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HIV-1-Induced Alterations of Claudin-5 Expression at the Blood–Brain Barrier Level

Ibolya E. András and Michal Toborek

Abstract

HIV-1 crosses the blood–brain barrier (BBB) early in the course of systemic infection and resides in brain macrophages and microglia. The integrity of the brain endothelium is regulated by intercellular tight junctions, which also play a critical role in HIV-1-entry into the brain. Disruption of tight junctions, including changes in claudin-5 expression, is common in HIV-1-infected patients. Recent evidence indicates that both exposure to HIV-1 and HIV-1 specific proteins, such as Tat protein, can contribute to alterations of expression and distribution of claudin-5 in brain endothelial cells and brain microvessels.

Keywords

Claudin-5; Brain microvascular endothelial cell; Blood–brain barrier; HIV-1

1. Introduction

HIV-1 crosses the blood–brain barrier (BBB) early in the course of systemic infection and resides in brain macrophages and microglia (1–5). However, the mechanisms of these effects are not fully understood and no productive HIV-1 infection of brain endothelial cells was observed in HIV-1-infected patients. It has been hypothesized that HIV-1 trafficking into the brain may be mediated through a “Trojan horse” mechanism, in which HIV-infected CD4⁺ T-lymphocyte and/or circulating monocytes enter the CNS through the BBB via interaction with inflammatory mediators (1, 2). In an experimental in vitro setting, this mechanism is mimicked by an exposure of brain endothelial cells to HIV-1-infected monocytes.

HIV-1 can induce a variety of inflammatory mediators in brain endothelial cells and other cell components of the CNS. Inflammatory responses were observed in the brain tissue of patients with HIV-1-related encephalitis (6). HIV-1-induced upregulation of chemokines, CCL2 in particular, may stimulate paracellular transendothelial migration of both normal and HIV-infected leukocytes via disrupted tight junctions between adjacent brain endothelial cells (7, 8).

Well-developed tight junctions are the most prominent feature of the brain endothelium and are responsible for the integrity of the BBB. They limit paracellular flux and restrict permeability across the brain endothelium (9–11). Transmembrane tight junction proteins, such as claudin-5, are responsible for sealing together brain endothelial cells. Disruption of tight junctions was described in HIV-1-infected patients (12–14). These changes were associated with the accumulation of HIV-1-infected macrophages in the brain, fibrinogen leakage, and apoptosis (12, 14).

HIV-1 does not infect rodent cells. Therefore, HIV-1-specific proteins, instead of live virus, are frequently used in experimental research. Several vascular effects of HIV-1 observed in the CNS can be reproduced by treatment with HIV-1 protein Tat. Tat, produced by HIV-1-infected cells, is found circulating in the blood of HIV-1-infected patients (15). Tat can be taken up by host cells, enter the nucleus, and transactivate genes linked to the HIV-1 long terminal repeat (LTR) (16). In addition, Tat is a strong pro-inflammatory agent that can recruit and induce transendothelial migration of monocytes (17). Tat also affects endothelial cell actin microfilament dynamics, causing actin cytoskeletal rearrangements such as membrane ruffling, peripheral retraction, and subsequent cytoskeletal disassembly (18).

The mechanisms of Tat interaction with the cell surface are not fully understood. It was suggested that Tat can mimic extra-cellular matrix proteins and bind to $\alpha v\beta 3$ and $\alpha 5\beta 1$ integrins through the arginine–glycine–aspartate domain (19). In addition, the VEGF receptor-2 (VEGFR-2) has been proposed to serve as a high-affinity receptor for Tat in endothelial cells (20). This notion was supported by our data that indicate that blocking VEGFR-2 can attenuate Tat-induced disruption of tight junctions (21). VEGFR-2 and a variety of other cell surface receptors that belong to the large family of G-protein-coupled receptors (GPCRs) are localized to caveolae in endothelial cells. Activation of these receptors can stimulate the heterotrimeric G-proteins, which then induce the Ras signaling (22, 23). Indeed, Tat binding to cell membranes resulted in activation of the Ras/MAPK signaling pathway and contributed to cell progress through the G1 phase in response to mitogen in primary and immortalized human umbilical endothelial cells (19). Our research identified that Tat-induced activation of Ras results in stimulation of the downstream kinases, leading to alterations of tight junction proteins, including claudin-5 (24, 25).

