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Original Article

The effect of nuclear factor of activated T-cells (NFAT) in kidney I/R mediated by C5a/C5aR

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Abstract: To investigate the relationship between NFAT and C5a/C5aR in C5a/C5aR-mediated kidney Ischemia/reperfusion (I/R) injury, the rats' NRK-52E cell line was used in this study and was distributed into 4 groups, I: the normal control (NC), II: the ischemia/reperfusion (I/R) injury cell model (MG), III: the ischemia/reperfusion (I/R) injury cell model treated with C5a (50 nmol/l) (MG + C5a), IV: the ischemia/reperfusion (I/R) injury cell model treated with C5aR antagonist (2.5 μmol/l) (MG + anti-C5aR). Reverse transcription polymerase chain reaction (RT-PCR), western blot, immunofluorescence and flow cytometry were performed. Nuclear Factor Activated T Cell (NFAT), tumor necrosis factor-α (TNF-α) and interleukin (IL-6) were detected in this study. The results of immunofluorescence showed that NFAT had a nuclear translocation phenomenon during the study. The RT-PCR and WB data indicated that the expression of TNF-α and IL-6 in group III were higher than any other groups. Apoptosis in group III was much serious than other groups. All the results in this study showed that NFAT plays an important role in ischemia/reperfusion injury, it can be induced to up-regulate the inflammatory factor TNF-α and IL-6 by the complement system member C5a/C5aR.

Keywords: Complement system, C5a/C5aR, NFAT, ischemia/reperfusion injury

Introduction

Ischemia/reperfusion (I/R) injury means although the perfusion or oxygen supply was regained after ischemia, tissue or organ has been injured finally. I/R injury is a common cause of a wide variety of illness and death, it's also an unavoidable event in organ transplantation and has a major effect on graft survival [1]. In renal disease, I/R is a common cause of acute renal failure and the major factor limiting the function and survival of the transplanted kidney [2]. Previous studies have shown that I/R injury is associated with activation of the complement system, and it leads to the release of anaphylatoxins, such as C5a [3]. The pathogenesis of I/R injury is very complex, and lots factors, for example, complement activation, coagulation system, chemokines, cytokines and so on, are thought to contribute to its development [4]. Renal ischemia/reperfusion (I/R) injury is a significant complication of vascular surgery of the aorta and kidney [1].

The complement system consists of numerous proteins which present as soluble form or bound form at local inflammatory sites and in cell membranes. Under normal conditions, the soluble proteins exist as an inactive form what can be rapidly activated and amplified by a series of sequential proteases that are tightly regulated, and activation of complement leads to the formation of the anaphylatoxins such as C3a, C4a, and C5a ultimately [5]. C5a/C5aR plays an important role in promoting the occurrence and development of I/R injury. It's reported that stimulated C5a induced inflammatory mediators which from renal tubular epithelial cells and macrophages after hypoxia/reoxygenation in vitro, it contributed to renal ischemia-reperfusion injury, and its pathogenic role was predominant in this injury; it final suggested that expression of C5aR on renal and circulating leukocytes contributes to the pathogenesis of renal I/R injury [4].

Nuclear factor of activated T-cells (NFAT), a group of transcription factors ubiquitously expressed in mammalian tissues, plays a critical role in orchestrating the intricate cellular interactions that characterize vertebrate development and morphogenesis, and accumulated

evidence points to an emerging role for NFAT transcription factors in cancer progression, various NFAT isoforms are remarkably functional in tumor cells and multiple compartments in the tumor microenvironment, promoting carcinogenesis andcancer invasion [6]. The NFAT family contains four members of calcium/calcineurin-regulated proteins particularly recognized for their central roles in gene regulation during T-lymphocyte activation [7]. This translocation process coupled to the subsequent active maintenance of NFAT in the nucleus compartment is critical for the induction of expression of several genes encoding cytokines and membrane proteins that modulate immune responses. The molecular cloning of the NFAT family of transcription factors has facilitated rapid progress in the understanding of the signalling mechanisms that control the activity of NFAT [8].

According to the three factors mentioned above, we tried to find out something that was there any connections among them? We hypothesized that there were some contacts between the C5a/C5aR and NFAT. Now we all know C5a/C5aR can promote the occurrence and development of I/R so that lead to Ca^{2+} imbalance in cell. Based on this, we stimulated kidney epithelial cell by using C5a and C5aR antagonist to detect intracellular changes of NFAT's nuclear translocation and the expression of related pro-inflammatory cytokines.

