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Herpes Simplex Virus Type 1 (HSV-1) and Alzheimer's Disease: HSV-1 Promotes Amyloid Precursor Protein (APP) Hydrolysis

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Abstract

Previous studies have shown that senile plaques and neurofibrillary tangles are the main features that cause abnormalities in Alzheimer's disease (AD). At the same time, herpes simplex virus is also involved in the occurrence of AD. Researchers have suggested that when the herpes simplex virus 1 (HSV-1) is present in the people whose brains with type 4 allele of the apolipoprotein E gene (APOE-ε4), this indicates that this will lead to an important risk factor for AD. And the infection of cultured neurons and glial cells by HSV-1 can cause the intracellular amyloid precursor protein to cleave into more fragments and promote its gene expression, which in turn leads to sharp levels of β-amyloid (Aβ) 1-40 and 1-42 increased, with the level of amyloid precursor protein (APP) in cells decreased. In addition, the use of anti-HSV drugs can reduce the level of Aβ by directly regulating the secretase activity, in order to reduce the risk and degree of AD. This work showed that HSV-1 promotes APP hydrolysis as a way of the causes to AD, and provided a possible treatment (anti-HSV drugs) of AD.

Keywords

Alzheimer's disease; Herpes simplex virus 1; Amyloid precursor protein hydrolysis.

1. INTRODUCTION

Alzheimer's disease is the most common form of dementia. More than 30 million people worldwide suffer from Alzheimer's disease. Unfortunately, there is currently no cure for the disease. There are only drugs to relieve patients' symptoms. Alzheimer's disease (AD) is a neurodegenerative disorder that causes cognitive decline and memory loss that ultimately affects daily executive function. Its signature features include the formation of senile plaques in the brain, tangles of nerve fibers, increased glial cells, and inflammation. Deposition of amyloid-beta peptide (Aβ) as β-amyloid plaques is a hallmark pathology of AD. About 90% belong to the Aβ1-40 subtype, and the other 10% belong to the Aβ1-42 subtype [1]. The Aβ1-42 subtype exists in the form of monomer or oligomer. In fact, Aβ1-42 monomers have neuroprotection and resistance to neuron inactivation caused by nutritional deficiencies and excitotoxicity, and these monomers aggregate into oligomers to generate fibrils that contribute to the formation of senile plaques. Age spots are formed and are associated with neurotoxicity in AD [2]. Traditionally, Aβ has been characterized as a functionless catabolic byproduct and pathways leading to β-amyloid

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generation as intrinsically pathological [1]. There is increasing evidence that pathogens are involved in the development of sporadic AD.

The AD pathogen hypothesis: A 3D human brain–like tissue model of herpes-induced Alzheimer's disease proposes that various pathogens can be used as inducers to induce and/or lead to the accumulation of Aβ1-42 monomers [2]. Many pathogens can evade the host's immune function, especially the immune response of the elderly and/or penetrate into the brain, causing latent and potentially chronic infections. In turn, these pathogens continue to induce reactive glial hyperplasia and pro-inflammatory responses, eventually leading to progressive neurodegeneration and dementia. HSV-1 is a neurotropic double-stranded DNA virus, usually in the latent state in the entire peripheral nervous system, with the ability to penetrate into the blood-brain barrier (BBB), more and more evidence shows that HSV-1 has potential causality in the development of AD [2].

HSV-1 can induce AD, but Aβ, which is hydrolyzed by APP by β- and γ-secretase, produces neurotoxicity after cytoplasmic matrix precipitation and aggregation. In the progression of AD plays a major role. In 1991, Kowall et al. injected Aβ into the cerebral cortex of rats or monkeys, and found that there was tissue necrosis, loss of peripheral neurons and hyperplasia of nerve keratin at the injection site, which had a significant correlation with the dose. Animal experiments show that the effect of Aβ on neurons is related to their state. Dissolved Aβ can promote the growth of neurites and improve the survival rate of neurons in a short time, while Aβ in the deposited state has the opposite effect on neurons, causing pathological changes similar to AD — neurites Withdrawal and neuronal degeneration, the most significant changes occur in the brains of aging mammals. Therefore, the main reason for HSV-1 to cause AD is that HSV-1 will induce APP to produce multiple APP fragments and promote APP cleavage to produce more Aβ. These findings demonstrate that HSV-1 infection of neuronal cells can generate multiple APP fragments with well-documented neurotoxic potentials. It is tempting to speculate that intra- and extra cellular accumulation of these species in the central nervous system (CNS) resulting from repeated HSV-1 reactivation could, in the presence of other risk factors, play a co-factorial role in the development of AD [3].

