Increased excretion of urinary podocytes in lupus nephritis

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ABSTRACT

Podocytes are highly specialized epithelial cells that form part of the filtration barrier in the kidney, and their loss reflects a malfunction in glomerular filtration, which is usually associated with the progression of the disease. Glomerulonephritis is a serious complication that develops in about 50% of the lupus patients and is characterized by proteinuria arising from direct or indirect podocyte injury. To assess the possible role of podocytes in the pathogenesis of lupus nephritis (LN). Urinary and glomerular podocytes were detected in the kidney biopsies of patients (n = 17) with lupus nephritis, and from control biopsies obtained during autopsies. The WT-1 protein was used as a podocyte marker. The cumulative excretion of urinary podocytes was detected in the urinary sediments of LN patients and normal healthy controls, and the specimens were analyzed by immunohistochemistry, immunofluorescence, and enzyme-linked immunosorbent assay. The apoptotic index was determined by terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling. Gross proteinuria in lupus patients was determined via 24-hour urine samples, and the results were analyzed by Student t test. Biopsy specimens from 17 patients with class-III or IV LN had lower levels of glomerular WT-1 expression than the levels found in normal kidneys ($P < 0.0001$). The reduction of glomerular podocytes in patients with lupus nephritis correlated with the cumulative excretion of urinary podocytes ($P < 0.0001$) and proteinuria. There was no correlation between the urinary podocytes and the apoptotic index in the LN urinary sediments. A decrease in glomerular podocytes is associated with their cumulative excretion in urinary sediments; therefore, such findings correlate with proteinuria in lupus nephritis patients.

Key words: Lupus nephritis, podocytes, proteinuria, WT-1 marker

Introduction

Systemic lupus erythematosus (SLE) is a multi-organ autoimmune disease that involves the kidney. Consequently, lupus nephritis represents a management challenge, and its outcome determines the prognosis of SLE patients. The pathogenesis of SLE includes a deregulation of humoral and cellular immune activity, reflected in a broad production of autoantibodies, cytokines, and tissue damage. Among the nephritogenic autoantibodies are anti-dsDNA, anti-nucleosomes, and autoantibodies to laminin and collagen IV, which induce renal lesions by the *in situ* formation of immune complexes that cause complement activation.[1]

Podocytes, which are specialized cells of the visceral epithelia, are located in the glomerular membrane and are responsible for maintaining the flow of the glomerular filtrate via intercellular spaces. These cells are the terminal constituents of the ultra-filtration barrier, which impedes protein loss, and their injury results in the functional failure associated with proteinuria.[2,3] This symptom is frequently the initial clinical manifestation of glomerular disease, associated with severe lupus nephritis.[4]

Proteinuric diseases are classified into three categories: (1) those associated with diseases mediated by antibodies, such as lupus nephritis; (2) those related to metabolic disorders, such as diabetes, amyloidosis, and Fabry

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disease, and (3) diseases primarily caused by abnormal glomerular cell function.[5]

The comprehension of podocyte biology in lupus injury is essential for understanding the pathogenic mechanism of lupus nephritis. For this reason, we examined the issue of whether podocyte cells are affected in lupus nephritis. To answer this question, podocytes have been detected in the renal tissue by immunohistochemistry and immunofluorescence and in the urinary sediment by enzyme-linked immunosorbent assay (ELISA), using a monoclonal WT-1 antibody. The apoptotic features were determined by TUNEL.

Materials and Methods

Biopsies from 17 patients with systemic lupus erythematosus (SLE) (15 females and 2 males) were studied; the ethnicity of the subjects was Latin American Mestizos. The patients had a mean age of 25.9 years (range 14 – 40), with a mean disease duration of 4.8 years (range 1 – 10). All the patients met the revised American College of Rheumatology (ACR) classification criteria for SLE,[6] and they had one or more serologic criteria, such as a positive antinuclear antibody test, low serum complement or anti-dsDNA antibodies (*Crithidia luciliae* test). In all the patients, kidney biopsies were performed percutaneously, and a segment of each biopsy was stained for hematoxylin and eosin (H and E) and evaluated under light microscopy. The biopsies were classified according to the ISN / RPS (The 2004 International Society of Nephrology [ISN] / Renal Pathology Society [RPS]) 2004 classification of lupus nephritis.[7]

