

Original Article

Significance of C4d expression in minimal change disease and focal segmental glomerulosclerosis

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Abstract: C4d is a by-product of the lectin complement pathway. Glomerular C4d deposition is related to poor prognosis in various forms of immune-mediated glomerulonephritis. The aim of this study was to elucidate the role of the lectin pathway in minimal change disease (MCD) and focal segmental glomerulosclerosis (FSGS). C4d immunohistochemical staining was performed on 130 renal biopsy tissue specimens from patients diagnosed with MCD or FSGS at Seoul National University Hospital. C4d expression was lower in the control group than in the MCD and FSGS groups (0.32 ± 0.415 vs 1.31 ± 0.912 and 1.52 ± 1.05 , respectively, $P < 0.001$ for each). Additionally, C4d expression was higher in cases with nephrotic syndrome than without nephrotic syndrome (1.64 ± 1.020 vs 1.09 ± 0.841 , $P = 0.002$). Furthermore, the mean C4d scores were related to the creatinine levels at the final visit in both disease groups ($P = 0.010$, $R^2 = 0.051$). These results suggest that the lectin pathway might be associated with the pathogenesis and prognosis of MCD and FSGS.

Keywords: C4d, lectin pathway, prognosis, minimal change disease, focal segmental glomerulosclerosis

Introduction

The lectin complement pathway is initiated by that mannose-binding lectin (MBL) or ficolins recognise carbohydrate ligands on the surface of microorganisms. MBL binding to the ligands activates MBL-associated serine protease-2 (MASP-2) and subsequent cleavage of C4 [1, 2]. C4d is a split by-product of C4 activation. Although C4d is mainly interpreted as an indication of classical pathway activation, it is also evidence of activation of the lectin pathway when C1q deposition is not observed in involved tissues [3-5]. C4d is a stable molecule and could be easily detected with immunohistochemical staining. Therefore, C4d immunohistochemistry is used as a marker of renal damage in various renal diseases.

C4d deposition is associated with the prognoses of various forms of immune-mediated glomerulonephritis. Complement and immune complex deposition in glomerular disease causes local inflammation and proteinuria by podocyte disruption [6-8]. Glomerular deposition of C4d in IgA nephropathy is associated with more severe renal diseases [9, 10]. Circulating

IgA1 obtained from patients with IgAN induces aberrant glycosylation of O-linked glycans, which are potentially involved in recognition by lectins [9]. Glomerular MBL deposition in Henoch-Schonlein purpura nephritis is also associated with increased progression of renal disease [10]. C4d and MBL expression in pauci-immune crescentic glomerulonephritis is linked to poor prognosis [11, 12]. In lupus nephritis, C4d could be a useful marker for disease activity [13, 14].

Minimal change disease (MCD) and focal segmental glomerulosclerosis (FSGS) are well-known, non-immune-mediated glomerular diseases whose pathogenesis has been associated with podocyte injury [15-20]. An immunofluorescence study in MCD or FSGS patients did not show deposition of C3 or C1q, which are known as footprints of the classical complement pathway. Most investigators believe that the complement systems are not involved in MCD and FSGS [21, 22]. However, recent studies have shown that C4d, a by-product of the lectin pathway, is expressed in some patients with MCD or FSGS [11, 23].

