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Research on the correlation between toxic aggregation in brain and cell-cell fusion caused by SARS-CoV-2 infection

Yanyi Lyu

Shanghai Pinghe School, Shanghai, 201206

yanyilyu31415@163.com

Abstract. Since the outbreak of the COVID-19 pandemic, much research has been done to understand SARS-CoV-2's and COVID-19, the disease it cause. The pathway that the virus relies on to infect cells is through binding of the viral protein to the ACE2 receptor on the surface of host cells. After replication, the virus most often exits the cell by lysosomal exocytosis, which releases the virus into the extracellular space, and the cycle repeats. However, cell-cell fusion caused by SARS-CoV-2 can promote viral spread by fusing neighboring cells to form syncytia, leading to the infection of neighboring cells. One of the major concerns regarding SARS-CoV-2 is the neuroinvasive potential the virus exhibits. Cell-cell fusion is observed in neuronal cells as well, potentially compromising the overall integrity of neuronal activities. Thus, in this research proposal, cell-cell fusion and its impact on brains will be investigated by assaying its influence on protein aggregations in brains. The proposal plans to apply both brain organoids and animal models for assessment. Hypothetically, once cell-cell fusion and formation of protein is observed in brain tissue samples, more information can be revealed towards comprehending the mechanisms of SARS-CoV-2 activities during infection.

Keywords: SARS-Cov-2, Brain, Cell-Cell Fusion, Neurodegenerative Disease, Protein Aggregation.

1. Introduction

Coronaviruses belong to the subfamily Coronavirinae, which is consigned to the family Coronavirdae [1]. As a coronavirus, SARS-CoV-2 is equipped with the characteristic crown-shaped spike glycoprotein and single-stranded RNA. Coronavirus has four genera: Alphacoronavirus, Betacoronavirus, Gammacoronavirus and Deltacoronavirus, with SARS-CoV-2 being classified as a Betacoronavirus [2]. Interestingly, two other coronaviruses that have caused serious pandemic outbreak, MERS-CoV and SARS-CoV, also fall into this genus.

The structure of SARS-CoV-2's spike glycoprotein is a trimer composed of three S2 components and three S1 components covering the top each S2 subunit. Parts of S1 function as the receptor-binding domain (RBD) and an N-terminal domain (NTD) [3]. Early analysis suggested that a potential pathway by which SARS-CoV-2 could infect human cells is through the binding of SARS-CoV-2 RBD with angiotensin-converting enzyme 2 (ACE2) receptor by associating its genetic similarity with SARS-CoV [4]. This was confirmed by Peng Zhou et al. confirmed ACE2 to be the cell-binding receptor for viral entry. What enhances the viral replication rate is that compared to SARS-CoV, the RBD of SARS-CoV-2 has higher affinity for ACE2 and can evade the immune system through bending and hiding itself [5].

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After binding to ACE2, the virus will enter the cell. The most common cell entry pathway is through membrane fusion at the surface of plasma membrane. Membrane fusion, in essence, is when enveloped viruses fuses their lipid bilayer with host cell plasma membrane [6]. SARS-CoV-2 spike protein is a fusion protein. Cleavage can change the conformation of the protein from a pre-fusion to a fusion state [7].

Once SARS-CoV-2 has entered the cell, viral RNA replication, transcription and translation will take place, which includes the production of viral surface spike glycoprotein. Viral components will be assembled into virus in the ER-to-Golgi intermediate compartment (ERGIC) before leaving the host cell [8]. However, during this process, some SARS-CoV-2 spike proteins might leave the vesicles and accumulate at the surface of the host cell [9].

Because spike proteins, as a type of viral fusogens, can bind with the ACE2 receptors on the surface of other cells, the membranes of infected cell and its neighboring cells can fuse together. Thus forming a virus-infected syncytia, which usually consists of multiple nuclei and contents from the cells that were once separate. This phenomenon can be observed in any tissue infected with SARS-CoV-2 [10]

Due to the prevalence of ACE2 receptors in human cells, SARS-CoV-2 infection and the resulting cell-cell fusion can occur anywhere in the body. This also includes the central nervous system, which is usually not prone to viral infection. The unique features of SARS-CoV-2 enables it to pass through the blood-brain barrier using extracellular vesicles and infect neural cells [11, 12]. Therefore, even though SARS-CoV-2 is widely known as a causative agent of respiratory illness, studies has implied its capability to jeopardize central nervous system. Risks includes the increased risk of cerebrovascular accidents, viral encephalitis and meningoencephalitis [13].

