C5aR antagonist inhibits occurrence and progression of complement C5a induced inflammatory response of microglial cells through activating p38MAPK and ERK1/2 signaling pathway

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Abstract. – OBJECTIVE: To discuss the effect of complement C52 (C5a) and complement C5a receptor (C5aR) antagonists on inflammatory status of mouse microglial cells.

MATERIALS AND METHODS: Primary culture was performed on mouse microglial cells. 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) assay was used to detect effect of C5a and C5aR antagonists on vitality of microglial cells. The effect of C5a and C5aR antagonists on mRNA expression of p38MAPK and ERK1/2 was determined using quantitative PCR (qPCR). Enzyme linked immunosorbent assay (ELISA) was used to measure expression of interleukin-6 (IL-6) and tumor necrosis factor-α (TNF-α) in cells.

RESULTS: According to quantitative fluorescent PCR, relative expression of p38MAPK and ERK1/2 mRNA in C5a antagonist treatment group was significantly higher compared to normal group and C5a+C5aR antagonist treatment group (p<0.05). However, the relative expression of the C5a+C5aR antagonist treatment group was significantly lower compared to that of the normal group (p<0.05). Expression of Iba1, p-p38MAPK and p-ERK1/2 proteins in C5a antagonist treatment group was significantly higher than normal group, in C5a+C5aR antagonist treatment group was lower than C5a antagonist treatment group (p<0.05). There were significant differences for IL-6 and TNF-α levels among 5 groups (p<0.05). Expression of cytokines was the highest in 100 nM C5a antagonist treatments and lowest in normal group.

CONCLUSIONS: Complement C5a upregulated expression of inflammatory factors in mouse microglial cells, while C5aR antagonist inhibited occurrence and progression of inflammatory status. This was achieved by affecting transcriptional and translational processes of different factors in p38MAPK and ERK1/2 signaling pathway.

Key Words: C5a, C5aR Antagonist, Microglial cell, p38MAPK, Cytokine.

Introduction

Complement system consists of over 30 proteins and exists extensively in blood, tissues and on cell surface. Capable of extremely precise regulation, the complement system is a very important component of non-specific immunity. Complements are responsible for eradicating exogenous pathogenic microbes by promoting the phagocytosis and lysis of the target cells by phagocytes. Complements act as the bridge between adaptive immunity and innate immunity. C5a is the activation fragment of complement C5. In human C5a is a glycoprotein comprising 74 amino acids, and the α-helix is stably held together by 3 disulfide bonds. C5a and its receptor play a very important role in metabolic disorder and inflammatory response. An end pro-inflammatory product of complement activation, C5a not only binds to C5aR, but also fulfills its function by binding to C5L2. C5aR is the receptor of C5a, containing seven-transmembrane domain of G protein-coupled receptors. C5a-C5aR activation mediates intense inflammatory response via the MAPK and PI3K/PKB signaling pathways. For example, the chemotaxis of neutrophil granulocytes and phagocytes will be induced to the inflammatory regions with complement activation. As a result, a large amount of inflammatory cytokines such as interleukin-1 (IL-1), interleukin-6 (IL-6) and
C5aR antagonist inhibits inflammation of microglial cells

The central nervous system is the site where all types of complement factors are expressed. Under normal conditions, the complements can be hardly detected in the human brain. But in case of inflammatory changes of the central nervous system, the neurons and gliocytes will synthesize and release C5a and activate the classical receptor \(\text{C5aR}^{8,9}\). Such changes are similar to those seen in peripheral inflammatory diseases. Although it is generally agreed that the \(\text{C5a-C5aR} \) axis is involved in the central nervous system diseases, the mechanism of the involvement remains unclear. Study shows that the inflammatory response induced by C5a activation also causes damage to the organisms, in the form of acute lung injury, sauriaemia, glomerular nephritis and tumor\(^{10-11}\). In clinical practice, \(\text{C5aR} \) antagonists can be used to treat C5a-induced pathological injury due to over-treatment. This study used mouse microglial cells as the model. Primary culture was performed to analyze the effect of C5a and \(\text{C5aR} \) antagonists on the inflammatory status of the microglial cells. The working mechanism of the two was investigated. The research findings provide clues for the clinical application of \(\text{C5aR} \) antagonist in inflammatory response of the nervous system.

