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Claudin-1 and Nephryn Label Cellular Crescents in Diabetic Glomerulosclerosis

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Abstract

Cellular crescents are typically inflammatory and associated with rapidly progressive glomerulonephritis. Their pathogenesis involves glomerular basement membrane rupture due to circulating or intrinsic factors. Crescents associated with diabetic glomerulosclerosis are rarely reported. Furthermore, the nature of cells forming crescents in diabetes is unknown. To investigate the nature of crescents in diabetes, we examined renal biopsies from diabetic patients with nodular glomerulosclerosis and crescents ($n=2$), diabetes without crescents ($n=5$), non-diabetic renal biopsies ($n=3$) and crescentic glomerulonephritis with inflammatory crescents ($n=5$). Electron microscopy and confocal immunofluorescence analysis with antibodies against nephryn (a podocyte marker) and claudin-1 (parietal epithelial cell marker) were performed. Diabetic glomeruli with crescents contained a mixture of crescentic cells expressing either claudin-1 (11 ± 1.4 cells/glomerulus) or nephryn (5.5 ± 3.0 cells/glomerulus). Rare crescentic cells co-expressed nephryn and claudin-1 (2.5 ± 1.6 cells/glomerulus). In contrast, inflammatory crescents were almost exclusively composed of claudin-1 positive cells (25 ± 5.3 cells/glomerulus). Cells co-expressing claudin-1 and nephryn were absent in inflammatory crescents and all cases without crescents. Electron microscopy showed podocyte bridge formation between the glomerular basement membrane and parietal basement membrane but no glomerular basement membrane rupture as in inflammatory crescents. Crescents in diabetes may occur in diabetes in the absence of a secondary etiology and are composed of a mixture of parietal epithelial cells and visceral podocytes. Cells co-expressing parietal epithelial and podocyte markers suggest that parietal epithelial cells may transdifferentiate into podocytes in response to severe glomerular injury.

Keywords

Diabetes; crescent; nephryn; claudin-1; podocyte; parietal

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Introduction

Cellular crescents are typically associated with various forms of rapidly progressive glomerulonephritis (RPGN) such as anti-glomerular basement membrane (GBM) disease, pauci-immune glomerulonephritis, and lupus nephritis [1,2]. A diagnosis of crescentic glomerulonephritis is typically treated aggressively with potent immunosuppressive agents. Crescents, however, may also be observed in other conditions. They have been documented in a wide variety of glomerular diseases including IgA nephropathy and Alport syndrome [2,3]. Rare case reports have described crescents in patients with diabetic nephropathy [4-6]. This finding, however, has received little attention in the published literature to date.

Glomerular crescents appear to be a ubiquitous response to glomerular injury. In RPGN the inflammatory response results in GBM rupture, fibrin leakage into Bowman's space and crescent formation [2]. Such crescents typically contain inflammatory cells and fibrin within the proliferating epithelial cells. GBM rupture, however, may not be essential for crescent formation [7]. The nature of cells comprising crescents is an actively debated subject [8]. Some investigators identified cells of podocyte lineage within glomerular crescents [9-12]. Previous studies in mice suggested that visceral podocytes form bridges between the GBM and the parietal basement membrane (PBM) in early stages of crescent formation, perhaps triggering parietal epithelial cell (PEC) proliferation [7]. More recent studies using a transgenic mouse model of selective PEC depletion demonstrated that PECs are essential for crescent formation [13]. Investigators have argued that PECs may transdifferentiate into podocytes, serving as a reservoir capable of replenishing damaged glomeruli [14-17].

The study of glomerular crescents has largely been limited to examination of human biopsies from patients with RPGN or animal models of glomerulonephritis. There are only rare reports describing crescent formation outside of these scenarios [3-6]. When confronted with a biopsy containing crescents, it is critical to distinguish a pseudocrescent from the more aggressive inflammatory crescents associated with RPGN. Such a distinction is important in order to determine the appropriate treatment.

In the current study, two cases of diabetes with crescent formation were examined. Although crescent formation in diabetes is rare, their presence raises the suspicion for a co-existing disease such as pauci-immune glomerulonephritis. Distinguishing pseudocrescents associated with diabetes from true inflammatory crescents is essential to exclude this possibility. Comparing the nature of cells comprising diabetic crescents with the cells of inflammatory crescents may provide insights into the mechanism of glomerular injury in diabetes and provide pathologists with techniques to distinguish these entities. This is the first study to date to investigate the nature of crescent formation in diabetic glomeruli.