2. Materials

2.1. HIV-1 and HIV-1-Specific Proteins

2.1.1. HIV-1 Stock

1. PYK-JRCSF plasmid DNA (clone of HIV-1_{JR-CSF}, a primary isolate from cerebral spinal fluid obtained from an AIDS patient) available from the NIH AIDS Research & Reference Reagent Program (<http://www.aidsreagent.org>).
2. HB101 competent cells, 5 × 200 µL (Promega, Madison, WI).
3. LB broth base (Invitrogen, Carlsbad, CA).
4. LB agar (Invitrogen).
5. Dulbecco's modified Eagle medium (DMEM) (Sigma, St Louis, MO).
6. RPMI 1640 medium supplemented with 10% FBS, 100 U/mL penicillin, and 100 µg/mL streptomycin.
7. HEK 293 T/17 cells (American Type Culture Collection, Manassas, VA) cultured in DMEM with 10% FBS, 100 U/mL penicillin, and 100 µg/mL streptomycin.
8. Calcium phosphate transfection kit (Invitrogen).
9. Sac I restriction enzyme (Promega).
10. p24 ELISA kit (ZeptoMetrix, Buffalo, NY).
11. Agarose (Fisher Scientific).
12. Tris/borate/EDTA (TBE) buffer.

13. Plasmid isolation kit (Mini) (Qiagen, Valencia, CA).
14. Plasmid isolation kit (Maxi) (Qiagen).

2.1.2. Infection of U937 Cells

1. U937 cells (American Type Culture Collection, Manassas, VA) cultured in RPMI 1640 (Invitrogen, Carlsbad, CA) with 10% FBS (Hyclone), 100 U/mL penicillin, and 100 µg/mL streptomycin.
2. Phosphate-buffered saline (PBS) solution prepared from 10× PBS (80.6 mM Na₂HPO₄, 19.4 mM KH₂PO₄, 27 mM KCl, and 1.37 M NaCl, pH 7.4; USB Corp., Cleveland, OH).
3. RIPA cell lysis buffer (TBS, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS, 0.004% sodium azide, PMSF in DMSO, protease inhibitor cocktail, and sodium orthovanadate).
4. 1% Triton X-100 in lysis buffer to inactivate HIV-1.
5. p24 ELISA kit (ZeptoMetrix, Buffalo, NY).
6. T-25 flasks (Corning Costar, Corning, NY).
7. 6-well Transwell plates (pore size 0.4 µm, Corning Costar, Corning, NY).
8. Cell counting tubes with cap (TPP-US, St. Louis, MO).
9. Polyethylene cell lifters (Corning Inc., Corning, NY).

2.1.3. HIV-1-Specific Proteins—Several HIV-1-specific proteins are commercially available, e.g., from Diatheva (Fano, Italy). We produce and isolate HIV-1 Tat protein as described by Ma and Nath (26). Lyophilized Tat is then stored in siliconized Eppendorf tubes at −20 °C. Stock solution of Tat (100 mM) is prepared by dissolving lyophilized Tat in sterile conditions in cell culture medium without serum and then stored at −80 °C.

2.2. Analysis of Claudin-5 Expression by Western Blotting

2.2.1. SDS–Polyacrylamide Gel Electrophoresis

1. Ready 4–15% Tris–HCl mini gels with 50-µL 10-well comb (Bio-Rad, Hercules, CA).
2. Running buffer (10×): 250 mM Tris, 1.92 M glycine, and 1.0% (w/v) SDS, pH 8.6 (USB Corp.).
3. Pre-stained molecular weight marker: kaleidoscope marker (Bio-Rad).

2.2.2. Western Blotting

1. Transfer buffer (10×): 250 mM Tris, and 1.92 M glycine, pH 8.3 (USB Corp.).
2. Nitrocellulose membrane and blotting filter paper (Bio-Rad).
3. TBS-T solution: dilute 20× TBS (500 mM Tris, 60 mM KCl, and 2.8 M NaCl, pH 7.4 [USB Corp.]) to 1× TBS and add 0.05% Tween-20.
4. Blocking buffer: 5% milk in TBS-T. Dissolve skim milk (Difco BD, Sparks, MD) in TBS-T. Store for 1 week maximum at 4 °C.
5. Primary antibody: mouse anti-claudin-5 antibody (Invitrogen/ Zymed, cat No. 18–7364, Carlsbad, CA).