Materials and Methods

Primary Culture of Mouse Microglial Cells

Clean-grade mice newly born within 24 h were purchased from Laboratory Animal Center of the Third Military Medical University (Chongqing, China). The mice were disinfected with 75% alcohol (Tiangen Biotech Co. Ltd., Beijing, China) and decapitated under aseptic conditions. The scalp and skull were cut open to harvest the brain tissues, which were placed into a Petri dish containing precooled D-Hank’s solution (Gibco, Grand Island, NY, USA) without calcium and magnesium (pH 7.2). The cerebellum, hippocampus, and cerebral medulla were removed, and the cerebrum cortex was isolated. Using iris scissors, the tissues were cut into blocks of about 1 mm\(^3\) and digested with 0.125% trypsin at 37°C for 20 min. The supernatant was discarded, and the cells were washed twice and gently blown with a straw to disperse them. After standing for 2 min, the suspension was collected into a new centrifuge tube and centrifuged (1000 r/min, 10 min and 4°C). The supernatant was discarded. The cells were re-suspended in complete culture medium and passed through a 200-mesh stainless steel screen. The cells were cultured at 37°C in a 5% CO\(_2\) incubator. The culture medium was first replaced after 24 h, then at a frequency of once every 3 days. The cells were incubated for about 14-16 d until cell stratification was observed in the mixed gliocyte culture under the inverted phase contrast microscope. The cells were digested with 2-3 ml of 0.05% trypsin (Beyotime Biotech., Shanghai, China). When the microglial cells adhering to the astrocytes fell off, the digestion fluid containing the floating microglial cells was transferred into a 10 mL centrifuge tube. Centrifugation was performed at 1000 r/min for 5 min. The supernatant was discarded, and the cells were gently blown in complete culture medium into a suspension. The cells were inoculated to the pre-coated 24-well plate with the cover glass. The cells were cultured in a CO\(_2\) incubator at a constant temperature for 24 h. The culture medium was discarded, the non-adherent oligodendrocytes were removed. Complete culture medium was replenished to further incubate the cells.

Immunohistochemical Detection of Iba1 (Specific Antibody for Microglial Cells)

The cells were digested and suspended in complete culture medium to adjust the density to 2×10\(^4\)/ml. The cells were then inoculated to the sterile 12-well plate. The suspension was added dropwise onto the disc to initiate cell adhesion for 30 min. Complete culture medium was replenished into the dish, and the cells were cultured at 37°C in a 5% CO\(_2\) incubator. The dish was washed out and washed with phosphate buffered saline (PBS, Beyotime Biotech. Shanghai, China) twice. The cells were fixed in 4% paraformaldehyde (Sigma-Aldrich, St. Louis, MO, USA) for 20 min. After washing with phosphate-buffered saline (PBS), they were treated with 1% Triton X-100 (Beyotime Biotech. Shanghai, China) for 15 min and washed again with PBS. The cells were further treated with 3% \(\text{H}_2\text{O}_2\) for 15 min and washed with PBS. The cover slip was sealed with goat serum (Gibco, Grand Island, NY, USA) at room temperature for 60 min. Then, the cover slip was dried by shaking. The cells were cultured with primary antibodies (dilution 1:200) at 4°C overni-
ght. For negative control, PBS was added instead of the primary antibodies. The cells were washed with PBS for three times and incubated with working solution of biotin-labeled secondary antibodies at 37°C for 30 min. The cells were washed again with PBS and incubated with working solution of ALP-labeled streptavidin (Sigma-Aldrich, St. Louis, MO, USA) at 37°C for 30 min. After washing with PBS, 3, 3-diaminobenzidine (DAB) substrate (Beyotime Biotech. Shanghai, China) was added in the dark for 10 min until the cells turned brown under the microscope. The cells were washed with distilled water twice and counterstained with hematoxylin (Beyotime Biotech., Shanghai, China) for 5 min. After washing with running water, the cells were dehydrated in gradient alcohol (75%, 85%, 95%, 100%), for 3 min each. The cells were transparentized with xylene twice, and the cover slip was sealed with neutral balsam.

**Detection of Effects of C5a and C5aR Antagonists on Vitality of Microglial Cells Using 3-(4,5-dimethyl-2-thiazolyl)-2,5-Diphenyl-2-H-Tetrazolium Bromide (MTT) Assay**