The activity and chronicity were determined by the indicators proposed by Austin *et al*.[8] The activity index (AI) was based on the following histological features: glomerular proliferation, leukocyte exudation, karyorrhexis / fibrinoid necrosis (x 2), cellular crescents (x 2), hyaline deposits, and interstitial inflammation. Scales of 0 (absent), 1 (mild), 2 (moderate), and 3 (severe) were applied to each histological feature of the biopsy specimens, in which six or more glomeruli were examined. Accordingly, the maximum AI score was 24. The chronicity index (Cl) was the sum of the individual scores of the following features: glomerular sclerosis, fibrous crescents, tubular atrophy, and interstitial fibrosis, using the scale of 0 (absent), 1 (mild), 2 (moderate), and 3 (severe). The maximum CI score was 12.

Seventeen normal kidney biopsies of the controls were obtained during necropsy, from subjects without renal pathology, who died in accidents as a result of head trauma. The protocol for the research project was approved by the Ethics Committee of each institution, and therefore, patients and relatives of the controls were informed and written consent was obtained in each case.

The WT-1 marker[9] was used for the analysis of a number of podocytes per glomerulus by immunohistochemistry of 4-μm-thick sections of renal tissues mounted on microscope slides. The specimens were dewaxed, permeabilized with 0.01% Triton X-100 / phosphate buffered saline (PBS), and then washed thrice with PBS. Endogenous peroxidase was blocked for 10 minutes with 3% H_2O_2 dissolved in methanol. After an additional wash, the tissues were incubated for 12 hours with a monoclonal anti-WT-1 antibody (sc-7385 Santa Cruz, Biotechnology Inc., Santa Cruz, CA) and diluted 1 : 100 in 10% FBS-PBS. Cell proliferation in the glomerulus was monitored by anti-PCNA diluted 1 : 200 (Zymed cat 13-3900). The tissues were then washed in several changes of PBS, and the bound antibodies were identified with HRP-goat anti-mouse IgG (Zymed, Laboratories Inc., San Francisco, CA). The color reaction was induced by 3,3'-diaminobenzidine-0.06% H_2O_2 (Sigma, St Louis, MO), and the reaction was stopped with 0.5 M sulfuric acid. The slides were then examined under a light microscope. The assays were performed in triplicate and evaluated by two pathologists in a blinded fashion.

The tissues were blocked with 3% fetal bovine serum in PBS for 30 minutes and incubated for one hour with the monoclonal anti-WT-1 antibody diluted 1 : 100 in 10% FBS-PBS. After the washes with PBS, presence of the bound antibody was identified with FITC-goat anti-mouse IgG (Cat # 50695148, Zymed, Laboratories Inc., South San Francisco, CA). In addition, following the washes, some slides were counterstained with 4',6-Diamidino-2-phenylindole (DAPI). Finally, the slides were mounted and examined under a confocal scanning microscope.

TUNEL was performed according to the manufacturer's instructions (Cat # 11684795910, Roche Molecular Biochemicals. Penzberg, Germany). Nuclear stripping was performed on the kidney biopsies by immersing the slides for five minutes in 10 mM Tris-HCl, pH 8.0, followed by 15 minutes in 20 µg/ml proteinase K dissolved in the same buffer, and finally they were washed with PBS. Elongation of the DNA fragments was performed by incubation for 60 minutes, at 37ºC, with 50 μl of the reaction mixture (DDW, 10X TdT buffer [30 mM Tris base, 140 mM sodium cacodylate, pH 7.2, 1 mM cobalt chloride, 1 mM DTT]; 10% of the final volume), fluorescein-11-dUTP (0.5 mg dissolved in 1 ml of 10 mM Tris-HCl, pH 7.0), and TdT enzyme (0.3 enzyme units /µl). The reaction was

terminated with a stop solution (300 mM NaCl, 30 mM sodium citrate, pH 8.0). To differentiate the true green tag of apoptotic cells from the background incorporation, the tissues were counterstained with 0.5% propidium iodide, giving the non-apoptotic nuclei a red stain. Finally, the slides were washed in PBS and evaluated under a confocal scanning microscope LSM (Axiovert 200M, Carl Zeiss. Gottingen, Germany). Combinations of fluorescence filters with excitations of 450 – 490 nm and rhodamine filters with emissions of 515 – 565 nm were used; the objectives were LCI 'Plan-Neofular', and image processing was done with a Zeiss LSM image examiner.

