich) with 2% FBS. Endothelial cells were isolated from the digested tissue using magnetic Dynabeads (Dyna-Invitrogen, Oslo, Norway) coated with mouse monoclonal anti-human CD31 antibody (BD Pharmingen, San Diego, CA), per the manufacturer's instructions. Cells were cultured at 37 °C and 3.5% to 5% CO₂ in MCDB-131 medium (Sigma-Aldrich) with up to 10% FBS and EGM-2 SingleQuots supplement, omitting gentamicin, hydrocortisone and FBS (Lonza-Clonetics, Walkerville, MD). To generate sufficient cells for study, the cells were transduced with the mouse recombinant amphotropic retrovirus, LXS16E6E7 (gift of Denise A. Galloway, PhD, Fred Hutchinson Cancer Institute).

siRNA blockade

UCHL1-targeted small interfering (si)RNA were designed using siDIRECT¹⁷ and synthesized by Life Technologies-Invitrogen: sense = 5'-GAAGUAGUCCUAAAGUGUAC-3'; and anti-sense = 5'-ACACUUUAGGACUACUUCUU-3'. Control siRNA were designed by scrambling the constituent nucleotides to generate non-targeted sequence: sense = 5'-CCUUUAAAUCCCCAAAGGGUU-3'; and anti-sense = 5'-CCC UUUGGGAUUUAAAAGGAA-3'. Human choroidal or retinal endothelial cells were grown to at least 80% confluence in 6 or 10 cm diameter dishes, and subsequently incubated for 24 hours with UCHL1 or non-targeted siRNA, in complex with Targefect-siRNA Transfection Kit reagents (Targeting Systems, El Cajon, CA), according to the manufacturer's instructions. After transfection, the endothelial cells were rested at 37 °C in modified MCDB-131 medium with 5% FBS for 24 hours, ahead of use in cell proliferation or cell migration assays. For every cell proliferation or cell migration assay, protein was extracted from parallel, identically transfected cultures of human choroidal or retinal endothelial cells, and interrogated by Western blot using rabbit polyclonal anti-human UCHL1 antibody (Cedarlane Laboratories) and mouse monoclonal anti-human β -actin

antibody (clone AC-15, isotype IgG1; Sigma-Aldrich), to verify UCHL1 protein knock-down.

Cell proliferation assay

Transfected human choroidal or retinal endothelial cells were plated in 96-well plates at 6×10^3 or 9×10^3 cells/well, and subsequently incubated at 37 °C in modified MCDB-131 medium with 5% FBS. After 48 hours, cell proliferation was measured using the CyQuant NF Cell Proliferation Assay Kit (Life Technologies-Molecular Probes). Instructions of the manufacturer were followed exactly with the exception that the volume of dye binding solution applied to each well was 50 μ l. Fluorescence intensity was measured on a microplate reader (VICTOR³ Multilabel Counter 1420, PerkinElmer Life, Waltham, MA) at room temperature within 2 hours of equilibration of dye-DNA binding, with excitation set at 485 nm and emission detected at 530 nm.

Cell migration assay

Transfected human choroidal or retinal endothelial cells in modified MCDB-131 medium with 10% FBS (0.3 ml aliquots containing 7.5×10^4 or 15×10^4 cells) were added to polyethylene terephthalate transwells with 0.3 cm² diameter membranes that were pierced with 8 micron pores (BD Falcon, Franklin Lakes, NJ). Transwells were subsequently placed into wells of a 24-well plate, containing 1.2 ml of the same medium. After overnight incubation, membranes were fixed with 4% paraformaldehyde, washed with phosphate buffered saline and air-dried. They were excised from the transwells and mounted on glass slides in ProLong Gold Antifade Reagent with DAPI (Life Technologies-Molecular Probes). Lower surfaces of the membranes were imaged under 200 $\times$ magnification at the confocal microscope (Fluoview FV1000 confocal microscope, Olympus America, Center Valley, PA). Number of migrated cells was averaged across 6 photographic fields for each membrane.

Statistical methods

Data obtained in cell proliferation assays and cell migration assays were presented as mean $\pm$ standard error of mean. Data from two groups were compared by unpaired two-tailed Student t-test, using GraphPad Prism (GraphPad Software, La Jolla, CA). A significant difference was defined as one yielding a *p*-value less than 0.05.

Ethics

The human tissue used in the reported work was purchased from the “Lions VisionGift” eye bank. Since the US Office for Human Research Protections does not consider the deceased to be human subjects, institutional review board clearance did not apply to this work.

