Synthesis and Secretion of Hemoglobin by Retinal Pigment Epithelium

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Purpose. To demonstrate the production of hemoglobin by human retinal pigment epithelium (RPE).

Methods. Proteomic analysis using 10 donor eyes identified hemoglobin as a major constituent of soluble human RPE proteome. Western blot analysis, RT-PCR, and immunocytochemistry were used to confirm the results. The presence of erythrocyte-specific proteins within primary human RPE cytosol was investigated to rule out phagocytosis as the source of hemoglobin. ELISA was used to determine the rate of hemoglobin secretion from human RPE cells. Globin mRNA expression of human RPE was studied in comparison with a human erythroblast cell line and a spontaneously transformed human RPE cell line (ARPE-19).

Results. Hemoglobin is a regular constituent of soluble human RPE proteome. RT-PCR and Western blot analysis confirmed the presence of hemoglobin in human RPE. No other erythrocyte-specific proteins were detected within human RPE cytosol. Hemoglobin expression persisted up to seven passages in vitro. Human RPE globin expression exceeded that in human erythroblast and ARPE-19 cells. Immunohistochemistry revealed the presence of hemoglobin within RPE and Bruch's membrane. The hemoglobin release rate was calculated to be 1.9 ± 1.2 attomoles per cell per hour.

Conclusions. Hemoglobin expression by human RPE brings a new perspective to the understanding of oxygen transport to the outer retina. Malfunction of RPE-hemoglobin production may underlie the pathophysiology of ocular diseases characterized by subfoveal hypoxia and VEGF upregulation, such as age-related macular degeneration and diabetic retinopathy. Pharmacologic modulations of local hemoglobin production in RPE cells will create new opportunities to interfere with the course of these diseases. (*Invest Ophthalmol Vis Sci.* 2009;50: 1911–1919) DOI:10.1167/iovs.07-1372

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Age-related macular degeneration is the leading cause of vision loss among the elderly population in the United States and Western Europe. It is expected to affect the sight of more than 6 million people within the next decade as the "baby-boomer" generation ages.[1]

Within the past couple of years, intravitreal injection of various pharmacologic agents has become a standard treatment of exudative age-related macular degeneration. The first generation of such agents target vascular endothelial growth factor (VEGF) due to its central role in the development of choroidal neovascularization (i.e., exudative age-related macular degeneration).[2] Already an RNA-based aptamer (pegaptanib) and two modified antibody molecules (ranibizumab and bevacizumab) have been introduced for clinical use,[3] and several similar drugs are in development.

Hypoxia is the main trigger of VEGF upregulation in exudative age-related macular degeneration,[4] even though several cytokines, growth factors, hormones, and tumor-suppressor genes[5] can also induce VEGF synthesis. All major risk factors for age-related macular degeneration, such as aging, the presence of age-related maculopathy, smoking, and systemic hypertension are associated with impaired subfoveal blood flow and hypoxia.[6]

The main oxygen consumers in the outer retina are photoreceptors. Their oxygen demand is the highest in the whole body and even exceeds cerebral neurons. This high oxygen demand is supplied mainly by the choroidal circulation via diffusion.[7] Unlike the retinal vessels, the choroidal vasculature lacks autoregulation and cannot compensate for periods of increased oxygen demand (e.g., in the dark).[8] Paradoxically, even in severe systemic hypoxemia, photoreceptors continue to function normally in contrast to inner retinal neurons, indicating that an additional oxygen buffer or transport system may compensate for their oxygen need.

Hemoglobin is the most common oxygen-transporter in the human body. Its major form, hemoglobin A, is a tetramer consisting of two α- and β-polypeptide chains of approximately 17 kDa, each carrying a nonprotein heme group.[9] The classic concept that hemoglobin is produced only by cells with an erythroid lineage has recently been challenged by reports demonstrating its expression in alveolar epithelium, presumably for the purpose of increased oxygen transport.[10,11] Our studies of the human retinal pigment epithelial (RPE) proteome revealed that its major constituent is hemoglobin.

Materials and Methods

The experimental protocols applied for this study were approved and monitored by the Institutional Review Board of the University of Louisville. Animal maintenance and experimentation were performed in accordance with guidelines established by the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research and the Institutional Animal Care and Use Committee at the University of Louisville Medical School. Four- to 6-week-old C57BL/6 mice (*Mus musculus*; 20 eyes) and 8-week-old brown Norway rats (*Rattus norvegicus*) were purchased from Jackson Laboratories (Bar Harbor, ME). Freshly enucleated 10- to 12-week-old domestic porcine (*Sus domestica*) and

TABLE 1. Sequences of Oligonucleotides Used in RT-PCR

Gene	Accession Number	Forward	Reverse	Size (bp)
Experimental genes				
Human hemoglobin β	NM_000518	5′-GAGAAGTCTGCCGTTACTGCC-3′	5′-CCGAGCACTTTCTTGCCATGA-3′	188
Human hemoglobin α1	NM_000558	5′-GGTCCCCACAGACTCAGAGA-3′	5′-AGTGCGGGAAGTAGGTCTTG-3′	167
Human hemoglobin α2	NM_000517	5′-GCTCTGCCCAGGTTAAGGG-3′	5′-CAGTGGCTTAGGAGCTTGAAG-3′	160
Human hemoglobin F	NM_000184	5′-CTTCCTTGGGAGATGCCATA-3′	5′-GAATTCTTTGCCGAAATGGA-3′	161
Mouse hemoglobin β^{minor}*	NM_016956	5′-CTGCATGTGGATCCTGAGAA-3′	5′-ATCCACATGCAGCTTGTCAC-3′	231
Mouse hemoglobin β^{major}*	NM_008220	5′-GCTGGTTGTCTACCCTTGGA-3′	5′-ACGATCATATTGCCCAGGAG-3′	240
Rat hemoglobin β†	NM_033234.1	5′-TGCTGAGAAGGCTGCTGTTA-3′	5′-TGTTTCAGGCCATCATTGAA-3′	216
Control genes				
Human β-actin	NM_001101	5′-CCAACCGCGAGAAGATGACCC-3′	5′-GGAGTCCATCACGATGCCAGT-3′	125
Mouse β-actin	NM_007393	5′-GGCTGTATTCCCCTCCATCG-3′	5′-CCAGTTGGTAACAATGCCATGT-3′	154
Rat β-actin	AF_122902	5′-TGTCACCAACTGGGACGATA-3′	5′-GGGGTGTTGAAGGTCTCAAA-3′	165

* C57/B6 Mouse (*Mus musculus*).
† Brown Norway Rat (*Rattus norvegicus*).

bovine (>2 years old, *Bos taurus*) eyes were obtained from a local abattoir.