Materials and methods

Cell culture

In this study, the rats' NRK-52E cell line which purchased from ATCC (America) was used, the culture medium was DMEM in the normal.

Preparedness for cell I/R injury model

Ischemic and hypoxemic model's simulating: cells cultured in a airtight container with the condition of 5% CO_2, 1% O_2, 37°C by the Serum-free and low sugar medium for 90 min, after this replaced them in the 5% CO_2, 37°C with normal culture.

Immunofluorescence (NFAT nuclear translocation)

Cells in each group were washed three times with PBS and fixed in 4% paraformaldehyde for 15 min, then washed three times in PBS (5 min each) again. Cells were permeabilized with 0.2% Triton X-100 for 20 min, and washed three times (5 min each) with PBS further. The cells were blocked with 5% BSA (Solarbio, China) for 30 min at room temperature, after this incubated in 3% BSA containing primary antibodies (1:800 Abcam, USA) at 4°C overnight. The cells were washed three times (ways as before) with PBS and then incubated in PBS containing Goat Anti-Rabbit IgG/FITC (1:1000, Beyotime, China) for 1 h at 37°C. After this, cells were washed thrice with PBS (5 min each). Cells were observed by the fluorescence microscope (OLYMPUS, Japan).

Real-time quantitative PCR

Total RNA were prepared using the Trizol^R Reagent (invitrogen, USA) according to the manufacturer's protocol, and the integrity of RNA was analyzed by the electrophoresis (Bio-Rad, USA). RNA were reversely transcribed using the cDNA Reverse Transcriptase kit (Thermo, USA) following to the manufacturer's protocol. The following PCR primers were used: TNF-α: 5'-TGGCGTGTTCATCCGTTC-3'(F), 5'-CTACTTCAGCGTCTCGTGTG-3'(R); IL-6: 5'-CACCAGGAACGAAAGTCAAC-3'(F), 5'-CAGTGGCTGTCAACAACATC-3'(R); GAPDH: 5'-GTCGGTGTGAACGGATTTG-3'(F), 5'-TCCCATTCTCAGCCTTGAC-3'(R). RT-PCR amplification was performed using the SYBR Green PCR Kit (Thermo, USA). For each gene, the following PCR conditions were used: 95°C for 10 min, followed by 40 cycles of 95°C for 15 sec, 60°C for 45 sec. The mRNA level was analyzed by ABI-7300 (ABI, USA). The results were normalized to GAPDH and expressed relative to the control and calculated by the $2^{-\Delta\Delta Ct}$ method.

Western blot

After collecting the cells, put cells in lysis buffer homogeneously and lyse completely at 4°C. Centrifugation at 12,000× g for 30 min at 4°C, and the protein content determined using a BCA Protein Assay Kit (Thermo, USA). From each sample, 15% SDS-PAGE was selected in the electrophoresis. Proteins were transferred to nitrocellulose filter membrane (NC) using a Trans-blot apparatus (Bio-Rad, USA). Primary antibodies of TNF-α and IL-6 were purchased from Abcam (USA), except for GAPDH which was purchase from Epitmics (USA). The dilutions of the antibodies were as follows: TNF-α (1:1000), IL-6 (1:500), GAPDH (1:1500). The