Results

Human choroidal and retinal endothelial cells express UCHL1 *in vivo*

Reports that human endothelium expresses UCHL1 are based solely on studies with cultured endothelial cells.^{10–13} However, *in vitro* culture may impact cell phenotype, and thus we

examined UCHL1 expression *in vivo*, using intact choroid and retina dissected from human cadaver eyes (Figure 1). In comparison to tissue stained with control antibody, tissue stained with specific antibody detected UCHL1 in choroidal endothelial cells (Figures 1A and 1B) and retinal endothelial cells (Figures 1C and 1D). There was some non-specific uptake of the antibody within vessels. Consistent with the neuroscience literature,⁷ positive staining was observed in the nerve fiber, ganglion cell and inner and outer plexiform layers of the retina, and some staining also was noted in the choroidal stroma. These results confirm that human choroidal and retinal endothelial cells express UCHL1 *in vivo*.

Targeted knockdown of UCHL1 consistently inhibits human choroidal and retinal endothelial cell proliferation

Endothelial cell proliferation is one key process in neovascularisation. We studied the effect of siRNA-mediated UCHL1 silencing on human retinal and choroidal endothelial cell proliferation, using an assay in which cellular DNA was fluorescently tagged for quantification by microplate reader (Figure 2). This assay does not evaluate cell viability. UCHL1-specific knockdown resulted in significant reduction in mean cell proliferation by 18.1% to 59.5% for 6 of 6 human choroidal or retinal endothelial isolates (Figure 2A and 2C). Western blot of protein extracts from siRNA-treated cells confirmed knockdown by specific siRNA (Figure 2B and 2D). This result suggests that UCHL1 may be involved in ocular endothelial cell proliferation in most persons.

Targeted knockdown of UCHL1 variably inhibits human choroidal and retinal endothelial cell migration

Endothelial cell migration is a second key process in neovascularisation. We examined the impact of siRNA-mediated UCHL1 silencing on human retinal and choroidal endothelial cell migration, counting the number of endothelial cells moving across a perforated membrane (Figure 3). UCHL1-specific knockdown resulted in significant reduction in cell migration for 2 of 3 human choroidal endothelial isolates and 1 of 3 human retinal endothelial isolates; where significant, mean migration was reduced by 36.3% – 76.6% (Figure 3A and 3C). Western blot of protein extracts from siRNA-treated cells confirmed knockdown by specific siRNA (Figure 3B and 3D). This result suggests that UCHL1 also may be involved in ocular endothelial cell migration in some individuals.

Discussion

Pharmacological inhibition of VEGF-A represents a major advance in the treatment of intraocular neovascularisation. However, because this treatment approach is not effective in all cases and may cause toxicity to the intraocular tissues, there is interest in identifying additional regulators of neovascularisation for therapeutic targeting. This study identifies UCHL1 as one such molecule. UCHL1 is generally considered a neuron-specific protein,⁷ but our profiling work has identified expression of UCHL1 in cultured ocular endothelial cells^{12,13} and other groups have reported expression by human umbilical vein and aortic endothelial cells,¹⁰ as well as cells with the potential to differentiate into endothelial cells.¹¹ Applying immunohistochemistry to human choroid and retina, we provide the important *in*

vivo confirmation of these previous cell culture studies, which have suggested UCHL1 is synthesized by human endothelial cells.

This is the first report to implicate UCHL1 in neovascularisation within the eye or in any body location. Evidence in the scientific literature suggested UCHL1 might influence blood vessel growth. Human choroidal endothelial sprouting is potently inhibited by the proteasome inhibitor, epoxomicin.¹⁸ Since UCHL1 is a deubiquitinating enzyme, this result suggests UCHL1 might inhibit neovascularisation. On the other hand, work in cancer biology indicates UCHL1 counteracts von Hippel-Lindau tumour suppressor protein (VHL)-chaperoned ubiquitination of HIF-1 α .¹⁴ This should promote vessel growth by stabilizing HIF-1 α , which activates transcription of VEGF-A. Indeed, using targeted siRNA to reduce cellular expression of UCHL1 in cultured cells, we show that UCHL1 promotes the proliferation and migration of human choroidal and retinal endothelial cells; UCHL1-specific knockdown resulted in significantly less proliferation and migration for 6 of 6 and 3 of 6 human choroidal or retinal endothelial cell isolates, respectively. This suggests that in the human eye, choroidal and retinal endothelial UCHL1 promotes neovascularisation.

