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Gingipain of Porphyromonas Gingivalis Exacerbates Features of Alzheimer's Disease and Affects the Level of TREM2 via Amyloid-β

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Abstract

In the last decade, periodontitis has been reported as a risk factor for the development of Alzheimer's disease (AD). Recently, it has been suggested that gingipain, a toxic protease secreted by Porphyromonas gingivalis, plays critical roles in host colonization, inactivation of host defenses, nutrient acquisition and tissue destruction. However, we do not have any evidence that gingipain exacerbates features of AD and affects the level of TREM2 via amyloid β -protein (Aβ). In this work, it is examined that gingipain helps the brain colonization of P. gingivalis and increases the production of Aβ(1-42). The work measures the concentrations of bacterial endotoxin and the concentrations of Aβ(40 and 42) in the brains of different groups of P. gingivalis-inoculated Tg mice. CoIP (Co-Immunoprecipitation) is used to prove the interactions between amyloid-β and TREM2. Also, the work employs the knock-out of TREM1 and TREM2 in mice' s brains. All mice with different knock-out conditions are divided into four groups, 'with TREM1 & with TREM2', 'with TREM1 & without TREM2', 'without TREM1 & with TREM2', and 'without TREM1 & without TREM2', and gingipain is induced into the brains of all mice. Furthermore, the mice are divided into 4 groups with one TREM-1 knock-out, one TREM2 knock-out, one both TREM1 and TREM2 knock-out, and one no knock-out to do 3 mouse behavior experiments (maze, boxes with road A with electron shock and road B, and 2 boxes covered with different odor, one with food).

Keywords

Alzheimer's Disease(AD); Porphyromonas Gingivalis; Gingipain; Periodontitis; Amyloid β-Protein (Aβ); AB(1-42); Aβ40; Aβ42; Triggering Receptor Expressed on Myeloid Cells 1 (TREM1); Triggering Receptor Expressed on Myeloid Cells 2 (TREM2).

1. Introduction

Alzheimer 's disease (AD) is a primary degenerative encephalopathy that occurs in geriatric and pre-geriatric. The characteristic pathological changes of AD are cortical atrophy with Aβ deposition, neurogenic fiber tangles, reduction in the number of memory neurons, and the formation of senile plaques. Lines of evidence have suggested that the neurotoxic effect of amyloid β-protein (Aβ), which is derived from the hydrolysis of β-amyloid precursor protein (APP), plays a major role in the progression of Alzheimer's disease. The Aβ hypothesis suggests that an excess of Aβ42 is produced due to a disruption of the metabolism of APP, which rapidly aggregates to form oligomers, causing the deposition of Aβ and the formation of senile plaques. It has been shown that there is a severe inflammatory response at the lesions of AD patients, and it is inferred that the pathogenesis of AD may be related to the abnormal release of inflammatory mediators. Aβ may be involved in the inflammatory response, in which it plays the role of inflammatory mediator. It has also been shown that Aβ may be involved in the synthesis and release of various inflammatory mediators, including

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interleukins and tumor necrosis factors. Thereby, Aβ may be involved in and exacerbate inflammatory response, thus exacerbating feature of AD.

The surface receptor, Triggering Receptor Expressed on Myeloid cells 1 (TREM-1), on immune cells amplifies inflammatory processes, which means that once being activated it increases the level of inflammation[2][3]. TREM-1 can be activated by bacterial infection, and studies have shown that it is regulated by gingipain of P. Gingivalis, inducing chronic inflammation in the brain, leading to neurodegeneration and cognitive decline. Triggering Receptor Expressed on Myeloid cells 2 (TREM-2) is a single transmembrane superimmunoglobulin that was originally identified in monocyte-derived dendritic cells and mouse macrophages[4]. TREM-2 shares homology with TREM-1, encoding a receptor expressed on immune cells such as macrophage and microglia. In contrast, studies have shown that TREM-2 protects the liver from hepatocellular carcinoma (HCC). Once activated by its ligand, TREM-2 reduces the level of inflammation in liver. Aβ is one of the ligands of TREM-2.