C4d in MCD and FSGS

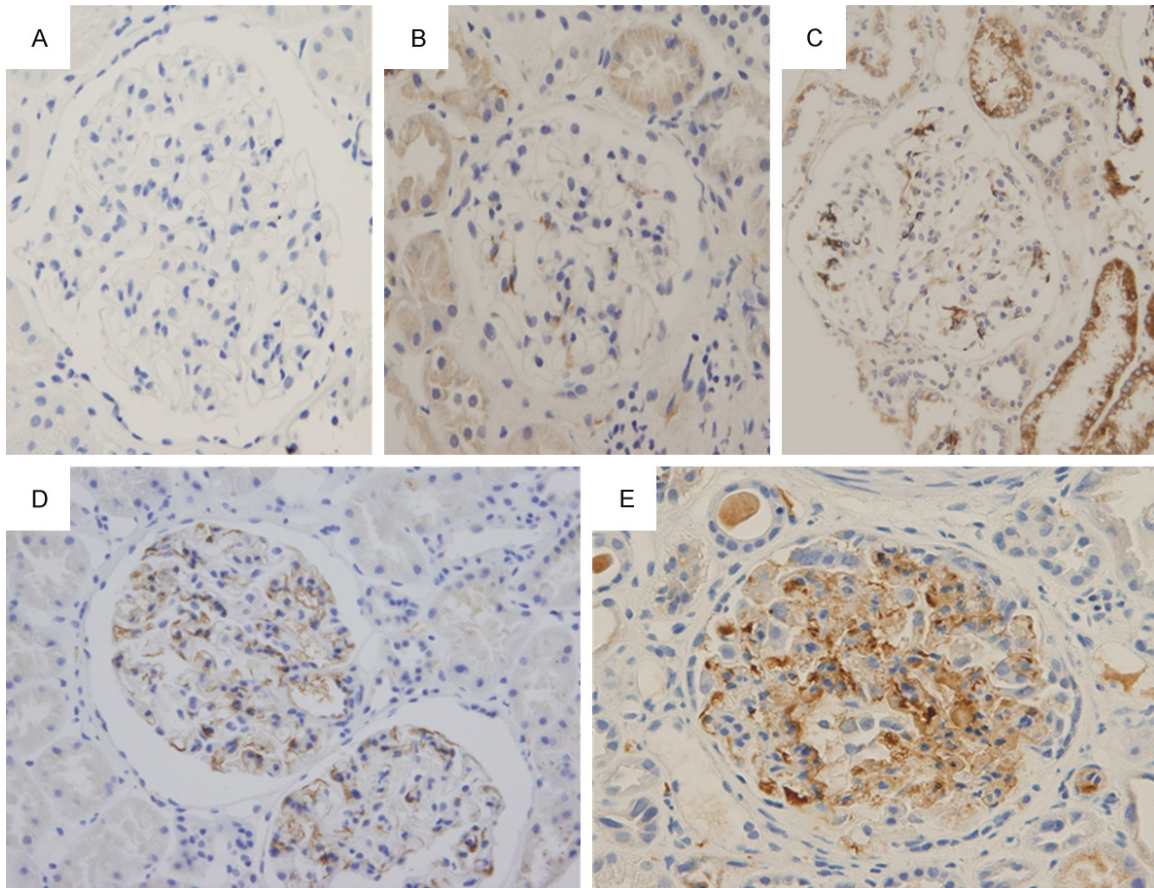


Figure 1. The scoring system of glomerular C4d deposition ($\times 200$). (A) 0 point: negative C4d, (B) 1 point: C4d staining of $< 25\%$ of the entire glomerular area, (C) 2 points: C4d staining of 25% - 50% of the entire glomerular area, (D) 3 points: C4d staining of $> 50\%$ of the entire glomerular area with moderate intensity, (E) 4 points: C4d staining of $> 50\%$ of the entire glomerular area with strong intensity.

The aim of this study is to elucidate the role of the lectin pathway in MCD and FSGS. We performed immunohistochemical staining for C4d using renal biopsy specimens that were obtained from the patients with MCD or FSGS to evaluate the role of the lectin pathway in pathogenesis of these diseases and evaluated the clinicopathologic correlation in MCD and FSGS.

Materials and methods

Patients and renal biopsy

Renal biopsy specimens were recruited from patients who were diagnosed with MCD or FSGS at Seoul National University Hospital between January 2000 and July 2011. A total of 130 patients were enrolled in this study (male patients, 61%; female patients, 39%).

The patients ranged in age from 2 to 77 years. Twenty-four children (18.5%) were younger than 15 years old. Sixty-four patients had MCD and 66 patients had FSGS. Eighteen patients were assigned to the control group; they underwent a renal biopsy for asymptomatic urinary abnormalities but there were no abnormal findings in the pathology reports.

We retrospectively reviewed the clinical characteristics of patients who were diagnosed with MCD or FSGS and followed up with them at least for over 6 months. This study was approved by the Institutional Review Board of Seoul National University Hospital.

