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Original Article

Coexistence of micro-inflammatory and macrophage phenotype abnormalities in chronic kidney disease

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Abstract: The heterogeneity of macrophages promotes renal fibrosis and plays an important role in the repair of kidney damage. The "microinflammation state" is closely related to accelerated mortality in patients with chronic kidney disease (CKD). The aim of this study was to investigate the relationship between microinflammation and macrophage polarization in CKD. The levels of high-sensitivity C-reactive protein (hs-CRP), interleukin-6 (IL-6), and tumor necrosis factor-α (TNF-α) in peripheral blood of 30 non-dialysis CKD-5 patients (CKD group) and 20 healthy subjects (Con group) were measured. Peripheral mononuclear cells (PBMC) of each group were obtained, induced to differentiate into mature macrophages, and the expression of CD206 on the surface of macrophage M2 was detected. The expression of IL-10, TGF-β1 and TNF-α in the supernatant of macrophage culture medium was detected by real time RCR and ELISA. We found that the levels of hs-CRP, IL-6 and TNF-α in peripheral blood of patients with CKD were significantly higher than those of the control group. The expression of CD206 in macrophages was significantly decreased in CKD patients. The anti-inflammatory cytokines IL-10 and TGF-β1 in the supernatant of CKD macrophages decreased significantly, while the pro-inflammatory factor TNF-α did not change significantly. Our results demonstrate that the expressions of macrophage phenotype and anti-inflammatory cytokine in CKD patients are abnormal, which may be related to the microinflammation state prevalent in CKD patients.

Keywords: Microinflammation, macrophage phenotype, C reactive protein, chronic kidney disease

Introduction

Chronic kidney disease (CKD) has a high incidence worldwide, a high incidence of cardiovascular and cerebrovascular complications, and a high mortality rate. Society has a low awareness of CKD and a low rate of prevention and treatment, which causes severe physical and psychological shocks to patients, and brings huge financial burdens to patients' families and governments. In the past ten years, many studies have shown that "microinflammatory states" are common in CKD patients. This pathologic state runs through the disease and participates in the occurrence and development of anemia, malnutrition, and atherosclerotic cardiovascular and cerebrovascular diseases. It is closely related to the accelerated progression of CKD and the high mortality rate of CKD [1]. With a decline of renal function, the incidence of microinflammatory state gradually increases, and the microinflammatory state is more obvious in maintenance hemodialysis patients [2]. Microinflammatory state is a mild, slow and persistent immune inflammatory reaction centered on activation of the monocyte macrophage system, which is caused by non-pathogenic microorganism infection. The main manifestations are slight increase of circulating proinflammatory cytokines such as interleukin-1, IL-6, tumor necrosis factor α, serum amyloid A, fibrinogen, and C-reactive protein (CRP). Macrophages are differentiated from monocytes in circulating blood and are important cells for the immune response. Their main functions are tissue immune monitoring and immunosuppression. They conduct immune surveillance of their surroundings, identify signals of invasive microorganisms and tissue damage, stimulate activated lymphocytes and other immune cells, and defend against microbial infections. Macrophages have strong plasticity and change their physiological charac-