The protein claudin-1 is expressed exclusively in parietal epithelial cells [18]. In contrast, nephrin is expressed exclusively in podocytes [19,20]. Using antibodies to these two proteins, we investigated the cellular composition of diabetic crescents and compared the expression profile with cases of diabetes without crescents, inflammatory crescents and normal appearing glomeruli. Additional examination of the crescents using electron microscopy was performed.

Materials and Methods

All cases were selected from the archives of the Lauren V. Ackerman Laboratory of Surgical Pathology at Washington University School of Medicine in St. Louis. Human studies were approved by the internal review board for human studies of Washington University School of Medicine. Renal biopsies from patients with diabetic nodular glomerulosclerosis with crescents ($n=2$), diabetes with no crescents ($n=5$), crescentic glomerulonephritis with inflammatory crescents ($n=5$) and control kidneys from patients with minimal change disease ($n=1$) and uninvolved kidney adjacent to resected renal tumors ($n=2$) were stained for nephrin and claudin-1.

Immunofluorescence

Immunofluorescence staining was performed on formalin fixed paraffin embedded tissue ($3\mu\text{m}$). Following deparaffinization, the slides were subjected to antigen retrieval using 10mM sodium citrate, 0.05% Tween20, pH 6.0 for 15 minutes. All primary antibodies were applied at 4°C overnight. Samples were probed with a Cy3 conjugated anti-sheep (Jackson ImmunoResearch Lab., Inc. #713-165-147) or Alexa Fluor 488 conjugated anti-rabbit secondary antibodies (Jackson ImmunoResearch Lab., Inc. #711-545-152) for 1-3 hours followed by bis-benzamide (Sigma) staining. The primary antibodies used were a commercial sheep polyclonal anti-nephrin antibody (1:20, R&D systems #AF4269) and a rabbit polyclonal anti-claudin-1 antibody (1:200, Abcam #AB15098).

Imaging

A Nikon 80i upright microscope with an attached Nikon C-1 Confocal system (Nikon) was used to capture the immunofluorescence images. The images were analyzed using Nikon Elements software (Nikon). Z-stack immunofluorescence confocal images were used to count labeled cells in the glomerular tuft, lining the PBM and in the crescents. Cell counts were compared between groups using ANOVA statistical analysis. An Olympus BX51 microscope equipped with an Olympus DP 71 camera was used to capture light microscopic images.

Clinical data

Demographic and clinical data are presented in Table 1. Briefly, the diabetic renal biopsies with crescents were from two African American men ages 43 and 25 years with an 18-year history of diabetes mellitus type II and type I respectively. The first patient was hospitalized for pneumonia and treated with vancomycin. His highest vancomycin trough was 20. During his hospital stay, his serum creatinine rose from a baseline of 1.9 mg/dL to 3.4 mg/dL with a concomitant increase in BUN from 32 mg/dL to 74 mg/dL. A urinalysis showed proteinuria (3+) and microhematuria. Serum anti-GBM and ANCA tests were negative. Serum complements C3 and C4 were within normal limits. The second patient was referred for evaluation of a serum creatinine of 3.7 mg/dL, increased from a baseline of 1.28 mg/dL. Urinalysis demonstrated cloudy urine with 4+ albumin, 4+ glucose, and 2+ blood. Urine microscopy showed 5 red blood cells, 10 white blood cells, trace bacteria, and 2 hyaline casts. Tests for serum complements C3 and C4 were within normal limits. Serum tests for

ANCA and anti-GBM antibodies were negative. Testing for HIV, Hepatitis C, and Hepatitis B was performed and was negative.

Results

Patient #1—The renal biopsy showed 16 glomeruli with diabetic nodular glomerulosclerosis 30% of which contained crescents (3 cellular and 2 fibrous). Kimmelstein-Wilson nodules were apparent with PAS and silver stains. Cellular crescents consisted exclusively of epithelial cells without admixed inflammatory cells or fibrin deposits. Representative cellular crescents are shown in Figure 1. Acute tubular necrosis and mild interstitial chronic inflammation were also present. No glomeruli were available for immunofluorescence evaluation. Electron microscopy showed GBM thickening, mesangial expansion and diffuse podocyte foot process effacement. The GBM appeared intact without rupture in all areas examined by electron microscopy. Focal podocyte bridging between the GBM and the PBM were seen (Figure 2A). No electron dense deposits were present.