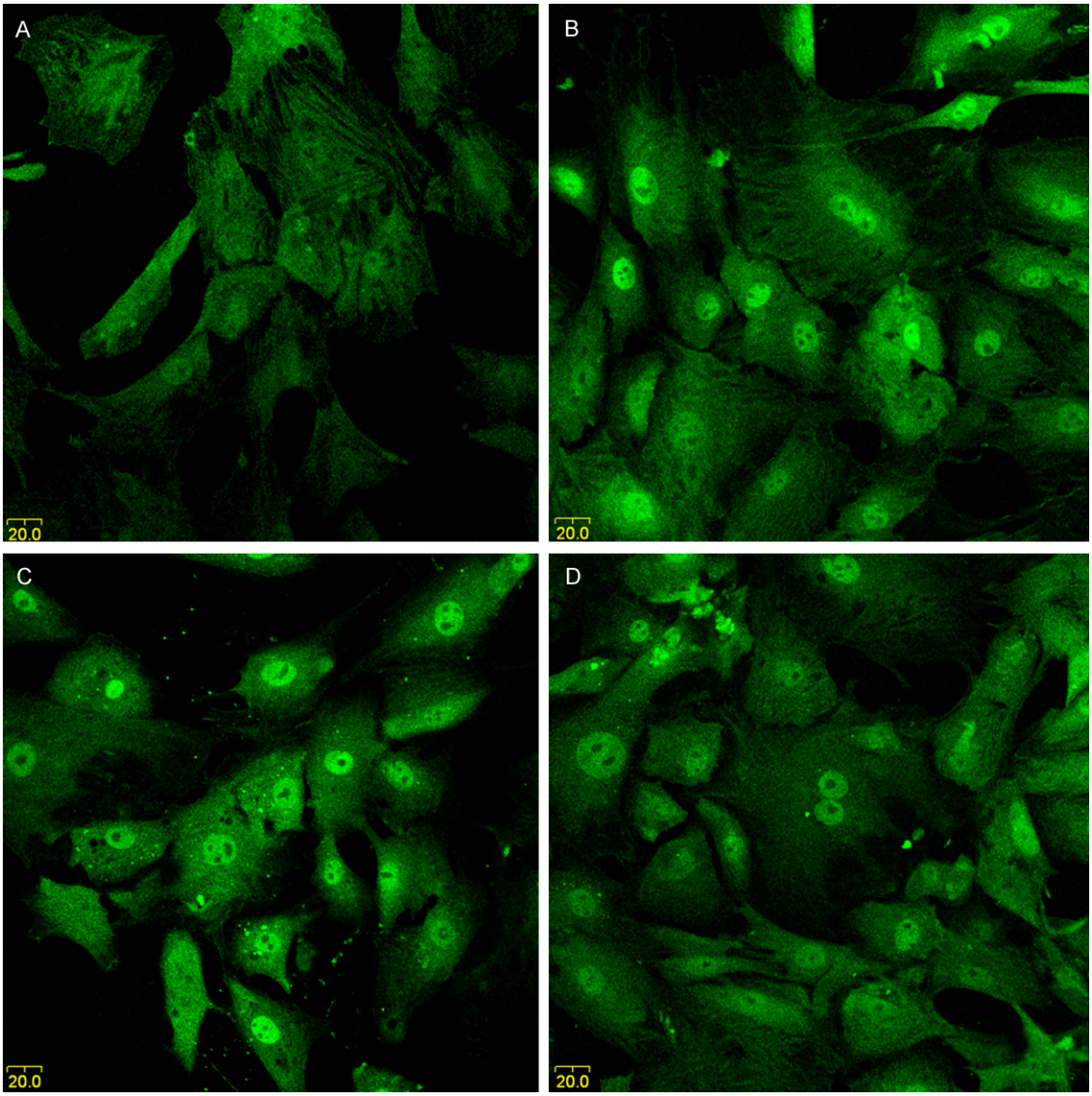

Figure 1. Expression of NFAT nuclear translocation which detected by immunofluorescence (indicated by green fluorescent) after treatment for 24 h. A. The normal control (NC), B. The ischemia/reperfusion (I/R) injury cell model (MG), C. The Ischemia/reperfusion (I/R) injury cell model treated with C5a (50 nmol/L) (MG + C5a), D. The ischemia/reperfusion (I/R) injury cell model treated with C5aR antagonist (2.5 μmol/L) (MG + anti-C5aR).

secondary antibody: anti-rabbit IgG (1:1000, Beyotime, China) was used for TNF-α's and GAPDH's primary antibodies, except for IL-6 which used anti-mouse IgG (1:1000, Beyotime, China).

Flow cytometry

Apoptosis was assessed via flow cytometric analysis of each group that was stained with FITC-Annexin V and PI using the Annexin V-FITC Apoptosis Detection kit according to the manufacturer's protocol (Becton, Dickinson and Company, BD, USA). NRK-52E cells were seeded onto sterile 25 cm^2 culture flasks (1.0×10^5 cells per flask). After cultured and dealt separately, the cells were collected, washed in PBS and resuspended in 190 μl of Annexin V-FITC and 10 μl propidium iodide (PI) at 1.0×10^5 cells ml^{-1}. Cells were then incubated for 10 min at room temperature with Annexin V-FITC and PI stain under the conditions of protection from light. Samples were immediately analyzed via flow cytometry. Annexin V staining was detected as green fluorescence and PI as red fluorescence.