6. Secondary antibody: anti-mouse IgG conjugated to horse-radish peroxidase (Santa Cruz, Santa Cruz, CA).
7. Anti-actin antibody conjugated to horseradish peroxidase (Sigma, St Louis, MO).
8. Enhanced chemiluminescence (ECL) reagent (GE Healthcare, Buckinghamshire, UK) and blue basic autoradiographic film (ISC Bioexpress, Belgium).

2.3. Analysis of Claudin-5 Expression by Immunofluorescence Microscopy

2.3.1. Cell Cultures

1. Collagen I-coated chambered glass slides (BD Biocoat, Bedford, MA). Permanox plastic 4-well chambered slides (BD, Bedford, MA) can also be used; however, fluorescence signal is stronger and autofluorescence of the plastic slides is avoided when cells are grown on glass slides. Both types of dishes are additionally coated with collagen IV and fibronectin as described in Subheading 3.2.1.
2. Microscope coverslips (1½ 22 mm²) (Corning).
3. Absolute ethanol (Sigma) stored at 4°C.
4. Blocking buffer: 3% (w/v) bovine serum albumin (BSA, Sigma) in PBS, sterile filtered and stored at 4°C.
5. Primary antibody: mouse anti-claudin-5 antibody (Invitrogen/ Zymed, cat No. 18-7364).
6. Secondary antibody: anti-mouse IgG conjugated to FITC or Texas Red (Santa Cruz Biotechnology) or anti-mouse IgG conjugated to AlexaFluor 488 or AlexaFluor 568 (Invitrogen).
7. Mounting medium: Anti-fade mounting medium with DAPI for visualizing the nuclei (Invitrogen).

2.3.2. Microvessels

1. ProbeOn™ Plus microscope slides (charged and pre-cleaned, Fisher Scientific).
2. Liquid blocker super Pap pen (Daido Sangyo, Japan).
3. Paraformaldehyde: 4% (w/v) solution in PBS freshly prepared for each experiment from a 10% paraformaldehyde solution (Fisher Scientific).
4. Permeabilization solution: 0.1% (v/v) Triton X-100 in PBS, stored at 4°C.
5. Blocking buffer: 1% (w/v) BSA (Sigma) in PBS, sterile filtered and stored at 4°C.
6. Microscope coverslips (22 × 40–1) (Fisher Scientific, cat No.12-548-5C).
7. PBS, mounting medium, primary antibody, and secondary antibody are the same as that used for cell cultures.

3. Methods

3.1. Exposure to HIV-1 and HIV-1-Specific Proteins

Endothelial cells lack a CD4 receptor (27) and are not productively infected with HIV-1. Therefore, the experiments are performed using co-cultures of human brain endothelial cells (e.g., hCMEC/ D3 cells) with HIV-1-infected leukocytic cells (e.g., primary human monocytes, Jurkat cells, or U937 cells). We first describe the preparation of HIV-1 stock and infection of U937 cells, followed by co-culture of hCMEC/D3 cells with HIV-1-infected

U937 cells. Both hCMEC/D3 and mouse brain endothelial cells are susceptible to Tat treatment (see Note 1), which will also be described.