Cell Harvest and Culturing

Ten human donor eyes (age, 28–89 years) were obtained from the Kentucky Lions Eye Bank within 24 hours of death and were managed in accordance with the guidelines of the Declaration of Helsinki for research involving human tissue. Donors' medical and ophthalmic histories were obtained from their physicians, and globes with a previous history of any chorioretinal disease including diabetic retinopathy, age-related macular degeneration, myopia > 6D, or inherited retinal degeneration were excluded from the study. Likewise, any donor eye that had undergone any major ocular surgery including laser photocoagulation was not included in the study. RPE cells were harvested as described before, and cell count was determined (Coulter counter; Coulter Scientific, Hialeah, FL).[12] Cell viability was determined (Live-Dead Assay; Invitrogen, Carlsbad, CA) and immunostaining with RPE 65 and cytokeratin antibodies (Chemicon, Temecula, CA) was used to determine the purity. Only samples with >95% viability and >99% purity were used for further studies.

Effects of cell culturing on RPE hemoglobin expression was also studied for up to seven passages. Cell culturing methods were described previously.[13] First-, third-, and seventh-passage RPE cells were used to investigate the effects of globin expression in vitro.

Expression of globin chains in the RPE of C57BL/6 mouse (20 eyes) and brown Norway rat (10 eyes) were also studied. For this purpose, the eyes of the animals were enucleated with the animals under deep anesthesia. The anterior segment was discarded and the sensory retina was peeled off. Exposed RPE cells were washed three times with Hanks' balanced salt solution and, under a dissecting microscope, loosened RPE cells were scraped off of Bruch's membrane with a nonstick-coated spatula. Care was taken not to contaminate the harvested cell pool with any choroidal cells.

The mRNA expression of α1-, α2-, β-, and γ-globin mRNA and the globin content of primary human RPE cells from three donors (ages: 25, 70, and 64 years) was also studied in comparison with that in a human erythroblast cell line (HEL 92.1.7; ATTC, Manassas, VA) and a spontaneously transformed human RPE cell line (ARPE-19, ATTC). HEL 92.1.7 cells are known to express globin RNA and protein. HEL 92.1.7 cells were maintained in RPMI 1640 medium with 2 mM L-glutamine (Sigma-Aldrich, St. Louis, MO) containing 10 mM HEPES, 1 mM sodium pyruvate, 4.5 g/L glucose, 1.5 g/L bicarbonate, 10% fetal bovine serum, 100 IU/mL penicillin G, 100 μg/mL streptomycin, 5 μg/mL gentamicin, and 2.5 μg/mL amphotericin B. ARPE-19 cells were maintained in the RPE cell culture medium described earlier. For protein and mRNA determination, the same number of viable cells was studied.

Protein Extraction

Harvested cells were homogenized in sample buffer containing 7 M urea, 2.0 M thiourea, 65 mM CHAPS (3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate hydrate), 58 mM DTT, 1.78% carrier ampholytes (pH 3–10) and 1× protease inhibitor cocktail (Sigma-Aldrich). The mixture was shaken at 4°C for 1 hour and centrifuged at 15,000*g* for 20 minutes at 4°C. The supernatant containing the soluble proteins was removed, and protein concentration in the supernatant was determined by spectrophotometry with protein microassay based on the method of Bradford (Bio-Rad, Hercules, CA). Samples were then stored at −80°C.

Two-Dimensional Gel Electrophoresis

Thirty micrograms of protein from each sample was rehydrated overnight with immobilized pH gradient (IPG) strips (nonlinear pH 3–10), followed by isoelectric focusing (ZOOM IPG Runner; Invitrogen) and then separated in the second dimension on 4% to 12% Bis-Tris gels (Invitrogen), as described in detail previously.[14] After electrophoresis, gels were fixed in 10% methanol and 7% acetic acid and stained with a red protein stain (Sypro Ruby; Bio-Rad) overnight. The gel images after staining were captured (ProXPRESS 2D Proteomic Imaging System; Perkin Elmer Life Sciences, Boston, MA) at various exposure times, to achieve equal gray-level intensities for all gels.

Image Analysis

Quantification and comparison of protein spots on gel images were performed (Phoretix 2D Image Analysis Software; Nonlinear Dynamics, Newcastle-upon-Tyne, UK). This software was used to estimate the M_r and pI coordinates of the proteins, as well as to match protein spots between gels and analyze the relative pixel intensity of protein spots. The average mode of background subtraction was used to normalize the intensity before calculating spot volumes. Total spot volume was calculated to estimate the proportion of analyzed spots within the 2-D gel.

In-gel Trypsin Digestion, MALDI-TOF, and Peptide Mass Fingerprinting

Protein spots were excised with a clean scalpel and subjected to in-gel trypsin digestion as described before.[15] Mass spectral data were obtained using (TOFSpec-2E mass spectrometer; Micromass, Manchester, UK), and a 337-nm N_2 laser at 20% to 35% power in the positive ion reflectron mode. A monoisotopic mass spectrum was obtained by averaging 10 spectra, each of which was the composite of 10 laser firings. The mass axis was calibrated by using known peaks from tryptic autolysis. Protein identification from tryptic fragment sizes was made by using a commercial search engine (Mascot; Matrix Science, Boston, MA) based on the entire National Center for Biotechnology Information (NCBI, Bethesda, MD) protein database and using the assumption that the peptides were monoisotopic, oxidized at methio-

nine residues, and carboxamidomethylated at cysteine residues. Up to one missed trypsin cleavage was allowed. A mass-accuracy tolerance of a maximum of 150 ppm was used for matching tryptic peptides. A probability-based MOWSE score defined as $-10 \cdot \log(P)$, where P is the probability that the observed match is a random event, was determined.[16] A score of >70 was considered significant ($P < 0.05$). The validity of the mass spectrometry data was ascertained by comparing the expected molecular weight and pI of the identified proteins with their position in the 2-D gel.

Western Blot Analysis

Extracted RPE proteins were denatured at 70°C for 10 minutes, and equal amounts (20 μg in 20 μL/lane) of total proteins were subjected to electrophoresis on 4% to 12% gels (NuPAGE Novex; Invitrogen). Gels were then transferred onto nitrocellulose membranes (Invitrogen) electrophoretically and immunoblotted overnight at 4°C with horseradish peroxidase–conjugated sheep polyclonal anti-human panhemoglobin antibody (Bethyl Laboratories, Montgomery, TX) diluted 1:2000 in PBS. Native human hemoglobin was used as an antigen in the production of this antibody, and it is known to react specifically with human hemoglobin.[10,17,18] In control samples, Western blot analysis were developed with the chemiluminescence (ECL; Amersham Biosciences, Piscataway, NJ) and exposed to autoradiograph film (BioMax XAR film; Eastman Kodak, Rochester, NY). A human erythroblast (HEL 92.1.7; ATTC) and a human lymphoblast (RPMI 6666; ATTC) cell line were used as positive and negative control cultures, respectively. Hemoglobin F production was determined with a sheep polyclonal anti-human hemoglobin F antibody (Bethyl Laboratories). Intact human hemoglobin F was used as an antigen during the production of this antibody and it was solid-phase adsorbed against hemoglobin A to ensure its specificity. The presence of other erythrocyte markers was probed with antibodies against glycophorin A and B, as well as blood group H and AB antigens (Abcam, Cambridge, UK) at 1:500 dilution.