For neural cells, fusion can lead to compromised neural activities. Previous research has proved the presence of cell fusion in SARS-CoV-2 infected neural cells [14]. While focusing on the lasting impact of the formation of syncytia induced by SARS-CoV-2 in brain, the study of protein aggregation can reveal the possible correlation between this and neurodegenerative disease. Protein aggregation, which is one of the hallmarks of many neurodegenerative diseases, will be assayed in this study [15]. Additionally, a deeper comprehension of the possible correlation between SARS-CoV-2 and neurodegenerative diseases currently remains uninvestigated. Therefore, in this proposed study, the correlation between the formation of toxic aggregation and cell fusion in the brain will be investigated.

2. Methodology

In order to study the relationship between cell fusion and toxic aggregations, brain organoids in culture will be infected with SARS-CoV-2. The cells will then be examined microscopically using fluorescent antibodies to visualize toxic aggregates. Mice will be infected with SARS-CoV-2. Brain tissue will be obtained and analyzed using fluorescent microscopy to observe protein aggregations. Additionally, behaviors will be observed to correlate toxic aggregates formation with neurodegenerative disease.

The entire experiment process involving SARS-CoV-2 will be conducted under biosafety level 3 precautions.

2.1. Virus

The SARS-CoV-2 isolate that will be used in this study is hCoV-19/USA/MD-HP40900/2022 (XBB.1.5; Omicron Variant) obtained through BEI Resources, NIAID and NIH.

2.5×10^5 Vero E6 cells will be purchased from American Type Culture Collection for growth of the virus. The cells will be cultured in a humidified atmosphere at 37℃. The maintenance medium of the cells will consist of Dulbecco's modified Eagle's medium (DMEM; Gibco–Thermo Fisher Scientific) supplemented with 10% fetal bovine serum (Gibco–Thermo Fisher Scientific), 1× GlutaMAX (Gibco–Thermo Fisher Scientific), penicillin (100 U/ml)–streptomycin (100 μg/ml; Sigma-Aldrich–Merck) and puromycin (30 μg/ml; Sigma-Aldrich–Merck) [14].

The virus will then be introduced to the Vero E6 cells. First, 100 μl of SARS-CoV-2 will be first diluted with 900 μl maintainance medium with the same composition of that in the Vero E6 cell culture. The 1 ml of inoculum will then be added to the Vero E6 cell culture. The infected Vero cells will then be

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incubated for on a laboratory shaker in a humidified incubator for 1 hour under a temperature of 37℃. 100 μl of supernatant will be harvested as the first sample (0 h post-inoculation) and every 24 h for 4 days post-inoculation [16]. The supernatant will be used to infect.

2.2. Cell culture

Induced pluripotent stem cells (iPSCs) are stem cells that are derived from differentiated somatic cells. Through retroviral transduction, Yamanaka factors, Oct3/4, Sox2, Klf4, and c-Myc, causes the somatic cells to dedifferentiate and continuously renew themselves [17].

Fibroblasts (ATCC) will be infected with four types of retroviruses, each encodes Oct3/4, Sox2, Klf4, and c-Myc. Five days since the initial infection, the cells will be transferred and divided into six-well plates pre-seeded with irradiated mouse embryonic fibroblasts (MEF, GlobalStem). Then, seven days after the transfer, the original medium in the well plates will be replaced by human embryonic stem cell (hESC) medium, which will be mainly composed of KO-DMEM (Gibco), which is supplemented with 20% KO-Serum Replacement (Invitrogen), 2 mM of Glutamax (Invitrogen), 50 μM of β-mercaptoethanol (Invitrogen), non-essential amino acids (Cambrex) and 10 ng ml−1 b-FGF (Peprotech) [18].

