

Podocyte Expression of Osteopontin and FSP-1/S100A4 in Human Crescentic Glomerulonephritis

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Background: Osteopontin (OPN) is a cytokine associated with a cell-matrix via integrins. Fibroblast specific protein-1 (FSP-1), known as S100A4, has been implicated in cell migration by non-muscle myosin. We investigated whether the role of OPN and FSP-1/S100A4 expression in their contribution to the podocyte phenotype change to form podocyte bridge and cellular crescent. **Methods:** Glomerular expression of OPN and FSP-1/S100A4 in renal biopsies of 16 patients with crescentic glomerulonephritis (CrGN) and 13 normal renal biopsies were studied by immunohistochemistry. **Results:** The expression of OPN and FSP-1/S100A4 was increased in the podocytes of glomeruli, with and without crescents, in patients with CrGN. Neither OPN nor FSP-1/S100A4 was expressed in glomeruli from the normal controls ($p < 0.01$). A significant positive correlation was found between the expression of OPN in glomerular tufts and cellular crescents, and the expression of OPN and FSP-1/S100A4 in glomerular tufts ($p < 0.05$). **Conclusions:** The results suggest that OPN plays a role in early podocyte attachment to Bowman's capsule, and FSP-1/S100A4 potentiate podocyte contribution to cellular crescent formation by inducing cellular migration and growth.

Key Words: Osteopontin; FSP1 protein; S100A4 protein; Podocytes; Crescentic glomerulonephritis

Crescents have been described as a mixture of monocytes/macrophages, glomerular parietal epithelial cells, and myofibroblasts; however, the cellular components of crescents still remain controversial. Podocytes have not been thought to be involved in crescents; this is because markers for the podocyte phenotype have been rarely reported in crescents.¹ However, studies have shown that podocytes form bridges between the glomerular capillary tufts and Bowman's capsule during the early phase of disease in an anti-glomerular basement membrane (GBM) nephritis mouse model.² Both in Wilms tumor suppressor protein 1 (WT1) and synaptopodin, podocyte phenotype markers have been reported to be negative. However, the galactosidase gene-transfected podocytes have been shown to populate cellular crescents in an anti-GBM nephritis mouse model.³ In addition, nestin-positive podocytes contribute to crescent formation in human crescentic glomerulonephritis (CrGN).⁴ Such observations suggest that podocytes play a central role in early cellular crescent formation by migrating and adhering to Bowman's capsule.

Osteopontin (OPN) is a secreted or a matrix type, Ca^{2+} -dependent and highly acidic phosphorylated glycoprotein. The

secreted OPN is cleaved by thrombin into two soluble segments, but one, interacting with integrin $\alpha\beta_3$, is six amino acids to the COOH-terminal side of Arg-Gly-Asp (RGD) sequences.⁵ OPN mediates cell-matrix adhesion and delays survival by intracellular signaling that changes the focal adhesion kinase status by interacting with $\alpha\beta_3$ integrin, in a RGD sequence dependent manner.⁵ The other one, produced by thrombin cleavage, that is congruous with the COOH-terminal motif of OPN, is also detected in certain physiological and pathological conditions. The autocrine incursion of CD44v6 by soluble OPN, can induce the loosening of cell-matrix adhesion and contributes to cell migration via Rho family GTPase activation.⁶ As matrix components, OPN and hyaluronate (HA) competitively interact with CD44; HA, but not OPN, induces standard CD44 (CD44s)-dependent cell-matrix adhesion and cell-cell aggregation.

In human CrGN, integrin $\alpha\beta_3$ expression has been reported to increase in the podocytes of glomerular capillary tufts and in distal tubular epithelial cells;^{7,8} otherwise, CD44s expression has been shown in cellular components of the crescents, as well as in a rat model.^{9,10} In murine models of CrGN, OPN expres-

sion has been shown in podocytes and cellular components of crescents;^{10,11} however, it has not yet been determined in human CrGN.¹² Therefore, to date, whether podocytes express OPN to form podocyte bridges between glomerular tufts and Bowman's capsule or to form cellular crescents has not been confirmed in human CrGN.