Evaluation of renal tissue

Immunohistochemical staining was performed as follows: $2\text{-}\mu\text{m}$ thick sections were prepared from formalin-fixed, paraffin-embedded renal

C4d in MCD and FSGS

Table 1. The demographic and clinical characteristics of the patients

Variable	Value
Male sex (%)	61.5
Age at biopsy (yrs)	33 ± 20.3
Focal segmental glomerulosclerosis (%)	50.8
^a eGFR (ml/min/1.73 m ²)	93.06 ± 45.71
^b Renal insufficiency (%)	22.3
Serum albumin (g/dL)	2.89 ± 0.92
Serum cholesterol (mg/dL)	303.6 ± 148.57
Proteinuria (g/day)	5.3 ± 5.1
Nephrotic syndrome (%)	59.2
Hypertension (%)	22.7

Values are mean ± SD. ^aeGFR was calculated using the CKD-EPI equation. ^bRenal insufficiency was defined as the Cr level > 1.5 mg/dl.

tissue blocks and were dried at 60°C overnight. The sections were placed in a Bond Max Automated Immunohistochemistry Vision Biosystem (Leica Microsystems GmbH, Wetzlar, Germany) according to the following protocol. First, renal tissues were deparaffinised and pretreated with Epitope Retrieval Solution 2 (EDTA-buffer, pH 8.8) at 98°C for 20 min. After washing, peroxidase blocking was carried out for 10 min with a Bond Polymer Refine Detection Kit DC9800 (Leica Microsystems GmbH). Tissues were again washed and then incubated with rabbit polyclonal C4d antibody at 1:100 dilutions (Cell Marque, Rocklin, CA, USA) for 15 min. Thus, renal tissues were incubated with a polymer for 8 min and developed with DAB-Chromogen for 10 min.

We performed immunohistochemical staining for C4d in the MCD, FSGS, and control groups. Each glomerulus is scored as follows (**Figure 1**): 0 points = negative C4d staining, 1 point = C4d staining < 25% of the entire glomerular area, 2 points = C4d staining 25-50% of the entire glomerular area, 3 points = C4d staining > 50% of the entire glomerular area with moderate intensity, and 4 points = C4d staining > 50% of the entire glomerular area with strong intensity. Sclerotic lesions or unapparent staining of the glomerulus was excluded from the counting. Tissues with sclerotic lesions or only a few glomeruli were discarded. The mean C4d score was calculated semiquantitatively.

Double immunohistochemical staining was conducted to determine the location of the C4d

expression. CD10 (1:200, ready-to-use, mouse monoclonal antibody) (Novocastra, Newcastle, UK) and CD 34 (1:200, ready-to-use, mouse monoclonal antibody) (DAKO, Copenhagen, Denmark) were added to the aforementioned C4d protocol.

Statistical analysis

The mean C4d scores were non-parametrically distributed in the MCD, FSGS, and control groups. The Kruskal-Wallis test was used for between-group comparisons, and the Mann-Whitney test was used for comparisons between pairs of groups, when appropriate. Bonferroni's correction was applied to the *post hoc* analysis of between-group or within-group comparisons to adjust for the number of comparisons. The mean C4d scores, which were parametrically distributed in the MCD and FSGS groups, were analysed using Student's *t* test. A *p* value of less than 0.05 was considered statistically significant. All data were analyzed by software SPSS version 19.

Results

Patient characteristics

C4d immunohistochemical staining of 130 renal biopsy tissue specimens from patients who were diagnosed with MCD or FSGS at Seoul National University Hospital were analysed. The mean serum creatinine level of patients was 1.18 mg/dl and ranged from 0.2 to 7.4 mg/dl at the time of renal biopsy for the enrolled patients. The mean eGFR was calculated as 93.06 ml/min/1.73 m² by using the CKD-EPI equation and ranged from 6.8 to 258 ml/min/1.73 m². At the time of biopsy, 14.6% of the patients had asymptomatic urinary abnormalities, 54.6% of patients had nephrotic syndrome, and 25.4% of the patients had non-nephrotic range proteinuria. Additionally, 5.4% of the patients had renal insufficiency when it was defined as a serum creatinine level > 1.5 mg/dl. Patients had been followed up for 90 ± 47.8 months (mean ± S.E) at the outpatient clinic. Detailed clinical features are shown in **Table 1**.