3.1.1. Generation of HIV-1 Stock

1. Prepare LB agar plates (20 mL agar with 100 µg/mL ampicillin per one P100 plate) and dry them at 37°C overnight with the lids off. Prepare also LB broth containing 100 µg/mL ampicillin.
2. Transformation of HB101 cells:
 - a. Cool down a polypropylene tube on ice,
 - b. Remove HB101 cells from a freezer and place them on ice for 5 min,
 - c. Transfer 100 µL cell suspension to the polypropylene tube, add 1–4 µg plasmid DNA, and leave on ice for 10 min,
 - d. Heat shock the cells for 50 s at 42°C in a water bath without shaking,
 - e. Put the tube on ice for 2 min,
 - f. Add 900 µL cold LB broth and place the tube at 37°C for 1 h on a shaker.
3. Spread the transformed HB101 cells on the LB agar plates and incubate at 37°C overnight.
4. Next morning, choose at least five clones from the bacterial colonies (mark the colony locations on the bottom of the plate or on the lid) and place bacteria using sterile pipette tips from these colonies in 5 mL LB broth with ampicillin. Shake overnight at 37°C, seal the plates, and store at 4°C.
5. Extract plasmid DNA from the chosen expanded clones using the Plasmid Isolation Mini kit. Digest the plasmid DNA with Sac I restriction enzyme according to the instruction provided by the manufacturer, and perform electrophoresis (0.7% agarose gel in 0.5× TBE buffer; 100 V for 45 min) to identify the proper bacterial clones.
6. With a sterile loop, transfer bacteria from the selected clones into 250 mL LB broth with ampicillin and incubate on a shaker at 37°C (150 rpm) for 24–48 h.
7. Extract plasmid DNA with the Plasmid Isolation Maxi kit.
8. Transfect HEK 293T/17 cells at ~70% confluence with the calcium phosphate transfection kit following the protocol provided by the manufacturer.
9. Following transfection, incubate the cells in RPMI 1640 medium with 10% FBS and penicillin plus streptomycin for 24 h in a cell culture incubator. Collect cell culture supernatant, pass it through 0.45-µm filters (Millipore, Bedford, MA), and freeze in aliquots at –80°C.
10. HIV-1 p24 levels in the supernatant are determined by ELISA (ZeptoMetrix) as the indicator of HIV-1 infection (see Note 2).

¹Exposure to Tat is performed in media without serum or other supplements to avoid binding of Tat to serum proteins. In addition, working with Tat requires the use of low-adhesion pipette tips (Corning, Pittsburgh, PA) in order to prevent adherence of Tat to the plastic.

²It is necessary to dilute the samples ~5,000× because of high p24 levels resulting from this protocol.

3.1.2. Infection of U937 Cells

1. Suspend 5×10^6 U937 cells in 10 mL RPMI 1640 complete medium and place in a T-25 flask. Add viral isolate from the HIV-1 stock (we routinely use 80 ng p24/mL). Control cultures of U937 cells are prepared by adding RPMI 1640 complete medium instead of viral isolate.
2. The cells are incubated overnight at 37°C and 5% CO₂, followed by centrifugation and resuspension in 10 mL of complete RPMI 1640 medium.
3. The next day, count the cell number of HIV-1-infected and uninfected U937 cells. Use the cell counting tubes with the cap due to HIV-1 biohazard.
4. Measure p24 levels by ELISA in a 0.5-mL aliquot as the indicator of HIV-1 infection.

3.1.3. Exposure to HIV-1—hCMEC/D3 cells are exposed to HIV-1-infected U937 cells in 6-well Transwell systems (see Note 3). Uninfected control or HIV-1-infected U937 cells are cultured on the filter inserts in 1.5 mL of complete media, and hCMEC/D3 cells are cultured in the bottom chamber. The ratio of hCMEC/D3 cells to U937 cells is 1:1. Following HIV-1 exposure, the filter inserts are removed and the media are discarded. This procedure can only be performed in an HIV-1-designated biosafety cabinet and all waste materials are discarded into a bleach solution. The cells are then washed twice with PBS, each time removing PBS into the bleach solution. Following the final wash, hCMEC/D3 cells are lysed by adding 100 µL/well of RIPA cell lysis buffer (with freshly added PMSF, sodium ortho-vanadate, and protease inhibitor cocktail) and 1% Triton X-100 to inactivate HIV-1. Cells are scraped off the dish; the lysates are pipetted up and down ten times on ice, and then left for 30 min on ice. After use, the scrapers are dipped into the bleach solution and discarded in the biohazard cardboard box.

Because HIV-1 is inactivated in the cell lysates, the sample tubes (disinfected from the outside with 70% ethanol) can be safely taken out from the HIV-1 biosafety cabinet and then centrifuged at $15,000 \times g$ for 15 min at 4°C. The supernatants can be stored at −80°C until analysis.

