Expression of the SARS-CoV-2 Receptor ACE2 in Human Retina and Diabetes—Implications for Retinopathy

Lingli Zhou,[1] Zhenhua Xu,[1] James Guerra,[1] Avi Z. Rosenberg,[2] Paride Fenaroli,[2,3] Charles G. Eberhart,[1,2] and Elia J. Duh[1]

[1]Department of Ophthalmology, Johns Hopkins University School of Medicine, Baltimore, Maryland, United States
[2]Department of Pathology, Johns Hopkins University School of Medicine, Baltimore, Maryland, United States
[3]Nephrology Unit, Parma University Hospital, Department of Medicine and Surgery, Parma, Italy

Correspondence: Elia J. Duh, Department of Ophthalmology, Wilmer Eye Institute, Johns Hopkins University School of Medicine, 400 N. Broadway, Baltimore, MD 21287, USA;
eduh@jhmi.edu.
Charles G. Eberhart Department of Ophthalmology, Wilmer Eye Institute, Johns Hopkins University School of Medicine, 400 N. Broadway, Baltimore, MD 21287, USA;
ceberha@jhmi.edu.

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Purpose. To investigate the expression of angiotensin-converting enzyme 2 (ACE2), the receptor for SARS-CoV-2 in human retina.

Methods. Human post-mortem eyes from 13 non-diabetic control cases and 11 diabetic retinopathy cases were analyzed for the expression of ACE2. To compare the vascular ACE2 expression between different organs that involve in diabetes, the expression of ACE2 was investigated in renal specimens from nondiabetic and diabetic nephropathy patients. Expression of TMPRSS2, a cell-surface protease that facilitates SARS-CoV-2 entry, was also investigated in human nondiabetic retinas. Primary human retinal endothelial cells (HRECs) and primary human retinal pericytes (HRPCs) were further used to confirm the vascular ACE2 expression in human retina.

Results. We found that ACE2 was expressed in multiple nonvascular neuroretinal cells, including the retinal ganglion cell layer, inner plexiform layer, inner nuclear layer, and photoreceptor outer segments in both nondiabetic and diabetic retinopathy specimens. Strikingly, we observed significantly more ACE2 positive vessels in the diabetic retinopathy specimens. By contrast, in another end-stage organ affected by diabetes, the kidney, ACE2 in nondiabetic and diabetic nephropathy showed apical expression of ACE2 tubular epithelial cells, but no endothelial expression in glomerular or peritubular capillaries. Western blot analysis of protein lysates from HRECs and HRPCs confirmed expression of ACE2. TMPRSS2 expression was present in multiple retinal neuronal cells, vascular and perivascular cells, and Müller glia.

Conclusions. Together, these results indicate that retina expresses ACE2 and TMPRSS2. Moreover, there are increased vascular ACE2 expression in diabetic retinopathy retinas.

Keywords: COVID-19, SARS-CoV-2, diabetic retinopathy

In the past year, the importance of angiotensin-converting enzyme 2 (ACE2) has emerged as the critical cell-surface receptor for SARS-CoV-2[1] via binding to the viral spike protein,[2] in addition to its well-recognized role as a critical regulator of the renin-angiotensin system.[3] With respect to its cardiovascular effects, ACE2 is known to have a major protective role in multiple disease settings, including diabetic cardiovascular complications.[4] ACE2 is especially pertinent to diabetic vascular complications including retinopathy. An imbalance in the renin-angiotensin system (RAS) has been implicated in progression of diabetic retinopathy.[5,6] Indeed, a meta-analysis of clinical trials indicates that RAS inhibitors such as ACE inhibitors and angiotensin receptor blockers reduce the risk of diabetic retinopathy.[7] As a negative regulator of RAS, the role of ACE2 has received great attention. In experimental animal models, increase in ACE2 is protective against diabetic retinopathy.[8] Conversely, ACE2 deficiency exacerbates diabetic cardiovascular complications in experimental models.[9] Interestingly, in light of its importance for diabetic retinopathy, expression of ACE2 in the human retina, including retinal vascular cells, has not been reported.