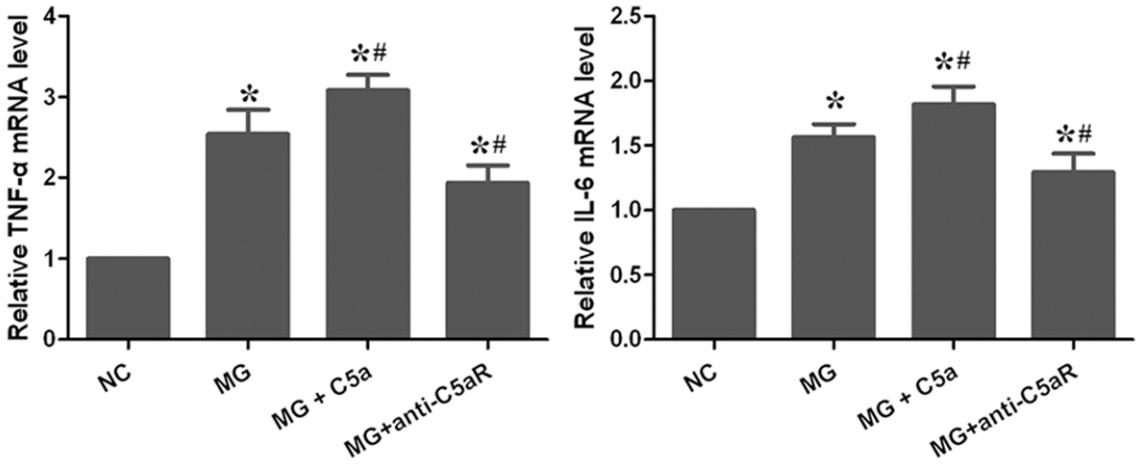

Figure 2. Expression of TNF-α and IL-6 mRNA detected by Real-time PCR after treatment for 24 h. Data are presented as mean ± SD, n=3. *Compared with normal control (NC), $P<0.05$. #Compared with ischemia/reperfusion (I/R) injury cell model (MG), $P<0.05$.

Statistical analysis

In this paper, all the experimental data were presented as means ± standard deviation (SD) of the number of experiments indicated in the legends. Analysis of variance (ANOVA) was performed using SPSS 17.0 software. $P<0.05$ was considered statistically significant.

Results

NFAT nuclear translocation detected by immunofluorescence

NFAT presents in the cytoplasm normally until dephosphorylation has happened. In this result (**Figure 1**) we can find the NFAT crossed the nuclear membranes and into the core obviously in group II, group III and group IV (vs group I). The level (fluorescence intensity) of nuclear translocation in group III was higher than group II and group IV, and contrast with group II, the nuclear translocation level in group IV showed a decrease. The results told us C5a can promote nuclear translocation of NFAT, and the existence of C5aR antagonist can reduce the level of NFAT's nuclear translocation.

Related mRNA and protein expression

In this study, we investigated the TNF-α mRNA and protein expression in cultured NRK-52E cell in each group. We also detected another factor--IL-6, which can participate in the inflammatory reaction express in several kinds of cells, such as activated T-cell and B-cell, epithelial cell, endotheliocyte, macrophages, and so on. TNF-α and IL-6 mRNA and protein expression were both increased obviously when C5a was added to the culture (**Figures 2** and **3**). The results told us C5a can promote the expression of the inflammatory factor TNF-α. Oppositely, when the C5a receptor-C5aR antagonist was added to the culture, we detected that the expression of TNF-α, contrasted with group II, was decreased. At the same time, TNF-α in group I, which is the normal cells group, was weakly expressed than any other groups.

Cell apoptotic situation analysis by flow cytometry

To further intuitively show the effect of each group that was treated with differently, flow cytometry was carried out here. The results (**Figure 4**) showed us clearly that the apoptotic level was much higher than any other groups in group III, even the group II. But in group IV, which C5aR antagonist was added into, the apoptotic level was lower than group II and group III. All above told us that the C5a/C5aR must plays an important role in this process, and they can induce the apoptotic during relevant complement or antagonist was added into.