RNA Preparation and Reverse Transcription–Polymerase Chain Reaction

Expression of α- and β-globin genes in human and rat RPE was determined by RT-PCR. In brief, human RPE mRNA was extracted from four fresh donor (ages, 48–65 years; death-to-harvest time, <24 hours) eyes, as well as 20 C57BL/6 and 10 brown Norway rat eyes (TRIzol reagent kit; Invitrogen).[19] Any possible DNA contamination was removed by incubation with amplification grade DNase I (Invitrogen) and confirmed by PCR analysis of total RNA samples before reverse transcription. After isolation, the integrity and size distribution of the RNA samples was checked by agarose gel electrophoresis. The yield and purity of total RNA was determined by measuring the absorbance (260/280 nm) of ethanol precipitated aliquots of the samples. Oligo-dT primed first-strand cDNA was synthesized (Superscript 3 First-Strand Synthesis System for RT-PCR; Invitrogen) using 1 μg of total RNA per reaction. PCR amplification of cDNAs was performed (Mx3000P; Stratagene, La Jolla, CA) system using *Taq* DNA polymerase (Promega, Madison, WI) and primers corresponding to conserved sequences of known human, mouse, and rat globin genes. All the primers were designed to cross exon–intron borders, to exclude the amplification of genomic templates (Table 1). Denaturation was started initially at 95°C for 4 minutes, followed by 40 amplification cycles of 95°C for 15 seconds, 60°C for 20 seconds, and 72°C for 20 seconds, for primer annealing, and 7 minutes at 72°C for primer extension. Control reactions for each primer pair were performed with the transcription control reaction without reverse transcriptase and with water instead of the cDNA template. PCR products were then separated on 2% agarose gels and bands were visualized with ethidium bromide.

The β-, α1-, α2-, and γ-globin expression of primary human RPE cells from three donors was also compared with that in HEL 92.1.7 and ARPE-19 cells. Data were normalized to a viable cell count and expressed as the log-fold difference in reference to HEL 92.1.7 cells. Globin contents of these cells types were also compared by using an ELISA specific for α-globin.

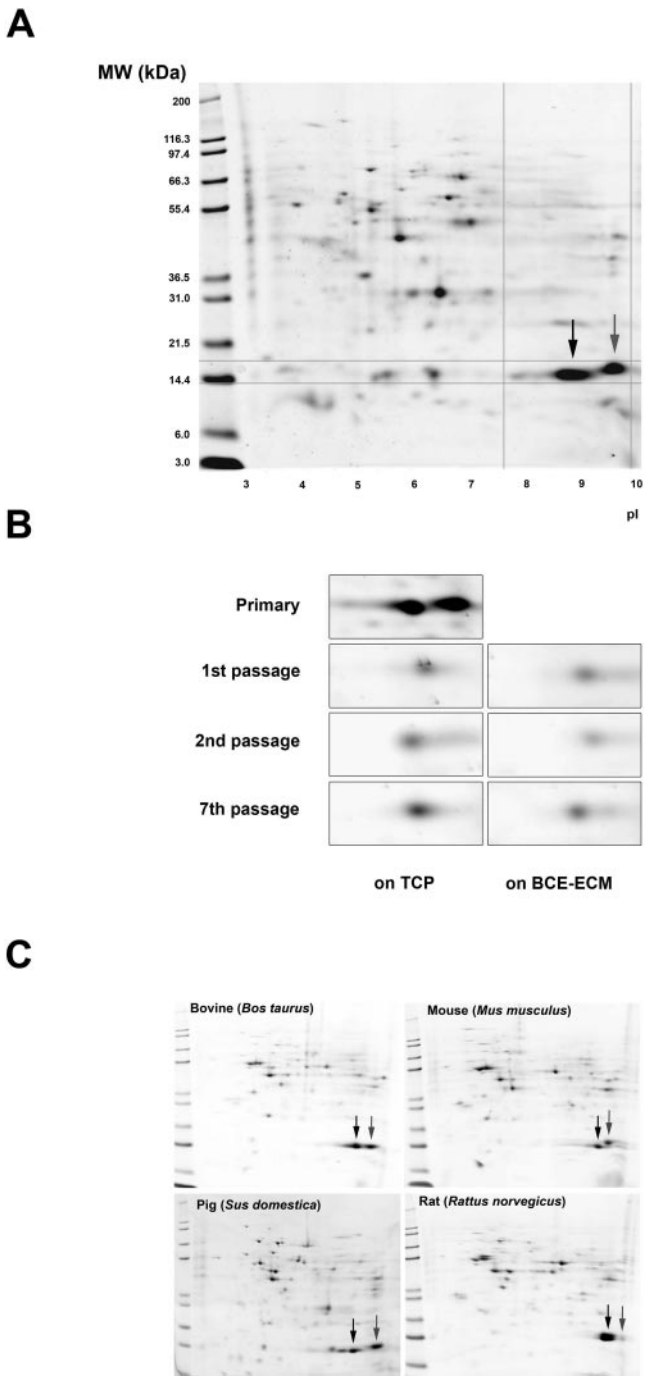

Figure 1. Hemoglobin proteins were a major part of mammalian RPE proteome. (**A**) A representative 2-D gel of human RPE proteins. Globin monomers cluster at around 15 kDa between the pI zones of 6.5 and 9.0. Mass spectrometry was used to identify β (*black arrow*)- and α (*gray arrow*)-globin. (**B**) Hemoglobin expression of human RPE persisted through seven passages in vitro both on tissue culture plastic (TCP) and bovine corneal endothelium-extracellular matrix (BCE-ECM)–coated plates. (**C**) Both β (*black arrows*)- and α (*gray arrows*)-globin are major components of the RPE proteomes of other mammalian species. Representative 2-D gels from nine different experiments are presented.