Five groups were set up: A was the normal control, B was C5a 50 nM treatment, C was C5a 100 nM treatment, D was C5a 50 nM+PMX53 100 nM treatment, E was C5a 100 nM+PMX53 100 nM treatment. The microglial cells were isolated and cultured in the Dulbecco’s modified eagle medium F12 (DMEM-F12, Gibco, Grand Island, NY, USA) supplemented with 15% fetal bovine serum (FBS, Gibco, Grand Island, NY, USA), penicillin (1 U/ml, Gibco, Grand Island, NY, USA) and streptomycin (0.1 mg/ml, Gibco, Grand Island, NY, USA). The cells were cultured at 37°C in a humidified 5% CO₂ incubator. The purified microglial cells were harvested and adjusted to 1×10⁵/ml. The cells were inoculated to the 96-well plate for 100 μl per well. Each treatment had 3 replicates. The 96-well plate was transferred to the incubator (37°C, 5% CO₂). After different treatment for appropriate time, 20 μl 5 mg/ml MTT (Sigma-Aldrich, St. Louis, MO, USA) was added into each well. The cells were cultured in the incubator for 4-6 h, and the reaction was terminated. Into each well 150 μl of dimethyl sulfoxide (Sigma-Aldrich, St. Louis, MO, USA) was added and the cells were oscillated on a shaker at low speed for 10 min. The absorbance was measured in each well at 490 nm using a microplate reader (Thermo Fisher Scientific, Waltham, MA, USA).

**Quantitative Real-Time PCR (qRT-PCR)**

Detection of mRNA Expression of p38MAPK and ERK1/2

The same grouping scheme above was used. SYBR Green I Real-time RT-PCR assay was performed, with the primers provided in Table I (β-actin as internal reference). Total RNA extraction was performed using TRizol reagent (Beyotime Biotech. Shanghai, China), and RNA was reversely transcribed into complementary DNA (cDNA). The 20 μl reverse transcription system was used: 25°C 10 min, 42°C 50 min, 85°C 5 min. The fluorescence quantitative PCR system was 50 μl, and the reaction conditions were as follows: 94°C 4 min, 94°C 20 s, 60°C 30 s, 72°C 30 s, 35 cycle. Fluorescence signals were detected at 72°C.

**Western Blot Detection of Protein Expression of Iba1, p38MAPK, ERK1/2, p-p38MAPK and p-ERK1/2**

Western Blot was performed to detect the changes in the expression of Iba1, p38MAPK, ERK1/2, p-p38MAPK and p-ERK1/2 proteins after C5a and C5aR antagonist treatment. The same grouping scheme was used as above. The cells were lysed with radioimmunoprecipitation assay (RIPA) buffer (Tiangen Biotech Co. Ltd., Beijing, China) and total protein was extracted. Protein concentration was quantified by using bicinchononic acid (BCA, Sigma-Aldrich, St. Louis, MO, USA) assay. The proteins were analyzed by sodium do-

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**Table I. Primers for fluorescent quantitative PCR.**

<table>
<thead>
<tr>
<th>Gene</th>
<th>ID</th>
<th>Primers 5'-3'</th>
<th>Length</th>
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<tr>
<td>p38MAPK</td>
<td>26416</td>
<td>Forwards AGACCGTTTCTAGTCATCATTG</td>
<td>150 bp</td>
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<tr>
<td></td>
<td></td>
<td>Reverse GGTCAACAGTGACACGTTCTTGG</td>
<td></td>
</tr>
<tr>
<td>ERK1/2</td>
<td>26417</td>
<td>Forwards CTTCCAATCTGCATTACACAC</td>
<td>173 bp</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Reverse ATGGATTGATGTAGCCCTTG</td>
<td></td>
</tr>
<tr>
<td>β-actin</td>
<td>11461</td>
<td>Forwards GAGACCTTCAACACCCCAGC</td>
<td>263 bp</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Reverse ATGTCAACGCACGATTTCCC</td>
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decyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE), using 4% stacking gene and 10% separating gel. The sample was mixed with 5× SDS loading buffer at 4:1 ratio and boiled in water for 5 min for denaturation. The proteins were run through the stacking gel at 80 V, and then the voltage was changed to 120 V until the bromophenol blue dye reached the bottom of each gel. After electrophoresis, the proteins were transferred to the polyvinylidene difluoride (PVDF, Amresco Inc., Solon, OH, USA) membranes at a constant current of 200 mA for about 1 h. The primary antibodies were diluted with the blocking buffer to a certain concentration (1:500). The final concentration of dilution with the primary antibody for the internal reference was 1: 3000. The cells were incubated for 1.5 h and washed with Tris-buffered saline and Tween-20 (TBST) three times, 5 min each time. The secondary antibodies were diluted with the blocking buffer (1:3000) and incubated for 1.5 h. The cells were then washed with TBST four times, 5 min each time. Reagents A and B were mixed in equal volume in a tube and then applied to the upper side of the PVDF membrane. The cells were incubated for about 2 min and transferred to a dark room. The PVDF membrane was covered with the plastic wrap and the excess enhanced chemiluminescence ECL reagent (Tiangen Biotech Co. Ltd., Beijing, China) was removed. The gel was placed into the developing solution and exposed to X-ray for different durations depending on the intensity of chemiluminescence. When the bands appeared, they were immediately placed into the fixing solution. The gels were washed with running water and air-dried. The gels were scanned and the grayscale values of the target bands were acquired through UVP Bio-imaging Systems (UVP, Sacramento, CA, USA) and analyzed using Labworks 4.6 software (UVP, Sacramento, CA, USA).