COVID-19 and ACE2 have drawn considerable attention with respect to their interplay with diabetes. Patients with diabetes are known to have more adverse clinical outcomes, and preexisting diabetes increases risk of COVID-19-induced kidney damage, as well as cytokine storm.[10,11] An important component of this may be COVID-19 tropism caused by tissue expression of ACE2. Patients with diabetes exhibit increased expression of ACE2 protein in bronchioles and alveoli,[12] as well as liver.[13]

The retinal involvement of SARS-CoV-2 has drawn increasing attention. COVID-19 is well known to induce systemic damage beyond respiratory manifestations,[14] leading to enhanced understanding of this condition as a multiorgan disease,[14] affecting the brain, heart, kidney, digestive system, as well as the ocular surface.[15] In recent months, an expanding number of reports have emerged regarding retinal abnormalities in COVID-19 patients. These studies document retinal pathology including hemorrhages and

cotton-wool spots, venous dilation, vascular tortuosity, and retinal sectorial pallor.[16–19] Several investigators have reported alterations in the retinal microvasculature in COVID-19 patients as assessed by OCT angiography, including reduced retinal and foveal-centered vessel density.[20–22] Postmortem ocular tissue studies have demonstrated evidence of intraocular SARS-CoV-2 in some cases of COVID-19.[23,24] Documented severe visual loss related to COVID-19 has been reported, including acute retinal necrosis[25] and acute viral retinitis,[26] with confirmed SARS-CoV-2 positivity on vitreous testing. Greater attention to potentially pathogenic retinal damage and infection is warranted. An important question regarding retinal COVID-19 manifestations is whether the retina could be infected by SARS-CoV-2. Notably, in brain, the SARS-CoV-2 receptor ACE2 is expressed in choroid plexus[27] and endothelial cells,[28] potentially facilitating infection and damage of blood-brain barriers.

Strikingly, whether the human retina expresses the host susceptibility factors for infection, particularly ACE2 and TMPRSS2, the cell-surface–associated protease that facilitates viral entry after binding of the spike protein to ACE2,[1,29] has not been reported. In addition, the effect of diabetes on ACE2 expression has not been studied, an important issue because diabetes is known to exacerbate multiorgan involvement of COVID-19. ACE2 expression in diabetes is of further interest given the likely importance of ACE2 in progression of DR and other complications. In this study, we therefore examined ACE2 and TMPRSS2 protein expression in postmortem human eyes with an immunohistochemical approach similar to our previous study of the ocular surface.[30] We also studied ACE2 expression in specific vascular cell types using cultured human retinal endothelial cells and pericytes.

Research Design and Methods

Patients and Specimens

Postmortem human globes from autopsy specimens of individuals at Johns Hopkins Hospital were collected from 13 nondiabetic controls without ocular disease and from 11 diabetic individuals with diabetic retinopathy. The tissues were fixed in 10% formalin and embedded in paraffin as described.[30] Clinical details pertaining to study patients are described in the Table. Study protocols were approved by the Institutional Review Board at the Johns Hopkins School of Medicine.

Immunohistochemistry Staining

Immunohistochemistry staining was performed as previous described.[30] Briefly, deparaffinized sections were boiled in 100 × antigen retrieval buffer (ab93678, Abcam, Cambridge, MA) for 20 minutes. After blocking in 5% normal goat serum diluted in PBS, the sections were incubated with anti-ACE2 antibody (ab108252, 1: 100; Abcam, Cambridge, MA, USA), anti-TMPRSS2 antibody (ab92323, 1:2000; Abcam), and isotype- and concentration-matched normal IgG control (ab172730, Abcam) overnight at 4°C. The sections were incubated with secondary biotinylated goat anti-rabbit IgG (1:2000) for one hour at room temperature. ACE2 staining was then detected using the alkaline phosphatase detection system (Vectastain ABC-AP kit, Vector laboratories, Burlingame, CA), and a blue reaction product was produced by incubating sections with alkaline phosphatase substrate (Vector blue AP substrate kit III; Vector Laboratories, Burlingame, CA, USA) for 20 to 30 minutes. The sections were counterstained with nuclear fast red (Vector Laboratories), mounted with mounting medium (H-5501, Vector Laboratories), and examined under a light microscope. For quantitation of ACE2 positive blood vessels, figures at magnification ×10 were taken from four consecutive fields adjacent to the optic nerve. The number of ACE2 positive vessels was quantified by two independent observers in a masked fashion.

