

Primary Culture of Human Blood–Retinal Barrier Cells and Preliminary Study of APOBEC3 Expression: An In Vitro Study

Haotian Lin,¹ Zhenping Zhang,¹ Hui Zhang,² Pisong Yan,¹ Qilin Wang,¹ and Ling Bai³

PURPOSE. To develop methods for primary culture of human blood–retinal barrier (BRB) cells and to explore the expression of *APOBEC3* (apolipoprotein B mRNA-editing enzyme, catalytic polypeptide-like 3) family gene, novel host-defense factors to HIV-1.

METHODS. Cellular components of human BRB (human retinal capillary endothelial cells [HRCECs], human retinal capillary pericytes, and human retinal pigment epithelial cells) were isolated separately and subjected to primary culture according to procedures modified in our laboratory. Immunocytochemistry and immunofluorescence were used to identify specific markers of the primary cells and to analyze their purity by flow cytometry. RNA of the three different cells was isolated, and primers were designed to probe expression of the *APOBEC3* gene by reverse transcription–polymerase chain reaction (RT-PCR) and real-time PCR. For further confirmation, *APOBEC3F* and *APOBEC3G* proteins were detected in the cultured cells and fresh retina tissue through Western blot analysis. In the end, HRCECs were treated with IFN- γ , and change of *APOBEC3G* expression was displayed.

RESULTS. Pure BRB cells (>95% purity) were primary cultured according to procedures modified in our laboratory. Qualitative test of RT-PCR and semiquantitative examination of real-time PCR demonstrated the presence of *APOBEC3B*, *-3C*, *-3F*, and *-3G* genes and the absence of *APOBEC3A* and *-3D* genes in all cellular components of the BRB. Finding of the *APOBEC3G* and *APOBEC3F* proteins expressed in the three primary cultured cells and different layers of retinal tissue by Western blot analysis further confirmed the PCR results. Moreover, IFN- γ could upregulate the expression of *APOBEC3G* in HRCECs.

CONCLUSIONS. Major cellular components of human BRB could be primary cultured in vitro according to procedures optimized in our laboratory. Different expression of *APOBEC3* in human blood–retinal barrier gives a clue to further research in intrinsic antiviral immunity in HIV-1–related retinopathy. (*In-*

vest Ophthalmol Vis Sci. 2009;50:4436–4443) DOI:10.1167/iov.08-3169

The apolipoprotein B mRNA-editing enzyme, catalytic polypeptide-like 3 (*APOBEC3*) family, consisting of *APOBEC3A-3H*, consists of cellular proteins with cytidine deaminase activity that induces dC-to-dU mutations in minus-stranded DNA formed during reverse transcription to disable a broad range of retroviruses. Most members of this family, including *APOBEC3A*, *-3B*, *-3C*, *-3D*, *-3F*, and *-3G*, have recently been identified to potentially inhibit the replication of various retroviruses, among them human immunodeficiency virus type 1 (HIV-1), simian immunodeficiency virus, equine infectious anemia virus, murine leukemia virus, foamy virus, and hepatitis B virus.^{1–12} In the case of HIV-1 and many other lentiviruses, virion infectivity factor is encoded to effectively neutralize the antiviral effect of *APOBEC3G*, *APOBEC3F*, and others by facilitating the degradation of these cytidine deaminases.^{10,13–16}

Recent studies have demonstrated that *APOBEC3G* exists in two different forms in various cell systems. A low molecular mass form is associated with HIV-1 restriction, and a high molecular mass complex lacks enzymatic activity and anti-HIV-1 activity.^{17,18} The distinct existing forms and the different gene expression amount and function in various tissues and cells are the characters of the *APOBEC3* family. It is reported that *APOBEC3* primarily expresses in testicle, ovary, spleen, granule cells, and lymph cells of peripheral blood.¹⁰ However, recent studies from Hui Zhang's laboratories demonstrate that *APOBEC3B*, *-3C*, *-3F*, and *-3G* are expressed in all major cellular components of the central nervous system. Moreover, the studies show that interferon- α (IFN- α) and IFN- γ significantly enhance the expression of *APOBEC3G*, *-3F* and drastically inhibit HIV-1 replication in primary human brain microvascular endothelial cells, the major component of the blood–brain barrier (BBB).¹⁹ As we know, human retinal and brain tissue arise from the same embryonic origin, which means the blood–retinal barrier (BRB) and the BBB should have similar tissue structure and function.²⁰ Like the BBB, the BRB is an important virus reservoir that has been researched for a quarter century without breakthrough.²¹ We hypothesize that such a “molecule barrier” of the *APOBEC* family may also exist in the BRB, and the difference in the expression and distribution of this important host-defense factor may be the real reason for the discrepant intrinsic antiviral immunity among persons.

Effective prevention and treatment of HIV-1–related retinopathy are important for the quality of life of HIV-1–positive patients. Although the pathogenesis of this disease remains unclear, it has been believed that disruption of the BRB is the primary cause. The development of convenient and practical methods for primary culture of human BRB cells provides a good foundation for in vitro study of HIV-1–related retinopathy. Here we try to modify procedures and develop techniques for the primary culture of major cellular components of human BRB (human retinal capillary endothelial cells [HRCECs]; human retinal capillary pericytes [HRPCs]; human retinal pigment epithelial cells [HRPEs]) and to study preliminarily the

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Supported by National Natural Science Foundation of China (30471851) and Guangdong Provincial Science and Technology program (2008B060600026).

Submitted for publication November 17, 2008; revised February 23 and March 22, 2009; accepted June 15, 2009.