C4d expression was higher in MCD and FSGS

C4d immunostaining was semiquantitatively scored (**Figure 1**). The mean C4d scores were

C4d in MCD and FSGS

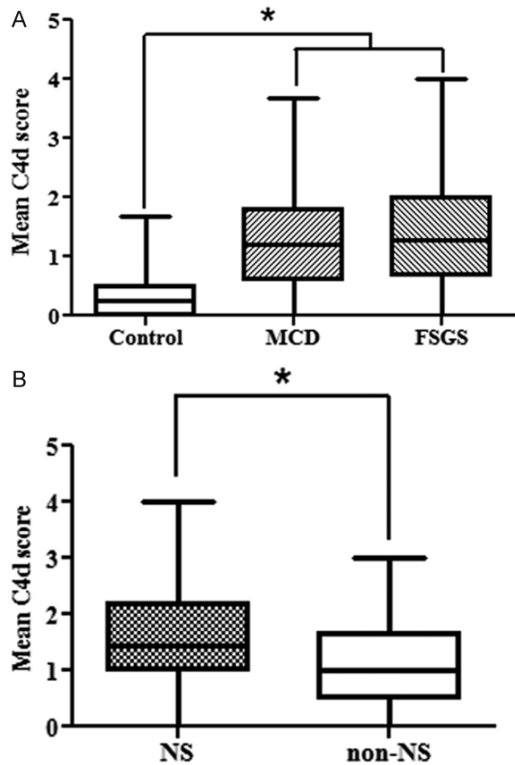


Figure 2. A: C4d scores in the minimal change disease (MCD) and focal segmental glomerulosclerosis (FSGS) groups. Mean C4d scores were higher in the MCD and FSGS groups (1.31 ± 0.912 and 1.52 ± 1.05 , respectively) than in the control group (0.32 ± 0.415) ($*P < 0.001$, Kruskal-Wallis test). B: Mean C4d scores according to the presence of nephrotic syndrome (NS). Patients with nephrotic syndrome (NS) had a higher mean C4d score than those with non-nephrotic range proteinuria (non-NS) (1.64 ± 1.020 vs 1.09 ± 0.841) ($*P = 0.002$, Student's *t* test).

0.32 ± 0.415 in the control group, 1.31 ± 0.912 in the MCD group, and 1.52 ± 1.05 in the FSGS group. C4d expression was elevated in the MCD and FSGS groups compared to the control group ($P < 0.001$). The mean C4d scores were higher in the FSGS group than in the MCD group, but the difference was not significantly different ($P = 0.6$) (Figure 2A).

The albumin levels were negatively related to the mean C4d scores in the MCD and FSGS groups ($R^2 = 0.056$, $P = 0.007$). The cholesterol levels were positively related to the mean C4d scores ($R^2 = 0.053$, $P = 0.012$). The mean C4d scores were higher in patients with nephrotic syndrome than those with other manifestations (1.64 ± 1.020 vs 1.09 ± 0.841 , $P = 0.001$) (Figure 2B); however, there was no significant difference in the 24-hour urine protein levels

and mean C4d scores ($P = 0.059$). Additionally, the protein levels measured with the spot urine stick test were not related to the mean C4d scores ($P = 0.507$). The mean C4d scores were higher in patients with nephrotic syndrome who had more than 3.5 g of proteinuria compared to those who had non-nephrotic range proteinuria ($P = 0.035$).

C4d expression might be related to the prognosis

The mean C4d scores were not significantly related to the random urine protein-to-creatinine ratios at the last follow-up ($P = 0.980$). The final treatment outcomes were classified as complete remission (proteinuria < 0.3 g/day), frequent response (2 or more recurrences), partial remission (proteinuria < 3.5 g/day), or non-response (> 3.5 g/day). The mean C4d scores did not influence the treatment outcomes ($P = 0.536$). The serum creatinine levels at biopsy were not related to the mean C4d scores ($P = 0.139$). The estimated GFR values, as calculated by the CKD-EPI equation, were also not related to the mean C4d scores ($P = 0.078$). However, the creatinine levels at the final visit were related to the mean C4d scores in both disease groups ($P = 0.010$, $R^2 = 0.051$). Changes in the creatinine levels between the initial and final visits were related to the mean C4d scores in the FSGS group ($R^2 = 0.066$, $P = 0.037$) (Figure 3). The creatinine levels at the final visit were related to the mean C4d scores after adjustment for age, sex, type of disease, initial creatinine level, and 24-hour protein level at biopsy ($P = 0.032$). Patients with a C4d score higher than 3 had higher creatinine levels at the final follow-up ($P = 0.014$, Fisher's exact test).