Table. Demographic Information for Postmortem Eyes

Case Number	Age	Gender	Diabetes Status
1	92	F	ND
2	92	F	ND
3	65	M	ND
4	65	M	ND
5	44	M	ND
6	81	F	ND
7	82	F	ND
8	80	M	ND
9	80	M	ND
10	74	M	ND
11	74	M	ND
12	62	M	ND
13	46	M	ND
14	57	M	NPDR
15	68	F	NPDR
16	87	M	NPDR
17	60	F	NPDR
18	62	F	NPDR
19	60	M	NPDR
20	55	M	PDR
21	55	M	PDR
22	68	F	PDR
23	89	F	PDR
24	86	M	PDR

ND, nondiabetic control.

Immunofluorescence Staining

After boiling in 100 × antigen retrieval buffer (ab93678; Abcam) for 20 minutes, sections were blocked in 5% normal donkey serum diluted in tris-buffered saline (TBS) with 1% triton X-100. Sections were then incubated with anti-ACE2 antibody (AF933, 3 µg/mL; R&D, Boston, MA, USA) and anti-CD31 (ab76533, 1:500; Abcam) overnight at 4°C. After thoroughly washing with TBS, the sections were then incubated with Alexa 488 donkey anti-rabbit IgG (1:400) and Alexa 594 donkey anti-goat IgG (1:400) for one hour at room temperature. DAPI was used for counterstain. Images were taken with confocal (LSM 880; Zeiss, Oberkochen, Germany).

Cell Culture

Human retinal pericytes (HRPs; Cell Systems, Kirkland, WA) were cultured in complete medium formulated at normal blood glucose level with serum and CultureBoost (Cell Systems) and were used at passage 5 to 7. Human retinal endothelial cells (HRECs; Cell Systems) were cultured in EGM2-MV medium (Lonza). HRECs were grown on fibronectin (Invitrogen, Carlsbad, CA, USA) coated dishes and were used at passages 6 to 10. HRPs and HRECs were

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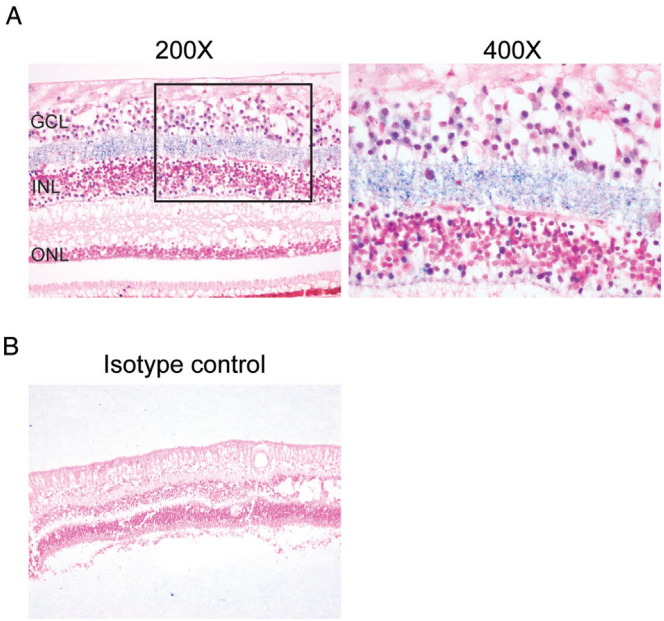

Figure 1. ACE2 expression in human retina. (**A**) Characteristic ACE2 expression in retina specimens from nondiabetic retina. ACE2 expression was found in some cells in the retinal ganglion cell layer, inner plexiform layer, and inner nuclear layer. (**B**) Isotype IgG control was negative for staining.

cultured in a humidified 5% CO_2 incubator at 37°C, and medium was changed every two to three days.