Periodontitis is a chronic inflammation of periodontal supporting tissues caused by periodontopathic bacteria. Porphyromonas gingivalis is an example. Gingipain is a toxic protease secreted by P. gingivalis which is important for P.gingivalis survival and pathogenically. Recently, it has been suggested that gingipain plays significant roles in host colonization, inactivation of host defenses, nutrient acquisition and the destruction of tissues[1]. It is possible that P. gingivalis colonizes in the brain and exacerbates features of AD via its gingipain. In addition, the activation of TREM-2 reduces inflammation level and protects neurons from degeneration.

In this work, to investigate that gingipain helps the brain colonization of P. gingivalis and increases the production of Aβ(1-42), porphyromonas gingivalis wild-type W50 strain and gingipain knock-out mutant K1A and E8 strains are used. The work measures the concentrations of bacterial endotoxin and the concentrations of Aβ(40 and 42) in the brains of different groups of P. gingivalis-inoculated Tg mice. CoIP (Co-Immunoprecipitation) is used to prove the interactions between amyloid-β and TREM2. Also, the work employs the knock-out of TREM-1 and TREM-2 in mice' s brains. All mice with different knock-out conditions are divided into four groups, 'with TREM-1 & with TREM-2', 'with TREM-1 & without TREM-2', 'without TREM-1 & with TREM-2', and 'without TREM-1 & without TREM-2', and gingipain is induced into the brains of all mice. Furthermore, mouse behavior is tested. The mice are divided into 4 groups with one TREM-1 knock-out, one TREM-2 knock-out, one both TREM-1 and TREM-2 knock-out, and one no knock-out to do 3 mouse behavior experiments (maze, boxes with road A with electron shock and road B, and 2 boxes covered with different odor, one with food).

2. Methods

2.1 Mice

Transgenic mice (Tg mice) with FAD gene insertion are used as models to study AD. The mice are housed in a pathogen-free facility with up to five mice per cage until the beginning of the study, when the mice are housed separately. The mice maintain a 12 hour light / dark cycle and are provided with food and water. All mice are bred and maintained in specific pathogen-free conditions in the animal facility, and all experiments are performed in accordance with institutional guidelines of the Animal Experiment Committee and Gene Recombination Experiment Committee.

2.2 Wild-Type Gingivalis, Gingipain Knock-Out Mutant and E8 Strains

Porphyromonas gingivalis wild-type W50 strain and gingipain knock-out mutant K1A and E8 strains is used in this study. E8 strain is deficient in both Arg-gingipain A and Arg- gingipain B (rgpA&rgpB), whereas K1A strain is deficient in Lys- gingipain (kgp). All three strains are cultured with 5% Brucella agar supplemented with rabbit blood with hemin (2.5μg/ml), menadione (5μg/ml), and dithiothreitol (0.1 mg/ml) and in Trypticase soy broth with yeast extract (2.5 mg/ml), hemin (2.5 μg/ml), menadione (5 μg/ml), and dithiothreitol (0.1 mg/ml). Bacterial cells are collected, washed,

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and suspended in phosphate-buffered saline (PBS). The concentration of bacteria is determined with a spectrophotometer at an optical density of 660 nm. Live P. gingivalis (109 CFU) is resuspended in 100 µl of PBS with 2.5% carboxymethylcellulose[5].

2.3 Development of Periodontitis in Mice

To develop experimental periodontitis in Tg mice, sulfamethoxazole (700µg/ml) and trimethoprim (400 µg/ml) are used for Tg mice for 10 days. After 3 days, 10^9 CFU of live P. gingivalis W50 strain, gingipain-knock-out mutant K1A and E8 strains, each in 100 µl of PBS with 2.5% carboxymethylcellulose, is applied to the gingival margin of each mouse under brief isoflurane anesthesia. After inoculation, the mice are fasted for 1 h. 100 µl PBS with 2.5% carboxymethyl cellulose is administered to the control group[5]. After 5 weeks, the mice are sacrificed, and the brains are collected.