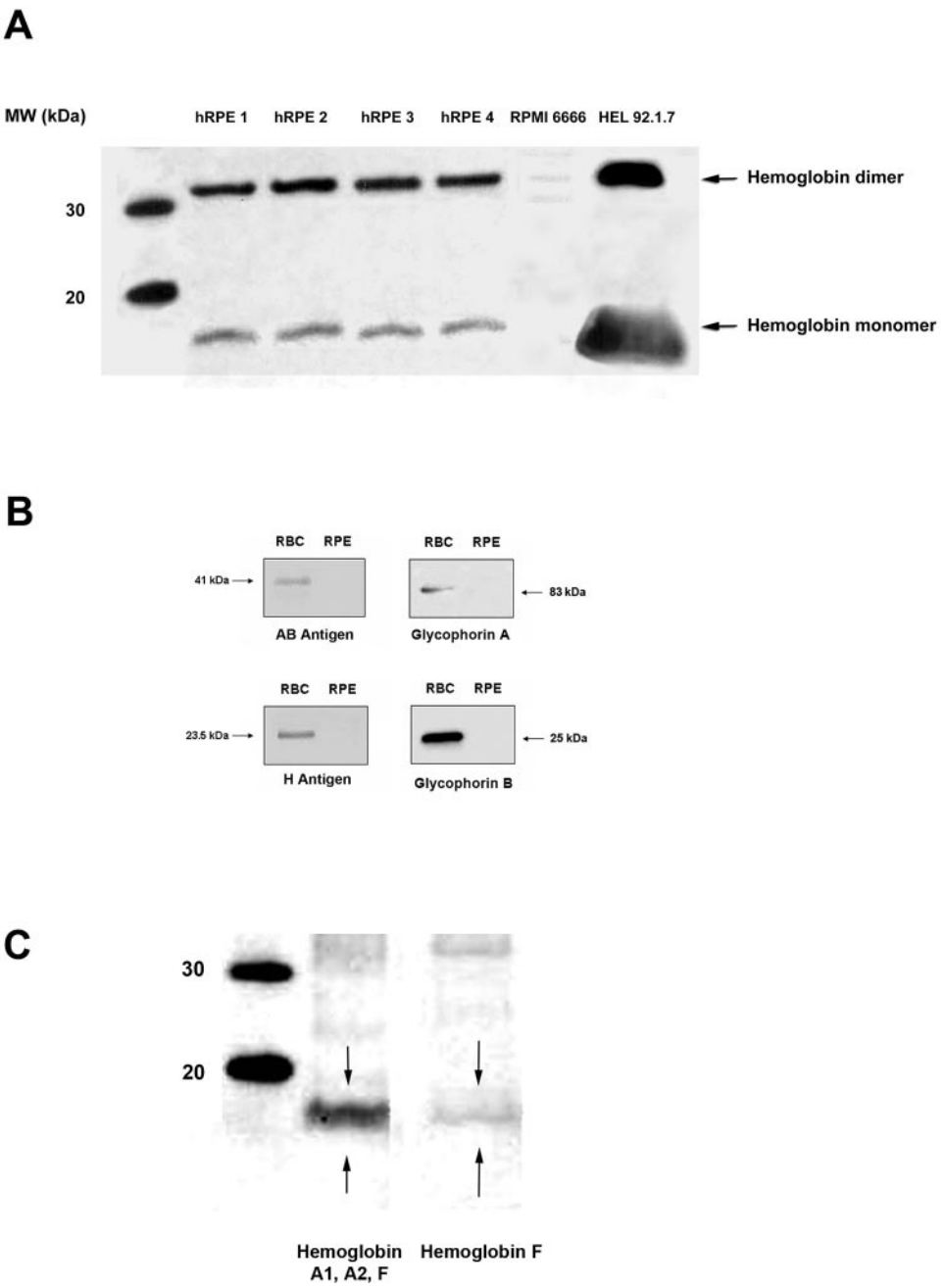

FIGURE 2. Hemoglobin was present in the human RPE cytosol. However, the RPE cells did not express erythrocyte-specific markers, indicating that the source of the hemoglobin was not phagocytosis of erythrocytes. (**A**) Western blot analysis of human RPE proteins from four different donors (hRPE 1–4) with anti-human panhemoglobin antibody confirmed the presence of hemoglobin monomer and dimers within the RPE cytosol. Control human lymphoblast cells (RPMI 6666) did not reveal any hemoglobin, whereas abundant amounts of hemoglobin were present in human erythroblast cells (HEL 92.1.7). (**B**) The absence of erythrocyte-specific proteins, such as glycophorin-A and -B, and blood group antigens H and AB within the human RPE cytosol indicated that the source of hemoglobin is not the phagocytosis of erythrocytes. Representative gels from three different experiments are shown. (**C**) Western blot analysis for different hemoglobin types reveals that minute amounts of hemoglobin F were also present within the RPE–hemoglobin pool.

Metabolic Labeling with [^{35}S] Methionine/Cysteine and Immunoprecipitation

Metabolic labeling and immunoprecipitation were performed as previously described.[20] Briefly, 2×10^{-6} primary human RPE cells from three donors (age range: 67–72 years) were starved in methionine/cysteine-free DMEM H16 (Sigma-Aldrich) for 2 hours and then metabolically labeled with 100 μCi/mL of L-[^{35}S] methionine/cysteine (PRO-MIX; Amersham). After labeling, the cells were lysed in 50 mM Tris-HCl buffer (pH 7.4) containing 0.25 M sucrose, 0.1% SDS, 1% Triton X-100, 0.5% sodium deoxycholate, 50 μg/mL each of leupeptin and pepstatin, and 0.5 mM PMSF (lysis buffer) for 6 hours at 4°C. The cell lysates were centrifuged for 20 minutes at 14,000 rpm in a microcentrifuge, and the supernatants were used for immunoprecipitation. Proteins in the cell lysates were immunoprecipitated with anti-goat anti-human panhemoglobin antibody (Bethyl Laboratories) for 16 hours at 37°C. The immunoprecipitates were analyzed by SDS/PAGE (3%–12% [wt/vol] gels) and visualized by autoradiography. The same number of HEL 92.1.7 cells was used as the positive control.

Immunohistochemistry

Immunohistochemistry for the presence of hemoglobin in human and mouse eyes were performed at the light and electron microscopy levels, as previously described.[21] For this purpose, goat anti-human panhemoglobin antibody (Bethyl Laboratories) and mouse monoclonal anti-hemoglobin antibody (Abcam) were used at dilutions of 1:500 and 1:100, respectively. Both antibodies are known not to cross-react with other heme-containing respiratory proteins, such as neuroglobin or cytoglobin (personal communication with Abcam, 2008). A secondary Cy3-conjugated anti-sheep antibody was used to visualize the binding sites. For control sections at light microscopy level an irrelevant polyclonal sheep IgG (anti-human PSA; Abcam) coupled with the same