ELISA for the urinary sediment

We established our staining system using urine samples from patients with nephrotic-range proteinuria. Seven millilitres of urine (collected during 24 hours) was centrifuged at 1,500 rpm; the pellet was resuspended in 500 µl of lysis buffer (0.1 M Tris-HCl, pH 7, 10% SDS supplemented with protease inhibitors) (Complete, Roche), and then the sample was spun at 5,000 rpm and the supernatant was saved and used for ELISA assays. The urinary sediment from 17 healthy controls matched by age and sex were used as controls.

The assay was carried out using polystyrene microwell plates coated overnight at 4°C with 100 µl of anti-WT-1 monoclonal antibody diluted 1 : 200 in PBS. The active sites were blocked for 30 minutes with 3% bovine serum albumin dissolved in PBS-0.05% Tween 20, pH 7.2. After washes with PBS-Tween, endogenous peroxidase was blocked for 30 minutes with 3% H_2O_2 dissolved in methanol, and after three washes with PBS, 100 µl of extract was applied to each well, and the samples were incubated for one hour at room temperature. After additional PBS-Tween washes, the micro wells were incubated for one hour with 100 µl of anti-WT-1 diluted 1 : 200, and after three washes with PBS, an additional 60-minute incubation with 100 μl HRP-goat anti-mouse IgG (Zymed) was performed. Additional washes were performed, and then the color reaction was developed by incubation with the TMB substrate solution (3,3',5,5'-tetramethylbenzidine dihydrochloride / hydrogen peroxide, Sigma-Aldrich, St. Louis, MO) for 30 minutes. The reaction was stopped with 100 µl of 0.5 M sulfuric acid. The optical density was measured at 450 nm.

The data were processed by Student t test using the Prisma program.

Results

Histology

The lupus nephritis biopsies included ten patients with class IV LN and seven with class III LN. Patients with class IV nephritis displayed the highest activity and chronicity scores, while the control kidneys had no histological evidence of renal disease [Table 1].

Proliferation assessment

The total number of glomerular cells was determined by staining with 4',6-diamidino-2-phenylindole and correlated well with the cell number count obtained by hematoxilin and eosin staining. In healthy controls the cell number had a mean of 93.5 ± 2.0, in class III patients

Table 1: Clinical and histological data of lupus nephritis patients

Gender	Age	Disease duration in years	Glomerular filtration rate (ml/min)	Nephritis		
				Class	Activity	Chronicity
Female	33	5	CrCl 108	IV-	4	4
Male	19	8	CrCl 49	IV-	4	5
Male	40	8	CrCl 109	III-	5	1
Female	26	7	CrCl 130	IV-	8	4
Female	15	7	CrCl 79.8	III-	6	3
Female	35	5	CrCl 124	III-	6	2
Female	24	10	CrCl 103	III-	8	0
Female	34	9	CrCl 38	IV-	14	12
Female	32	4	CrCl 103	IV-	4	3
Female	14	1	CrCl 47	III-	16	11
Female	16	4	CrCl 89	IV	6	0
Female	30	4	CrCl 96	IV-	4	5
Female	36	1	CrCl 108	IV-	5	2
Female	26	2	CrCl 86	III-	8	1
Male	16	2	CrCl 55	IV-	10	1
Female	26	2	CrCl 86	III-	8	5
Female	19	4	CrCl 97	IV	7	5

Sd = Standard deviation, CrCl = Creatinine clearance (24-hour collection)

it was 128 ± 20, and in patients with class IV it was 137 ± 8.7; therefore, it was an increased cell number in lupus nephritis patients. The proliferation rate measured by a proliferating cell nuclear antigen (PCNA) marker was 6.4 ± 0.5 cells / glomeurulus. Such a number was significantly superior in class IV lupus nephritis [Table 2 and Figure 1].

WT-1 is expressed in all glomeruli

The total number of glomerular cells was obtained by determining the number of DAPI-positive nuclei (stained in blue), as stated in Table 2. WT-1 positive cells with double staining (turquoise) were quantified separately to obtain the number of podocytes per glomerulus. The slide analysis was carried out by two independent investigators who were blinded to the manner of the study.