Western Blot Analysis

Proteins from HRECS and HRPs were extracted using RIPA lysis buffer (Sigma-Aldrich Corp., St. Louis, MO, USA). Total protein concentration was measured using DC protein assay kit (500-0112; Bio-Rad Life Science, Hercules, CA, USA). Protein 80 µg from cell lysates was subjected to 7.5% SDS-PAGE and transferred to Hybond ECL nitrocellulose membrane (Amersham Biosciences, Piscataway, NJ, USA). After incubation with appropriate primary and secondary antibodies, the blots were detected with the Supersignal Femto Chemiluminescent Substrates (Thermo Fisher Scientific, Waltham, MA, USA). For reprobing, blots were washed in Western blot stripping buffer (Thermo Fisher Scientific) for 15 minutes before proceeding with new blotting. Anti-ACE2 (ab108252, 1:1000) antibodies were purchased from Abcam. The β-actin detected by its antibody (no. 4970, 1:2000; Cell Signaling) was used for loading control. Horseradish peroxidase-tagged secondary anti-rabbit IgG was from Cell Signaling (no. 7074, 1:2000).

Results

ACE2 Expression in Human Retina

We first examine the ACE2 expression in non-diabetic control retinas that lack ocular abnormalities. In all specimens, we found that the ACE2 was expressed in multiple non-vascular neuroretinal cells, including the retinal ganglion cell layer, inner plexiform layer, inner nuclear layer, and photoreceptor outer segments (Fig. 1A). Staining of inner nuclear layer was especially prominent in the inner and outer fringes, including adjacent synapses (Fig. 1A). Isotype IgG control was negative for staining (Fig. 1B).

Increased ACE2 Expression in Diabetic Retinopathy Retinas

We further investigated the expression of ACE2 in diabetic retinopathy specimens. We found that neuroretinal ACE2 expression in both non-proliferative diabetic retinopathy (NPDR) specimens and proliferative diabetic retinopathy (PDR) was similar in comparison with that in the non-diabetic control specimens (Fig. 2A). Strikingly, ACE2 staining was prominent in retinal blood vessels, in both superficial and deep plexuses, in both the NPDR and PDR specimens as compared to nondiabetic. We quantified the number of ACE2 positive blood vessels in four consecutive 10 × fields adjacent to the optic nerve and found significantly more ACE2 positive vessels in the NPDR and PDR specimens compared to nondiabetic (Fig. 2B). Among the five PDR cases, only two cases showed microscopic lesions consistent with prior laser therapy. One of the cases showed less ACE2 + staining compared with other cases. However, on the basis of the limited case numbers, we were not able to conclude whether laser photocoagulation might alter ACE2 expression. Isotype IgG control was negative for staining (Fig. 2D).

ACE2 Expression in Nondiabetic and Diabetic Kidneys

Diabetic nephropathy is another well-known microvascular complication of diabetes. We further investigated ACE2 expression, including vascular ACE2 expression, in kidney specimens. Consistent with previous reports,[31] ACE2 expression was observed in the renal tubular epithelial cells in