3.1.4. Exposure to HIV-1-Specific Proteins in the Example of HIV-1 Tat—Prior to Tat treatment, cells are washed twice with sterile PBS solution, followed by replacing normal cell culture medium with a serum-free media (see Note 3). Cells are typically exposed to Tat at the final concentration of 1–100 nM; Tat is added directly into the media. Gentle shaking of the dishes helps to distribute Tat evenly in the media. Repeated freezing-thawing of Tat solution results in loss of biological activity of Tat and should be avoided. Negative controls include exposure to heat-inactivated Tat (100°C for 5 min, followed by centrifugation of denaturated protein) and immunoabsorption by Tat anti-sera conjugated to protein-A beads.

3.2. Analysis of HIV-1-Induced Changes in Claudin-5 Expression by Western Blotting

We routinely analyze claudin-5 expression in rat and mouse brain endothelial cells (RBMEC and MBMEC, respectively) and in the human cell line (hCMEC/D3 cells). Handling of rodent and human cells is different and will be described separately.

³To synchronize cellular responses, hCMEC/D3 cells can be serum starved overnight prior to HIV-1 or Tat exposure. However, the primary cells are more sensitive and do not withstand such conditions. When removing culture medium and PBS from HIV-1-exposed cells, pipette the entire solution into bleach.

3.2.1. Cell Culture and Lysis

1. Primary cultures of MBMEC (the same approaches are used for RBMEC). Cells are cultured on dishes coated with collagen type IV and fibronectin. Coating solution constitutes of 800 μ L sterile water, 100 μ L fibronectin, and 100 μ L collagen type IV (both from Sigma, St Louis, MO). The whole surface of cell culture dishes is covered with the coating solution and removed immediately before seeding the microvessel fragments (28). MBMEC are cultured in DMEM (Sigma, St Louis, MO) with 20% plasma-derived bovine serum (PDBS, Animal Technologies Inc., Tyler, TX), 40 mg/mL endothelial cell growth supplement (BD Biosciences Pharmingen, San Jose, CA), 100 mg/mL heparin, 2 mmol/L glutamine, 5 mg/mL insulin, 5 mg/mL human transferrin, 5 ng/mL Na-selenite (Insulin-transferrin-sodium selenite media supplement, Sigma, St Louis, MO), and 25 mg/mL gentamicin (see Note 4).
2. hCMEC/D3 cultures. hCMEC/D3 cells are cultured on dishes coated with collagen type I (BD Biosciences). Collagen I is diluted 1:50 in sterile distilled water, sterile filtered with a 0.45- μ m syringe filter, and stored at 4°C. The whole surface area of cell culture dishes is covered with collagen I solution, quickly removed, incubated with the complete medium for at least 1 h, and discarded immediately prior to seeding the cells. hCMEC/D3 cells are cultured in EBM-2 medium (Lonza, Walkersville, MD) supplemented with the bullet kit (1 kit/2 L medium) supplied with the EBM-2 medium (29). The bullet kit contains vascular endothelial growth factor, insulin-like growth factor-1, epidermal growth factor, basic fibroblast growth factor, hydrocortisone, ascorbate, gentamicin, and 2.5% fetal bovine serum (FBS). hCMEC/D3 cells are passaged with trypsin-EDTA (0.25%; Invitrogen, Carlsbad, CA), typically twice a week from one dish to four dishes of equal size or from one P100 dish to four 6-well plates. In order to pass the cells, a confluent culture in a P100 dish is rinsed twice with PBS, followed by adding trypsin-EDTA solution to cover the whole area (we use 1 mL trypsin-EDTA per one P100 dish and remove the excess with a Pasteur pipette). The dish is then placed in a cell culture incubator at 37°C for 5 min. When the cells are detached, 12 mL of complete EBM-2 medium is added; the cells are washed off the dish by repeated pipetting and transferred in equal aliquots into new dishes.

3.2.2. Sample Preparation for Claudin-5 Western Blotting—One well of a 6-well plate or one 35-mm dish of confluent MBMEC or hCMEC/D3 cells provides sufficient sample size to determine claudin-5 expression by Western blot. After HIV-1 or Tat treatment and measuring protein concentrations, add 6 $\times$ gel loading buffer to each sample, followed by boiling for 5 min. Before boiling the samples, make a hole in the tube cap with a syringe needle. Place the tubes from ice directly into the boiling water. After boiling, cool the samples on ice. Following centrifugation at 15,000 $\times$ g for 3 min at room temperature, the samples can be used for SDS-polyacrylamide gel electrophoresis (SDS-PAGE).