Disclosure: **H. Lin**, None; **Z. Zhang**, None; **H. Zhang**, None; **P. Yan**, None; **Q. Wang**, None; **L. Bai**, None

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distribution and expression of the APOBEC3 family in human BRB.

MATERIALS AND METHODS

Reagents

Rabbit anti-hu *APOBEC3G* and rabbit anti-hu *APOBEC3F* polyclonal antibodies obtained from the AIDS Research and Reference Reagent Program (Division of AIDS, National Institute of Allergy and Infectious Diseases, National Institutes of Health; catalog numbers 10084 and 11226, respectively) were a generous gift from Hui Zhang (Thomas Jefferson University, Philadelphia, PA). Except for those indicated in the text, all other chemicals and reagents were obtained from Invitrogen/Gibco (Grand Island, NY).

Tissue Source

This study was performed in accordance with the ethical standards laid down in the 1964 Declaration of Helsinki for Research Involving Human Tissue. After receiving consent for research use and Sun Yatsen University ethics committee approval, seven postmortem eyes (donor age range, 24–32 years) were obtained from the Eye Bank of Zhongshan Ophthalmic Center (Guangzhou, China) 8 to 24 hours after death. Only eyes free of ocular disease were used to establish primary cultures or other experiments. The research was conducted according to the tenets of the Declaration of Helsinki.

Primary Human Retina Cells Isolation and Cultures

Donor eyes were washed with calcium- and magnesium-free (CMF) Hanks balanced salt solution (HBSS) containing 100 U/mL streptomycin and penicillin, after which the anterior segment was removed by hemisection of the globe at the ora serrata. The retina was detached from the pigment epithelium with the vitreous and dissected away from the optic disc. Then the retina with the vitreous and the detached pigment epithelium were collected separately.

Primary HRCECs were isolated, maintained, and passaged in vitro under strict conditions, as described previously, and optimized in our laboratory as described.^{22–26} The retina and the vitreous were rinsed with CMF HBSS and transferred to 10 mL (per retina) dispersal solution I (2% trypsin; Sigma, St. Louis, MO). After 20-minute incubation at 37°C, the vitreous and supernatant were discarded. The retinas were dispersed by gentle pipetting in 10 mL dispersal solution II (0.066% Collagenase II). After another 20-minute incubation at 37°C, the resultant cell suspension was centrifuged at 1200g, resuspended in culture medium consisting of human endothelial-SFM with 10% (vol/vol) fetal bovine serum, additional 0.348% (wt/vol) insulin-transferrin-selenium, β -ECGF (Sigma), and 100 U/mL streptomycin and penicillin, seeded into three 25-cm² tissue culture flasks or into 32 (2-cm²) wells with 13-mm sterile coverslips (per pair of retinas) precoated with human fibronectin (0.5 μ g/mL), and maintained at a humidified, CO₂-regulated (95% air/5% CO₂), and 37°C constant-temperature incubator (Shel Lab CO₂ Incubator; Sheldon Manufacturing, Cornelius, OR) atmosphere. After 2 days in culture, the medium was replaced to remove lightly adherent cells and vascular fragments. The cultures were fed weekly until confluence, usually 2 to 3 weeks. Then the confluent colonies were isolated and dissociated with trypsin (0.25% trypsin + 0.02% EDTA) in cloning cylinders. With antibodies against von Willebrand factor (Maixin Bio, Fuzhou, China) and zonula occludens (ZO)-1, a tight junction-associated protein characteristic of endothelium, the purity of HRCECs was analyzed by immunofluorescent staining and immunocytochemistry microscopy and confirmed by flow cytometry.²⁷

HRCPCs were isolated by slight modification of an established method.²⁸ Briefly, each retina was vigorously pipetted, incubated with dispersal solution I (2% trypsin + 2% EDTA) for 30 minutes at 37°C, and filtrated through a stainless steel sieve. Cells were washed and

cultured to confluence in DMEM/F-12 containing 2 mM L-glutamax I and 10% fetal bovine serum (FBS). Purity of HRCPCs was identified by their characteristic morphology under phase-contrast microscopy and by their expression of glial fibrillary acidic protein and cytoskeletal structural protein (α -actin), as demonstrated by immunocytochemical staining and immunofluorescence.^{29,30}

HRPEs were isolated and cultured briefly as follows.³¹ The detached pigment epithelium was immersed in a trypsin (0.25%)-EDTA (0.02%) solution at 37°C for 1 hour. Then DMEM with 10% serum was added, and the RPE was isolated and collected with a fire-blown Pasteur pipette under direct observation with a dissecting microscope. Isolated cells were centrifuged, resuspended, and seeded to a culture flask. The culture medium used for culturing the RPE was DMEM with high glucose (4.5 g/L) containing 10% FBS, penicillin (100 U/mL), and streptomycin (100 U/mL) and was changed three times a week. Once cells reached confluence, they were detached from the culture dish using the trypsin (0.25% trypsin + 0.02% EDTA) solution. The cell suspension was diluted at 1:3 and seeded into culture flasks for subculture. Purity of HRPEs was identified by rabbit anti-hu keratin multiclone antibody (Zhongshan Ltd., Beijing, China). All cell lines of passages 3 to 5 were used in the following studies.