In addition, experiments conducted by other scientists have shown that HSV-1 can disrupt Ca2+ in the body, thereby promoting the increase of Aβ content and deepening AD. Studies have shown that HSV-1 infection causes neurodegenerative changes, AD-like phosphorylation of tau protein, and activation of the arachidonic acid cascade. Neuropathological changes of AD type. On the contrary, some studies have shown that the production and accumulation of amyloid-β peptide (Aβ), which is associated with the increase of neuronal excitability and synaptic activity, leading to intracellular Ca2+ signaling and regulation abnormal [4]. Therefore, based on the former experimental ideas and related methods, in this work, we used the virus to explore the effects on the electrophysiological properties and calcium treatment of cultured rat cortical neurons. According to the results, HSV-1 produced significant changes in neuronal excitability and intracellular Ca2+ signaling, which affected the processing of amyloid precursor protein (APP) and led to Aβ [4].

At the same time, in order to explore how to reduce the degree of AD, from this work anti-HSV drugs were used to prove whether such drugs reduce the content of Aβ in the body, thereby reducing the risk of AD. Recent studies have shown that scientists use classic memory experiments to find future treatments for improving AD.

2. EXPERIMENTS

2.1. Immunofluorescence Detection of AB Expression

Immunofluorescence is used to prove that HSV-1 can cause an increase in Aβ. Culture human SH-SY5Y neuroblastoma, and HSV-1. Divided SH-SY5Y into 2 groups. Infect 10 MOI HSV-1 in one

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group, and infect the same volume of PBS in control group at time 0. After 12 hours, discard the culture medium and wash 3 times with PBS, 3 minutes each time. Cells were fixed with 4% paraformaldehyde for 15 minutes, and then washed 3 times with PBS. 0.5% Triton X-100 (prepared with PBS) for 15 minutes to permeate through the cell membrane, and wash 3 times with PBS. Add 10% goat serum at room temperature, and recover the blocking solution after 1 hour. Add primary antibody for incubation overnight at 4°C. Then, recover the primary antibody, washed 3 times with PBST, and added with fluorescent secondary antibody and incubated at room temperature. After 2 hours, the secondary antibody was recovered and wash 3 times with PBST. Add DAPI staining solution and incubate for 5 minutes, wash 3 times with PBST. Cover the film with an anti-fluorescence quencher and take pictures under a fluorescence microscope, using software to analyze data.

Predictions were made, as the experiments cannot be done. The most likely result is described as follows. Immunofluorescence marks different particles in different colors. HSV-1 is marked in green, Aβ is marked in red, and cells are marked in blue. There are only a few red spots (Aβs) and no green spot (HSV-1) in the picture of control group. And the red spots coincide with the blue spots (cells). While in the picture of group infected HSV-1, there are several green spots surrounded by lots of red spots. And some red spots are outside the blue spots, which means that Aβ escapes from cells and forms clusters around virus particles that are outside of cells. Meanwhile, the number of red spots is more than that in the control group. It does show that HSV-1 can cause an increase in Aβ.

2.2. Detection of β- and γ-secretase Expression in Cells by Western Blot

The first experiment can only be displayed intuitively and lacks persuasion. So, the second experiment was designed as a supplement. This experiment can quantitatively prove that HSV-1 can increase the content of Aβ.

The cleavage of APP into Aβ requires the activity of two enzymes: β- and γ-secretase. Therefore, the experiment is designed to determine whether HSV-1 infection can increase the levels of these enzymes, using Western blot with antibodies that bind to BACE, the β-secretase, and nicastrin, an essential component of the γ-secretase [5].

Human neuroblastoma SH-SY5Y cells are also divided into 2 groups. Infect 10 MOI HSV-1 in one group, and infect the same volume of PBS in control group. 24 hours after infection, the total protein of SH-SY5Y cells was lysed and extracted with RIPA lysate, and the total protein concentration was determined by BCA method. The sample load was 30 μg per well. After SDS-PAGE (sodium dodecyl sulfate-polyacrylamide gel electrophoresis), the membrane was transferred to a polyvinylidene fluoride membrane, and 5% bovine serum albumin was added at room temperature for 1 hour. Then, add primary antibody and incubate at 4°C overnight. Before adding the corresponding secondary antibody labeled with horseradish peroxidase, recover primary antibody and wash 3 times with TBST, 10 minutes each time. And then, incubate together with the secondary antibody at room temperature for 2 hours, and wash 3 times with TBST. Develop with chemiluminescent solution, and use ImageJ software to do grayscale analysis.