Fibroblast specific protein-1 (FSP-1) protein is a part of the large S100 family of Ca^{2+} binding proteins. The internal activity of FSP-1/S100A4 is mediated by numerous intracellular protein partners, especially non-muscle myosin, which is associated with cell motility. The external paracrine effects of extracellular FSP-1/S100A4 are mediated by interactions with, for example, the receptor of advanced glycation end-products, annexin II, and heparan sulfate proteoglycans.¹³ FSP-1/S100A4, as a marker of 'epithelial-to-mesenchymal transition,' has been reported to be expressed in distal tubular epithelial cells in a rat tubular injury model.¹⁴ Rossini *et al.*¹⁵ reported that FSP-1/S100A4 expression increased in the podocytes of glomeruli with cellular crescents from patients with lupus nephropathy. Both OPN and FSP-1/S100A4 appear to be associated with an increase in cell motility, phenotype differentiation, and the inhibition of cell death. However, it is not clear what role they play in the podocyte adhesion to Bowman's capsule and in the podocyte migration into cellular crescents.

This study was designed to investigate whether there is any change in the cell-matrix adhesion and migration-related factors in glomerular cells that are associated with cellular crescent formation. The expression of OPN and FSP-1/S100A4 was studied in the glomeruli with or without cellular crescents from patients with CrGN and compared to normal controls (NCs). We investigated whether the expression of OPN and FSP-1/S100A4 was localized to the podocytes and contributed to the formation of podocyte bridges or cellular crescents in patients with CrGN.

MATERIALS AND METHODS

Study group

Sixteen patients with CrGN presented with the acute clinical features including: generalized edema, proteinuria or hypertension from 2001 to 2006. All 16 patients had typical clinical features of rapidly progressive glomerulonephritis and were diagnosed with CrGN based on the following criteria: presence or absence of perinuclear or cytoplasmic antineutrophil cytoplasmic antibody (ANCA), presence or absence of immunofluores-

cent deposition in glomerular capillary tufts, and the presence of morphological characteristics of CrGN. Tests for blood urea nitrogen concentrations, serum creatinine concentrations, and urinalysis were performed using standard laboratory procedures. Prior to a renal biopsy, each patient underwent a 24 hour urine collection to calculate the glomerular filtration rate (GFR) and urinary protein excretion. Renal insufficiency was defined as a serum creatinine levels in excess of 1.5 mg/dL or as a GFR lower than 80 mL/min/1.73 m². The NCs were selected from patients presenting for health screening for military service or a job. Thirteen biopsies had non diagnostic abnormalities in patients that initially presented with an abnormal routine urinalysis. In addition, one normal renal cortical section from a patient with renal cell carcinoma was selected to control the technical problems.

Immunohistochemistry

A portion of the tissue was placed in a 10% phosphate buffered formalin fixative solution for more than 12 hours, washed in tap water, and embedded in paraffin. Immunohistochemistry was performed on 16 formalin-fixed paraffin-embedded renal biopsy specimens from patients with CrGN and 13 NCs.

An avidin-biotin-peroxidase procedure for antibody localization was added to each serial 3 μm -paraffin section as previously described. In brief, the sections were placed in 0.1 N citrate buffer (pH 6.0) for antigen unmasking in a microwave at 600 W (5 minutes). The evaporated volume was replaced with sodium citrate buffer and this procedure was repeated twice. To prevent endogenous peroxidase reactivity and biotin activity, the sections were incubated in methanol containing 3% H_2O_2 for 30 minutes to block endogenous peroxidase activity and in an avidin biotin blocking kit (Vector Laboratories, Burlingame, CA, USA) for 30 minutes, respectively. The sections were then incubated in blocking antibody for 30 minutes at room temperature, followed by the removing excess serum, and then incubated the slides for 18 hours at 4°C in a humidified chamber with mouse anti-human C-terminal motif OPN (1 : 600, ARP, Belmont, MA, USA) and rabbit polyclonal anti-human FSP-1/S100A4 (1 : 1,200, DakoCytomation, Glostrup, Denmark) antibodies. Biotinylated horse anti-mouse immunoglobulin and biotinylated goat anti-rabbit immunoglobulin were used for the primary mouse monoclonal antibody and for the primary rabbit polyclonal antibody, respectively. Detection was carried out with a Vectastatin Elite ABC kit (Vector Laboratories). The sections were labeled with 3,3'-diaminobenzidine tetrahydro-