Immunocytochemistry and Immunofluorescence

To analyze retinal cell markers expression by immunocytochemistry, primary cultures of human retinal cells (HRCECs, HRCPCs, and HRPEs) were established on 13-mm glass coverslips in 32-well culture plates. When cultures became confluent (3–4 weeks), the coverslips were rinsed in CMF HBSS and fixed in 4% buffered paraformaldehyde for 20 minutes at 4°C. The coverslips were washed in 50 mM Tris HCl, pH 7.5, and 150 mM NaCl (Tris-buffered saline [TBS]), permeabilized in TBS with 0.1% (vol/vol) Triton X-100 (TBS/Triton) for 20 minutes, and washed again in TBS. Endogenous peroxidase activity was quenched by a 5-minute incubation of coverslips in 3% (vol/vol) hydrogen peroxide in water and again rinsed in TBS. Nonspecific binding sites were blocked by a 15-minute incubation in 6% (wt/vol) nonfat dry milk in TBS. After a brief wash in TBS, further blocking of nonspecific avidin-binding sites was accomplished with 15-minute incubations in avidin solution followed by another brief wash in TBS and incubation for 15 minutes in biotin solution. The coverslips were washed in TBS and incubated overnight at 4°C in a humidified chamber with primary antibodies diluted in 3% (wt/vol) nonfat dry milk in TBS/Triton. They were then washed (4 \times 5 minutes) with TBS/Triton and incubated in biotinylated second antibody (diluted 1:200 in 3% nonfat dry milk in TBS/Triton) at room temperature for 1 hour. This was followed by successive washes in TBS/Triton (4 \times 5 minutes) and TBS (3 \times 5 minutes), after which they were incubated in avidin-biotinylated horseradish peroxidase complex for 45 minutes. The coverslips were then washed (4 \times 5 minutes) in TBS and developed for 5 minutes in diaminobenzidine solution (0.5 mg/mL). After the color developed, they were rinsed in water, and some were counterstained with hematoxylin. The coverslips were dehydrated through alcohol and xylene and mounted on glass slides for light microscopy.

Cultures also were stained by single- or double-label immunofluorescence. Simply, cells were washed with PBS, fixed for 30 minutes with 4% paraformaldehyde, permeabilized with 1% Triton-PBS (10 minutes at room temperature), and blocked with 2% BSA-PBS (1 hour at room temperature). Cells were then incubated with one or two primary antibodies as described and then by the appropriate fluorescein-conjugated secondary antibody (diluted 1:50 in 1% BSA-PBS). Cells were rinsed three times with PBS, mounted in antifade solution, sealed, and analyzed by confocal microscopy (TCS NT; Leica, Wetzlar, Germany).

RNA Isolation and Reverse Transcriptase–Polymerase Chain Reaction

Total RNA from HRCECs, HRCPCs, and HRPEs grown in six-well plates (confluent) and H9 and 293T cells were extracted with reagent (Trizol;

TABLE 1. Primer Sequence Used for PCR

Gene Name	Accession Number	Forward	Reverse
<i>APOBEC3A</i>	NM_145699.2	5'-GAAGGGACAAGCACATGGAAGC-3'	5'-ATCTACTTGATCGGGAGCATAC-3'
<i>APOBEC3B</i>	NM_004900	5'-TCGAGGCCAGGTGTATTTC AAG-3'	5'-CTCATAGCACAAAGTAGGTCTGG-3'
<i>APOBEC3C</i>	NM_014508	5'-AACCTATGGGAAGCCAAGGATC-3'	5'-CCTCCTGGTAACATGGATACTG-3'
<i>APOBEC3D</i>	NM_152426	5'-ACGTCAGTCGAATCACAGGCAG-3'	5'-CTGGTCTCCTGGCTGTCAAGTTG-3'
<i>APOBEC3F</i>	NM_145298	5'-CGGCCTGTCTTTATCAGAGGT-3'	5'-CAGGTGAGTGGTGGTTTACAAC-3'
<i>APOBEC3G</i>	NM_021822	5'-GGTGTATTCCGAACTTAAGTAC-3'	5'-CAAGGAAACCGTGTTTATGTGG-3'
<i>GAPDH</i>	NM_002046	5'-AAGAGCACAAAGGAAGAGAGAGAC-3'	5'-GTCTACATGGCAACTGTGAGGAG-3'

Invitrogen). RNA (200–300 ng) from each sample was reverse transcribed with a cDNA synthesis kit (iScript; Bio-Rad, Hercules, CA). The cDNAs of *APOBEC3A*, *APOBEC3B*, *APOBEC3C*, *APOBEC3D*, *APOBEC3F*, and *APOBEC3G* were PCR amplified using specifically designed primers (Table 1). All PCR reactions were performed by 35-cycle amplification (94°C for 3 minutes; 35 cycles at 94°C for 45 seconds, 55°C for 45 seconds, and 72°C for 1.5 minutes; and extension at 72°C for 7 minutes). GAPDH was used as an input control, H9 cells were used as positive control, and 293T cells served as negative control.

Real-Time Reverse Transcriptase–Polymerase Chain Reaction

Real-time quantitative reverse transcriptase–polymerase chain reaction with SYBR (real-time qRT-PCR) was performed with a qRT-PCR kit (Super-Script III Platinum1 Two-Step; Invitrogen, Carlsbad, CA) on a sequence detection PCR system (ABI PRISM 7000; Applied Biosystems, Foster City, CA) according to the manufacturers' protocols. DNA polymerase was first activated at 95°C for 10 minutes, denatured at 95°C for 15 seconds, and annealed/extended at 60°C for 1 minute, for 40 cycles according to the manufacturer's protocol. The products were sequenced to ensure that the correct gene sequence was amplified. All PCR reactions were performed in triplicate. For each experimental sample, the amounts of target and endogenous reference were determined from the appropriate standard curves. Then the target amount was divided by the endogenous reference amount to obtain a normalized target value. Each of the experimental normalized sample values was divided by the normalized control sample value to generate the relative expression levels. We repeated examinations for every sample (three times for each) to obtain the mean values of all samples. GAPDH was used as an internal control, H9 cells were used as positive control, and 293T cells were used as negative control.