This experiment shows influence of HSV-1 infection on levels of enzymes involved in Aβ production from APP. It measured the content of BACE and nicastrin in uninfected and infected human neuroblastoma cells. And the most possible result is below. The amount of both proteins in the group infected HSV-1 is higher than uninfected group. Therefore, it can be inferred that HSV-1 can improve the content of both proteins, which means when virus was infected, the amount of β- and γ-secretase will increase, and there will be more Aβ.

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2.3. Quantitative and Proportional Analysis of Protein Aβ and APP

Following parts 2.1 and 2.2, after proving HSV-1 can increase the amount of Aβ, the potential explanation of how HSV-1 increases Aβ is discussed.

Assuming that HSV-1 can affect the processing of APP, HSV-1 promotes APP cleavage to produce more Aβ. So, this experiment is designed to determine whether the cutting of APPs is responsible for the increase of Aβ.

Prepare two sets of brain organoids in corresponding Petri dishes. Before injecting the virus into the experimental group, perform a Western blot to detect the concentration of related proteins (APP, ACID, Aβ and SAPPβ). Also, calculate the ratio between Aβ and APP (Aβ/APP) for comparison later. For the experimental group, inject 10 MOI HSV-1 into the petri dish. Starting at time 0, take a sample from the infected organoid, extract proteins and perform a Western bolt every hour to observe and record any changes. Compare the final concentration of each protein with its initial content and the ratio between Aβ and APP. In addition, the protein concentration will be determined by the spectrophotometric and turbidimetric methods [6].

There are three potential outcomes of the experiments, which are described as follows. The first one is that the amount of all proteins except APP increases, the amount of APP decreases and the ratio between Aβ and APP is higher than before. From this result, it seems that the injection of HSV-1 does increase the concentration of AICD, Aβ, and SAPPβ in the organoid while decreasing the APP concentration. Moreover, the ratio of Aβ over APP increases, which means that it is APP cutting affected by HSV-1 that makes the Aβ concentration increase. Furthermore, it is consistent with our hypothesis. The second one, the content of all proteins, including Aβ and APP, increases, and the Aβ/APP ratio stays the same to the initial data, which shows the hypothesis is incorrect, APP cutting is not the reason for the increase of Aβ.

Moreover, the third possible result is that only the concentration of Aβ increases with other proteins' concentration, hence rejecting the hypothesis. Then there is the possibility which, instead of promoting APP hydrolysis, HSV-1 may increase APP gene expression, hence synthesizing more APP to produce more Aβ.

2.4. Ca^{2+} Signaling Detection

The fourth experiment is designed to determine whether the HSV-1 virus cause the APP cutting and result in the accumulation of Aβ or not. There was a finding that the HSV-1 can cause the irregular Ca signaling during neuron impulse can increase the APP cutting and accumulate Aβ [4]. Therefore, this property will be used to design this experiment which help to make the conclusion. If HSV-1 virus can cause the Ca signaling irregularity, then the HSV-1 virus is the reason of Aβ accumulation in the brain of an AD patient.

First, this work need to incubate three brain organoids in three individual dishes at a temperature of 37°C. Then, inject GCAMP protein into three brain organoids, letting them bind with the Calcium in the brain. Before infecting the sample with HSV-1 virus, use Western blot to count the amount of Aβ in all three samples. This is for the comparison after the experiment. Set sample 1 as the control group and infect the sample 2 and 3 with HSV-1 virus. After that, inject Calcium channel block drug to sample 2. Put the sample 1, 2 and 3 under the microscope and observe the increase of fluorescents in the next 18 hours. Take a picture every 30 minutes to see the changes in the intensity of fluorescent spots. After 18 hours of observation, perform the Western blot on all three brain organoids and count the amount of Aβ left. Repeat the steps above for three more trails. Compare the amount of fluorescent spots created by the binding of GCAMP protein and Ca^{2+} in the three samples. Compare the changes of the initial Western blot data with the final data.

If the amount of fluorescent spots in sample 3 is higher than the amount in sample 1, it represents that the amount of Ca^{2+} signaling in sample 3 is higher than the ones in sample 1,

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proving that HSV-1 can cause irregular Ca^{2+} signaling. But if the fluorescent spots in sample 3 is equal to sample 1, it means that HSV-1 can not cause irregular Ca^{2+} signaling. For the Western blot comparison between sample 2 and sample 3, if the amount of Aβ in sample 3 is higher than the ones in sample 2, it proves that irregular Ca^{2+} signaling is the cause of the Aβ increase and HSV-1 infection is the cause of more APP cutting. But if the amount of Aβ in sample 3 is equal to the ones in sample 2, it means that Ca^{2+} signaling is not the cause of the Aβ increase and the HSV-1 is not the cause of APP cutting increase.