The patient was managed with inpatient hemodialysis, broad-spectrum antibiotics, and supportive care. His serum creatinine and BUN peaked at 8.2 mg/dL and 105 mg/dL, respectively. His urine output gradually improved along with a drop in his serum creatinine to 1.9 mg/dL over his admission. He was successfully weaned from mechanical ventilation and discharged 11 days after admission. His renal function, however, continued to deteriorate. He was started on hemodialysis within 2.5 years of the biopsy.

Patient #2—The renal biopsy contained 33 glomeruli with diffuse diabetic nodular glomerulosclerosis. Thirteen glomeruli were globally sclerotic; of the nonglobally sclerosed glomeruli 87% contained crescents. No fibrin deposition or inflammatory cells were noted in the crescents. Immunofluorescence showed linear staining for IgG (3+), albumin (3+), and IgA (2+). Electron microscopy of two glomeruli showed diffuse thickening of the GBM. The GBM appeared intact in all areas examined by electron microscopy. The podocyte foot processes were diffusely effaced. Cellular crescents captured within the material submitted for electron microscopy showed proliferation of parietal epithelial cells (Figure 2B). Rarely, fibrous bridges from the PBM to adjacent podocytes were noted (Figure 2C). No electron dense deposits were identified.

The patient's serum creatinine remained stable during his hospitalization. Because of this, he was managed conservatively and discharged 7 days after admission. His serum creatinine at the time of discharge was 3.36 mg/dL. Follow-up laboratory testing 1 month after discharge showed an increase in serum creatinine to 4.5 mg/dL. 2 months after discharge the patient was readmitted with a serum creatinine of 6.75 mg/dL and blurred vision secondary to diabetic retinopathy. The patient lost vision in one eye as a result. He was started on hemodialysis 2.5 months after the biopsy was performed.

Renal biopsies from patients with pauci-immune glomerulonephritis ($n=4$) and anti-GBM glomerulonephritis ($n=1$) were examined. The patient characteristics are summarized in table 1. All of these cases contained numerous cellular crescents. In contrast to the crescents

observed in patients with diabetes, the inflammatory crescents observed in these biopsies showed breaks in the GBM and inflammatory cells in Bowman's space (Figure 2D).

Immunofluorescence

Anti-nephrin and anti-claudin-1 antibodies were used as podocyte and PEC markers, respectively, to explore the glomerular cell types present in diabetes with crescents ($n=2$), crescentic glomerulonephritis ($n=5$), and diabetes without crescents ($n=5$). These were compared with unremarkable appearing glomeruli from renal cortex adjacent to tumor ($n=2$) and minimal change disease ($n=1$). Confocal images were used to count claudin-1 positive cells lining Bowman's capsule or within crescents and nephrin positive cells in the glomerular tuft, in crescents or lining Bowman's capsule.

Unremarkable appearing glomeruli showed strong and diffuse nephrin staining within the glomerular tuft (Figure 3A). Five representative glomeruli were counted and showed 23.8 ± 4.0 nephrin positive cells/glomerulus (mean $\pm$ SEM; Figure 4A). Claudin-1 antibodies highlighted a single layer of PECs in unremarkable appearing glomeruli (Figure 3A). The number of claudin-1 positive cells was counted and revealed an average of 4.2 ± 1.7 claudin-1 positive cells/glomerulus lining the PBM (Figure 4B). Rare nephrin positive cells were observed lining the PBM of unremarkable appearing glomeruli (0.9 ± 0.4 cells/glomerulus; Figure 4C). No cells staining for both claudin-1 and nephrin were observed (Figure 4D).