teristics according to different environments. According to their activation function, they can be divided into 2 categories: classical activated macrophages (M1), and alternatively activated macrophages (M2). The function of M1 is to protect the host from microbial infection and anti-tumor. It can secrete inflammatory mediators such as TNF-α, IL-1, NO, IL-12, and IL-23. In addition to resisting microbial infection, it also participates in a variety of chronic inflammation and autoimmunity Disease pathogenesis. M2 has the function of inhibiting inflammation and promoting wound healing. It secretes TGF-β1 and PDGF, promotes the growth of fibroblasts, and has the function of regulating Th2 type inflammatory response. Macrophages can both play a pro-inflammatory and tissue damage role, and also control the inflammatory response and participate in tissue repair [3]. This "dual effect" of macrophages is closely related to its different activation states, which ultimately determine the development direction of kidney disease [4]. It was found that M1 macrophage was significantly related to the induction of renal injury, and the removal of M1 macrophage could reduce the degree of renal injury; on the contrary, M2 macrophage could reduce the inflammatory response and promote tissue repair, which might be a potential therapeutic tool for anti renal injury. Therefore, to study the change trend and role of macrophages with different activated phenotypes in the process of renal injury and repair plays a key role in elucidating the molecular mechanism of the disease and related treatment [5]. This biological effect difference of macrophages in kidney injury is related to its phenotypic heterogeneity. In different microenvironments, macrophages can be polarized into two distinct functional states that induce inflammatory damage (M1) or promote repair (M2) [6]. Different activated phenotypes of these two macrophages may be involved in the inflammatory damage and repair process of renal tissue. What is the relationship between the microinflammatory status and the macrophage polarization status in patients with CKD? In this study, we investigated the relationship between the phenotype of mature macrophages induced by peripheral mononuclear cells (PBMC), the expression of inflammatory cytokines and the state of microinflammation in non-dialysis CKD-5 patients.

Materials and methods

Research objects

CKD is defined as chronic renal structure and dysfunction caused by various reasons (the history of renal damage is more than 3 months), including normal or abnormal pathological damage of GFR, abnormal blood or urine and imaging abnormalities, or the unexplained decrease in GFR (< 60 ml/min·1.73 m^2) for more than 3 months [7]. The CKD-5 stage was GFR < 15 ml/min·1.73 m^2. A total of 30 cases of CKD-5 non dialysis patients in the Department of Nephrology, Affiliated Hospital of Nantong University were selected as the study object, and 20 healthy persons matched in gender and age were selected as the control group. Exclusion criteria: ① there are diseases of heart, liver, lung and other important organs; ② there is a history of infection or vaccination in the past month; ③ various secondary kidney diseases; ④ there are mental diseases or a patient cannot cooperate. This study was approved by the ethics committee of Affiliated Hospital of Nantong University. All participants or their families have signed informed consent.

General indicator

The enrolled patients received peripheral venous blood on an empty stomach in the morning, and the blood routine test was performed using a fully automatic Sysmex Xe 2100 hematology analyzer. The Roche Cobas 8000 automatic electrochemiluminescence immunoassay analyzer was used for blood biochemistry. Another 4 mL of fasting venous blood was centrifuged, and the serum was separated and stored at -80°C. The expression levels of hs-CRP, IL-6 and TNF-α were detected by ELISA according to the kit (American R&D Systems).

Detection of macrophage surface marker CD206

About 5 ml of fresh anticoagulated peripheral blood was taken from the two groups. Peripheral blood mononuclear cells (PBMC) were isolated by density gradient centrifugation (Ficoll method). CD14+ monocytes were obtained by magnetic bead sorting, and macrophage colony-stimulating factor (M-CSF) (Pepro Tech) was added for mature macrophages inducing. We

Table 1. Real-time PCR primers

Primer	Primer sequence (5′→3′)
IL-10	F: GAGATGCCTTCAGCAGAGTGAAGA
	R: AGTTCACATGCGCCTTGATGTC
TGF-β	F: GCGACTCGCCAGAGTGGTTA
	R: GTTGATGTCCACTTGCAGTGTGTTA
TNF-α	F: TGCTTGTTCCTCAGCCTCTT
	R: CAGAGGGCTGATTAGAGAGAGGT
GAPDH	F: AGGCTAGCTGGCCCGATTTC
	R: TGGCAACAATATCCACTTTACCAGA

exchanged the culture solution, and removed M-CSF culture for 2 days and then set it aside. Human FcR Blocking was incubated with macrophages at room temperature, labeled with anti-human CD14-PE and anti-human CD206-APC (eBioscience) antibody, and washed with PBS and detected by flow cytometry.

Macrophage-associated cytokine mRNA expression

RNA was extracted with a kit (TIANGEN, Beijing) according to the operating instructions, and cDNA was reverse transcribed using the TaKaRa Rrime ScriptTM RT kit. Real-time PCR was performed using SYBR Green MasterMix reagent with GAPDH as an internal reference. The expression of the target genes IL-10, TGF-β, and TNF-α was detected. Each primer sequence is shown in **Table 1**.