3.2.3. SDS-PAGE

1. The analyses are performed using the ready 4–15% Tris-HCl mini gels and the Mini-Protean electrophoresis system (both from Bio-Rad).
2. Prepare the running buffer by diluting 100 mL of the 10 $\times$ running buffer with 900 mL of water.

⁴All experiments are performed only on primary cultures, because primary endothelial cells lose their phenotypic properties even after one passage. Nevertheless, MBMEC at passage one express high immunoreactivity for tight junction proteins, including claudin-5, as determined by immunofluorescence microscopy.

3. Carefully remove the comb from the mini gel. Place the gel in the holder and in the tank.
4. Add running buffer to the upper and lower chambers of the gel unit and wash the wells with the running buffer from the tank using a 10-mL syringe with a 22-gauge needle.
5. Load 20 μ L of sample into each gel well. Include one well for a pre-stained molecular weight marker. Do not place sample in the last well; load it with the loading buffer instead.
6. Complete the assembly of the gel unit and connect it to a power supply. Perform electrophoresis for 1 h at 100 V (see Note 5).

3.2.4. Western Blotting

1. The samples separated by SDS–PAGE are then transferred onto pure nitrocellulose membranes using the Mini TransBlot system (Bio-Rad). A tray of transfer buffer has to be large enough to lay out a transfer cassette with a sponge and a blotting paper pad on the top of the black side of the cassette. The second sponge, the nitrocellulose membrane, and the blotting paper are submerged in the transfer buffer in separate smaller trays. The nitrocellulose membrane is cut to the size of the blotting paper.
2. The gel unit is disconnected from the power supply and disassembled. The gel is laid on the top of the blotting paper; the nitrocellulose membrane is placed on the gel, followed by another sheet of the blotting paper and the second sponge. Avoid the formation of any air bubbles between these layers. A plastic pipette (or a similar device) is used as a rolling pin to press out any air between the layers. The amount of transfer buffer in the tray should be sufficient to cover the blot entirely. The cassette is carefully closed after the assembly.
3. The cassette is placed into the transfer tank with transfer buffer in such a way that its black part is facing the black side of the transfer unit. It is important to ensure this orientation; otherwise, proteins will not be transferred onto the nitrocellulose membrane. Then, an icepack is placed in the transfer system. The entire transfer tank is placed in a bigger tray filled with ice to ensure low temperature during transfer. The lid is put on the transfer tank and the protein transfer is performed at constant 250 mA for 1 h 45 min.
4. Once the transfer is complete, the cassette is taken out of the tank and carefully disassembled (black side down). The nitro-cellulose membrane is carefully removed and placed in a smaller tray with TBS-T buffer and washed for 5 min with gentle shaking. The colored molecular weight markers should be visible as sharp and strong bands on the membrane. The gel can be discarded.
5. The membrane is then incubated in 10 mL blocking buffer (5% milk in TBS-T) for 1 h at room temperature on a shaker.
6. The blocking buffer is discarded and the membrane is incubated with the primary anti-claudin-5 antibody diluted in 5% milk in TBS-T (antibody dilution 1:1,000) overnight at 4°C on a shaker.

⁵If claudin-5 bands are not sharply and uniformly visualized using the described electrophoresis conditions, shorten the running time to approximately 45 min.

7. The primary antibody is removed and the membrane is washed three times for 10 min with ~50 mL TBS-T.
8. The membrane is incubated with the secondary antibody (diluted 1:2,000 in 5% milk in TBS-T) for 1 h at room temperature on a rocking platform.
9. The secondary antibody is discarded and the membrane is washed three times for 10 min with TBS-T. Note that the next steps are performed in a dark room where the film can be quickly placed over the membrane.
10. The washed membrane is placed on a sheet of plastic foil; 1 mL of reagent A and 1 mL of reagent B of the ECL reagent are mixed together and poured over the blot to cover it completely for 1 min.
11. The blot is drained from the ECL reagents and placed upside down on a sheet of plastic foil (avoid the formation of air bubbles and wrinkles), wrapped in the foil, and placed in an X-ray film cassette.
12. One of the film corners is cut for easier orientation, and the film is placed promptly over the membrane (without moving it over the membrane to avoid shifted ECL signals) for the duration of the exposure time (typically a few minutes). An example of the results produced is shown in Fig. 1.