Western Blot Analysis

Human primary cultured retina cells, neural epithelium layer, pigment epithelium layer, and whole retina were lysed, and total proteins were

extracted with a protein extraction reagent (CytoBuster; Novagen, EMD Chemicals Inc., Darmstadt, Germany) and quantified by a bicinchoninic acid protein assay reagent kit (Pierce Chemical Co., Rockford, IL). Up to 30 µg total protein was used for electrophoresis (10% polyacrylamide gel) and transferred onto polyvinylidene difluoride membranes. After blocking with 10% milk in phosphate-buffered saline (PBS) with 0.05% Tween-20, membranes were probed with rabbit polyclonal anti-*APOBEC3G* or anti-*APOBEC3F* antibodies at 1:1000 for 2 hours at room temperature. Loading control was detected with anti-GAPDH monoclonal antibody (Sigma). Membranes were washed with PBS three times, followed by incubation with secondary antibodies conjugated with horseradish peroxidase (Santa Cruz Biotechnology, Santa Cruz, CA) for 1 hour at room temperature, and were visualized by chemiluminescence (Pierce Chemical Co.). We used H9 cells, which are known to express high levels of *APOBEC3G* and *APOBEC3F*, as controls and included 293T cells as negative control.¹⁰

Treatment of HRCECs with IFN-γ and Effect on *APOBEC3G* Expression

HRCECs are the major component of the blood–retinal barrier, and *APOBEC3G* is one of the earliest recognized powerful antiretroviral factors; therefore, we tested the direct effect of exogenously added IFN-γ (Sigma) on *APOBEC3G* protein expression in primary human HRCECs. To determine whether IFN-γ affects *APOBEC3G* protein expression, HRCECs were similarly treated with single-dose increasing concentrations (0, 150, 300, 600, and 1200 U/mL) of IFN-γ, and cell lysates were collected and subjected to Western blot analysis at different time points (6, 12, 24, and 48 hours) after IFN-γ treatment.

Statistical Analysis

Differences between groups were assessed with the use of one-way ANOVA and a statistical software program (SPSS 13.0; SPSS, Chicago, IL). $P < 0.05$ was considered indicative of statistical significance.

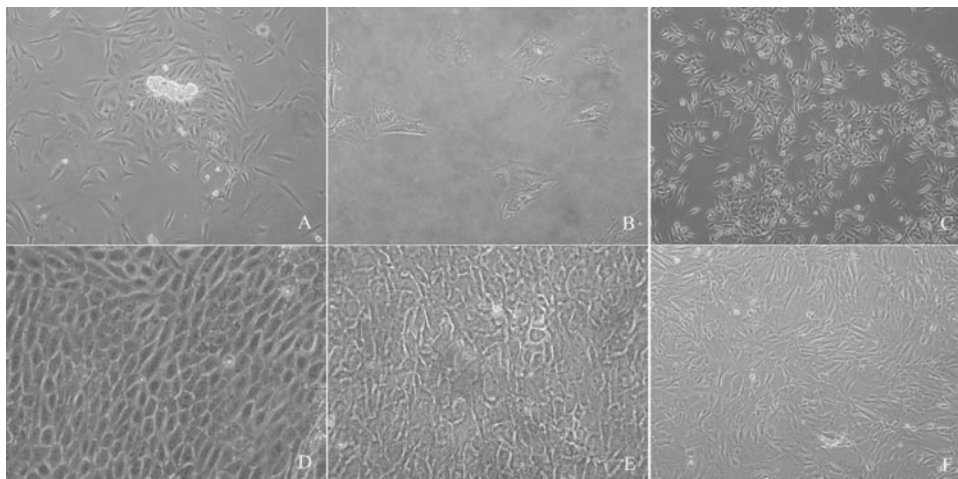


FIGURE 1. Cultured primary human BRB cells. All primary cell systems were cultured and maintained. (A) Primary HRCECs were isolated and cultured on the third day and were mixed with capillary fragments and some other lightly adherent cells. (D) Medium was replaced every 3 to 4 days to purify and feed the HRCECs until confluence occurred, usually at 2 to 3 weeks. HRCPCs (B, E) and HRPEs (C, F) were cultured in the same manner as the HRCECs.