2.5. Anti-Herpes Virus Drugs (Acyclovir) Testing

In the above experiments, the relationship between HSV-1 and Aβ was proved. And in this experiment, the work is verify whether anti-Herpes virus drugs, such as acyclovir, can decrease the content of Aβ caused by HSV-1.

Before the main experiment, two preliminary experiments were designed. The first one is to see what concentration works well to stop infection. If the concentration is too high, kidney damage will form. And if the concentration is too low, the effect of the drug cannot be seen obviously. Immunofluorescence was used to mark HSV-1 in red, Aβ in blue, and β-amyloid plaques in green. First, inject HSV-1 to mice with Aβ antibodies that activate blue fluorescence and antibodies that can detect HSV-1 and activate red fluorescence (at least 10) and wait for 12 hours. Second, inject different amount of acyclovir drug to the same mice every 8 hours for 21 days (could be 0.00 mg, 0.05 mg, 0.15 mg, 0.25 mg, 0.35 mg, 0.45 mg, 0.55 mg...). Next, cut a slice of each mouse's brain (control & drug group), using microscope to see the number of dots with red fluorescence and blue fluorescence in each mouse's brain. Finally, determine the concentration of acyclovir drug that match the fewest red fluorescence and blue fluorescence dots shown under the microscope.

The second preliminary experiment is to see when to give the drug before the Aβ form and can hardly be eliminated. If the time is to short, the effect of the drug cannot be seen at all. If the time is too long, Aβ plaques already form and drug cannot influence the number of Aβ plaques that already form. The first step is to inject HSV-1 to mice with antibodies that can detect Aβ (blue), HSV-1 (red), and β-amyloid plaques (green), waiting for different hours (could be 0h, 20h, 40h, 60h, 80h, ...240h) before injecting the same amount of acyclovir drug to each mouse. Second, cut a slice of each mouse's brain. Them, use microscope to see the number of dots with blue, red, and green fluorescence in each mouse's brain. Finally, determine the time when there are fewest blue dots and red dots in the brain.

After determining the most suitable concentration of acyclovir drug and the time that works to prevent Aβ plaques from the two preliminary experiments, set up three groups of mice for the main experiment: control group with healthy adult mice; HSV-1 group, healthy adult mice injected with HSV-1; anti-HSV-1 group, healthy adult mice injected with HSV-1, but will be treated with acyclovir drug. For the control group, inject 0.9% saline with antibodies that can detect Aβ (blue) and HSV-1 (red) into mice brains, waiting for 12 hours, cutting the brains of mice, using microscope to see the number of red and blue spots in mice brains and collecting data. For the steps of HSV-1 group, the only change compared with the control group is to replace the same amount of 0.9% saline for control group to HSV-1 with antibodies that can detect Aβ (blue) and HSV-1 (red) into mice brains. For the steps of anti-HSV-1 group, compared with HSV-1 group, add one step: inject the amount of acyclovir drug obtained from the preliminary experiments to the mice. After doing all the experiments, the possible result can be shown in the picture. The number of blue and red dots shown from the microscope in the anti-HSV-1 group are fewer than them in HSV-1 group, so the acyclovir drug could be effective to prevent the form of Aβ protein caused by HSV-1[1].

For the main experiment, all the experiments done are just a beginning of searching for ways to reduce the amount of Aβ caused by HSV-1. There are other mechanisms that are involved in

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propagating the Aβ plaques when the acyclovir drug was added too late. For this reason, more researches should be done to totally reduce the number of Aβ plaques. One possible way is to achieve that goal is to use the antibody that matches APOE, a kind of protein inside the Aβ plaques. All in all, finding effective healing methods for AD is a long way to go. But hope more and more efficient treatments can be discovered for Alzheimer's disease patients.

3. DISCUSSION

This work can prove that HSV-1 can affect the content of Aβ by increasing the APP cutting through these experiments. In other words, HSV-1 can promote the hydrolysis of APP and cause accumulation of Aβ, which eventually worsens AD.