In renal biopsies from patients with diabetic nodular glomerulosclerosis and no crescents there was reduced nephrin staining of the glomerular tuft when compared with normal appearing glomeruli (Figure 3B). The nephrin stain localized to the periphery of the glomerular tuft and stained the visceral podocyte cytoplasm in a linear distribution (Figure 3B). No nephrin staining was observed outside of the glomerular tuft. Eight representative glomeruli were counted and revealed 10.1 ± 1.5 nephrin positive cells/glomerulus. Although this was decreased when compared with normal appearing glomeruli, the difference was not statistically significant. Claudin-1 staining highlighted a prominent but single layer of cells lining the PBM (Figure 3B). The staining was granular and localized to the cell membrane. No staining was observed within the glomerular tuft. Cell counts revealed an average of 5.6 ± 1.0 claudin-1 positive cells/glomerulus in cases of diabetic nodular glomerulosclerosis (Figure 4B). This was similar to the number of cells observed in normal appearing glomeruli. No cells showed co-staining with claudin-1 and nephrin (Figure 4D).

Renal biopsies from patients with pauci-immune glomerulonephritis ($n=4$) and anti-GBM glomerulonephritis ($n=1$) were examined for claudin-1 and nephrin expression in the glomeruli containing cellular crescents. The crescents were predominantly composed of claudin-1 positive cells (Figure 3C). Six representative glomeruli were counted and demonstrated 25 ± 5.3 claudin-1 positive cells/glomerulus in the inflammatory crescents. This was significantly increased when compared with normal appearing glomeruli and glomeruli from diabetes without crescents ($p<0.001$; Figure 4B). Although the number of claudin-1 positive cells/glomerulus was greater than those observed in the cases of diabetes with crescents, the difference was not statistically significant ($p=0.052$). Rare nephrin positive cells were seen within the crescents (0.3 ± 0.3 cells/glomerulus). Nephrin

highlighted native visceral podocytes attached to the capillary loops (Figure 3C). There were 19.3 ± 7.8 nephrin positive cells/glomerulus in the glomerular tuft (Figure 4C). This was similar to the number of nephrin positive cells observed in normal appearing glomeruli. No double-labeled cells were observed in the inflammatory crescents (Figure 4D).

Renal biopsies from two patients with diabetic nodular glomerulosclerosis and crescents were examined for claudin-1 and nephrin expression. In some of the crescents, a pure population of claudin-1 positive cells was seen. More commonly, however, crescents contained a mixed population of cells staining for either nephrin or claudin-1 (Figure 3D). Four representative glomeruli were counted and revealed 11.0 ± 1.4 claudin-1 positive cells/glomerulus (Figure 4B). Although the number of claudin-1 positive cells was increased in crescents associated with diabetes, this was not significantly different from the number of claudin-1 positive cells present in Bowman's capsule of normal appearing glomeruli or diabetic glomeruli without crescents. There was, however, a significant increase in the number of nephrin positive cells in the crescents associated with diabetes (5.5 ± 3.0 cells/glomerulus) compared with inflammatory crescents ($p < 0.05$; Figure 4C). Rare cells were identified within crescents associated with diabetes that stained with claudin-1 and nephrin (2.5 ± 1.6 cells/glomerulus; Figures 3E and 3F). These cells were present both at the periphery of the crescent and populating the glomerular tuft. Double-labeled cells were not observed in inflammatory crescents, normal glomeruli or diabetic glomeruli without crescents (Figure 4D).

Discussion

The presence of crescents in diabetic nephropathy has received relatively little attention in the literature. The report by Elfenbein and Reyes in 1975 is the most comprehensive analysis of this phenomenon [6]. In this study, the authors showed a correlation between the frequency of crescents and severity of diabetic renal disease. The percentage of crescents also correlated with increased blood urea nitrogen and serum creatinine. Unfortunately, it is unclear whether some of these patients represented cases of diabetes with superimposed crescentic glomerulonephritis secondary to ANCA, anti-GBM disease, or other etiologies. Since the study was completed in 1975, ANCA and anti-GBM testing were unavailable to the investigators.

Toth reported a case of crescent formation in diabetes in 1987 [5]. Fifty percent of the glomeruli contained crescents. No evidence of immunoglobulin or fibrin was found in the biopsies. Laboratory testing showed no detectable antinuclear or anti-GBM antibodies. Complement components C3 and C4 were also within normal limits. Thus, it is most likely that this case truly represents diabetes with crescents. Similar to the two cases reported here, Toth found no evidence of fibrin or GBM breaks.