Detection of IL-6 and TNF-α Using Enzyme Linked Immunosorbent Assay (ELISA)

ELISA was performed to detect the expression of IL-6 and TNF-α. The procedures were performed in strict accordance with the manufacturer’s instructions. The standard curves were plotted for each cytokine. The expression of each cytokine was calculated from the optical density (OD) value. The same grouping scheme was used in ELISA as above, with 3 samples in each group.

Statistical Analysis

Statistical analyses were performed using SPSS 17.0 software (SPSS Inc., Chicago, IL, USA). Qualitative variables were analyzed with \( x^2 \)-test, quantitative variables were analyzed with Student’ \( t \)-test. Tukey’s post-hoc test was used to validate the ANOVA for comparing measurement data among groups. \( p<0.05 \) represents a significant difference.

Results

Primary Culture of Microglial Cells and Iba1 Expression in Microglial Cells

Primary culture of microglial cells was successfully performed, as shown in Figure 1. The cells were harvested for subsequent experiments. Immunohistochemistry indicated that Iba1 protein was widely present in the primary microglial cells (brown particles in Figure 2). The microglial cells proliferated exuberantly.

MTT Assay

OD values were determined in the normal group and experimental group. Inhibition rate (%) was calculated as follows: inhibition rate=(OD value of normal group-OD value of experimental group)/OD value of normal group. From the curves it can be known that the optimal treatment duration of C5a and C5aR antagonists was 30 min (Figure 3). This optimal duration was used in subsequent experiments.

qRT-PCR Detection of p38MAPK mRNA and ERK1/2 mRNA

As indicated by fluorescence quantitative PCR, the relative expression of p38MAPK mRNA was higher significantly in the C5a antagonist treatment group than in the C5a+C5aR antagonist treatment group (\( p<0.05 \)). However, the relative expression in the C5a+C5aR antagonist treatment group was lower than that of the normal control. There was significant difference between the five groups (Figure 4, \( t=6.313, p<0.05 \)).

Western Blot

According to Western blot (Figure 5A), the expression of Iba1 was higher in the C5a antagonist treatment group than in the normal group (\( p<0.05 \)). Iba1 was also upregulated in the C5a+-C5aR antagonist treatment group as compared with the C5a antagonist treatment group (Figure 5B, \( t=14.427, p<0.05 \)). However, the expression of p-p38MAPK protein was not significantly different among the 5 groups (\( p>0.05 \)). Its expression in the C5a antagonist treatment group was higher than that in the normal group, but it was lower
in the C5a+C5aR antagonist treatment group than in the C5a antagonist treatment group. There was no significant difference between the 5 groups (t=13.732, p<0.05). The ERK1/2 protein expression was not significantly different between the 5 groups; its expression was higher in the C5a antagonist treatment group than in the normal control (Figure 5B, p<0.05), the expression was lower in the C5a+C5aR antagonist treatment group than in the C5a antagonist treatment group (Figure 5B, p<0.05). The difference between the 5 groups was of statistical significance (Figure 5B, t=12.850, p<0.05).

**ELISA**

IL-6 expression was significantly different between the 5 groups (p<0.05), with the highest found in the C5a antagonist 100 nM treatment group and the lowest in the normal control. Pairwise comparisons indicated significant difference in the IL-6 expression (p<0.05), as shown in Figure 6A. The expression of TNF-α showed a similar variation pattern as that of IL-6 among the 5 groups. There was also significant difference between the five groups in terms of TNF-α expression (p<0.05). The expression was the highest in the C5a antagonist 100 nM treatment group and lowest in the normal control. Pairwise comparisons indicated significant difference in TNF-α expression among the five groups (Figure 6B, p<0.05).