The control kidney tissues broadly expressed the podocyte marker WT-1, which was exclusively detected along the glomerular structures in normal kidney biopsies. In normal biopsies, the number of podocytes had a mean of 53 / glomerulus, which represented 56% of the glomerular cell population. In lupus nephritis biopsies, there was a 39% decrease of the WT-1 tagged cells, because there were 22 podocytes / glomerulus, which represented 22% of cell population, and furthermore, few podocytes were observed scattered along the glomerular tissues in lupus nephritis patients and significant differences between the biopsies of controls and lupus nephritis patients were found ($P<0.0001$) [Tables 2 and 3 and Figure 2].

Proteinuria is associated with podocyte decreases in lupus nephritis

Protein traces were found in three urine samples from 17 normal individuals, without clinical evidence of renal disease. In sharp contrast, patients with lupus nephritis had a mean of 2.08 g proteinuria in their 24-hour collected urine, and the difference between the groups was significant ($P<0.0001$) Table 3.

Patients with lupus nephritis show increased excretion of urinary podocytes

In healthy controls, the number of urinary podocytes was irrelevant, because only one podocyte was found in ten microscopy fields and the optical density obtained by ELISA was 0.1. Conversely, patients with lupus nephritis exhibited 10 to 70 podocytes per microscopic field, and ELISA revealed a significant increase in the optical density, up to 0.5, suggesting a cumulative excretion of podocytes in the urine. Furthermore, a statistical difference ($P<0.0001$) between controls and lupus nephritis patients was

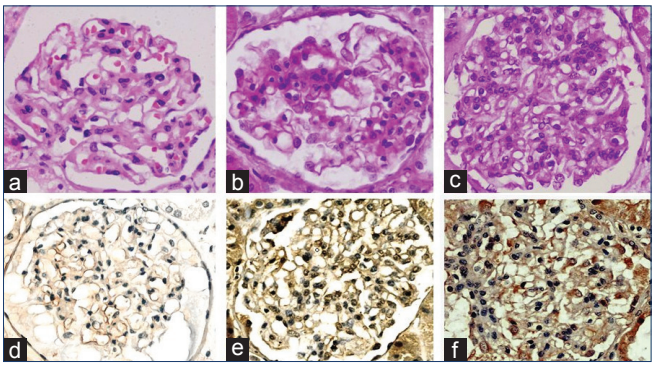

Figure 1: Proliferative changes assessed by the proliferating cell nuclear antigen (PCNA) in Lupus Nephritis (LN). Superior panel stained by H and E. Inferior panel, immunohystochemistry for PCNA. a and d: Control biopses. b and e: Class III nephritis. c and f: Class IV nephritis (40×).

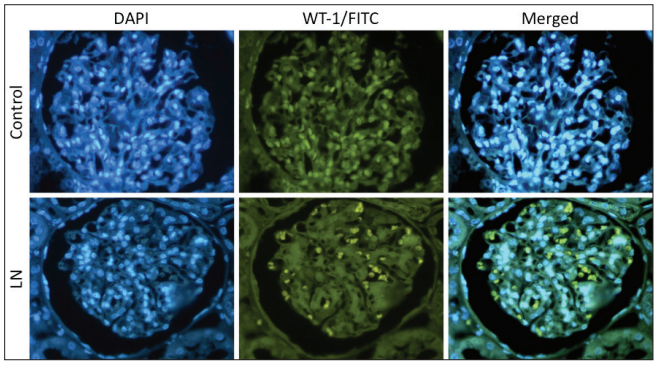

Figure 2: Podocytes detected by immunofluorescent WT-1 expression in the glomeruli. Control tissues are represented in the left panel staining in blue by DAPI; the center panel shows the WT-1 podocytes marked in green, and the right panel shows a merged figure, where the podocytes are tagged in turquoise. The upper panel shows the control tissue, and the lower panel shows a representative lupus nephritis biopsy (40×)

Table 2: Glomerular cell number and proliferation marker PCNA

Group	Control	Lupus nephritis III	Lupus nephritis IV	*P* value
Cell number	93.5 ± 2.0	128 ± 20	137 ± 8.7	C vs. III = 0.01; C vs. IV = < 0.0001; III vs. IV = 0.6
PCNA	1.58 ± 0.1	5.9 ± 0.2	6.4 ± 0.5	C vs. III and IV = < 0.001 III vs. IV = 0.5