The fibroblast-derived iPSCs will be induced to form brain cortical organoids. iPSCs will first be dissociated using a solution that will be prepared using Dulbecco's phosphate-buffered saline (DPBS, ThermoFisher) and StemPro Accutase (ThermoFisher) according to a 1:1 ratio. After removing the solution, the cells will then be centrifuged and resuspended in mTeSR1 supplemented with 10 μm SB431542 (SB; Stemgent) and 1 μm Dorsomorphin (R&D Systems). The cells will then be moved into a well plate, where they will be supplemented with 5 μM of ROCK inhibitor (Y-27632; Calbiochem) and maintained in suspension under a rotation speed of 95rpm for 24 hours. Forty-eight hours later, the medium will be removed and replaced by neural induction medium which sonsits DMEM/F12 (Life Technologies), 1% Glutamax (Life Technologies), 1% N2 Neuroplex supplement (Gemini Bio), 1% non-essential amino acids (NEAA, Gibco), 1% Pen-Strep (PS; ThermoFisher), 10μM SB431542 and 1μM of Dorsomorphin. For seven days, the cells will be kept inside this medium, and the medium will be replaced with new medium of the same composition every day. Then, neural proliferation medium will be introduced to substitute the neural induction medium. The neural proliferation medium will be composed of Neurobasal media (Life Technologies), 2% Gem21 Neuroplex, 1% non-essential amino acids, 1% Glutamax, and 20 ng/mL basic fibroblast growth factor (bFGF; Life Technologies). Again, the cells will have to be maintained in this medium that will be changed daily for seven days. Then, additional supplement of neural proliferation media supplemented with 20 ng/mL of epidermal growth factor (EGF, Peprotech) will also be added to the plate during the daily medium change, which will last for another seven days. For neural maturation, the medium will be switched to Neurobasal GlutaMAX, 1% Gem21 NeuroPlex (Gemini Bio), 1% NEAA and 1% PS; supplemented with 10 ng/mL of BDNF, 10 ng/mL of GDNF, 10 ng/mL of NT-3 (PeproTech), 200 mM L-ascorbic acid and 1 mM dibutyryl-cAMP (Sigma-Aldrich). For seven days the medium of the same composition will be changed daily. Eventually, the iPSC-derived organoids formed from these cells are ready for infection, and the organoid maintenance media, consisting of Neurobasal with GlutaMAX, 1% Gem21 NeuroPlex (Gemini Bio), 1% NEAA, and 1% PS, will be replacing the neural maturation medium [19].

iPSC-derived brain organoids will receive a dose of 10^2 plaque forming units (PFU) per ml. The inoculation will take place by directly transferring the virus into the medium that maintains brain organoids.

2.3. Animals

k18-hACE mice are genetically modified mice that express the human ACE2 under the direction of the cytokeratin promoter [20]. Forty-eight k18-hACE mice, twenty-four male (twelve infected and twelve uninfected) and twenty-four female (twelve infected and twelve uninfected), will be purchased from Jackson Laboratory. Using a random control trial, the mice will be split into two groups. One will be the experimental (infected) group, and the other will be the control (uninfected) group. One will be the

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experimented (infected) group and the other will be the control (uninfected) group. Both groups of mice will be housed under a 12h light and 12h dark cycle, food and water provided. The two groups will remain separated throughout the entire study. All procedures involving animals follow the National Institutes of Health Office of Laboratory Animal Care and Use Committee guidelines.

The virus will be delivered into the nostrils of the mice using a pipette. 2×10^1plaque forming units (PFU) of SARS-CoV-2 will be administered to each mouse in the infected group to ensure 30% of recovery rate while showing implications of viral influences in brain tissues [21].

3. Analysis

3.1. Fluorescence microscopy

Brain tissue of both experimented and control mice will be observed and compared. Immunofluorescent staining will be used to examine the protein aggregation present in brain organoids. Two types of protein will be examined: alpha synuclein and tau proteins, associated with Parkinson disease and Alzheimer's disease respectively. Cells will also be stained with DAPI to visualize nuclei, which act as an indicator of multi-nuclear syncytia. These procedures will be used to process both brain organoids and tissues from mice brain cortex.