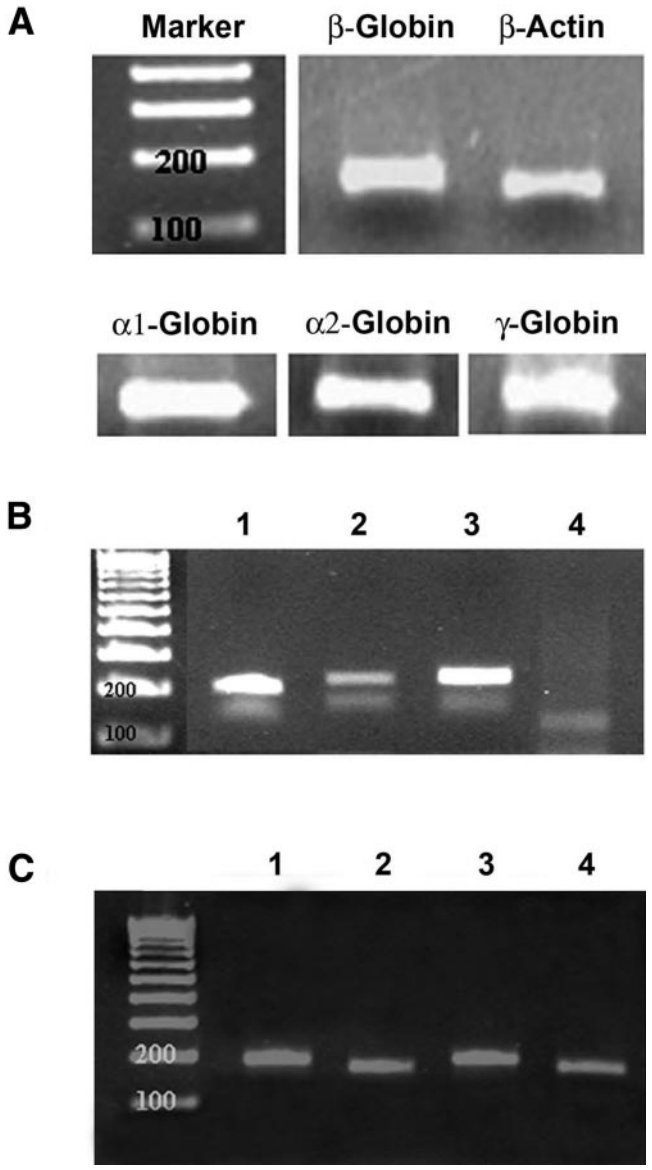

FIGURE 3. Globin expression in mammalian RPE. (**A**) RT-PCR amplification products of different globin genes in human RPE separated by 2% agarose gel electrophoresis. Human RPE cells expressed β (188 bp)-α1 (167 bp)-, α2 (160 bp)-, and γ (161 bp)-globin genes. During the amplification, β-actin (123 bp) was used as the internal control. (**B**) RT-PCR for globin gene expression in mouse (C57BL6) RPE. *Lane 1*: mouse β-minor globin (Hb 2b, 231 kb); *lane 2*: mouse b-minor globin (Hb 2a, 239 kb); *lane 3*: mouse β-major globin (Hb 1, 240 kb); and *lane 4*: mouse β-actin (154 kb) as the internal control. (**C**) RT-PCR for β-globin in the RPE cells of Brown Norway rats. Ethidium bromide–stained gel showing RT-PCR products for β-globin (*lanes 1* and *3*) and β-actin (*lanes 2* and *4*). Bands of the expected size (185 bp for β-globin and 165 bp for β-actin) were present in the amplification reaction. Representative gels from nine different experiments are presented.

secondary antibody was used. For immunoelectron microscopy, a gold-conjugated goat anti-mouse IgG (Bioassay Works, LLC, Ijamsville, MD) was used as the negative control.

Hemoglobin Release from Human RPE

The amount of hemoglobin released from the human RPE was determined with an enzyme-linked immunosorbent assay (ELISA; Bethyl Laboratories).[22] For this purpose, seven pairs of freshly enucleated (within 24 hours of death) human globes were obtained from the eye bank (age, 57–70 years). Anterior segment, vitreous, and retina were removed exposing the RPE cells. Cyanoacrylate glue was used to seal the suprachoroidal space. Eye cups were then washed gently three times and incubated in a humidified atmosphere of 5% CO_2 and 95% air at 37°C and maintained in Dulbecco's modified Eagle's medium (DMEM H16; Invitrogen), 100 IU/mL penicillin G, 100 μg/mL streptomycin, 5 μg/mL gentamicin, and 2.5 μg/mL amphotericin B for 16 hours. At the end of the incubation period, culture media were collected from all experimental groups and centrifuged, and the amount of hemoglobin in the media was determined with an ELISA kit, according to the manufacturer's instruction.[17] A similar method has been used to determine human extracellular hemoglobin amounts reliably.[23] Briefly, microtiter plates were coated with sheep anti-human panhemoglobin antibody (1:50,000 dilution; Bethyl Laboratories), washed, blocked, and incubated with culture medium and incubated for 1 hour at room temperature. After incubation samples were removed, and plates were incubated with horseradish peroxidase–conjugated sheep anti-human hemoglobin antibody at 1:50,000 dilution for 60 minutes. Detection was accomplished adding the chromogenic substrate 3,3′,5,5′,-tetramethyl-benzidine (Bethyl Laboratories) to the wells. The chromophore development was terminated after 30 minutes with 2 N H_2SO_4. The plates were read at a wavelength of 450 nm with a microplate reader (VERSAmax; Molecular Devices, Sunnyvale, CA). Amount of hemoglobin was calculated using the normalization curve obtained from wells containing dilutions of human hemoglobin ranging from 16 ng/mL to 1 mg/mL.

The fellow eye was used to determine the number of RPE cells and also the effect of incubation on cell viability. For this reason, RPE cells within the fellow eye were exposed similarly. A 2.0 × 2.0-mm area outside the temporal arcades was removed to determine the cell viability with a cell-viability assay (Live-Dead Assay; Invitrogen). At least 250 cells from five different areas were examined under 100× magnification, and the viability was expressed as the average ratio of live cells to the total number of cells. Initial RPE cell viability was then compared with the viability of RPE cells from a similar area 16 hours after incubation. The remaining RPE cells of the fellow eye were washed three times with calcium-magnesium–free Hanks' balanced salt solution. Loosened RPE cells were collected by gentle scraping with a Teflon spatula without disrupting the integrity of the underlying Bruch's membrane, and the number of cells was determined (counter model Z-1; Coulter Scientific). Fellow eye cups devoid of RPE were also incubated with the culture medium for 16 hours to determine the baseline hemoglobin levels that would accumulate within the eye cups within the incubation period. This amount was subtracted to determine the globin release from the RPE into the medium. Obtained data were further corrected to live RPE cell count to estimate the hemoglobin release from each RPE cell.

Statistical Analysis

Experiments were performed in triplicate on three different occasions. Data were expressed as the mean ± SD. Differences in cell viability were compared by using Mann-Whitney *U* test.[24] Globin expression and α-globin content of cells were compared pair-wise by the Tukey test (Sigma-Stat 2.03; SPSS, Chicago, IL). $P < 0.05$ was considered to be statistically significant.