2.4 Measure the Concentrations of Bacterial Endotoxin

The work measures the concentrations of bacterial endotoxin in the brains of different groups of P. gingivalis-inoculated Tg mice to see if gingipains affect the ability of brain colonization of its bacteria. The endotoxin in mouse brain lysate is measured by a Limulus assay using an Endospecy ES-50M kit.

2.5 Measure the Concentrations of A-Beta Protein (40 and 42)

The work measures the concentrations of A-beta protein (40 and 42)in the brains of different groups of P. gingivalis-inoculated Tg mice to see if A-beta accumulation in the brain is caused by gingipains of P. gingivalis.. The brain sections are immunostained using anti-Aβ antibody 82E1 and visualized by the avidin–biotin–peroxidase complex procedure using diaminobenzidine as the chromogen. Then the sections are observed with a microscope. The number of immunopositive pixels in the hippocampus are counted, and the values are converted to the area as the amount of Aβ deposition[5].

2.6 Preparation of Cell Lysates

1) Aspirate the medium. Add fresh medium containing conditioning molecules to the cells for a predetermined amount of time.

2) Collect the cells, remove the medium and wash the cells once with ice-pre-cooled 1 X PBS.

3) After removing PBS, ice-pre-cooled Lysis buffer is added to each cell plate (10 cm). It is incubated on ice for at least 5 min.

4) Scrape the cells from the plate and transfer the extract to a microcentrifuge tube. Place on ice.

5) Ultrasonic destruction is carried out on ice for 3 times, each lasting for 5 seconds.

6) Microcentrifuge at 14000 x g for 10 minutes at 4 ° C and transfer the supernatant to a new tube. The supernatant is cell lysate. The pyrolysis liquid is stored at - 80 ° C.

2.7 Immunoprecipitation

Antibody: Abeta antibody.

Antigen (protein A) : Abeta.

Negative control groups	Possible false positive results
Agarose beads + Aβ antibody + TREM2 antibody	Non-specific binding of agarose beads to Aβ
Agarose beads + TREM2 antibody	Non-specific binding of agarose beads to TREM2
Agarose beads + Aβ antibody + TREM2	Non-specific binding of Aβ antibody to TREM2
The cell lysates + Aβ antibody + TREM2 antibody	Aβ antibody or agarose beads bind with other proteins of in the cell lysates
Normal IgG + Aβ + TREM2	Non-specific binding of antibodies

Figure 1. Negative control groups and possible false positive results

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Protein B: trem2.

1) Take about 20ul of supernatant from cell lysis and boil it for 5min with 2 x loading buffer as input group.

2) Divide agarose beads equally into new EP tubes, using a gun with the tip cut off to aspirate the beads, and ensure that the amount of beads in each tube is the same. Aspirate the supernatant and add Aβ antibody and cell lysis supernatant. 1mg of protein lysate is added to 25ul of suspension including 1:1 S beads with 2ug of Aβ antibody.

3) Incubate for 4h at 4°C in a shaker.

Input group	The presence of protein Aβ & TREM2 in the cell lysates is verified by direct WB assay using Aβ & TREM2 antibody.
Output group	The supernatants after IP are subjected to WB assays for protein X and protein Y, respectively.

Figure 2. Input group and output group

4) At the end of Binding, centrifuge at 1400r x 1min, 4°C.

5) Use vacuum pump or pipette gun to aspirate the supernatant, add 800ul NETN, mix upside down to ensure that the bottom of the precipitate is suspended.

6) Centrifuge, repeat 4) and 5), wash beads for three times.

7) Discard the supernatant, and aspirate the residue with a spotting gun. Add 15ul 2xloading buffer and boil for 5 min as a spotting sample for the Co-IP group, add 10ul of 2 x loading buffer into the remaining and boil to be the IP group.