Macrophage-associated cytokine protein expression

The macrophage culture solution was replaced, cultured at 37°C for 48 hours, and the culture supernatant was collected and stored in a -80°C refrigerator for use. After collecting the supernatant of the cells, the contents of IL-10, TGF-β, and TNF-α in the cell supernatant were measured by an ELISA kit (American R&D Systems, Inc.) according to the relevant instructions.

Statistical analysis

Normally distributed variables are expressed as mean ± standard deviation ($\bar{x} \pm s$) and continuous variables using Student's t test. The categorical variable uses a chi-square test. Statistical analysis was performed using SPSS 22.0 analysis software. $P < 0.05$ was considered significant.

Results

Patient characteristics

There were no significant differences in gender and age between the two groups ($P > 0.05$). Compared with the control group, the levels of BUN, Scr, UA, and PTH in the CKD group were increased, and the levels of Hb, Alb, and GFR were decreased, and the differences were significant ($P < 0.05$), detailed clinical characteristics were shown in **Table 2**.

Comparison of inflammation indexes between the two groups

Compared with the control group, hs CRP (12.32 ± 4.57 mg/L vs. 3.51 ± 1.02 mg/L), IL-6 (32.09 ± 9.05 pg/ml vs. 17.64 ± 5.24 pg/ml), TNF-α (25.55 ± 8.03 pg/ml vs. 10.61 ± 3.34 pg/ml) in CKD group were significantly increased ($P < 0.01$), as shown in **Figure 1**.

CD206 expression on macrophage surface

CD206 is an important surface marker of macrophage M2. We induced monocytes in CKD-5 patients and control group to differentiate into mature macrophages. The expression of macrophage surface marker CD206 was detected by flow cytometry. The results showed that compared with the control group, the expression of CD206 in macrophages of CKD-5 patients was significantly lower ($P < 0.01$), as shown in **Figure 2**.

Macrophage related factor expression

To investigate the presence of proinflammatory and anti-inflammatory cytokines secreted by macrophages in CKD patients, we examined the levels of IL-10, TGF-β, and TNF-α in macrophage culture supernatants. After 48 hours of culture in the differentiation-inducing system, we examined the mRNA and protein expression of macrophage-associated factors in the culture supernatant. The results showed that the macrophage anti-inflammatory factors IL-10 and TGF-β were significantly decreased in CKD patients, but the expression level of pro-inflammatory cytokine TNF-α was not statistically different, suggesting that the function of some

Table 2. Comparison of general data between the two groups

	n	Men/women	Age (years)	Hb (g/L)	Alb (g/L)	BUN (mmol/L)	Scr (μmol/L)	UA (μmol/L)	GFR (mL/min)	PTH (pg/mL)
Con group	20	13/7	54.19 ± 18.32	142.09 ± 16.28	45.02 ± 4.55	5.56 ± 1.53	72.16 ± 13.22	358.33 ± 67.79	105.78 ± 13.13	28.51 ± 6.45
CKD group	30	18/12	53.21 ± 15.73	101.22 ± 22.17*	37.92 ± 6.48*	12.70 ± 6.02*	721.4 ± 55.08*	517.67 ± 99.55*	10.56 ± 3.71*	468.52 ± 89.06*

Note: compared with the control group, *P < 0.05.

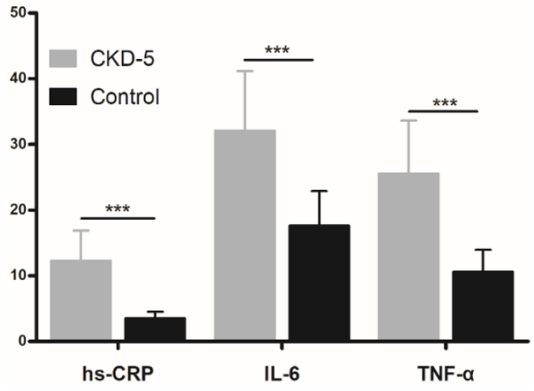

Figure 1. Comparison of inflammation indices between CKD group and control group.

anti-inflammatory factors in macrophages of CKD patients is abnormal (**Figures 3**, **4**).