RESULTS

Presence of Hemoglobin within the RPE

We studied the human RPE proteome of 10 human donor eyes (age, 28–89 years). Globin chains (α and β) were clearly detectable in all the eyes, regardless of gender and age. Human hemoglobin proteins were clustered at approximately 15-kDa and a pI zone of 6.5 to 9.0 (Fig. 1A). Image densitometry revealed that α- and β-globin constituted a major proportion of the human RPE proteome, as detected by 2-D gel electrophoresis (18. 0% ± 3.3% and 18.0% ± 6.5%, respectively). Within the

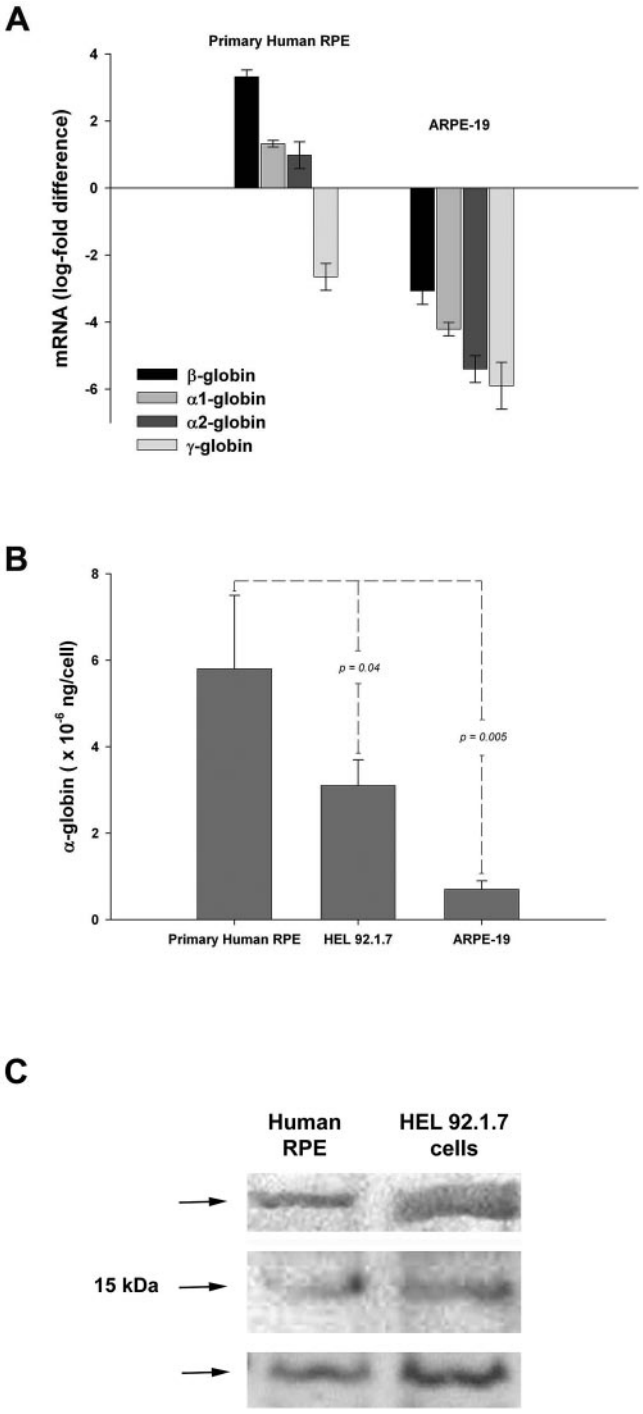

FIGURE 4. Human RPE expressed β-, α1-, and α2-globin mRNAs and synthesize globins in vitro. (**A**) qRT-PCR analysis of β-, α1-, α2-, and γ-globin expression of primary human RPE, HEL 92.1.7, and ARPE-19 cells. Results are given as log-fold differences relative to HEL.92.1.7 cells. Primary human RPE expression of β-, α1-, α2-globins exceeded that in HEL 92.1.7 and ARPE-19 cells. Although primary human RPE γ-globin expression was higher than that in ARPE-19 cells, it was significantly less than that in HEL 92.1.7 cells ($n = 9$ per experiment, $P < 0.001$ by Tukey test). Error bars, SD. (**B**) ELISA for α-globin reveals that the primary human RPE cell contained a higher amount of intracellular α-globin than did the HEL 92.1.7 and ARPE-19 cells ($n = 9$ per experiment, Tukey test). Error bars, SD. (**C**) Immunoprecipitation of [^{35}S] methionine/cysteine-labeled human RPE proteins confirmed globin synthesis in vitro. As expected, control human erythroblasts (HEL 92.1.7) also synthesized globins. A representative gel from three separate experiments is shown.

globin cluster, the α- and α2-globin chains (molecular weight, 15.2 kDa; pI, 8.72), the β chain (molecular weight, 15.9 kDa; pI, 6.7), as well as a third[25] and recently identified quaternary structure of α-globin (molecular weight, 15.9 kDa; pI, 6.8)[26] were identified with mass spectrometry (Fig. 1A). Hemoglobin production of human RPE cells declined on in vitro culture, but persisted through seven passages both on tissue-culture plastic and bovine cornea-endothelial cell matrix–coated culture plates (Fig. 1B). Hemoglobin was also present in the proteomes of the RPE from other mammals, including the rat, mouse, pig, and bovine (Fig. 1C).

Western blot analysis confirmed the presence of monomers and dimers of hemoglobin proteins within the RPE cytosol (Fig. 2A). It failed to show the presence of erythrocyte-specific proteins, such as glycophorin-A and -B, as well as blood group antigens H and AB, suggesting that the presence of hemoglobin in the RPE was not simply from phagocytosis of erythrocytes (Fig. 2B). The hemoglobin pool of RPE cells contained minute amounts of hemoglobin F, as well (Fig. 2C).

mRNA Expression of Globins by RPE

RT-PCR from primary human RPE revealed α1-, α2-, γ-, and β-globin mRNA expression by RPE cells (Fig. 3A). Similarly, mouse and rat RPE cells expressed globins (Fig. 3B).

Primary human RPE cells expressed higher amounts of β-, α1-, and α2-globin mRNA than did human erythroblast cells (HEL 92.1.7; ATTC; 3.3 ± 0.2-, 1.3 ± 0.1-, and 1.0 ± 0.4-log-fold difference) and a spontaneously transformed human RPE cell line (ARPE-19, ATTC; 6.4 ± 0.3, 5.5 ± 0.2, and 6.4 ± 0.4-log-fold difference, Fig. 4A). γ-Globin expression of primary RPE cells was also higher than that of ARPE-19 cells (3.3 ± 0.4-log-fold difference); however, it was less than that of HEL 92.1.7 cells (−2.7 ± 0.4 log-fold difference). Accordingly, the intracellular α-globin content of primary human RPE cells ($5.8 \pm 1.7 \times 10^{-6}$ ng/cell) was higher than that in both HEL 92.1.7 ($3.1 \pm 0.6 \times 10^{-6}$ ng/cell, $P = 0.04$) and ARPE-19 ($0.7 \pm 0.2 \times 10^{-6}$ ng/cell, $P = 0.005$) cells (Fig. 4B).

Metabolic Labeling

We investigated the synthesis of hemoglobin by human RPE cells in pulse/chase experiments with [^{35}S] methionine/cysteine. We observed that the globins expressed by primary human RPE cells were metabolically labeled, confirming the synthesis of globins by human RPE cells (Fig. 4C). We could not detect any metabolic labeling in passaged RPE and ARPE-19 cells.