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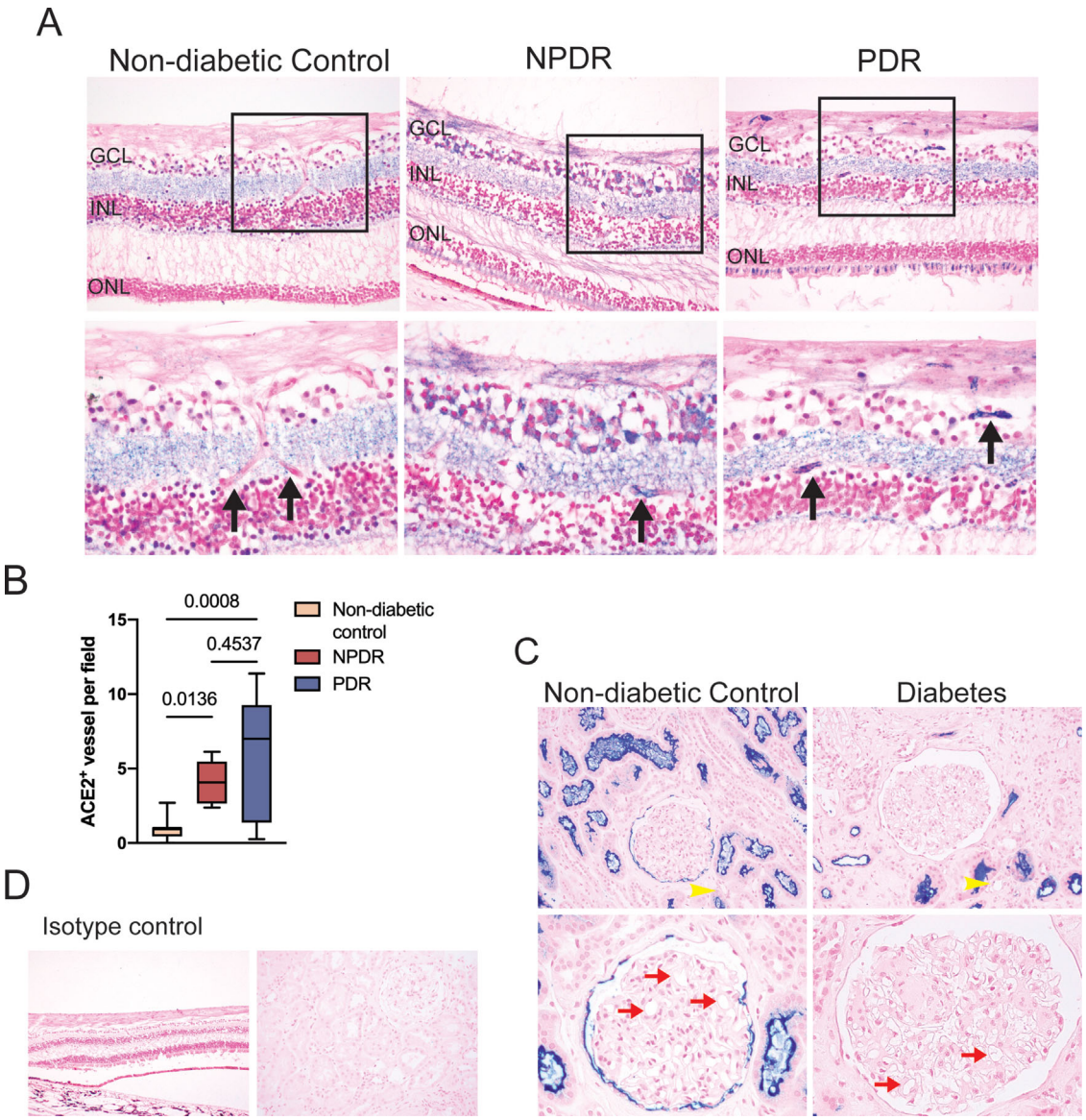