**Discussion**

We found that C52 upregulated the inflammatory factors in mouse microglial cells, while C5aR antagonist inhibited the occurrence and development of inflammatory status. This process is related to the effect on the p38MAPK and

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**Figure 1.** Microscopic findings of primary culture of microglial cells.
C5aR antagonist inhibits inflammation of microglial cells

ERK1/2 signaling pathways at the transcriptional and translational level, in addition to the up-regulation of the inflammatory cytokines. Our findings lay the basis for understanding the inflammatory response of the central nervous system. C5a achieves its function only after binding to C5aR. The existing studies demonstrate that there are two types of C5aR, CD88 and second C5a receptor (C5L2), both of which are widely present in the tissues and immunocytes. CD88 is a G protein-coupled receptor with seven transmembrane domains and its binding to C5a can induce the chemotaxis of neutrophil granulocytes and promote the release of myeloperoxidase and secretion of inflammatory cytokines such as IL-6 and IL-8. CD88 is the most important receptor of C5a.

C5L2 was first discovered in the early neutrophil granulocytes and immature dendritic cells. Because C5L2 does not contain the G protein, it is also called G protein receptor 77 (GPR77). Cyclic hexapeptide (PMX53) is a C5aR inhibitor and its cyclic amino acids are formed by the amino group on the side chain of ornithine and carboxyl group the main chain of arginine. PMX53 has proven effect in many animal experiments by mitigating the inflammatory status and reducing the secretion of cytokines. It was also found through experiments that PMX53 effectively inhibited the inflammatory response induced by C5a. Its binding to C5a will suppress inflammatory signaling pathways at the transcriptional or translational level. Moreover, the inflammatory

![Figure 2. Immunohistochemistry of Iba1 in microglial cells.](image)

![Figure 3. Curves of the inhibition rate in different treatment. *p<0.05 vs. Group D or Group E.](image)
cytokines were down-regulated, thus inhibiting the inflammatory response. Ingersoll et al.\(^{21}\) shows that in the central nervous system, C5a can induce the release of inflammatory and chemotactic factors by activating the AKP ERK1/2 pathway in the astrocytes and microglial cells. The activated gliocytes will generate such harmful substances as oxygen free radicals and NO\(^{22}\), thus amplifying the local inflammatory response and leading to secondary damage to the brain tissues\(^{23}\). If left uncontrolled, these harmful substances will create a neurotoxic environment and further damage the neurons and aggravate the lesions of the nervous system. The role of C5a/C5aR axis

**Figure 4.** Changes of mRNA expression of p38MAPK and ERK1/2 in different treatment. *\(p<0.05\) vs. Group D or Group E.

**Figure 5.** Western blot assay for detecting expression of proteins. **A**, Western blot assay images. **B**, Statistical analysis for the western blot *\(p<0.05\) vs. Group D or Group E.
C5aR antagonist inhibits inflammation of microglial cells

in the central nervous system diseases has been proven by the use of C5aR-specific antagonist PMX205/PMX53 and in the C5aR-knockout (C5aR-/-) mice. The gliocyte activity and Aβ accumulation decreased after PMX53 treatment of Alzheimer’s disease (AD) for 12 weeks, while the memory improved. The C5s content in the brain tissue increased dramatically following ischemic stroke, with an up-regulation of C5aR in cerebral cortical neurons and an increase in neuronal apoptosis. This resulted in a dramatic reduction in neuronal apoptosis and alleviation of brain injury in C5aR-/- mice and in mice treated with C5aR antagonist. Our research findings coincide with the above. Mitogen-activated protein kinase (MAPK) cascade is one of the most important intracellular signaling pathways and related to the regulation of various physiological processes. In mammals at least 4 MAPK pathways have been identified: extracellular signal-regulated kinase (ERK) pathway, c-Jun N-terminal kinase (JNK) pathway, p38MAPK pathway and ERK5 pathway. p38MAPK pathway is one of the most important members of the MAPK family. It can be activated by different stimuli, cytokines, bacterial lipopolysaccharides (LPS) and cell wall constituents, which results in a series of biological effects that affect transcription and protein synthesis.

Conclusions

We showed the effect of C5a and C5aR antagonists on the inflammatory status of mouse microglial cells. It was revealed that the two antagonists worked mainly by inhibiting the transcription of p38MAPK and ERK1/2, as well as by inhibiting the translation of phosphorylated p38MAPK and ERK1/2. In addition, the antagonists suppressed the secretions of the inflammatory cytokines IL-6 and TNF-α, thus relieving the inflammation. Although we aimed to clarify...
the working mechanism of C5a/C5aR axis in the inflammatory response of the central nervous system, the role of C5L2, another C5aR was not investigated. Besides, the function of C5a in the inflammatory response of the central nervous system induced under specific conditions was not analyzed. In the future, whether new types of inhibitors can suppress the inflammation will be the research topic.

**Conflict of Interest**

The Authors declare that they have no conflict of interest.

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