Immunohistochemistry

Antibody staining of hemoglobin within human RPE using light microscopy demonstrated small granular deposits, near the basal membrane (Fig. 5A). Hemoglobin was also present within the inner layers of Bruch's membrane, and as granular deposits within the erythrocytes in the choriocapillaris. A similar staining pattern was also observed in rat and mouse eyes; however, the intensity of the hemoglobin signal was lower than that in human eyes (Fig. 5B).

Immunoelectron microscopy confirmed the presence of hemoglobin within RPE cells (Fig. 6). Immunogold particles attached to antibodies against hemoglobin were present within the RPE cytosol (Fig. 6A), mainly in multiple round, intermediate- to high-density cytoplasmic granules (Fig. 6B). These vesicles docked and fused to plasma membrane mainly along the basolateral plasma membrane and formed typical porosomes[27] (Figs. 6C, 6D); they were present as well within the inner layers of Bruch's membrane (Figs. 6E, 6F). No specific labeling was seen in the negative control experiments (data not shown).

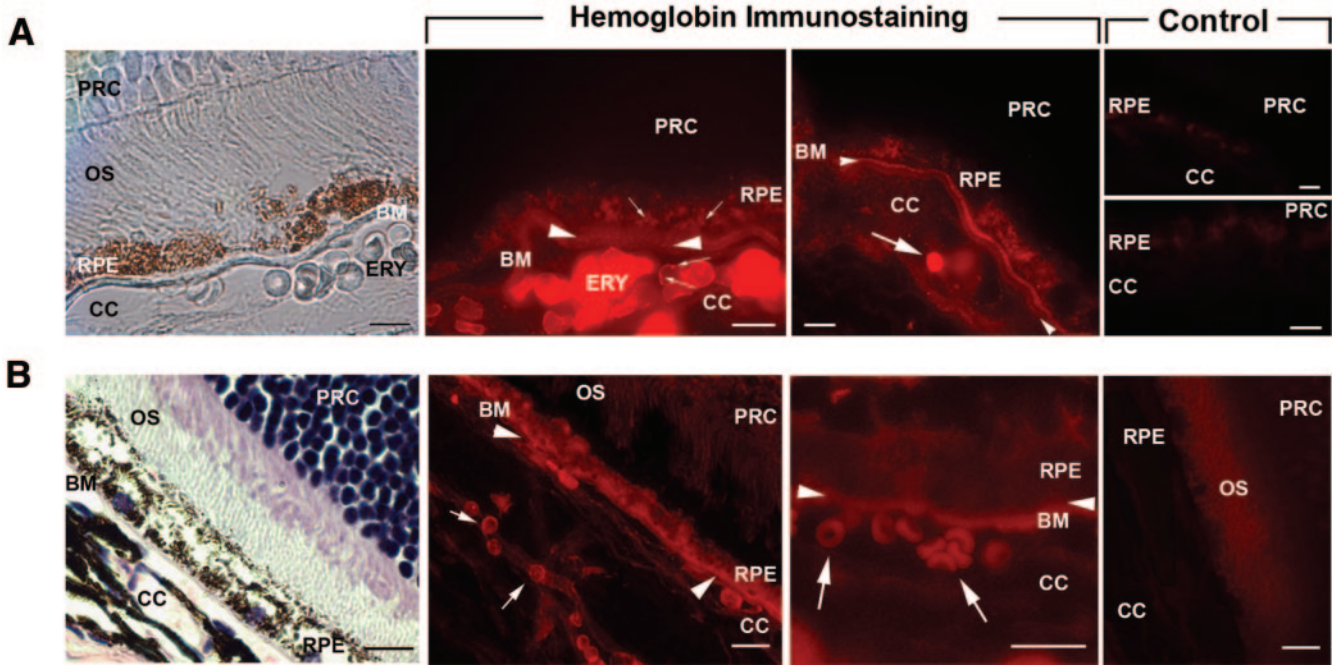

FIGURE 5. Immunostaining with anti-human panhemoglobin antibody revealed the presence of hemoglobin in mammalian RPE and Bruch's membrane. (**A**) Immunohistochemistry for hemoglobin in human outer retina. *Left*: unstained paraffin-embedded section of human outer retina and choroid at the level of choriocapillaris (CC), retinal pigment epithelium (RPE), and photoreceptors (PRC). Outer segments (OS) were in close contact with RPE. Bruch's membrane (BM) is a pentalaminar extracellular matrix between the RPE and underlying choriocapillaris, which is a fenestrated capillary bed filled with erythrocytes (ERY). *Middle*: a granular staining for hemoglobin was seen both on human RPE cells and erythrocytes within the choriocapillaris (*arrows*). The erythrocytes yielded a stronger signal due to higher hemoglobin concentration. A similar granular hemoglobin signal was also present along Bruch's membrane (*arrowheads*). *Right*: control sections revealed faint diffuse autofluorescence originating from RPE. (**B**) Hemoglobin immunostaining of the mouse (C57B6) outer retina. *Left*: hematoxylin-stained section showing RPE, Bruch's membrane, choriocapillaris, and photoreceptors. *Middle*: hemoglobin immunostaining revealed diffuse staining within and along basal membrane of RPE and Bruch's membrane (*arrowheads*) with some intense focal staining of the erythrocytes within the choriocapillaris (*arrows*). At higher magnification, immunostaining revealed the presence of hemoglobin along the basal membrane of RPE, Bruch's membrane (*arrowheads*), and within the erythrocytes of choriocapillaris (*arrows*). *Right*: control section showing faint autofluorescence originating from the photoreceptor outer segments. Scale bar, 10 μm.

Quantification of RPE-Hemoglobin Release with ELISA In Vitro

The release of hemoglobin from adult human RPE cells was determined using eye cups from freshly enucleated eyes (<24 hours of death). The eye cups from seven pairs of eyes were filled with medium and the exposed RPE monolayer was incubated for 16 hours. RPE cell viability was not affected (99.3% $\pm$ 6.7% vs. 98.6% $\pm$ 5.3%, $P > 0.05$) throughout the incubation period. The amount of hemoglobin released into the media was quantified with ELISA (Bethyl Laboratories). On average, the RPE cells released 0.46 $\pm$ 0.29 pg of the hemoglobin within 16 hours, which corresponded to an average secretion rate of 1.9 $\pm$ 1.2 attomoles of hemoglobin released per cell per hour.

DISCUSSION

We undertook proteomic analysis of normal human RPE with the expectation that we might gain insight into the pathogenesis of the leading cause of blindness after age 55—namely, age-related macular degeneration. Surprisingly, we observed that the major protein in the RPE proteome of the normal human, as well as other species (i.e., mouse, rat, bovine, and pig), is hemoglobin. The possibility of phagocytosed erythrocytes as a source of the hemoglobin is remote because of the lack of other erythrocyte markers and the persistence of hemoglobin expression by the RPE after several in vitro passages.