C4d is expressed in both endothelial cells and podocytes

C4d was expressed in the glomeruli in the MCD and FSGS groups (Figure 1). We further identified the expression site of C4d using double immunohistochemistry. C4d expression was co-localised with an endothelial cell marker, CD34, and podocyte marker, CD10 (Figure 4), which means that C4d could be expressed in both endothelial cells and podocytes.

Discussion

Since complement deposition was observed near podocytes in Heymann's nephritis model

C4d in MCD and FSGS

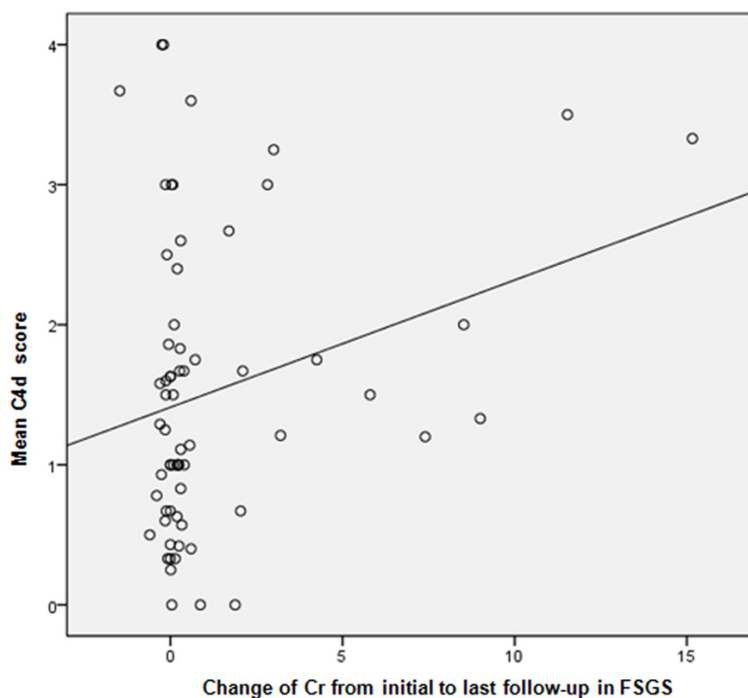


Figure 3. Correlation of C4d scores with change of serum creatinine levels in FSGS. Mean C4d scores were positively related to creatinine level change from initial to last follow-up (Simple regression test, $R^2 = 0.066$, $P = 0.038$).

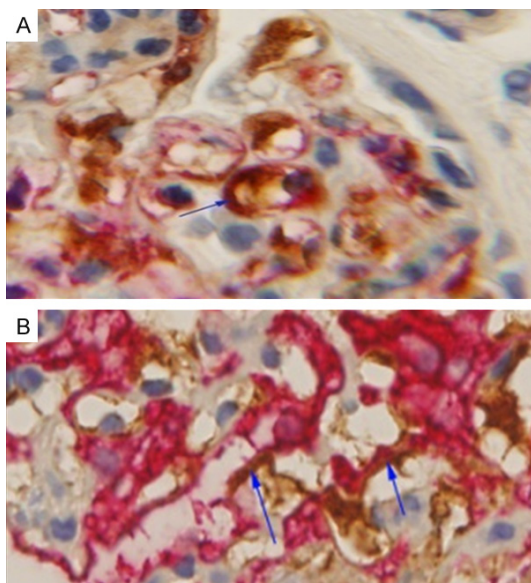


Figure 4. Localization of C4d expression in the glomerulus by double immunohistochemical staining ($\times 1000$). A: Co-localization of brown staining for C4d and red staining for CD34 (endothelial marker). B: Co-localization of brown staining for C4d and red staining for CD10 (podocyte marker).