Discussion

Our study showed that compared with the control group, hs CRP, IL-6, and TNF-α were significantly increased in the CKD group, and the difference was significant ($P < 0.01$), indicating a general microinflammatory state in CKD patients. The state of microinflammation is not the same as the inflammatory reaction caused by infection, but a slight inflammatory reaction caused by the stimulation of endotoxins, microorganisms, complement, various chemicals and immune complexes, activation of the monocyte-macrophage system, or release of inflammatory cytokines such as CRP, IL-6, IL-1, and acute phase proteins [8]. In 2000, Schoming and other scholars took the lead in proposing this concept. They believe that the increase in acute protein levels and pro-inflammatory mediator levels in patients with end-stage renal disease is an important signal indicating a chronic inflammatory response in patients [9]. For CKD patients, the persistent microinflammatory state is more harmful to the body than the traditional cardiovascular risk factors and uremic toxins. CRP, IL-6, TNF-α, and fibrinogen are the main markers of inflammation. At present, the pathogenesis of the microinflammatory state in CKD patients has not been fully understood, which may be related to immune disorders and inflammatory activation caused by various factors. With the decline of glomerular filtration function and the decrease in cytokine clearance, the production of inflammatory factors increases, which leads to their increase in the blood. Epigenetic changes such as genetic factors, lifestyle, eating habits and accumulation of uremic toxins in the body can further activate the inflammatory response [10, 11]. The changes of uremic toxin metabolism and dietary habits in CKD patients can lead to an imbalance of intestinal flora and dysfunction of the mucosal barrier, cause the transfer of intestinal bacteria and uremic toxins in vivo, and further promote the systemic inflammatory response [12]. In the late stage of CKD, many kinds of metabolites cannot be excreted normally, and remain in the body, stimulating cells to produce oxidative stress [13, 14].

Under physiologic conditions, macrophages can not only phagocytose, kill, and eliminate pathogens, but also process and present antigens, initiate immune response, and play an important role in maintaining tissue homeostasis. Macrophages have significant heterogeneity, differentiate in specific microenvironments, and acquire two independent activation phenotypes: classically activated macrophages (M1) and alternatively activated macrophages (M2) [15]. We induced mononuclear cells to differentiate into mature macrophages. Expression of the macrophage surface marker CD206 (surface marker of M2) was detected by flow cytometry. The results showed that the expression of CD206 in macrophages of CKD-5 patients was significantly lower than that of the control group ($P < 0.01$). This indicates that macrophage cells in the CKD-5 stage have a barrier to M2 transformation. We further examined the expression of mRNA and protein of macrophage-associated factors in culture supernatants. The results showed that the macrophage anti-inflammatory factors IL-10 and TGF-β were significantly decreased in CKD patients, but the expression of pro-inflammatory cytokine TNF-α was not significantly different, suggesting abnormal function of some anti-inflammatory factors in macrophages of CKD patients. M1/M2 are the two extremes of macrophage phenotypic differentiation. In vivo, macrophages simultaneously contact multiple microenvironmental signals at the same instant [13, 16, 17]. Therefore, the polarization process of macrophage phenotype in vivo is more complex than that stimulated by a single factor in vitro. Macrophages with different phenotypes can coexist in the same tissue, while macrophages can also express M1 and