Discussion

The C5a/C5aR, we introduced at the beginning of the paper, can promote the I/R injury. They belong to complement. Complement is composed of more than 30 kinds of proteins, widely present in the serum, tissue fluid and cell sur-

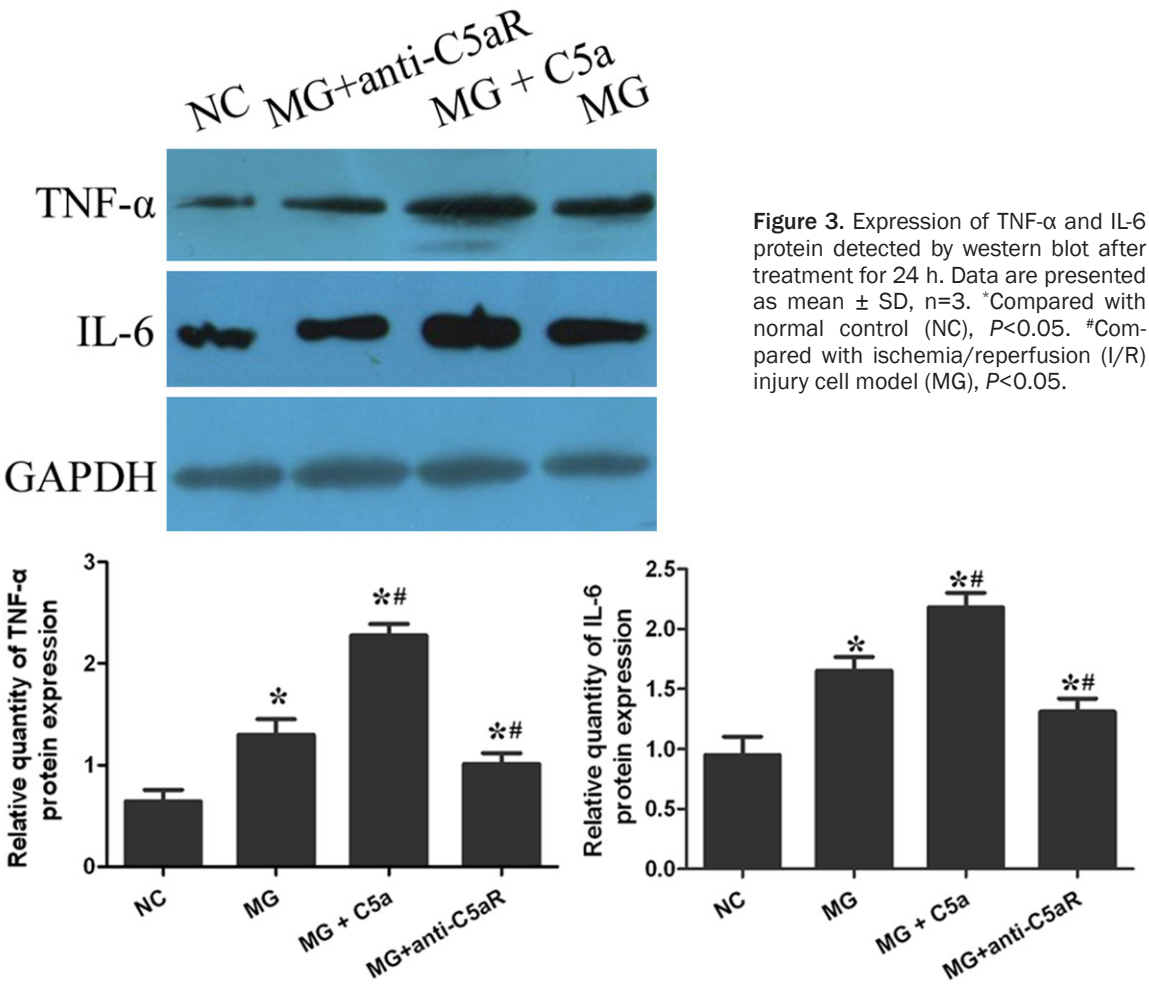

Figure 3. Expression of TNF-α and IL-6 protein detected by western blot after treatment for 24 h. Data are presented as mean ± SD, n=3. *Compared with normal control (NC), $P<0.05$. #Compared with ischemia/reperfusion (I/R) injury cell model (MG), $P<0.05$.

face, which equip protein response system with sophisticated control mechanism. The activation of complement includes a series of cascade of enzymatic reactions of serine proteases.