Figure 2. Increased vascular ACE2 expression in diabetic retinopathy retina. (**A**) Characteristic ACE2 expression in retina specimens from nondiabetic retina, NPDR, and PDR. *Black arrows* denote retinal capillaries, with significantly increased staining in diabetes. (**B**) ACE2 positive vessel staining was quantitated in retinas from nondiabetic retina, NPDR, and PDR. (**C**) Characteristic ACE2 expression in renal specimens from nondiabetic and diabetic nephropathy. ACE2 staining was absent in peritubular capillaries (*yellow arrowhead*) and glomerular capillary loop (*red arrow*). (D) Isotype IgG control was negative for staining.

nondiabetic kidney specimens. In the diabetic kidney, we observed similar ACE2 expression patterns compared with the nondiabetic kidney specimens (Fig. 2C). Interesting, no vascular ACE2 expression was observed in both nondiabetic and diabetic kidney (Fig. 2C).

Retinal Vascular Expression of ACE2 in PDR

We further evaluated which vascular cell types express ACE2. With immunofluorescent staining, we found that ACE2 colocalized with the endothelial cell marker CD31. As shown in Figure 3A, we observed double positive ACE2 and CD31 staining on cross-sectional and longitudinal cuts of capillaries. We also found ACE2 staining on CD31 negative cells, which could morphologically indicate pericytes or possibly astrocytes or microglial cells in the NFL. In high-magnification image of immunohistochemical staining, we also found ACE2 expression in capillary endothelial cells (Fig. 3B).

ACE2 Protein Expression in Primary Human Retina Endothelial Cell and Primary Human Pericytes

To further confirm expression of ACE2 in human retinal vascular cells, we examine ACE2 expression in primary HREC and primary human pericytes by Western blot analysis. As shown in Figure 4, ACE2 protein was expressed in both HREC and primary human pericytes.

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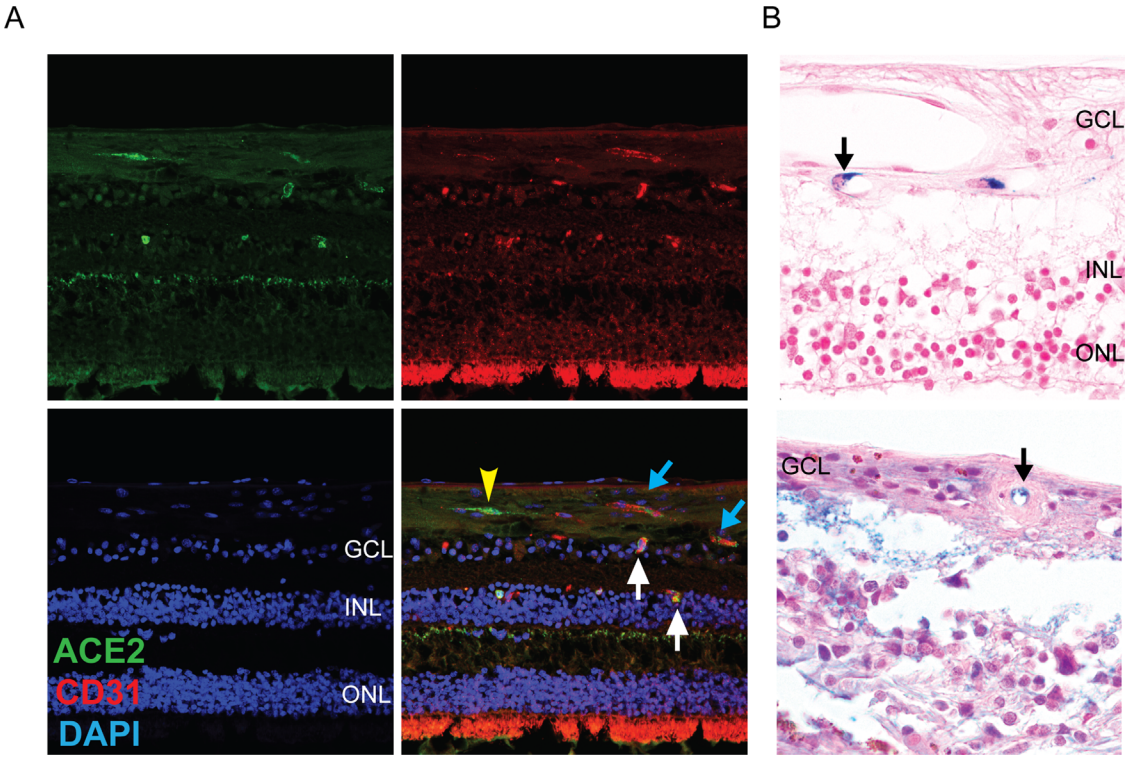