One strength of our work is its use of human cells and tissues. Information on endothelial cell involvement in intraocular neovascular disease derives largely from work conducted in mice. Mice provide important *in vivo* models, but the structure of the mouse eye and its circulation differ from those of the human, and scale disparities of multiple parameters likely result in differences in the level of oxygenation of cells and effects on vessel proliferation in disease.¹⁹ Studies focusing on basic disease processes that involve human ocular endothelial cells therefore have unique value even though models are necessarily *in vitro*. Our results, from experiments using multiple human isolates, highlight an important issue in working in a human system. We observed UCHL1 involvement in endothelial proliferation for all 6 isolates, but in endothelial migration for only half of isolates. While technical variation – including variation in the level of UCHL1 knockdown achieved by targeted siRNA – likely contributes to differences between results, gene expression varies across humans. Indeed, our previous work shows gene expression profiles of choroidal and retinal endothelial cells from different human donors are distinct.¹² Research is often based on cells derived from a single human donor. These data show the importance of studying multiple donors when investigating the role of human endothelial cells in disease.

Potential for toxicity to neurons is a concern in specifically targeting UCHL1, given the role of UCHL1 in axonal transport. It is possible to target blockade to the proliferating ocular endothelial cells that contribute to neovascularisation, and limit off-target expression in retinal neurons and non-dividing ocular vascular endothelial cells. For example, this could be achieved using UCHL1-specific shRNA, transcribed under control of a human Cdc6 promoter-multimerized mouse endothelin enhancer element, delivered by lentiviral construct. Cdc6 promotes the cell transition from resting to active division. The multimerized endothelin enhancer stimulates promoter activity within endothelial cells. Originally described by Szymanski et al,²⁰ this promoter-enhancer element achieves high and exclusive expression of reporter and therapeutic transgenes within proliferating endothelial cells in the mouse eye.²¹

In summary, our study indicates that UCHL1 is expressed by human choroidal and retinal endothelial cells *in vivo*, and suggests that UCHL1 may be involved in ocular endothelial proliferation in most persons and in ocular endothelial migration in some persons. The data support a hypothesis that UCHL1 promotes choroidal and retinal neovascularisation, and suggests UCHL1 may be a suitable target of a new treatment for neovascular eye disease. Studies using *in vivo* models – including laser-induced choroidal neovascularisation and oxygen-induced retinopathy in the mouse²² – would be an appropriate follow-up investigation, ahead of clinical application.

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Figure 1.

Human choroid (A and B) and retina (C and D) immunostained with rabbit anti-human UCHL1 antibody show positivity of choroidal and retinal vascular endothelium (arrows) and retinal neurons, which is not present in negative control sections (A and C inserts) stained with rabbit serum in place of primary antibody. Fast Red with haematoxylin counterstain. Original magnification: A and C = 400X; B and D = 1000X. Bar approximates length of 10 microns.

Figure 2.

Human choroidal or retinal endothelial cells (EC) were treated with UCHL1-targeted (UCHL1) or non-targeted (NT) siRNA for 24 hours. Proliferation over a 48-hour period was measured by DNA binding assay after 96 hours (n = 8 wells/condition). Western blot of protein extracts from siRNA-treated cells confirmed knockdown by targeted siRNA. (A and C) Graphs show results obtained for (A) 3 choroidal endothelial isolates and (C) 3 retinal endothelial isolates. Bars represent mean and error bars represent standard error of mean. Student t-test, 2-tailed. (B and D) Representative gel images of UCHL1 protein (25 kDa, green; β -actin as loading control at 42 kDa, red) in (B) human choroidal endothelial cells and (D) human retinal endothelial cells 48 hours after transfection. L = ladder.

Figure 3.

Human choroidal or retinal endothelial cells (EC) were treated with UCHL1-targeted (UCHL1) or non-targeted (NT) siRNA for 24 hours. Migration across a perforated membrane (8 μ m pores) over a 24-hour period was measured after 72 hours ($n = 4$ wells/condition). Western blot of protein extracts from siRNA-treated cells confirmed knockdown by targeted siRNA. (A and C) Graphs show results obtained for (A) 3 choroidal endothelial isolates and (C) 3 retinal endothelial isolates. Bars represent mean and error bars represent standard error of mean. Student t-test, 2-tailed. (B and D) Representative gel images of UCHL1 protein (25 kDa, green; β -actin as loading control at 42 kDa, red) in (B) human choroidal endothelial cells and (D) human retinal endothelial cells 48 hours after transfection. L = ladder.