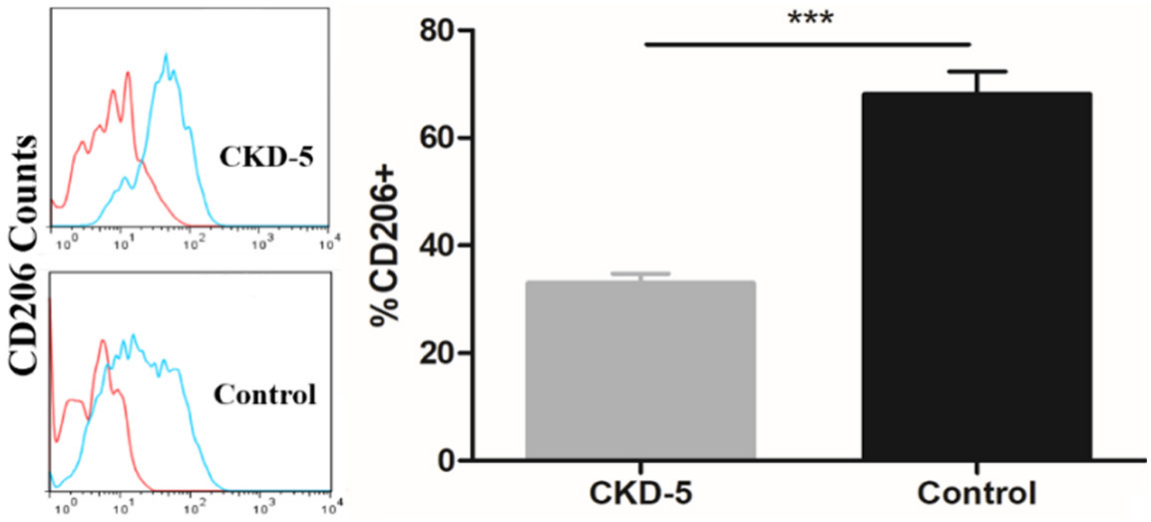

Figure 2. Expression of CD206 in macrophages of CKD group and control group.

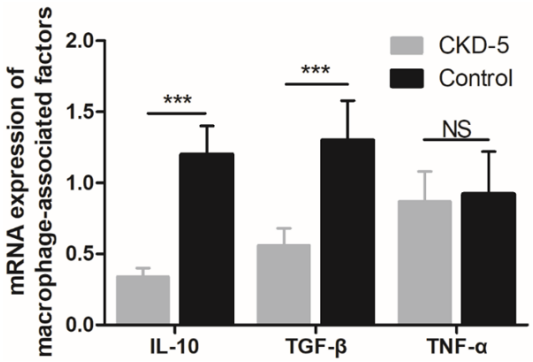

Figure 3. mRNA expression of macrophage-associated factors.

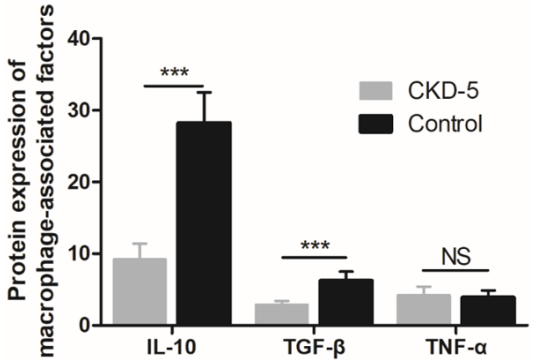

Figure 4. Protein expression of macrophage-associated factors.

M2 markers at the same time [15]. Under some conditions, polarized M1 and M2 can also be transformed into each other [18, 19]. We found that peripheral nucleated cells (PBMC) are more likely to differentiate into pro-inflammatory M1 type and show abnormalities in the production of partial anti-inflammatory factors by macrophages in CKD. With macrophage dysfunction, pathogenic bacteria cannot be effectively eliminated, producing a large number of pro-inflammatory factors such as TNF-α, IL-6, and TGF-β, eventually leading to systemic inflammation, and aggravating a systemic microinflammatory response [20]. At the same time, the presence of a microinflammatory state in CKD patients promotes macrophages to differentiate into pro-inflammatory M1, stimulates a systemic immune response, and ultimately leads to CKD complications.

In conclusion, macrophage phenotype and anti-inflammatory cytokine expression in CKD patients are abnormal, which may be related to the widespread microinflammatory state. Therefore, the study of macrophage function changes in CKD patients helps to understand the causes of microinflammation in vivo, and is of great significance for the treatment of microinflammation level and related complications.

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Disclosure of conflict of interest

None.

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