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Original Article

Clinicopathological significance of CD206-positive macrophages in patients with acute tubulointerstitial disease

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Abstract: Objective: To investigate the clinicopathological significance of CD206-positive macrophage expression in patients with acute tubulointerstitial disease, including acute tubular necrosis (ATN) and acute interstitial nephritis (AIN). Methods: Renal tissue samples from patients with ATN (n=10), AIN (n=10), and minimal change disease (MCD, as disease control, n=8) as well as tissue from normal control kidneys (negative control, n=3) were included in this study. The expression of CD206 and CD68 in renal tissues was detected by immunohistochemistry or immunofluorescence. Results: CD206-positive cells accumulated in areas around damaged tubular cells and regenerating tubules. Compared with AIN patients, ATN patients had lower serum albumin, lower proteinuria, lower urinary osmolality and higher plasma hemoglobin, (P=0.002; P=0.01; P<0.001; P=0.002, respectively). CD206-positive cells could also be observed in the tubular basement membrane and tubule lumen. Some CD206-positive cells infiltrated into the tubular cells in patients with AIN. Compared to patients with ATN, patients with AIN had more CD206-positive cells (P=0.005). In the ATN patients, there were more CD206-positive cells in ischemic tissue. CD206-positive cells were negatively correlated with hemoglobin (r=-0.565, P=0.009) and positively correlated with serum albumin (r=0.496, P=0.026), urinary osmolality (r=0.567, P=0.009) and proteinuria (r=0.460, P=0.041). There was no correlation between CD206-positive cells and eGFR. Conclusion: CD206-positive macrophages are involved in the pathogenesis of acute tubular necrosis and acute interstitial nephritis.

Keywords: CD206, macrophage, acute tubular necrosis, acute interstitial nephritis

Introduction

Acute kidney injury (AKI) is a well-recognized risk factor for the development of chronic kidney disease (CKD) in humans. The severity of AKI is correlated with the rate of progression to CKD. Tubular injury plays an important role in the progression of CKD [1]. Furthermore, recent studies showed that macrophage phenotypes were correlated with long-term AKI outcome [2, 3].

Macrophages are divided into classically activated (M1) and alternatively activated (M2) types based on their diverse functions and phenotypic plasticity [4]. M1 macrophages are characterized by a pro-inflammatory phenotype, and M2 macrophages have regulatory functions in tissue repair and remodeling [5-7]. One characteristic of M2 macrophages is increased expression of the mannose receptor (CD206) [5-7]. A previous study reported that mesenchymal stem cells ameliorate rhabdomyolysis-induced acute kidney injury via activation of macrophages to a trophic M2 phenotype, which supports a functional transition from tubule injury to tubule repair [8]. In animal models of rhabdomyolysis or ischemia/reperfusion-induced acute renal injury, aristolochic acid nephropathy and adriamycin nephropathy, reparative proliferation has been correlated with polarization from M1- to M2-type macrophages [9-12].

However, the clinicopathological significance of CD206-positive macrophages in patients with acute tubulointerstitial disease remains unknown. This study aims to investigate the clinicopathological significance of CD206-positive macrophages in patients with acute tubulointerstitial disease

erstitial disease, including acute tubular necrosis (ATN) and acute interstitial nephritis (AIN).

Subjects and methods

Subjects

Formalin-fixed, paraffin-embedded renal tissues from core needle biopsies derived from patients with ATN (n=10), AIN (n=10), and minimal change disease (MCD, as a disease control, n=8) as well as from normal control kidneys (negative control, n=3) were included in this study. The percentage of global glomerular sclerosis in all ATN patients was within the range of the corresponding age (i.e., no more than [(age/2) -10] %).

Pathologic characteristics of the ATN and AIN patients were scored. These characteristics included interstitial infiltration of inflammatory cells, renal tubular injury (vacuolization, granular degeneration, necrosis and detachment), naked tubular basement membrane, percentage of global glomerular sclerosis and focal glomerular sclerosis, and interstitial fibrosis.

Patients with acute inflammatory diseases, such as fever, urinary infection, pneumonia and sepsis were excluded. All biopsies were obtained before any medical intervention. Written informed consent was obtained from each patient. This study was in compliance with the Declaration of Helsinki. The study protocol was approved by the Ethics Committee of the Clinical Medical College of Yangzhou University.