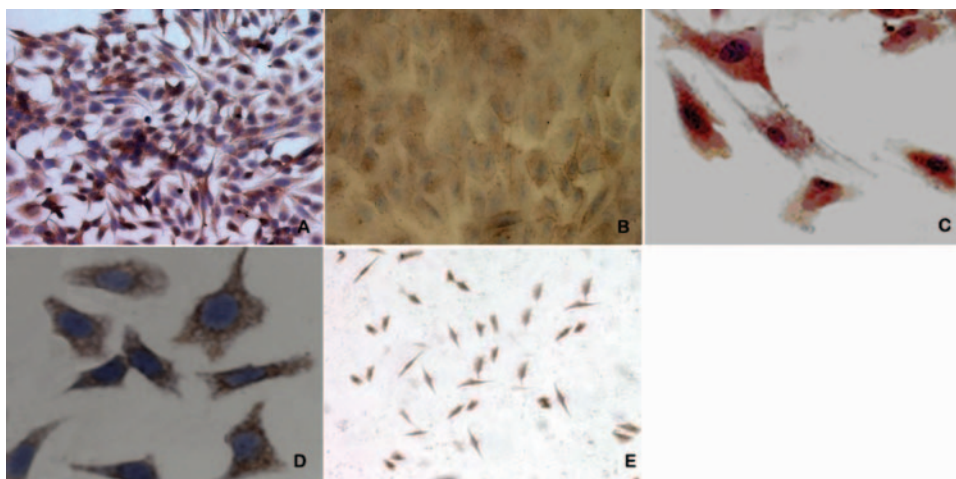


FIGURE 2. Primary human BRB cells in our studies were identified by immunohistochemical staining. Primary HRCECs expressing (A) von Willebrand factor and (B) ZO-1. Primary HRCPCs (C) expressing glial fibrillary acidic protein and (D) stained for cytoskeletal structural proteins (α -actin). (E) HRPEs immunostained for keratin.

RESULTS

Primary Cells of BRB (HRCECs, HRCPCs, HRPEs) Cultured and Identified

All BRB-based cell systems were cultured, maintained, and passaged (Figs. 1A–G) with the use of specially designed media and according to the optimized procedures in our research group, as described.

The purity of BRB cells (>95%) was analyzed by immunocytochemistry microscopy, immunofluorescent staining and was confirmed by flow cytometry with antibodies against specific markers. Primary HRCECs were positive for expressing von Willebrand factor (Figs. 2A, 3A) and ZO-1 (Figs. 2B, 3B). Primary HRCPCs were monitored for expressing glial fibrillary acidic protein (Figs. 2C, 3C) and cytoskeletal structural proteins (α -actin) (Figs. 2D, 3D). The morphology of HRPEs was monitored carefully by examining the expression of human epithelial marker protein (keratin; Figs. 2E, 3E).

Analysis of APOBEC3 Expression by Reverse Transcribed-PCR

To analyze the expression of different APOBEC3 family members in the human BRB, including primary HRCECs, HRCPCs, and HRPEs, we first evaluated the mRNA levels of various APOBEC3 family members in the BRB through semiquantitative RT-PCR by using specifically designed *APOBEC3A–G* primer-pairs. GAPDH

was used as an input control, H9 cells were used as positive control, and 293T cells served as negative control. The results, as shown in Figure 4, indicated strong mRNA expression of *APOBEC3B*, *APOBEC3C*, *APOBEC3F*, and *APOBEC3G* in all BRB cells. Conversely, *APOBEC3A* and *APOBEC3D* mRNA were not detected in any BRB cells, namely HRCECs, HRCPCs, and HRPEs.

Analysis of APOBEC3 Expression by Real-Time PCR

To confirm the mRNA levels of various APOBEC3 family members in BRB, we used real-time PCR to further evaluate quantitatively the different expression by making use of the same primer pairs. Real-time qRT-PCR demonstrated that in HRCECs, HRCPCs, HRPEs, and H9 cells, values of *APOBEC3B*, *-3C*, *-3F*, *-3G*, and GAPDH were more than 30 Ct, and expression quantity was significantly larger than negative control with statistical significance ($P < 0.05$), whereas *APOBEC3A* and *-3D* were not (Fig. 5). We concluded that *APOBEC3B*, *APOBEC3C*, *APOBEC3F*, and *APOBEC3G* were expressed in all BRB cells, which proved the results through RT-PCR. GAPDH was used as an input control, H9 cells were used as positive control, and 293T cells served as negative control. Expression levels of *APOBEC3* are presented relative to those in the control group. To validate our real-time qRT-PCR protocol, melting-curve analysis was performed to check for the absence of primer dimers.

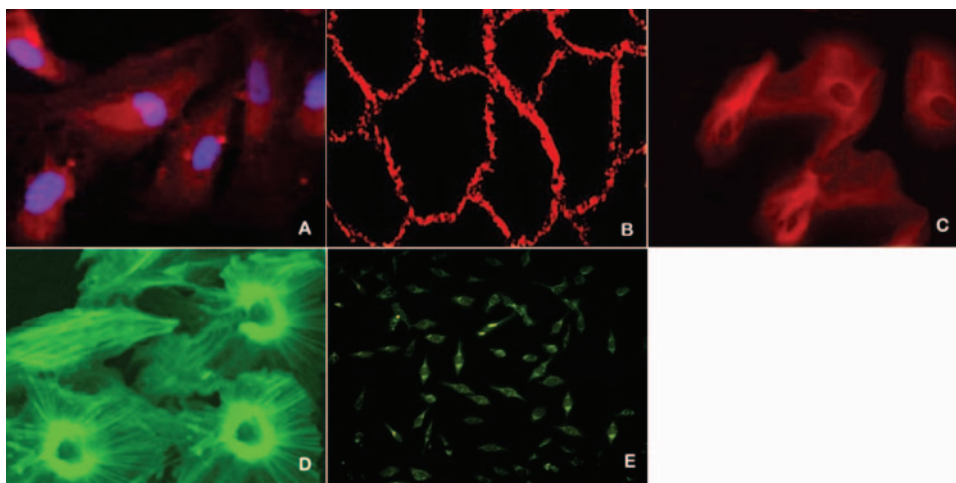


FIGURE 3. Primary human BRB cells were further identified by immunofluorescent staining. Primary HRCECs expressing (A) von Willebrand factor and (B) ZO-1. Primary HRCPCs (C) expressing glial fibrillary acidic protein and (D) stained for cytoskeletal structural proteins (α -actin). (E) HRPEs immunostained for keratin. To focus on specific markers immunofluorescent staining, DAPI nuclear staining on (B–E) was not displayed.