The complement system, involved in specific and nonspecific immune mechanisms, is an important effect system and effect of amplification system in organism, and the system performs as anti-microbial defense response, immune regulation and mediating immunopathology damage response [7]. The complement system, presents in a fresh normal serum of human and vertebrates, is a group of nonspecific globulin. And it is related to the activity of enzyme. It named because of it is recognized as a useful adjuvant substance to antibody's cytolytic effect during the 19th century of an immune lysis and immune hemolytic reaction's study. Complement contains 9 kinds of ingredients, which named C1, C2, C3, ..., C9 respectively. And most components present in the serum as a precursor form of the enzyme which needs to become the antigen-antibody complex or activate by other factors so that it can play the role of biological activity. And this is the classical pathway of complement. In recent years, scientists found an alternative activation pathway and some other activation pathway. They still found many other factors in the serum involved in the activation of these pathways too. In addition, many factors to inactivate complements have been found also. Therefore, the complement activity and related factors of regulation were collectively known as the complement system. The complement system is a multi-component system and at least has two or more different activation pathways.

As we known, when the renal ischemia and hypoxia has happened, oxidative phosphorylation in kidney cells began to stop, accompanied with the decrease of production of ATP, the

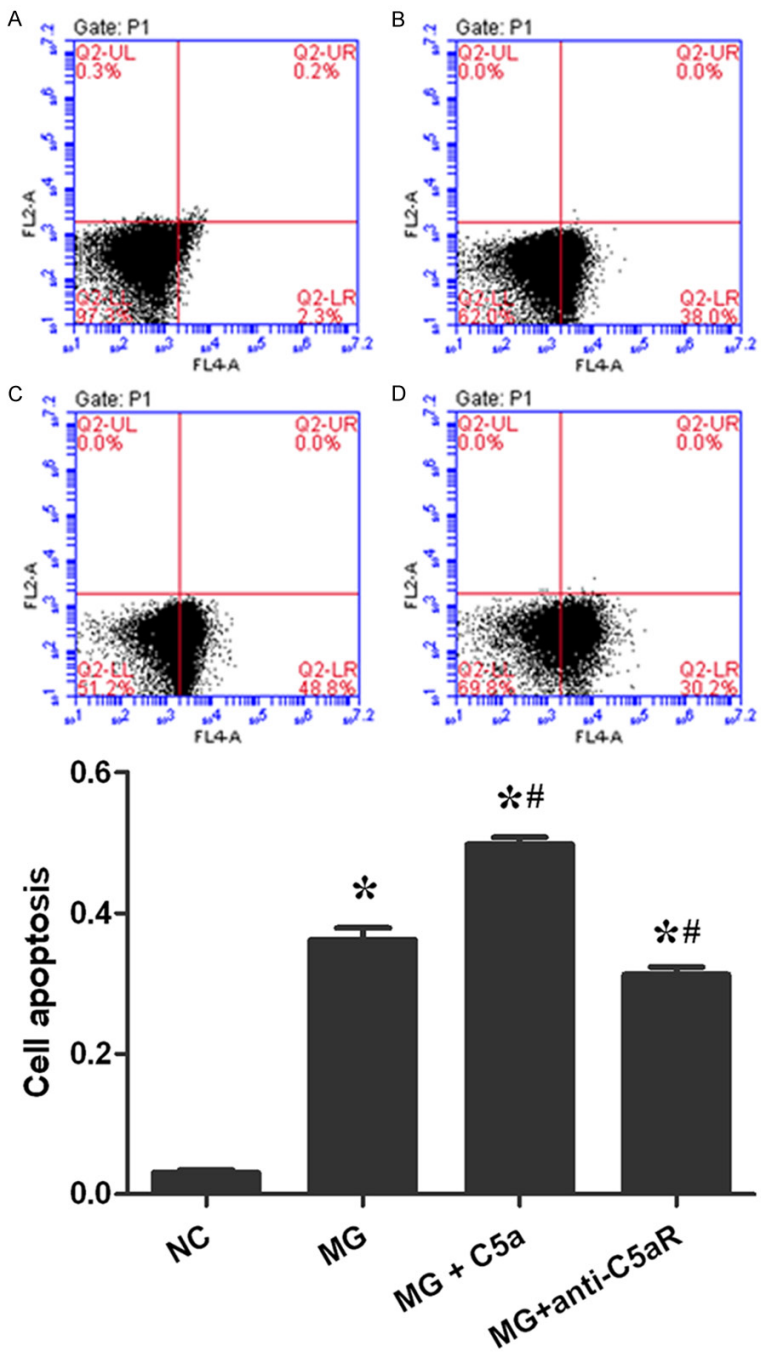

Figure 4. Effect on cell apoptosis with different addition in different group after treatment for 24 h. A: NC, B: MG, C: MG + C5a, D: MG + anti-C5aR. Data are presented as mean ± SD, n=3. *Compared with normal control (NC), $P<0.05$. #Compared with ischemia/reperfusion (I/R) injury cell model (MG), $P<0.05$.