Immunohistochemical detection of CD206 and CD68

Thin sections (2 μm) of the renal biopsies were deparaffinized and dehydrated using a series of xylene and alcohol washes. Following quenching endogenous peroxidase activity with 3% (vol/vol) H_2O_2 in methanol × 10 minutes, slides were immersed in buffer solution and placed in pressure cooker (100°C, 2 min) for antigen retrieval. Slides were blocked with 1% (wt/vol) bovine serum albumin (BSA) and incubated with monoclonal antibodies against CD206 (1:500, ab64693, Abcam, USA) and monoclonal antibodies against CD68 (1:50, ZM-0464, ZSGB-BIO, Beijing, China) at 4°C overnight in a humidified chamber. Goat anti-rabbit-IgG H + L (1:500, KPL, 474-1516, USA), anti-mouse antibody and anti-rabbit antibody (Ready to Use, GK500705, GENENTECH, USA) were applied for 30 minutes. A chromogen reaction was developed with 3,3' Diaminobenzidine (DAB). The slides were counterstained with hematoxylin and Periodic Acid Schiff (PAS) staining. Negative controls for all the antibodies were incubated with preimmune serum minus the primary antibody, which completely prevented immunostaining.

Immunofluorescence detection of co-localization of CD206 and CD68

Dual staining of paraffin sections was used to examine the co-localization of CD206 and CD68. Antigen retrieval was performed by heating sections to 100°C for 2 minutes in 10 mmol/L sodium citrate buffer (pH 6.0). Sections were first stained with mouse anti-CD68 monoclonal antibody as described above, followed by incubation with rabbit anti-CD206 polyclonal antibody (1:500, ab64693, Abcam, USA). Sections were sequentially incubated with Rhodamine (TRITC)-conjugated goat-anti-rabbit IgG (1:50, Jackson Immuno Research Inc, USA) and FITC-conjugated goat-anti-mouse-IgG (1:50, Jackson Immuno Research Inc, USA). Cover slips were placed on the sections using Glycergel containing 2.5% 1, 4-diazabicyclo octane (DABCO; Sigma) as fading retardant.

Immunohistochemical assessment of biopsies

The number of CD206-positive cells in the glomeruli and interstitium were counted at a magnification of ×400. The number of CD206-positive cells in the glomerular infiltrate was expressed as the number of positive cells/glomerulus. The number of CD206-positive cells in the interstitial infiltrate was expressed as the number of positive cells/high-power field (HPF). Ten fields were selected. The results were expressed as the mean ± standard deviation (SD).

Statistical analysis

The two groups were compared using Mann-Whitney U test for nonparametric data. Correlation analysis was performed using Spearman test for nonparametric data. *P* values <0.05 were considered significant.

Table 1. Clinical data of ATN and AIN patients before renal biopsy

	ATN patients	AIN patients	*P*
Gender (male/female)	6/4	5/5	0.824
Age (y)	45±13	41±11	0.472
Serum albumin (g/l)	35±5	43±2	0.002*
Hemoglobin	124±12	102±11	0.002**
Urinary protein (g/d)	0.2±0.1	0.4±0.1	0.01*
Urinary osmolality (mOsm/l)	397±56	561±47	<0.0001**
eGFR (ml/min)	28±11	30±9	0.821
CD206/tubulointerstitium	8±5	15±3	0.005**

Footnote: *P<0.05, **P<0.01; ATN: Acute tubular necrosis; AIN: Acute interstitial nephritis; eGFR: Estimated glomerular filtration rate.

Table 2. Pathologic data of ATN and AIN patients

	ATN	AIN	*P*
% Glomerular sclerosis	11.25	9.75	0.565
% Focal sclerosis	0	0	-
Interstitial fibrosis	8.33	11.5	0.166

Footnote: ATN: acute tubular necrosis; AIN: acute interstitial nephritis.

Results

Clinical and pathologic data from study patients before renal biopsy

The etiologies of ATN in ten patients included drug-associated (n=8) and ischemic (n=2). The renal pathologic signs included flat proximal renal tubular epithelial cells, loss of brush border, tubular epithelial cell vacuoles, granular degeneration and necrosis. Detachment of the tubular epithelial cells, cell debris in the tubule lumen and expansion of the tubule lumen were observed. In the patients with ischemic ATN, rupture of the renal tubular basement membrane and a naked tubular basement membrane were noted in the renal tissue. Regeneration of the renal tubular epithelial cells was noted in the 3 patients with drug-associated ATN. There was diffuse interstitial edema accompanied by scattered lymphocyte and mononuclear cell infiltration. In the patients with AIN patients, flat proximal renal tubular epithelial cells, a loss of brush border, epithelial cell vacuoles, and diffuse interstitial edema accompanied by a sheet of mononuclear cell infiltration were noted. The etiologies of AIN were drugs (including omeprazole, traditional Chinese medicine and amoxicillin). After the renal biopsy, the AIN patients received oral prednisone (0.5 mg/kg.d). One month after the renal biopsy during the follow-up period, the renal function of the 8 ATN patients returned to normal, except in 2 patients who had improved renal function. Three months after the renal biopsy during the follow-up period, the renal functions of all the AIN patients were improved.