2.8 Western Blot

1) The order of spotting samples follows Co-IP group, Input1, Marker, IP group, Input2, running gel.

2) Turn the membrane, and after closure, add TREM2 antiibody for the Co-IP group and Input1, and add Aβ antibody for the IP group and Input2 for incubation at 4°C.

3) Recover the primary antibody and incubate the secondary antibody at room temperature.

4) Develop and observe the experimental results.

2.9 The Knock-out of TREM1 and TREM2

1) Two LoxP sites are set surrounding Myeloid cells I/II (TREM1/TREM-2) gene in the cortex. Calmodulin-Dependent Protein Kinase II (CaMK-II) is put in mice' s brains with excitatory glutamate. In such a situation, CaMK-II can be activated, promoting cre-protein begin transcription. Then, the gene segment (TREM-1/TREM-2) between two LoxP sites can be knocked out.

2) Divide all mice with different knock-out conditions into four groups. In these four groups, mice are in the conditions "with TREM1 & with TREM2", "with TREM1 & without TREM2", "without TREM1 & with TREM2", and "without TREM1 & without TREM-2". Gingipain is induced into the brains of all mice according to the experiment mentioned above (experiment 1).

3) Use ELISA kits to measure the production of TNF-α and IL-1β and the amount of Aβ oligomer.

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2.10 Mouse Behavior Testing

Divide mouse into 4 groups with one TREM-1 knock-out, one TREM-2 knock-out, one both knock-out, and one does not do anything. Give each group of mice a sign, group A for the TREM-1 knock-out, group B for TREM-2 knock-out, group C for both knock-out, group D for the one does not do anything.

1) Design a maze with several turns that a normal mouse can walk out. Put each group into the maze individually to measure the time they need for the first time to find way out. Do the same thing for 3 times and measure the time each group need to get out. Compare the change in the need of time between different groups to conclude the result.

2) Put each group into a box with road A and B that lead to food, if the mice pass through road A, they will receive an electron shock of 5V. Make the mouse empty stomach for 5 hours and put each group into the box so that they will go for the food. Do the same thing for 10 times and measure the time they go for road A and B.

3) A training for all the mouse to open the box to find food will be done before do the surgery. After the surgery, put two sealed boxes in two directions with two different odor that is not related with mouse in any areas, one of the boxes contains food. Put each group in that area to find food for 10 times. Measure the time it takes for mouse to know the box with which odor contains food.

3. Results

3.1 The Concentrations of Bacterial Endotoxin

If gingipain can actually promote brain colonization of P. gingivalis, we would expect that the concentration of bacterial endotoxin in the brains of mice inoculated with W50 strain, is significantly higher than that in the brains of mice inoculated with K1A and E8 strains. If not, there will be no significant difference between the concentrations of endotoxin.

3.2 Measure the Concentrations of A-Beta Protein (40 And 42)

	Input	IgG (IP)	Aβ (IP)
IB: Aβ	+	-	+
IB: TREM2	+	-	+

Figure 3. Predicted results of Input group, IgG group and Aβ group

CoIP is used to determine the interactions between Abeta and TREM2. If Abeta is immuno-precipitated with Abeta antibodies, then TREM2 can also be precipitated. As shown in the figure, we verify the presence of Abeta and Trem2 in the Input group and IP group. We predict that both Abeta and TREM2 are present. In the IP group we perform precipitation experiments using IgG. If Abeta and Trem2 are not precipitated, Abeta and TREM2 can not bind to IgG. In the IP group we also perform precipitation experiments using Abeta. If Abeta is precipitated and TREM2 is precipitated, then there is an interaction between Abeta and TREM2.

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3.3 The Knock-out of TREM-1 and TREM-2 in Mice' s Brains

1) (mice with TREM-1 & TREM-2): The amounts of pro-inflammatory cytokines and Aβ deposition are raised gently (without obvious change)-inflammations are simultaneously accompanied with the exacerbation by TREM-1 and the inhibition by TREM-2.