Figure 3. ACE2 expression in vessels (**A**) ACE2 is colocalized with some CD31-positive endothelial cells in retina. White arrows show ACE2 positive capillaries in cross section. *Blue arrows* mark longitudinal cuts through a capillary with endothelial cells positive for both CD31 and ACE2; ACE2 expression in perivascular cells (*yellow arrowhead*); (**B**) *Upper panel*: ACE2 expression in a small capillary endothelial cell (*arrow*), with a larger caliber vessel above lined by ACE2-negative endothelial cells. *Lower panel*: ACE2 expression in a small capillary endothelial cell (*arrow*) in a hyalinized vessel.

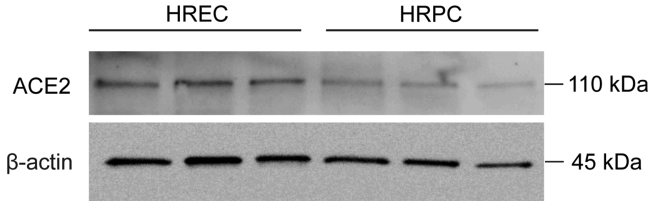

Figure 4. ACE2 expression in primary human retina endothelial cells and primary human pericytes. Western blot analysis demonstrated ACE2 protein expression in both primary human retina endothelial cells and primary human pericytes.

TMPRSS2 Expression in Human Retina

We further investigated the expression of TMPRSS2, an important cell surface–associated protease that allows the viral entry. As shown in Figure 5A, TMPRSS2 expression was found in some cells in the nerve fiber layer, retinal ganglion cell layer, inner nuclear layer, outer plexiform layer, and outer nuclear layer. TMPRSS2 is expressed in vascular/perivascular cells and radial processes morphologically consistent with Müller glia. Isotype IgG control was negative for TMPRSS2 staining (Fig. 5B).

Discussion

An increasing number of clinical studies are reporting retinal abnormalities in patients with COVID-19, but whether

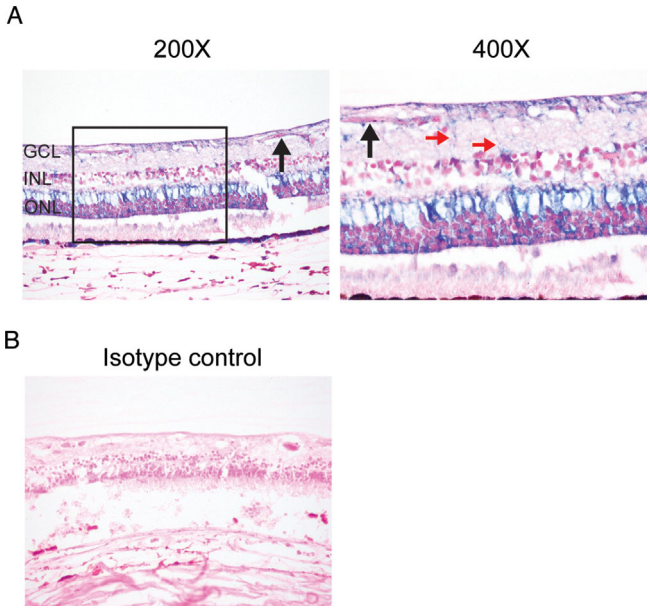

Figure 5. TMPRSS2 expression in human retina. (**A**) Characteristic TMPRSS2 expression in retina specimens from nondiabetic retina. TMPRSS2 expression was found in some cells in the nerve fiber layer, retinal ganglion cell layer, inner nuclear layer, outer plexiform layer, and outer nuclear layer. TMPRSS2 is expressed in vascular/perivascular cells (*black arrow*) and radial processes morphologically consistent with Müller glia (*red arrows*). (**B**) Isotype IgG control was negative for staining.