As noted in **Table 1**, compared with the AIN patients, the ATN patients had lower serum albumin, lower proteinuria, lower urinary osmolality and higher plasma hemoglobin (P=0.002; P=0.01; P< 0.0001*;* P=0.002). As noted in **Table 2**, there were no difference of chronic pathologic indices between AIN and ATN patients, including percentage of glomerular sclerosis, focal sclerosis and interstitial fibrosis.

*Expression of CD206 and CD68 in the renal tissue of the negative controls and MCD (**Figure 1**)*

As shown in **Figure 1**, in the normal kidneys, CD68 was occasionally expressed in the tubulointerstitial tissue, and the CD206 staining was almost always negative. In the negative controls (rabbit serum or mouse serum substituted for the primary antibody), CD206 and CD68 staining were both negative. In the MCD, CD206 and CD68 were occasionally expressed in tubulointerstitial tissue.

*Expression of CD206 in acute tubulointerstitial lesions (**Table 1; Figure 2**)*

As shown in **Figure 1**, CD206-positive cells accumulated in areas around damaged tubular cells and regenerating tubules. CD206-positive cells were also observed in the tubular basement membrane and tubule lumen. As shown in **Figure 2**, some CD206-positive cells infiltrated into the tubular cells in patients with AIN. There were more CD206-positive cells in the ATN patients with tubular regeneration.

As shown in **Figure 2**, dual staining showed that CD206 positive cells also expressed CD68.

As noted in **Table 1**, compared with the ATN patients, the AIN patients had more CD206-positive cells (P=0.005). There were more