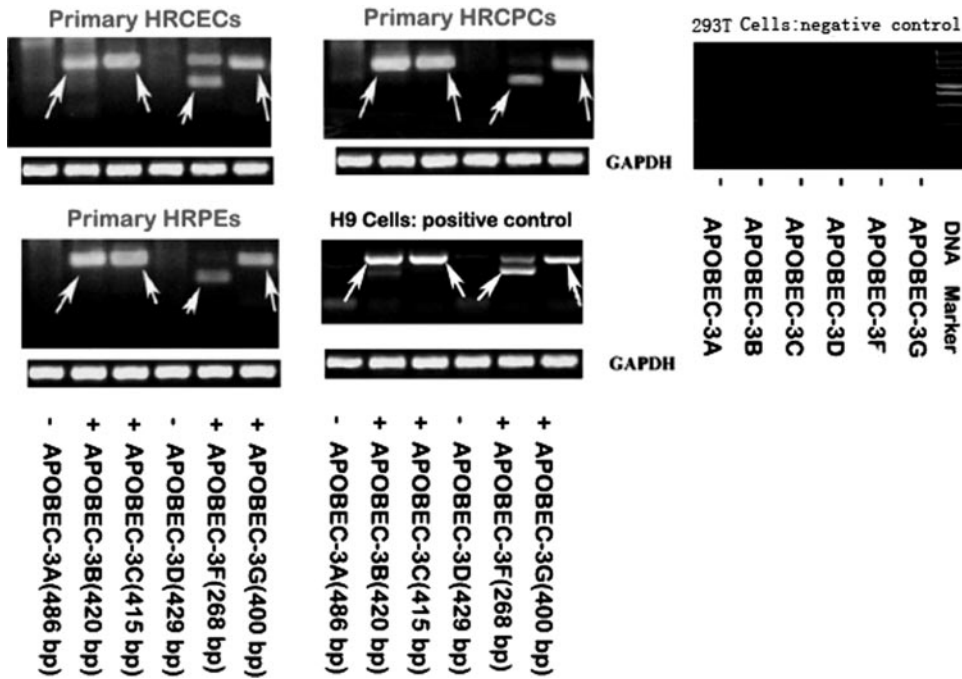


FIGURE 4. Expression of APOBEC3 in human BRB cells detected by RT-PCR. The presence of *APOBEC3A*, *-3B*, *-3C*, *-3D*, *-3F*, and *-3G* mRNA isolated from primary HRCECs, HRCPCs, and HRPEs, which are key components of the BRB, and positive control H9 cells and negative control 293T cells. Arrows: specifically amplified *APOBEC3* products. *APOBEC3B*, *-3C*, *-3F*, and *-3G* were detected (+, positive), but *APOBEC3A* and *-3D* were not detected (-, negative). GAPDH served as a positive control.

Evaluation of *APOBEC3* Expression by Western Blot Analysis

We evaluated the protein expression levels of *APOBEC3G* and *APOBEC3F*, which are shown to represent powerful antiretroviral host-restriction factors,¹³⁻¹⁶ by Western blot analysis in BRB cells, neural epithelium layer, pigment epithelium layer, and whole retina. In our studies, we used well-characterized rabbit polyclonal antibodies against APOBEC3G and APOBEC3F, which were obtained from the AIDS Research and Reference Reagent Program at the National Institutes of Health. As shown in Figure 6A, our findings clearly demonstrate strong protein expressions of *APOBEC3G* and *APOBEC3F* (~46 kDa) in all BRB-based cells. As shown, the detected *APOBEC3G* and *APOBEC3F* protein levels in HRCECs, HRCPCs, and HRPEs are somehow lower, yet clearly identified, compared with the stronger signal detected in the positive control, H9 cells. In Figure 6B, *APOBEC3G* and *APOBEC3F* protein levels in neural

epithelium layer, pigment epithelium layer and whole retina are higher than in BRB cells, and the signal of the pigment epithelium layer is stronger than the other tissue layers. These results suggest that the expressions of *APOBEC3* proteins in fresh tissues are stronger than in long-term cell cultures, and the level of *APOBEC3* proteins in the major cell component (HRPEs in fresh pigment epithelium layer of retina) of BRB may be higher than in miscellaneous cells (including BRB cells, neural cells, and others) of different layers of retinal tissue. As expected, neither *APOBEC3G* nor *APOBEC3F* protein expression was detected in 293T cells, which served as a negative control. Taken together, Western blot analysis confirmed findings of the RT-PCR and real-time PCR evaluations and further demonstrated the protein expression of *APOBEC3G* and *APOBEC3F* in the three cultured BRB cells and the different layers of fresh retinal tissue, including HRCECs, HRCPCs, HRPEs, neural epithelium layer, pigment epithelium layer, and