Otani et al. recently reported a case of diabetic nodular glomerulosclerosis with crescent formation [4]. Laboratory studies showed no evidence of ANCA, anti-nuclear antibodies, anti-GBM antibodies, hepatitis B or C. The biopsy showed no evidence of vasculitis, fibrinoid necrosis, or GBM breaks. No immune complex deposits were identified.

Similar to previous reports, the diabetic patients presenting with crescents had a more aggressive clinical presentation. Serum creatinine was elevated and both patients had hematuria and proteinuria. The aggressive clinical presentation in case 1, however, was complicated by the co-existence of acute tubular necrosis (ATN). This finding may have accounted for the abrupt increase in serum creatinine. Nonetheless, both patients went on to develop ESRD.

The pathogenesis of crescent formation in diabetic nephropathy is unknown. Two types of crescents are recognized, inflammatory crescents and pseudocrescents. The latter are a characteristic feature of collapsing glomerulopathy and diffuse mesangial sclerosis. Studies indicate that pseudocrescents may result from proliferating podocytes [21]. Animal models of crescentic glomerulonephritis and focal segmental glomerulosclerosis suggest that both visceral and parietal podocytes participate in crescent formation [8-12, 22]. Our results are in agreement with these studies. In cases of diabetes with crescents, the crescents were typically composed of cells expressing either claudin-1 or nephrin, or, rarely, both claudin-1 and nephrin. Inflammatory crescents, however, were almost exclusively composed of claudin-1 positive cells. Although rare nephrin positive cells were observed in the inflammatory crescents, no cells expressing both claudin-1 and nephrin were identified. Double-labeled cells were not observed in normal-appearing glomeruli or diabetic glomeruli without crescents. They may represent detached podocytes that contacted the PBM and were in the process of differentiating into PECs. Alternatively, they may represent PECs in the process of differentiating into podocytes, supporting the theory that PECs may replenish podocytes in injured glomeruli [23,24]. The findings may be of clinical utility. Distinguishing inflammatory crescents from pseudocrescents is critical for determining appropriate treatment. Further studies are warranted to explore this finding in a greater number and wider variety of cases.

Diabetic glomeruli with and without crescents showed decreased nephrin staining within the glomerular tuft, a finding previously noted by others [25,26]. Decreased nephrin staining was proposed to be due to podocyte loss secondary to hyperglycemia. When injured, podocytes, as post-mitotic cells, may attempt to re-enter the cell cycle but cannot divide and may die via mitotic catastrophe [25].

Using an animal model of crescentic glomerulonephritis, Le Hir et al. demonstrated that early crescents contain bridges between injured podocytes and the PBM [7]. The authors hypothesized that these bridges were essential to crescent formation. In the current study, podocyte bridges were observed. This observation indicates that the mechanism suggested by Le Hir's animal studies may underlie crescent formation in human diabetes.

This study is notably limited by small number of cases of diabetes with crescents. This is an inherent difficulty when studying rare entities. Because of this limitation, the suggestion that claudin-1 and nephrin staining be used to differentiate inflammatory crescents from pseudocrescents must be interpreted with caution. Further investigation using greater numbers of cases is needed to determine the clinical utility of this observation.

In summary, we presented two unusual cases of diabetic nodular glomerulosclerosis with crescents in the absence of another cause. We investigated the cellular composition of crescents in diabetes and compared expression profiles of nephrin and claudin-1 with glomeruli containing inflammatory crescents, diabetic glomeruli without crescents, and normal-appearing glomeruli. Interestingly, the crescents observed in diabetes contained a significantly greater proportion of nephrin positive cells compared with inflammatory crescents. Furthermore, diabetic crescents contained a small population of cells that stained for both claudin-1 and nephrin. These cells were not observed in inflammatory crescents. The findings suggest that claudin-1 and nephrin staining may help to distinguish inflammatory crescents from pseudocrescents. Furthermore, the results indicate that the crescent response in diabetes may represent an attempt of the injured glomerulus to heal itself by generating new podocytes via the proliferation of PECs.

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Abbreviations

GBM	glomerular basement membrane
PBM	parietal basement membrane
BUN	blood urea nitrogen

Figure 1.

A. and B. H&E (A) and PAS (B) stains of a representative glomerulus from a patient with diabetes. A cellular crescent is seen encasing the glomerular tuft. The glomerulus has a nodular appearance (400 $\times$).

Figure 2.