2) (mice with TREM-1 & without TREM-2): The amounts of pro-inflammatory cytokines and Aβ deposition are raised drastically-inflammations are exacerbated by TREM-1, while TREM-2 is absent.

3) (mice without TREM-1 & with TREM-2): The amounts of pro-inflammatory cytokines and Aβ deposition are balanced-inflammations are inhibited by TREM-2, while TREM-1 is absent.

4) (mice without TREM-1 & without TREM-2): The amounts of pro-inflammatory cytokines and Aβ deposition are raised gently (without obvious change)-both TREM-1 and TREM-2 are absent.

3.4 Mouse Behavior Testing

1) Group A will show an obvious decrease in the time it takes to pass the maze as the time of trying increases. Group B will need almost the same time during each time of trying. Group C shows a similar result as group B. Group D shows a constant decrease in time as the time it passes the maze increases.

2) As the time of the experiment increases, group A shows an obvious decrease in the chance it goes for road A. Group B shows a chance of about 50% that it will go for road A. Group C shows a similar result as group B. Group D shows a decrease in the chance it goes for road A as the time of experiment increases.

3) Group A will show a decrease in the time it needed to find food as the time of experiment increases and can be concluded that group A can link a kind of odor to food. Group B shows a similar time each time it needed to find food as the time of experiment increases so that it can be concluded that group B cannot link a kind of odor with food. Group C shows a similar result as group B. Group D shows a decrease in time as more experiment is done and can be concluded that group D can link a kind of odor to food.

4. Discussion

To prove that gingipain helps the brain colonization of p. gingivalis and increases the production of A-beta protein (1-42), we measure the concentrations of bacterial endotoxin in the brains of different groups of P. gingivalis-inoculated Tg mice to see if gingipains affect the ability of brain colonization of its bacteria. We also measure the concentrations of A-beta protein (40 and 42) in the brains of different groups of P. gingivalis-inoculated Tg mice to see if A-beta accumulation in the brain is caused by gingipain of P. gingivalis. There are large individual differences among mice that we use during experiments, and there are large differences among the abilities of mice to respond to the invading of gingivalis. The length of the experiments may also be a limitation. We need to cost several weeks to have a preliminary observation for suitable mice and material preparation. The cause of such length of time is that scientists still have not come up with the most adaptive range of parameters for experiments, including mice' s age, P. gingivalis' s dosage injected in mice, and the time we use for collecting data. In addition, using P. gingivalis would increase the death rate of mice. Although this is an expense that must be faced, we hope to find better ways to improve the survival rate of mice to provide us with lower costs and a longer time to observe the same targets.

For the CoIP experiment, which is used to determine the interactions between Abeta and TREM2, if Abeta is immuno-precipitated with Abeta antibodies, then TREM2 can also be precipitated. However, too low a concentration of antibodies will result in undetectable antigens, while too high a concentration of antibodies will not settle on beads. Besides, it is also important to design and proceed with more CoIP experiments for the other members in the myeloid cell (TREM) family.

According to other scientists' studies, we can know that Chronic inflammation is one of the most crucial factors to exacerbate the etiology of AD. To evaluate the level of inflammation, we have to measure the production of pro-inflammatory cytokines, including TNF-α and IL-1β. Furthermore, the

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amount of Aβ oligomer is also an indispensable feature for us to evaluate the degree of AD. In order to measure the amount of Aβ oligomer, TNF-α, and IL-1β, we can use a kind of instrument called enzyme-linked immunosorbent assay (ELISA) kits. In further studies, scientists had confirmed that TREM-1 can propagate a pro-inflammatory cytokine production named IL-8. Also, it is demonstrated that TREM-1 would motivate the release of IL-8 from Polymorphonuclear neutrophils (PMNs), the first wall that fights against the infection triggered by gingipain. Therefore, we cannot miss the measurement of the amount of IL-8 as key evidence.