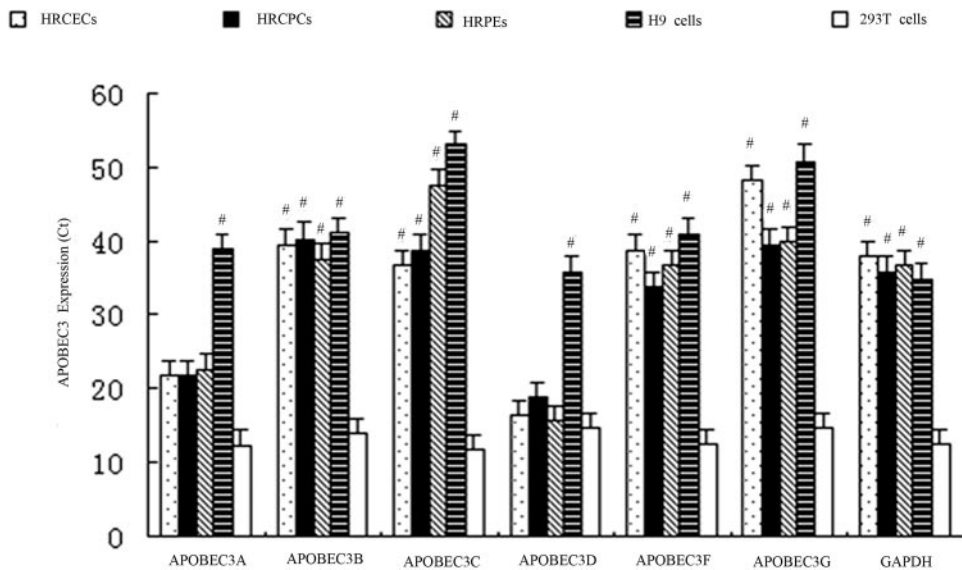
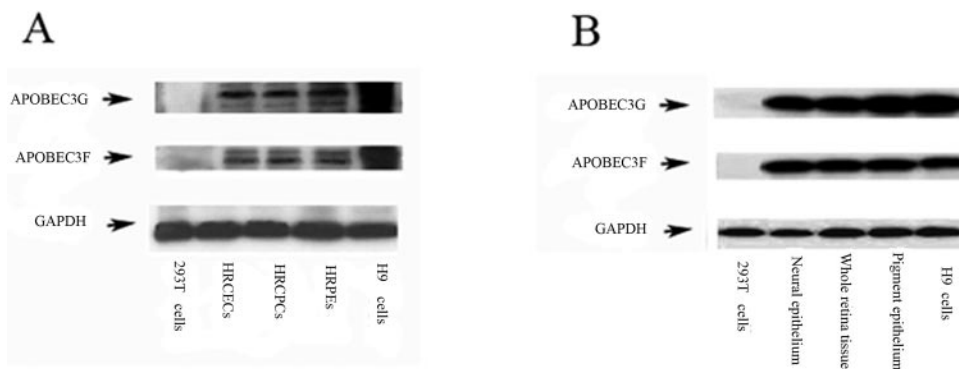


FIGURE 5. Expression of *APOBEC3* evaluated by real-time PCR. The relative expression level of each gene is detected in BRB cells compared with control groups. Data are mean \pm SD. Values (Ct) shown represent the mean \pm SD of three independent assays or experiments. The genes examined are shown on the x-axis. In HRCECs, HRCPCs, HRPEs, and H9 cells, values of *APOBEC3B*, *-3C*, *-3F*, and *-3G* and of GAPDH were greater than 30 Ct, and expression quantity was statistically significantly larger than negative control, but *APOBEC3A* and *-3D* were not. H9 cells, positive control; 293T cells, negative control. #*P* < 0.05 vs. negative control.

FIGURE 6. Expression of APOBEC3G and APOBEC3F proteins evaluated by Western blot analysis. (A) Cell lysates from primary HRCECs, HRCPCs, and HRPEs, (B) tissue lysates from neural epithelium layer, whole retina and pigment epithelium layer, and H9 (positive control) and 293T cells (negative control) were subjected to Western blot analysis with rabbit polyclonal antibodies against APOBEC3G (~46 kDa) and APOBEC3F (~46 kDa). GAPDH (~36 kDa) served as a positive control.



whole retinal tissue. Of note, given that anti-APOBEC3B and anti-APOBEC3C antibodies are not yet available, we could not detect these two proteins in our study.

IFN-γ Upregulation of APOBEC3G Expression in HRCECs

Recent studies have shown that many IFN family members can directly upregulate APOBEC3G expression in macrophages.^{18,32} In our study, a dose-dependent increase in APOBEC3G (Fig. 7) protein expression was detected as early as 12 hours on a single treatment with 300 U/mL IFN-γ. IFN-γ also exerted its maximum inducing effect on APOBEC3G protein expression in HRCECs at 600 U/mL, and no further protein induction was detected on treatment with higher concentrations. Moreover, a kinetic analysis revealed that single-dose treatment of 300 U/mL IFN-γ enhanced APOBEC3G protein expression within 12 hours and that it increased further at 24 hours but not at 48 hours (Fig. 7). It is important to mention that, on treatment of primary HRCECs even with high IFN doses, such as 1200 U/mL, we did not observe any toxic effects on the cells, and the morphology and cell numbers remained normal. Thus, our data indicate that IFN-γ cytokines can induce expression of APOBEC3G in primary human HRCECs.