Significant $P < 0.05$, PCNA = Proliferating cell nuclear antigen

Table 3: Biomarkers in lupus nephritis

Group	Proteinuria (g/L)	Glomerular WT-1 number	Urinary WT-1 (O.D.)	TUNEL in podocytes
Control	0.00785 ± 0.00712	53.94 ± 4.817	0.111 ± 0.005	2.3935 ± 0.4823
Lupus nephritis	2.3935 ± 0.4823	22.35 ± 2.201	0.543 ± 0.301	3.661 ± 2.373
P value	$P < 0.0001$	$P < 0.0001$	$P < 0.0001$	0.7744

Significant $P < 0.05$

observed. The apoptotic rate in urinary podocytes was not significantly different between healthy controls and lupus patients.

Discussion

The major findings of the present study demonstrate the following: (1) a decrease in glomerular podocytes associated with lupus nephritis; (2) an association between proteinuria and decreased podocyte numbers in lupus glomerulus, and (3) an increased excretion of urinary podocytes in patients with lupus nephritis. These three variables are the key indicators of renal disease progression in lupus patients.

The clinical differences between class III and IV nephritis were that class IV developed hypertension more frequently and the proteinuria was greater, also the SLEDAI index was superior (data not shown). There was an association in three patients to high index of histology, index with high anti-DNA titers, and hypocomplementemia. As expected the proportion of females was 7:1.

Proteinuria and hematuria are typical features of lupus nephritis, attributed to immune complex deposition and endocapillary proliferation. These abnormalities cause disruption of the glomerular filtration barrier; nevertheless, it is not clear whether podocytes are affected in the foot process dynamics (effacement), or whether this is caused by an inflammatory process. Normally, podocytes are attached to the Glomerular Basement Membrane (GBM). In crescentic glomerulonephritis, podocytes can move out of the GBM into areas of crescentic damage, and such movement may result in foot process effacement and proteinuria.[10]

Podocytopathy has been associated with lupus nephritis, particularly in those patients who develop the nephritic syndrome.[11] A probable explanation for this finding is that the reduced expression of podocytes is associated with the crescentic features of glomerulonephritis and results in proteinuria.[12] In this process, the podocytes are re-located outside of the crescents and lose their dedifferentiation markers.[13]

The present study shows a direct relationship between proteinuria and decreases in glomerular podocytes in lupus class III or IV nephritis, resulting in abnormal urinary sediments in these patients. Urine analysis does not routinely detect podocytes or glomerular cells, and consequently, a majority of lupus nephritis studies do not take into account the role of podocytes in disease progression. In this investigation, podocytes have been detected in renal tissues simultaneously with urinary sediments, demonstrating that the increased excretion of urinary podocytes in lupus nephritis reflects tissue damage, because patients exhibit class IV nephritis, with the major activity index scores associated with increased urinary podocytes. The present results confirm the findings reported by others and suggest the possible role of podocytopathy in severe forms of lupus nephritis.[10-13]

Angiopoietins (Ang-1 and Ang-2) are growth factors involved in angiogenesis and vasculogenesis. They are critical for normal vascular development; Ang-1 is normally expressed in glomerular podocytes, and thinly regulates the Ang-1 / Ang-2 level ratio (supporting Ang-1) and contributes toward the maintenance of the integrity of the glomerular filtration barrier, while Ang-2 may have a significant role in the pathophysiology of glomerular disease, therefore, Ang-2 is dependent on the VEGF-A levels; a reduction of glomerular expression of VEGF-A increases the Ang-2 / Ang-1 ratio; which turns down angiogenesis and triggers apoptosis. The decrease of glomerular podocytes may be associated with an increased balance of Ang-2 and decrease of VEGF-2.[14]

It has been reported that podocytes are excreted in the urine during activity flares of lupus nephritis. Most of podocytes in the urine are viable.[15] This observation is in agreement with the results of the present study. For this reason, the relationship between VEGF, podocyturia, and apoptosis needs to be further elucidated. In summary podocyturia is not a specific marker for lupus nephritis, but just indicates that there is renal injury in a lupus patient. The kidney biopsy together with renal function assays is a useful toll to define lupus nephritis flares.

In conclusion, a decrease in the number of glomerular podocytes is a cell marker of renal damage and would be a predictive factor of the loss of function of filtration in the kidneys of patients with lupus nephritis.

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