Experiments 1 and 2 complement each other. The immunofluorescence method can intuitively show that HSV-1 can cause an increase in Aβ, but it is not convincing enough. And the quantitative experiments to determine the content of the two enzymes indirectly showed that HSV-1 may increase Aβ. They intuitively and convincingly proved that HSV-1 can cause an increase in Aβ. And experiments 3 and 4 further demonstrated from two different angles, that HSV-1 can promote APP cleavage to produce more Aβ. In calcium experiment, using GCAMP Protein, it can be clearly seen the increase and decrease of the amount of Ca^{2+} and drawn a conclusion, but the use of calcium block drug will also affect the usual neuron impulse to produce some byproduct and reduce the accuracy of the experiment. We still need to work hard in this area and reduce shortcomings in the future. The anti-Herpes virus drugs (acyclovir) testing showed from another side that HSV-1 does increase the Aβ content, and provide us with a treatment for AD. However, the nervous system of human beings is more complex than mice, and anti-HSV-1 drugs may have some side effects like muscle pain and nausea. Therefore, it should be prudent to use the relative medicine and people should follow the doctor's advice.

As we all know, Alzheimer's disease is the most common dementia, and researchers have been looking for ways to improve or even eliminate this symptom. This latest study was published in the journal eNeuroon May 14. Scientists completed the transfer of memory by injecting RNA. "In the near future, we may be able to use RNA to improve the effects of Alzheimer's disease, post-traumatic stress disorder and other diseases on brain memory." David Glanzman, author of the article and professor of physiology and neurobiology, imagined.

David Glanzman's team chose Californica Aplysia as a model, and lightly shocked its tail (5 shocks every 20 minutes, and 5 more after 24 hours). This electric shock enhances the aplysia's defensive withdrawal reflex (a self-protection response).

Later, when the researchers patted the aplysia, the aplysia that had experienced the electric shock also showed a defensive contraction (average duration of 50 seconds). This simple way of learning is called "sensitization" [7]. And the contraction reaction of those aplysia that had not been shocked lasted only about 1 second.

Immediately afterwards, the researchers extracted RNA from the nervous system of two groups of aplysia (experienced electric shock and tapping, and the control group that never been shocked), and injected the two groups of RNA into seven aplysia who had never received electric shock.

Interestingly, the results show that the RNA of the aplysia who have completed the sensitization study will make 7 aplysia who have not received electric shocks show the behavior they have experienced in electric shocks: they will make a defensive lasting about 40 seconds shrink. However, the control group did not show long-term contraction. "It's like, we transferred memories!" [7]

However, there are some different views:

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At the same time, they also added two sets of RNA to Petri dishes, which contain the nervous system (sensory neurons or motor neurons) extracted from the aplysia (which has never been shocked).

When the aplysia is shocked, its sensory neurons will become excited. It is worth noting that the addition of RNA from the aplysia that experienced electric shock will increase the excitability of sensory neurons in the Petri dish, but it does not affect motor neurons. However, adding RNA without a shock to the apysia does not stimulate sensory neurons.

In the field of neuroscience, people have always believed that memories are stored insynapses. Glanzman put forward different views based on the new research. He believes that memory exists in the nucleus of neurons.

“If memory is stored in synapses, our experiment will not succeed.” Glanzman believes that this discovery may change the way scientists think about memory, which may be related to epigenetic changes induced by RNA. Glanzman believes that RNA may be used in the future to wake up and restore the memory of patients with early dementia. As early as 2014, he and his team published an article in the journal “eLife” showing that the lost memory can be recovered. Next, Glanzman hopes to figure out the type of RNA responsible for memory transfer.

4. CONCLUSION

In this work, it can be proved that when HSV-1 existed or was infected in the brain, the level of Aβ increased, usually turned into AD later. A possible reason is that the degree of hydrolysis of APP was increased, which might be promoted by HSV-1. And the anti-HSV drugs, like acyclovir, were showed to the inhibition of Aβ accumulation and reduction of the risk of AD.

This work mainly explores the effect of HSV-1 on AD, proves that HSV-1 is one of the causes of AD, and further explores the corresponding mechanism. At the same time, a method of AD treatment is proposed. Of course, the work is only a simple exploration, as an overture to the future HSV-1 and AD research direction.

People have spent decades in the study of the causes of AD, and have come up with many assumptions. So far, however, researchers have not yet found the true cause of AD, and have not come to a unified conclusion. This suggests that the key factors that triggered AD have not yet been discovered. So, every relevant factor we find is critical. It is believed that in the near future, people will be able to find the cause of AD and be able to effectively cure AD.

5. CONTRIBUTIONS

Yunxuan Wang straightened out the core logic of this work and the main experiments, as a leader. Xuedou Lu initiated the project, providing the original idea. Jie Xiong came up with a unique idea in the experimental section. Zihan Chen was involved in the overall recording of the work. Haixin Tan participated in the proposal of some experimental ideas. The whole work was completed by all of us and we wrote this article together.

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