A. Electron microscopy Patient#1. A reactive podocyte is seen (*) bridging the glomerular basement membrane and the parietal basement membrane (arrow). Several lipid vacuoles are identified within this podocyte. B. Parietal epithelial cell (*) extending a cytoplasmic projection towards an adjacent podocyte (arrow). The podocyte foot processes are diffusely effaced and the glomerular basement membrane is diffusely thickened. C. Electron microscopy Patient#2. A cellular crescent fills the Bowman's space. D. Inflammatory crescent shows ruptured glomerular basement membrane fragment and neutrophils within the Bowman's space. Neither of these features was observed in crescents associated with diabetes.

Figure 3.

Immunofluorescence staining for claudin-1 (green) and nephrin (red); cell nuclei stain blue with DAPI. A. Normal appearing glomerulus shows numerous podocytes (red) at the periphery of the capillary loops; rare parietal epithelial cells (green) line the Bowman's capsule (600 $\times$). B. Representative glomerulus from diabetes without crescents shows reduced nephrin staining and increased claudin-1 positive cells (parietal epithelial cells) along the Bowman's capsule (600 $\times$). C. Inflammatory crescent shows the majority of the cells within the crescent staining positive for Claudin-1. Numerous Nephtrin positive podocytes are present as well (600 $\times$). D. Patient #2, diabetes with cellular crescents: shows a mixture of nephrin and claudin-1 staining within the crescent in contrast to the inflammatory crescent. Similar to the diabetic glomerulus without crescent formation, there is loss of nephrin staining within the glomerular tuft (600 $\times$). E. Double immunolabeling for claudin-1 (green) and nephrin (red) in the same glomerulus shown in Figure 3C. A single cell (arrow, inset) located along Bowman's capsule stains with nephrin and claudin-1. These double-labeled cells were not observed in the inflammatory crescents (600 $\times$). F. A second glomerulus from a patient with diabetes and crescents shows total loss of nephrin staining within the glomerular tuft. The crescentic cells contain numerous double-labeled (yellow) cells (400 $\times$).

Figure 4.

Representative glomeruli from renal biopsies of containing normal appearing glomeruli (controls; 5 glomeruli, 145 cells), diabetes with crescents (DM Crescents; 4 glomeruli, 83 cells), diabetes without crescents (DM; 8 glomeruli, 126 cells), and with inflammatory crescents (Crescentic GN; 6 glomeruli, 268 cells) were used to quantify claudin-1 and nephrin staining. A. Nephrin positive cells per glomerular tuft. There was a significantly decreased number of nephrin cells/glomerulus in glomeruli from cases of diabetes with crescents ($p<0.05$). There was a trend towards increased numbers of nephrin positive cells in the crescents from diabetics, but this was not statistically significant. B. Claudin-1 positive cells were counted in Bowman's capsule and in Bowman's space. There were a significantly greater number of claudin-1 positive cells in the inflammatory crescents ($p<0.05$). The crescents associated with diabetes had an increased number of claudin-1 positive cells, but this was not statistically significant. C. Nephrin positive cells were counted in Bowman's capsule and in Bowman's space. There were a significantly greater number of nephrin positive cells in the crescents associated with diabetes ($p<0.05$). Rare nephrin positive cells were seen in the normal appearing glomeruli and in inflammatory crescents. D. Claudin-1 and nephrin co-labeled cells were counted. Cells expressing both markers were only present in the crescents associated with diabetes ($p<0.05$).

Table 1
Clinical information comparing the four groups of patient biopsies that were analyzed. SD – standard deviation. Age, and creatinine were compared using a Student's t-test. Categorical variables were compared using Fisher's exact test

	DM w/ Crescents (n=2)	DM w/o Crescents (n=5)	Inflammatory Crescents (n=5)	Controls (n=3)
Age (years ± SD)	34 ± 13	49 ± 14	66 ± 14	55 ± 11
Gender (M:F)	2:0	2:3	2:3	2:1
Race				
Caucasian	0%	40%	100%	100%
African American	100%	60%	0%	0%
Serum creatinine (mg/dL ± SD)	3.57 ± 0.24 *	2.78 ± 1.13 *	3.32 ± 1.76	0.90 ± 0.05
Hematuria	100%	80%	100%	33%
Proteinuria	100%	100%	80%	33%

* p<0.05