rapid consumption of the stored energy, the activity of Na^+-K^+-ATP enzyme has decreased and positioning has also changed, so all the changes lead to the activities inside and outside the cell disordered. Na^+, K^+, Ca^{2+} is limited transmembrane transport, Na^+ cells, Ca^{2+} increased the formation of Ca^{2+} imbalance, causing cell damage and cell dysfunction and pumps. Research have shown that inhibition of C5aR diminished in vivo production of the pro-inflammatory cytokine TNF-α and chemokines MIP-2 and KC, resulting in the reduction of neutrophils influx and cell necrosis in renal tissues [3], and in our study, we found that inhibition of C5aR also can decrease the expression of TNF-α and IL-6 in vitro.

Nuclear factor of activated T (NFAT) is widely expressed in mammalian cells with transcription activity, which involved in many signaling pathways, it can regulate evolution, growth and differentiation of cell cycle at the transcriptional level. So far, there are five members have been found totally, include NFAT1 (also known as NFATp or NFATc2), NFAT2 (also known as NFATc or NFATcl), NFAT3 (also known as NFATc4), NFAT4 (also known as NFATx or NFATc3) and NFAT5, which the first four members depend on the regulation of calcium signaling pathway. NFAT family proteins contain three functional domains: Rel-similarity domain (DNA-binding activity and interactions with AP-1); NFAT-homology region (intracellular localization); and transcriptional activation domain [8]. Calcineurin is a calcium/calmodulin-activated enzyme that transmits signals to the nucleus through the dephosphorylation and translocation of the NFAT. NFAT is mainly activated by Calcium signaling pathways. Under the resting-state, NFAT presents in the cytoplasm as the form of serine phosphorylation, so we examined the nuclear translocation of NFAT. Once the Ca^{2+} concentration in cytoplasm is increased, Calcineurin (CaN) will be activated

and combines with the highly conserved sequence regions of NFAT, and this will lead to the dephosphorylation of NFAT, exposure of the nuclear localization signal and nuclear translocation, and we found that the expression in nuclear increased gradually in each group with differences in the amount. When getting into the nucleus, NFAT can cooperated with multiple transcription factors and promote the expression of downstream proinflammatory factors. And this process is termination gradually varies with the decrease of Ca^{2+} concentration.

Hyperphosphorylated NFAT transcription factors are sequestered in the cytoplasm, but are immediately translocated to the nucleus after calcineurin-mediated dephosphorylation. Five NFAT genes, each with distinct regulated expression patterns [9, 10]. NFAT 1-4 activate gene transcription by integrating inputs from the calcium/calcineurin and protein kinase C/mitogen-activated protein kinase signalling pathways [11].

In this study, we found that the NFAT nuclear expression, which accompanied with cell apoptosis and changes of TNF-α and IL-6, got up or down with the addition of C5a or anti-C5aR. This suggested us the C5a/C5aR can regulate the apoptosis and TNF-α and IL-6 which are downstream factors of NFAT by regulating the expression of NFAT. And in this study, C5a/C5aR directly promoted the occurrence, deterioration and remission of I/R, and it's mechanism maybe that, C5a/C5aR can induce and promote the occurrence of ischemia-reperfusion injury, so as to promote the increase of local accumulation of calcium ions. The increased calcium promotes the occurrence of NFAT nuclear's translocation, and also cause the changes in the expression of the downstream inflammatory factors which resulting cause the changes of the pathophysiology in the body. The existence of C5a/C5aR and NFAT play a synergies effect in the occurrence of ischemia-reperfusion injury.

Conclusion

All data in our study showed that C5a/C5aR can induce the development of the I/R through regulating NFAT nuclear translocate in I/R vitro model.

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Disclosure of conflict of interest

None.

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