3.3. Analysis of HIV-1-Induced Changes in Claudin-5 Expression by Immunofluorescence Microscopy

3.3.1. Immunofluorescence Microscopy for Cell Cultures

1. Use only confluent cultures to detect positive claudin-5 immunoreactivity at the cell–cell borders. Following exposure to HIV-1 or Tat protein, experimental media are removed and the cells are rinsed twice with PBS (gently add PBS on the side walls of the chambers to avoid washing off the cells) (see Note 6).
2. Add cold absolute ethanol into each chamber (1 mL/chamber for a 4-well chambered slide) to cover the cells entirely and place the slides for 30 min at 4°C for fixation and permeabilization. No additional permeabilization step is necessary. Fixation with absolute ethanol also inactivates HIV-1; however, before the chambers can be safely removed from the biosafety cabinet, the outside walls have to be decontaminated with 70% ethanol.
3. The chambers and the gaskets are removed using the plastic tool provided together with the chambered slides. It is important to avoid touching the cell layer. The slides are drained by placing a corner on a paper tissue, then the slides are placed in 100-mm cell culture dishes containing 10 mL PBS (1 slide per dish, cells facing up). The slides are washed three times with PBS for 5 min with gentle shaking on a platform. Place the slides with the cells facing up on a paper tissue while removing PBS solution when washing the cells. Use the same technique for all washing procedures throughout the procedure (see Note 7).
4. Add blocking solution (3% BSA in PBS) for 30 min at room temperature (1 mL/slide to cover it completely).
5. After removing the blocking solution, the cells are incubated with anti-claudin-5 antibody diluted in blocking buffer (100 µL/chamber) overnight at 4°C in a

⁶Throughout the procedure, when removing solutions and/ or washing the cells with PBS, do not dry out the samples.

⁷When using plastic chambered slides, place them upside down during the washing procedure. The plastic slides float in PBS; therefore, the cells have to face down in order to be washed.

humidified chamber to prevent cells from drying out. Use 1:100 dilution if the secondary antibody is FITC conjugated, or 1:500 if the secondary antibody is AlexaFluor 488 or AlexaFluor 568 conjugated. To prepare a humidified chamber, place the slides on a platform in a plastic box with a cover and add distilled water to the bottom of the chamber. After closing the lid, the chamber is sealed with parafilm and placed overnight in a refrigerator.

6. The next day, the antibody solution is removed and the slides are washed three times (5 min each) with PBS with very gentle shaking on a platform. Then, add anti-mouse FITC (1:100) or AlexaFluor-conjugated secondary antibody (1:1,000) diluted in blocking buffer (typically 100 μ L per chamber) for 2 h at room temperature. This step can be done in the same chamber as that used for an overnight incubation with primary antibody. However, the chamber should be covered with aluminum foil to protect the slides from exposure to light.
7. The secondary antibody solution is removed and the slides are washed three times with PBS for 5 min each, with gentle shaking. Washing is done in a box covered with aluminum foil for light protection.
8. Following the final wash (avoid completely drying the cells), the samples are ready to be mounted. Place two drops of mounting medium containing DAPI on each slide and carefully place two coverslips per slide. Avoid formation of air bubbles. Excess mounting medium will make the coverslip move over the cells. Do not move or press the coverslip. The slides can be viewed immediately or stored in the dark at 4°C. Cells can be examined under an epifluorescence or confocal microscope. A variety of specialized software can be used to merge the fluorescence images for claudin-5 and DAPI. Examples of claudin-5 immunoreactivity are shown in Fig. 2.