Hemoglobin expression by the RPE is not surprising, since hemoglobin is among the proteins identified in drusen[28] and human RPE.[29] Furthermore, two recent reports indicate that mammalian cells with nonerythroid lineage can also express hemoglobin.[10,30] This finding suggests that the expression of hemoglobin by nonerythroid cells at interfaces where oxygen-carbon dioxide diffusion occurs may be an adaptive mechanism to facilitate oxygen transport. Since the RPE borders both the choriocapillaris and photoreceptors and high amounts of carbon dioxide and oxygen must be transported back and forth between these structures to meet the high metabolic activity requirements of the outer retina, a similar physiologic function may exist in the eye.

Hemoglobin is an ideal molecule for such a purpose due to allosteric control of oxygen binding and release without the need for any cellular process. The oxygen tension declines rapidly from 60 to 0 mm Hg within a short distance of 50 μm from the choriocapillaris to the inner segments of the photoreceptors,[7] with a decrease in pH from 7.40 to 7.20,[31] creating a perfect environment for allosteric oxygen delivery of hemoglobin molecules in the subretinal space.

The α- and β-globin chains constituted approximately 18% of the human RPE proteome as detected by 2-D gel electrophoresis. Keeping in mind that 2-D gels fail to resolve hydrophobic proteins and have strict limitations on detection of very low- and high-molecular-weight proteins, hemoglobin concentration within the RPE is at least four times less than the concentration within an erythrocyte. Furthermore, since the RPE cell is so much larger (6.2 times) than the erythrocyte,[32] hemoglobin within RPE can only produce a faint red tint that

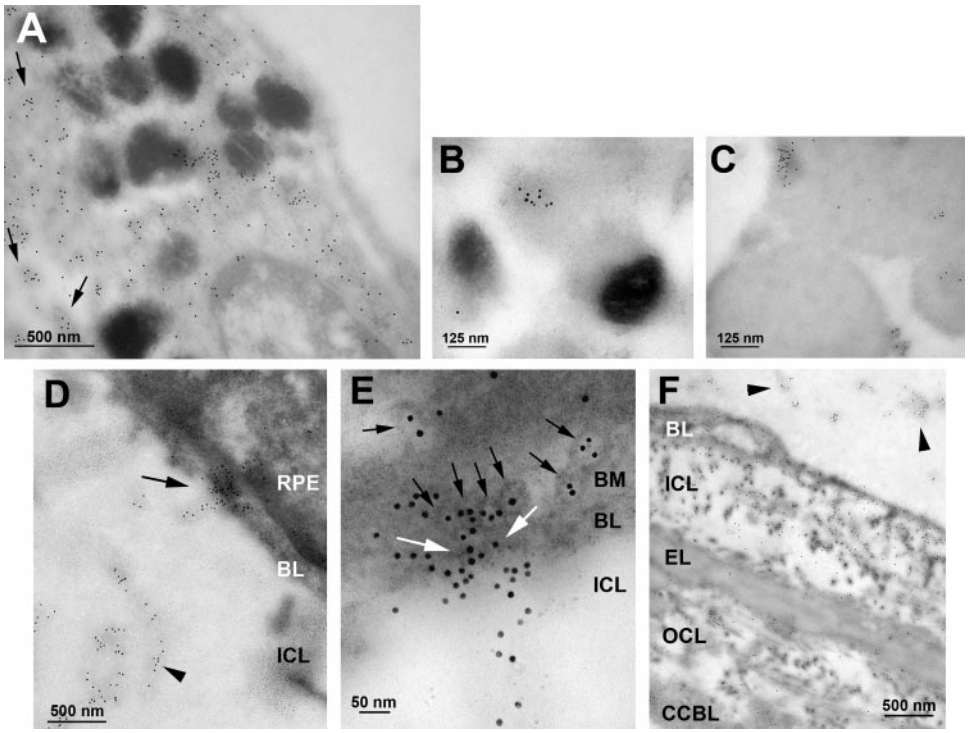

FIGURE 6. Immunoelectron microscopy of human RPE and Bruch's membrane. (**A**) Representative ultrathin sections (50 nm) of human RPE ($n = 2$, 81 and 64 years old) revealed the presence of hemoglobin in human RPE cytosol within multiple round, intermediate-density cytoplasmic granules (*arrows*). (**B**, **C**) At higher magnifications hemoglobin labeling was confined to the RPE cytoplasm, mainly as intermediate-density granules. (**D**) Human RPE cells excreted hemoglobin from the basal side toward Bruch's membrane (*arrow*). Excreted hemoglobin accumulated mainly within the inner collagen layer (ICL) of Bruch's membrane as electron-dense material (*arrowhead*). (**E**) At a higher magnification, hemoglobin immunostaining was observed within porosome-like vesicles (*arrows*) lining the basal infoldings at the RPE basal membrane (BM). Some vesicles fused with the BM and released hemoglobin toward the ICL (*white arrows*). (**F**) Secreted hemoglobin was seen as electron-dense clumps within the sub-RPE space at an area of focal RPE detachment (*arrowheads*). Sections through Bruch's membrane revealed the presence of hemoglobin within this pentalaminar extracellular matrix. Excreted hemoglobin accumulated mainly within the inner layers of Bruch's membrane. Replacement of anti-human panhemoglobin antibody with goat anti-mouse IgG resulted in the absence of gold labeling in control sections (data not shown). EL, elastic layer; OCL, outer collagen layer; CCBL, choriocapillaris basal lamina.

can barely be detected with only spectrophotometry.[33] The presence of high amounts of melanin and lipofuscin within the RPE cytoplasm also masks the red tint of hemoglobin. Bleaching melanin to visualize hemoglobin in the RPE is not helpful because routine histochemical methods[34] often digest hemoglobin.[35]

The rate of hemoglobin secretion by RPE cells approximated 1.5% of the intracellular hemoglobin pool per hour, indicating a high turnover (~2.7 days). It is quite possible that during our in vitro experiments, intercellular junctions of RPE are severed or RPE may become migratory in in vitro cultures. This possibility may explain how secreted hemoglobin can reach measurable levels in the culture medium. However, electron microscopy revealed that the main secretion route in vivo is through the basolateral membrane. Hemoglobin produced by the RPE cells may be secreted in a polarized fashion toward Bruch's membrane, where a concentration gradient between the inner and outer layers was observed.

Although the physiological importance of hemoglobin production by mammalian RPE is yet to be determined, it is quite possible that quantitative and qualitative defects in RPE hemoglobin expression underlie the hypoxia the leads to several macular diseases. For example, hypoxia-driven VEGF upregulation is responsible for subretinal neovascularization in exudative age-related macular degeneration,[4] and cigarette smoking, a major risk factor for age-related macular degeneration, is known to impair hemoglobin function by carboxylation,[36] ethylation, methylation, and/or nitration.[37,38]

In conclusion, our study showed that human RPE cells synthesize and secrete hemoglobin. We believe this finding may provide new insight into the mechanism of oxygen transport to the outer retina and the pathogenesis of age-related macular degeneration, as well as other ischemia-based macular diseases.

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