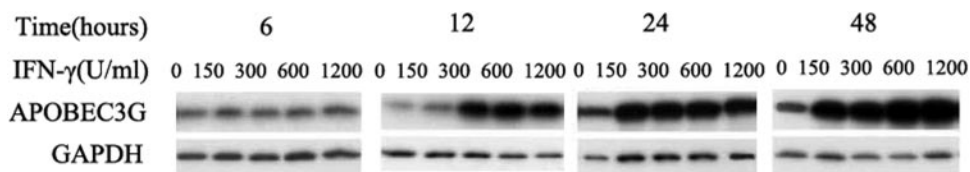
DISCUSSION

In the present study, we developed methods for primary culture of cellular components of human BRB and successfully obtained pure cells for further research. The culture of retinal cells and tissues is now a widely used tool, with broad applications in the fields of ophthalmology and other subjects. As with all in vitro systems, both advantages and potential limitations exist. Advantages include highly controllable conditions that allow for measurements on a cell-by-cell basis, isolation from confounding systemic/ocular effects, time course flexibility, and reduction in the number of animals used for research. Possible limitations include selective loss of specific cell phenotypes/functions, changes in tissue architecture, and the potentially questionable relevance of in vitro findings. For many

applications, the advantages of in vitro studies outweigh the potential limitations. Today retinal cell culture and tissue culture are routinely used for studies of cell growth, differentiation, cytotoxicity, gene expression, and cell death. Although many primary culture methods of retinal cells have been published,²²⁻³¹ no systemic and convenient methods for primary culture of BRB cells can be found. In our study, we tried to modify and integrate the advantages of the established culture methods to get optimized procedures of BRB cells. We described systemically how the entire human blood-retinal barrier cells (HRCECs, HRCPCs, and HRPEs) were cultured and identified separately in our laboratory.

With this precondition, we could evaluate the expression of different APOBEC3 family members in the BRB cells to probe the novel and mysterious host-defense factors in this important target (BRB) for viral invasion. Recent study of human blood-brain barrier¹⁹ demonstrates that APOBEC3B, -3C, -3F, and -3G are expressed in all major cellular components of the central nervous system and the manipulation of IFN-APOBEC3 signaling pathway could be a potent therapeutic strategy to prevent HIV invasion to CNS. This is obviously a breakthrough in the study of one of the important virus reservoirs (BBB) and even gives a clue for another significant virus invasion target, BRB. To probe such a “molecule barrier” of the APOBEC family in the BRB, we applied any available molecule detection methods. In our studies, by using specifically designed primers in RT-PCR detection assays, we were able to provide clear evidence of mRNA expression of different APOBEC3 family members known to have antiviral activity, such as APOBEC3G, APOBEC3F, APOBEC3B, and APOBEC3C, in all BRB cells. To confirm the results of RT-PCR, we used real-time PCR to quantitatively evaluate the different expression of various APOBEC3 family members by making use of the same primer pairs. Western Blot analysis further confirmed our findings on APOBEC3G and APOBEC3F protein expression in the BRB cell cultures and different layers of fresh retinal tissue by means of well-characterized rabbit polyclonal antibodies that we obtained from the AIDS Research and Reference Reagent Program. Immunohistochemical approaches for APOBEC family

FIGURE 7. IFN-γ upregulates the expression of APOBEC3G in human primary HRCECs. Time-course and dose-dependent studies on the upregulation of APOBEC3G (~46 kDa) by IFN-γ. IFN-γ (0, 150, 300, 600, and 1200 U/mL) was added to cultures of primary human HRCECs. The intracellular expression of APOBEC3G protein was assessed by Western blot analysis of cell lysate samples collected at 6, 12, 24, and 48 hours after IFN-γ treatment. GAPDH (~36 kDa) served as a positive control.



detection did not produce satisfactory results, possibly because *APOBEC3G/-3F* expression of BRB cells is too low to be detected (data not shown). This result may confirm study findings^{17,18,33} that indicate *APOBEC3G* exists in two forms in various cell systems, only one of which is a low molecular mass form and is associated with HIV-1 restriction.

The molecular mechanisms of viral entry through the BRB, infection, and spread in the retina and its involved cellular components remain unclear. Despite extensive research on HIV-1 ocular invasion, the mechanisms of initial entry into the retina and the precise causes of AIDS-related retinopathy, which leads to vision impairment in many HIV-1-seropositive patients, also remain enigmatic. For the first time, we demonstrate the various expressions of *APOBEC3* family members in BRB, which may have implications for discrepant intrinsic antiviral immunity among patients, and the diverse effects of antiviral treatment on HIV-related retinopathy, though more gene function and anti-HIV-1 replication studies are required to provide further direct evidence. Understanding the HIV-1 infection process in the human retina is critical for targeting this potential viral reservoir site. Recent data from different investigators^{17,18,32} on the expression and regulation of intracellular *APOBEC3* family members demonstrate that certain cytokines, including IFN- α , - β , and - γ , as well as other cytokines, can upregulate the expression of *APOBEC3G* through a unique and direct pathway in various primary cell systems, which results in a potent anti-HIV-1 mechanism. In our study, we have demonstrated that IFN- γ may upregulate the expression of *APOBEC3G* in HRCECs, though further work is required to fully characterize the pathways of HIV-1 replication into the retina and the regulation of intrinsic antiviral immunity of *APOBEC3*, which involves not only BRB cells but other critical retinal cellular components, such as neurons, microglia, and astrocytes.

In conclusion, the present study was conducted to systematically develop, with procedures optimized in our laboratory, convenient methods for primary culture of the cellular components of human BRB through which we identify pure (>95%) BRB cells characteristic of their specific markers. For the first time, differences in *APOBEC3* gene family expression are probed, and *APOBEC3B*, -*3C*, -*3F*, and -*3G* are proved to be expressed in all major BRB cells. Moreover, *APOBEC3G* expression was demonstrated to be upregulated by IFN- γ in HRCECs. The different expressions of *APOBEC3* in human BRB indicate the need for further study on the discrepant intrinsic antiviral immunity in HIV-1-related retinopathy.

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