After washing the organoids using D-PBS (STEMCELL), 5 ml per organoid of 4% paraformaldehyde solution (PFA) will be added and incubated overnight at 2 - 8℃. The organoids will then be washed and preserved in 0.1% PBS-T, which is prepared by adding 1mL of Tween 20 (Sigma-Aldrich) to 1 L of D-PBS. After storing the samples at 2 - 8℃ for seven days, the solution will be substituted with 5 ml per organoid of 30% sucrose solution. The organoids will be equilibrated until they stop floating in the solution. The sucrose solution will then be replaced by gelatin solution. The organoids will be maintained at 37% for one hour before being transferred to the embedding mold. The mold will then be submerged in a dry ice/ethanol slurry to be frozen. The organoids can be stored in -80℃ freezer. For sectioning of the tissues, the organoids need to warm to a temperature between -26 and -30℃. A new sharp, blade will be used for sectioning. The section thickness will be about 20 μm [22].

To obtain brain tissue from mice, the mice will first be anesthetized through injecting sodium pentobarbital (80 mg/kg). 50 ml of phosphate-buffered saline (PBS, 0.9% NaCl in 50 mM phosphate buffer, pH 7.4), 75 mL of acrolein, and 150 mL of PFA will sequentially pass into the mouse circulation via peristaltic pump for animal perfusion. After perfusion, the head of the mouse will be cut off. Brain will be removed from the skull and transferred into PBS [23]. The mice brains will then be treated following the procedures used to treat the organoids.

All sections will be incubated overnight with primary antibodies, which includes Tau Monoclonal Antibody (HT-7) and alpha Synuclein Monoclonal Antibody (Syn211; ThermoFisher). Then, secondary antibody conjugated with Alexa-555 fluorophore (Invitrogen) will be used to wash the samples. The samples will then be incubated overnight. Finally, samples will be counterstained for 10 minutes with DAPI (ThermoFisher) and mounted using FluorSave (Sigma-Aldrich). The samples will then be examined using epifluorescence microscopes.

3.2. Behavior

Both infected and healthy mice will be monitored 24 hours via motion sensors to identify abnormal circadian rhythms. Detection of motion is an indication of wakefulness, while devoid of motion implies both sleep and wakefulness. During this period, all of the mice will be housed separately at night to distinguish the circadian rhythm of each. The data of the rhythms will be documented and compared between the healthy and infected groups. Abnormal circadian rhythm is an implication of neurodegenerative diseases.

Gait and movements will be recorded in videos and compared between the two groups of mice. By comparing the difference in movements between the infected mice and healthy mice, abnormal gaits, for example, stumbling, can be found in infected mice if they are present.

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After the infected mice have recovered from infection, a T-maze involving food baits will be applied to test the working memories of both groups of mice. This involves a T-shaped maze with a 35 cm stem and two 28 cm arms. One arm will be supplemented with food. Each mouse will be deprived to 85% of ad libitum body weight in advance. Mice will first habituate the maze, choosing their preferred arm. The mice will then be trained 4 trials per day for three consecutive days, with food set at the arm not preferred [24].

4. Conclusion

It has been proved, indisputably, that the SARS-CoV-2 infection in brains is accompanied with damage and cell death. Many mechanisms have been deeply comprehended, yet viral-meditated cell-cell fusion was not fully understood. It is certain that formation of syncytia can lead to neural cell dysfunction or even cell death. However, when syncytia are accompanied with protein aggregates and symptoms of neurodegenerative diseases, the virus may be causing even more damage to the human brain then we thought.

One limitation of this study is that prominent results have not yet been yielded. However, aiming to shed light on certain mechanisms of SARS-CoV-2 activities in brains, the strength of this proposal is that the causation between neural cell fusion and toxic protein aggregates has not been studied.

This study aims to investigate the potential long-term impact of the virus on the human nervous system. Therefore, the results may shed lights on the future of the people suffering from their neurological disorders after recovering from SARS-CoV-2. For this reason, thorough research is encouraged to further comprehend the effect of or devise solutions for the influence of SARS-CoV-2 infection on the human nervous system.

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