[24, 25], complement systems were known to relate to podocyte injury in immune mediated

glomerulonephritis. The lectin pathway which was one of the complement systems was recently reported to be associated with prognosis in various types of immune-mediated glomerulonephritis [7, 12, 26]. MBL, a component of lectin pathway, can cause renal damage through increasing mesangial proliferation, extracapillary proliferation, glomerular sclerosis, interstitial infiltration, and proteinuria in IgA nephropathy [26]. Glomerular MBL deposition is associated with worse progression of renal disease in Henoch-Schonlein purpura nephritis [10]. MBL exerts a harmful effect on the host as a mediator of inflammation, ischemia/reperfusion injury [27] and diabetic nephropathy [28, 29]. MBL could also be linked to apoptosis and necrosis [30]. These previous studies suggest that the lectin pathway may be linked to various renal injuries in immune mediated glomerulonephritis.

Complement pathway, including lectin pathway, had hardly been evaluated in pathogenesis of non-immune mediated glomerulonephritis yet. It has recently noticed that C4d, a by-product of the lectin complement pathway, was expressed in some patients with MCD or FSGS, which were representative types of non-immune mediated glomerular diseases [11, 23]. In this study, C4d expression was significantly higher in MCD and FSGS groups than in control group of patients who were diagnosed with asymptomatic urinary abnormality, but there was no significant difference in the C4d expression between the MCD and FSGS groups. Furthermore, there was a significant difference in the C4d expression between patients with nephrotic range proteinuria and those with non-nephrotic range proteinuria ($P = 0.035$). Proteinuria beyond the nephrotic range in MCD or FSGS might affect the lectin pathway. Therefore, we suggest that C4d expression might be related to proteinuria amount rather than disease type. These results suggest that complement system, especially lectin

pathway can be related to podocyte injury in the development of MCD and FSGS.

Although the main pathogenesis of FSGS has been poorly understood, it has generally known to link with non-immunogenic causes, such as podocyte structural changes. Persistent podocyte foot process effacement leads to irreversible podocyte depletion and progressive glomerulopathy [15, 20]. As a result, proteinuria and nephrotic syndrome could be presented in patients who were diagnosed at FSGS. Inflammatory signaling in podocytes could contribute to podocyte damage and prolonged proteinuria [31, 32]. The complement system is known for its role as an inflammatory mediator; therefore, we suggest that higher C4d expression might indicate the involvement of inflammation in MCD and FSGS. The lectin pathway might also induce podocyte damage through inflammatory process.

The progression of FSGS has been known to link with oxidative stress, inflammation, and fibrosis [32-34]. The major source of inflammatory and profibrotic mediators play an important role in the progression of FSGS [35]. Blockade of oxidative stress can ameliorate renal sclerosis through anti-inflammatory and anti-apoptotic processes [36]. The inflammatory signal can amplify the apoptosis [37] and the activation of inflammatory pathway contributes to progression of chronic renal failure [38]. We observed a significant correlation between the mean C4d scores and renal function at the last visit, and the same result was seen after adjusting for the creatinine levels at the time of diagnosis. Patients with a C4d score of more than 3 points had deteriorated poor renal function at their final visit. The more C4d expression might be interpreted that inflammation was more happened in our clinical study group. We suggest that C4d expression might be used as a new prognostic factor. However, there was no significant correlation between the C4d expression and treatment outcome. It indicates that the treatment outcome may not be predicted based on the C4d expression degrees. This may be due to the biases that affect the treatment outcomes could not be eliminated or that the sample size of this study was too small to reach statistical significance. Future prospective studies with larger sample sizes are needed to confirm our results whether C4d expression might be related with final renal function.

In conclusion, this study suggests that the lectin pathway may play a role in the pathogenesis and progression of MCD and FSGS. Additionally, this study also suggests that glomerular C4d expression may correlate with the prognoses of MCD and FSGS. Future studies are needed to elucidate the exact pathogenic mechanism for the role of the lectin pathway.

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Disclosure of conflict of interest

None.

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