3.3.2. Immunofluorescence Microscopy for Microvessels

1. Freshly isolated brain microvessels are spread onto glass microscope slides by pipetting 5–15 μ L of the microvessel suspension on one side of a slide (mark the slides in advance with a pencil) and smearing the microvessels with a second slide by moving it continuously toward the other side of a slide to achieve a fine layer. The area of spread microvessels is outlined with a liquid blocker pen.
2. The samples are fixed by placing the slides on a preheated platform (95°C) for 10 min (see Note 8), followed by incubation with freshly prepared 4% paraformaldehyde in PBS (1 mL/ slide) for 10 min at room temperature. Remove paraformaldehyde by placing a corner of the slide on paper tissues and washing the slides five times with PBS (1 mL/slide each time).
3. The samples are permeabilized with 0.1% Triton X in PBS for 30 min at room temperature (1 mL/slide). Discard the solution as above and wash the slides five times with PBS. After the final wash, the samples are blocked with 1% BSA solution in PBS for 30 min at room temperature.
4. The blocking solution is removed and the slides are incubated with anti-claudin-5 antibody (1:500 dilution in 1% BSA in PBS; typically, 500 μ L per slide covering the microvessel smear) overnight at 4°C. A humidified plastic chamber (described in Subheading 3.3.1) should be used to avoid drying the slides (see Note 9).

⁸Leave the frosted part of the slide outside the heating platform, so it will not get hot and you can remove the slide quickly after fixation.

⁹Place the slides in the humidified chamber first and then add the antibody solution.

5. The slides are washed five times with PBS and incubated with anti-mouse AlexaFluor 488- or AlexaFluor 568-conjugated secondary antibody (1:1,000) diluted in the blocking solution (500 μ L per slide) for 2 h at room temperature. Cover the box with aluminum foil to protect the slides from light.
6. The slides are washed five times with PBS. Following the final wash (avoid completely drying the slides), the samples are ready to be mounted. Place a drop of mounting medium with DAPI in the center of each slide and carefully place a coverslip over it (avoid formation of air bubbles). Do not move or press the coverslip. Analyze the samples as described in Subheading 3.3.1. Examples of claudin-5 immunoreactivity in brain microvessels are shown in Fig. 3.

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Fig. 1.

Effects of Tat treatment on claudin-5 expression in brain microvascular endothelial cells. Confluent cultures were treated with vehicle (control) or 100 nM Tat for 24 h, and claudin-5 protein levels were detected by Western blot. In addition, selected cultures were pretreated for 15 min with U0126 (10 or 20 μM; an inhibitor of mitogen-activated protein kinase kinase1/2 [MEK1/2]), followed by a co-exposure to 100 nM Tat for 24 h (24).

Fig. 2.

Effects of Tat treatment on claudin-5 expression in mouse brain microvascular endothelial cells (MBMEC) as determined by immunofluorescence microscopy. Confluent primary cultures of MBMEC were derived from normal or from caveolin-1-deficient (caveolin-1^{-/-}) mice and treated with 100 nM Tat for 24 h. In control cultures (*upper left panel*), claudin-5 immunoreactivity (*green staining*) was restricted to junctional areas and some punctuate staining in the cytoplasm. In addition, short branches and/or ramifications from the cell border toward the cytoplasm were observed (*arrows*). Exposure of normal MBMEC to Tat (*upper right panel*) resulted in a slightly weaker junctional and cytoplasmic pattern of claudin-5 immunoreactivity. The short branching pattern of claudin-5-positive staining (*arrows*) was similar to that of the control cells. MBMEC isolated from caveolin-1^{-/-} mice exhibited markedly enhanced ramification (*lower left panel, arrows*) of claudin-5 immunoreactivity as compared to control cells. This effect was further potentiated by Tat treatment (*lower right panel, arrows*; 100 nM for 24 h) resulting in a dense brush-like image of the claudin-5 immunore-activity. DAPI (*blue staining*) was used to visualize the nuclei.

Fig. 3.

Claudin-5 expression in microvessels isolated from mouse brain. Claudin-5 immunoreactivity (*red staining*) exhibits a linear, sharply defined pattern along the endothelial cell–cell borders and some weaker immunoreactive patterns in the cytoplasm (*left panel*). DAPI (*blue staining*) was used to visualize the nuclei. Differential interference contrast (DIC) image was taken to visualize microvessels (*middle panel*). *Right panel* illustrates merged claudin-5 immunoreactivity and DIC images.