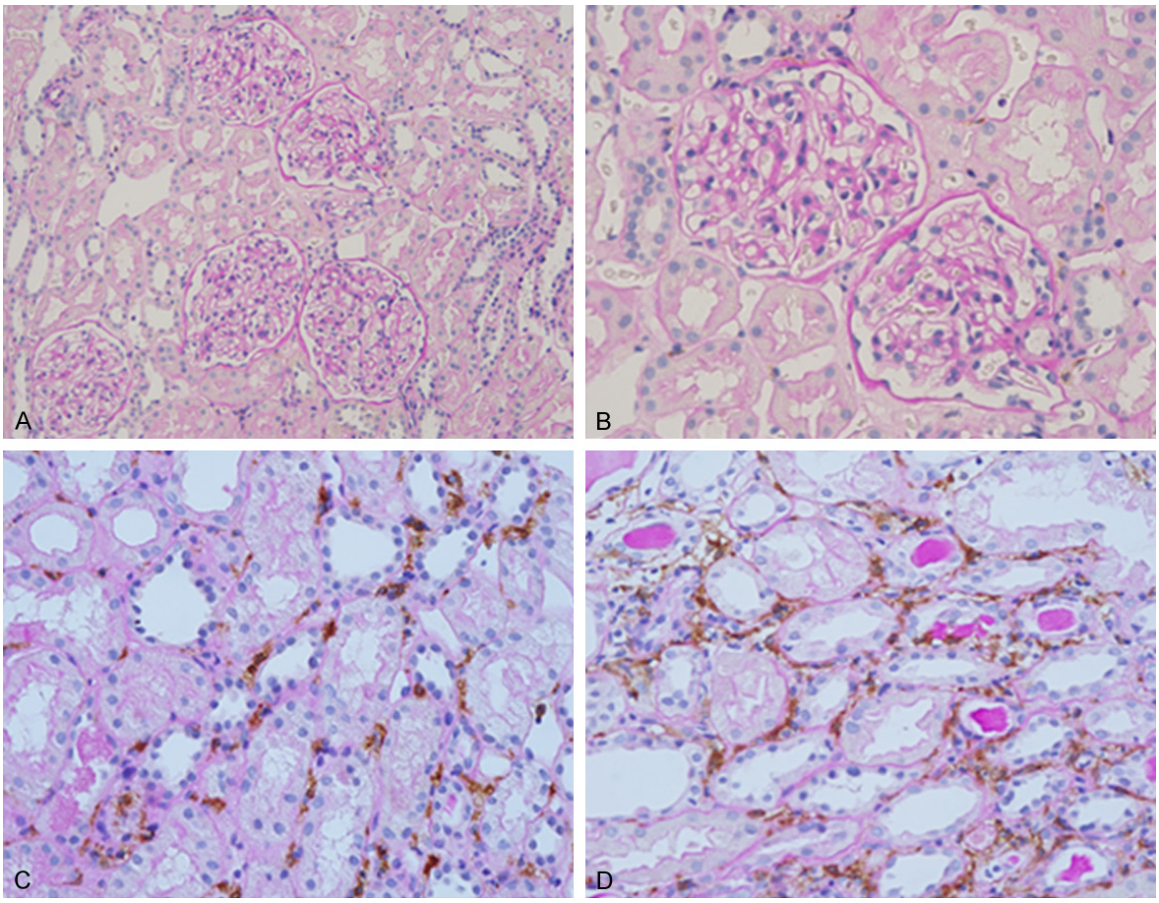

Figure 1. Location of CD206 in normal renal tissue and ATN. A. Location of CD206 in normal renal tissue. B. Location of CD206 in MCD. C. Localization of CD206 in drug associated ATN patients. D. Localization of CD206 in ischemic ATN. Immunohistochemistry (brown). (A. × 200, B-D. × 400).

CD206-positive cells in patients with ischemic ATN.

*Correlation of the number of tubulointerstitial CD206-positive cells with the clinicopathological index (**Table 3**)*

As noted in **Table 3**, the number of CD206-positive cells was positively correlated with serum albumin (r=0.496, *P*=0.026), urinary osmolality (r=0.567, *P*=0.009) and proteinuria (r=0.460, *P*=0.041) and negatively correlated with hemoglobin (r=-0.565, *P*=0.009). There was no correlation between CD206-positive cells and eGFR.

Discussion

Macrophages are key regulators involved in maintaining tissue homeostasis, regulating the immune response and injury repair. Current data suggest that macrophages perform both injury-inducing and repair-promoting tasks in different models of inflammation [4-7]. Macrophages express different surface molecules that have different functions during different stages of renal disease. Studies of AKI animal models support the notion that CD206-positive macrophages are involved in the repair of tubular cells. CD206, which is also called mannose receptor (MR), is a 180-kDa transmembrane protein. It has been identified as a marker of M2 macrophages. MRs on macrophages are involved in endocytosis and phagocytosis, innate host defense, signal transduction and the adaptive immune response [13]. In our study, dual staining showed that CD206-positive cells, which were expressed in acute tubulointerstitial lesions, are a subpopulation of macrophages.

In animal models of rhabdomyolysis-induced acute kidney injury, M2 macrophages are responsible for collagen degradation through a mannose receptor (CD206)-mediated pathway, which contributes to renal repair [14].