One of the previous studies had focused on the function of TREM-1 and TREM-2 in liver-related disease. In this research, the experimenters had concluded that TREM-1 would exacerbate inflammation during acute inflammation. By contrast, TREM-2 would actually prevent chronic inflammation. Lipopolysaccharide (LPS) is a kind of endotoxin that mainly compose the surface of P.gingivalis, which would enter mice' s brain with the entering of gingipain. LPS induces inflammation by Toll-like receptor 4 (TLR4), a receptor that can regulate the release of downstream inflammatory factors. TREM-1 regulates the TLR4 signaling pathway and expands the inflammatory response by activating myeloid differentiation factor 88 (MyD88), IL-1β, and IL-10 genes. The activated TREM-1/TLR4 signaling cascade increases the release of cytokines, such as TNF-α and IL-1β. Since the half-life of TREM-2 is short and TNF-α inhibits the expression of TREM2, it is reduced in acute inflammation. During chronic inflammation, TREM2 negatively regulates inflammation to reduce the organ injury caused by inflammation[6].

Based on the analysis above, we can infer that the inflammation would be exacerbated by TREM-1 with the increased amount of pro-inflammatory cytokines, while the inflammation would be inhibited by TREM-2 with the decreased amount of pro-inflammatory cytokines. While the inflammation is intensified, microglia would be activated and Aβ production would be promoted. At the same time, we cannot exclude that the condition of inflammation would still be increased even if TREM-1 and TREM-2 are both absent or TREM-1 and TREM-2 can counteract their influence on the level of inflammation. Whenever LPS of P. gingivalis enter mice' s brain, other receptors besides TREM-1 and TREM-2 would be activated, causing the increase of pro-inflammatory cytokines and deposition of Aβ.

We pay attention to the comparison between the function of TREM-1 and TREM-2, and we finally can find that TREM-1 would exacerbate chronic inflammation, while TREM-2 would inhibit chronic inflammation. Many researchers can indeed provide us with some experience about the function of TREM-1 in the brain circuit, but scientists have focused more on the function of TREM-2 in the condition of liver diseases. Although we can draw an analogy between the two cases (when TREM-2 have responses in the liver or brain), no one has confirmed that these two situations hold a necessary connection. Moreover, it is also limited that we cannot ensure that the presence of TREM-2 would have the same effects in animal's brains and human brains. AD is one of the most typical brain diseases, and there are still many common brain diseases waiting for researches by scientists.

For the mouse behavior experiment, we can assume that TREM-1 will exacerbate inflammation during acute inflammation, while TREM-2 will prevent chronic inflammation. According to Naoyuki Ishida, the inflammation is correlated with AD that when inflammation exacerbate, it will exacerbate the symptoms of Alzheimer's Disease. So that we can expect that the knock-out of TREM-1 will decrease the symptoms of AD, the knock-out of TREM-2 will exacerbate the symptoms of AD, while knock-out of both TREM-1 and TREM-2 will cause the deterioration of AD afterward. As the assumption we make, we can know that group A will present the state that AD is weakened. In group B, the present of AD will be exacerbated. In group C, the present of AD will be exacerbated. In group D, it is same as group C. We explore whether the cognitive abilities of mice would be influenced by the change of Aβ deposition, and we have set many invariable factors when we only change one variable---whether TREM-1/TREM-2 are knocked out. Nevertheless, there are still some X factors that would bring some flaws to results. For example, each mouse has different habits, some of these mice would not move in the maze or on the experiment table even if their conditions of TREM-

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1/TREM-1 knock-out are the same. Therefore, in many cases, it is difficult to generalize rules from the final results. To deal with these issues, we suggest having some training and screenings to ensure that the behavior of mice would not be affected by other unrelated factors.

5. Conclusion

Today, Alzheimer's Disease might be the most serious in the human brain, it would decline the ability to memorize and recognize, many patients are still suffered from it. Although many scientists have spent a great amount of time finding ways to prevent the spread of AD in the brain, there is still no efficient way. We are trying to explore whether using TREM-2 can inhibit the exacerbation of AD, and we hope that our research can make some contribution to the treatment of AD.

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