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the retina can be infected with SARS-CoV-2 is unknown. Notably, expression of ACE2, the critical SARS-CoV-2 receptor, in the human retina has not previously been reported. Here we report expression of ACE2 in multiple cell types in the human neuroretina, with marked upregulation of ACE2 in the retinal vasculature in diabetic retinopathy. These findings have potential implications for retinal manifestations of COVID-19 and for diabetic retinopathy.

The presence of ACE2 in the retinal endothelium supports the potential susceptibility of the retina to SARS-CoV-2 infection, serving as a portal for retinal invasion in the setting of systemic infection. In addition to direct infection of retinal endothelial cells, this expression could serve as a means for neuroretinal infection and damage, especially with concomitant cellular expression of TMPRSS2. Notably, in the brain, expression of ACE2 by choroid plexus cells and brain endothelial cells is thought to underlie infiltration of the blood-brain barrier and susceptibility of the CNS to SARS-CoV-2 infection.[28,32] In addition, the SARC-CoV-2 spike protein that binds ACE2 damages brain endothelial cells and impairs EC barrier integrity,[32] with implications for retinal endothelial cells and blood-retinal barrier integrity.

Diabetes is well known to be a predisposing factor for COVID-19 severity, including greater end-organ damage.[10,11] An important facet of this is tissue expression of ACE2, with higher levels observed in lung[12] and liver[13] in patients with diabetes. Greater expression of ACE2 in retinal endothelial cells in diabetes could indicate a greater susceptibility of retina to viral penetration, infection, and retinal damage.

Although our findings suggest the susceptibility of the retina to SARS-CoV-2 infection, it is important to be cognizant of additional considerations. It is quite conceivable that many retinal manifestations of COVID-19, including retinal hemorrhages and cotton-wool spots, may be a result of systemic inflammation and coagulopathy,[33] rather than direct viral infection of retinal cells. In addition, thus far, there have been fewer reports of patients with retinal involvement in COVID-19 compared to other organs. Although this may reflect in part the relative lack of ophthalmologic examinations of these patients, lower frequency of retinal manifestations could be due to additional important factors including a lower viral load to the retina or local factors for viral clearance.

As an important regulator of the renin-angiotensin system, ACE2 is a well-recognized protective factor for cardiovascular disease.[3] Specifically, ACE2 is a negative regulator of the ACE/angiotensin II/angiotensin type 1 receptor axis, a pathogenic are of the RAS for diabetic complications.[11] With respect to diabetic retinopathy, experimental studies have demonstrated gut barrier function in diabetic mice with ACE2 deficiency, with postulated pathogenic impact on retinal endothelial cell barrier function.[34] ACE2 deficiency in diabetic mice also compromised bone marrow function with impact on protective hematopoietic stem cells, with exacerbation of retinal capillary degeneration.[35] Therefore increased retinal vascular ACE2 expression may have important implications as a potential protective mechanism for diabetic retinopathy. Our studies further support the concept that ACE2 expression in diabetes could be a double-edged sword.[11] Whereas vascular ACE2 expression in diabetes could be a protective mechanism against diabetic retinopathy, it could potentiate retinal damage associated with COVID-19, which has been reported in previous studies. This is consistent with the general understanding of diabetes as an important co-morbidity in COVID-19. Future studies will be crucial to clarify retinal impact of COVID-19 in nondiabetic and diabetic population and whether it is directly due to the viral infection or secondary to the inflammation damage, similar to the CNS involvement that has been more extensively described.

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Investigative Ophthalmology & Visual Science

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