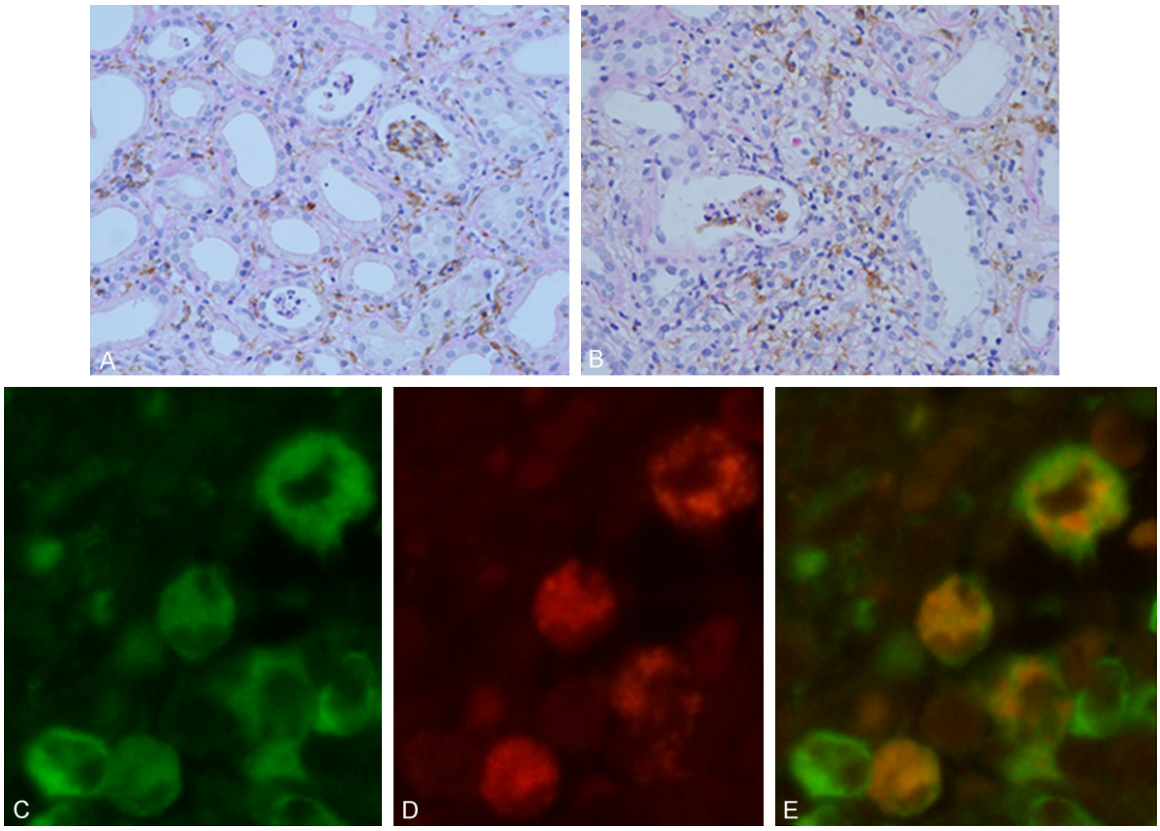

Figure 2. Localization of CD206 and CD68 in ATN and AIN patients. A, B: Localization of CD206 in AIN, Immunohistochemistry. C-E: Localization of CD68 (green), CD206 (red) and co-location (yellow) of CD68 and CD206 in tubulointerstitial lesions of ATN. Immunofluorescence. (A, B: × 400, C-E: × 1000).

Table 3. Correlation of CD206 counts (tubulointerstitial area/HPF) with clinicopathological data in ATN and AIN patients

Spearman's rho r *P*	CD206/ tubulointerstitium
Serum albumin (g/l)	0.496
	*0.026**
eGFR (ml/min)	-0.039
	0.872
Hemoglobin	-0.565
	*0.009***
Proteinuria (g/24 h)	0.460
	*0.041**
Urinary osmolality (mOsm/l)	0.567
	*0.009**

Footnote: **P*<0.05, ***P*<0.01; HPF: high-power field; ATN: acute tubular necrosis; AIN: acute interstitial nephritis; eGFR: estimated glomerular filtration rate.

In our study, infiltrating CD206-positive macrophages were noted in acute tubular necrosis, especially in tubular injury with regeneration. This implies that CD206-positive macrophages are involved in the pathogenesis of ATN, including regeneration. Because the renal function of most of the ATN patients returned to normal after one month, CD206-positive cells may assist in the repair of tubular cells. The mechanism might be as follows: ① negative regulation of migration through MR expression maintenance could help retain macrophages once they have been successfully recruited to sites of tissue damage [15]. ② M2 polarization and the increased expression of CD206 dramatically enhance the capacity for extracellular matrix turnover, cellular uptake and collagen degradation [14].

For AKI with different etiologies, there were different degrees of CD206-positive cell expression. There were more CD206-positive cells in ischemic ATN. CD206-positive macrophages help resolve ischemic ATN in animal models [10]. Therefore, we infer that these cells might play the same role in ATN patients.

In our study, AIN patients had lower plasma hemoglobin, which might be correlated with impaired EPO secretion by tubulointerstitial cells. Previous studies have reported that EPO helps in the recovery of AKI [16]. One study reported that the beneficial properties of EPO are related to its induction of beneficial macrophages and modulation of the immune system to promote anti-inflammatory responses in the peripheral nervous system [17]. Future studies are needed to investigate whether M2 macrophages are involved in AKI recovery by regulating the secretion of EPO.

No studies have previously reported on the expression of M2 macrophage surface markers in AIN. In our study, CD206-positive cells were more highly expressed in AIN. The renal function of the ten AIN patients improved after the prednisone treatment, which implies that CD206-positive cells are involved in acute tubulointerstitial lesions. Previous studies have reported that steroids are potent activators of M2 alternative macrophages [18]. Further studies are needed to investigate the efficacy of infusing of M2 macrophages or steroids in animal models of AIN.

In conclusion, CD206-positive cells accumulated in areas around damaged tubular cells and regenerating tubules, which implies that M2-type macrophages are involved in the pathogenesis of acute tubular necrosis and acute interstitial nephritis. In future research, we plan to investigate the efficacy of therapies with M2 macrophages or steroids in different AKI models and the effects of these therapies on the prognosis of AKI.

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Disclosure of conflict of interest

None.

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