chloride (DAB) for 1 minute. Then the sections were counter stained with 1% Mayer hematoxylin.

Double labeled immunohistochemistry

To determine whether podocytes express OPN or FSP-1/S100A4, sections of all renal biopsy specimens from patients with CrGN and NCs were prepared as described above. Then immune-labeling for the first primary antibodies was carried out as previously described.¹⁶ Detection for the anti-OPN antibody was performed with a Vectastatin ABC AP kit and a Vectastatin Elite ABC kit for the anti-FSP-1/S100A4 antibody. The sections were labeled with 5-bromo-4-chloro-3-indolyl phosphate/nitroblue tetrazolium (NBT/BCIP, Vector Laboratories) for 5 minutes and with DAB, respectively. For the second labeling, sections were placed in Tris-EDTA buffer (pH 8.0) for antigen unmasking in a microwave at 600 W (5 minutes, repeated 3×). The sections were performed for the endogenous peroxidase and biotin blocking procedure as described above and incubated in blocking antibody for 30 minutes at room temperature. The excess serum was removed from the slides and were incubated for 1 hour at room temperature in a humidified chamber with mouse monoclonal anti-human synaptopodin (1 : 400, Progen Biotechnik GmbH, Heidelberg, Germany) or mouse monoclonal anti-human WT1 (1 : 600, N-terminal truncated WT1 [6F-H2], Cell Marque Co., Rocklin, CA, USA) antibodies, to identify the podocytes. Biotinylated horse anti-mouse immunoglobulin or dextran-coupled goat anti-mouse immunoglobulin were used for the detection anti-OPN antibody and was carried out with a Vectastatin Elite ABC kit or with a EnVision™ AP kit (DakoCytomation). The sections were labeled with DAB or with Permanent Red (DakoCytomation) for 5 minutes and then counter-stained with 1 % Mayer hematoxylin.

Finally, to determine the 'tuft-to-capsule connections' in normal glomeruli as described previously,¹⁷ one normal cortical specimen from patients with renal cell carcinoma was prepared as described above. The immunostaining for the mouse anti-human WT1 antibody was carried out and the sections were labeled with DAB. To determine the parietal epithelial cells and 'capsule-to-tuft reflections (parietal epithelial cell-tuft podocyte)' in normal glomeruli, the immunostaining for the mouse anti-human cytokeratin (CK) antibody was carried out and the sections were labeled with Nova Red. For the double labeling of both mouse monoclonal anti-human WT1 and -CK antibodies, which were used to determine the 'podocyte-to-parietal epi-

thelial cell connections' because neither express WT1 nor CK, sections were labeled by DAB-Ni and DAB, respectively. All other procedures were performed as described above.

Assessment of OPN and FSP-1/S100A4 immunoreactivity

The presence of at least three or more layers of cellular accumulation between the glomerular capillary tufts and Bowman's capsule is referred to as a crescent. To quantify of expression of OPN or FSP-1/S100A4, OPN or FSP-1/S100A4 positive glomerular cells were counted per glomerular capillary tufts and cellular, fibrocellular, and fibrous crescents in each case. The extent of OPN or FSP-1/S100A4 expression in podocytes was assessed for the glomerular capillary tufts or crescents by a semi-quantitative scale: 0, negative; 1, when the immunoreactivity was present in less than 25% of the area examined; 2, when present in 25-50%; 3, when present in 50-75%; 4, when present in more than 75%. The relationship between the localization of OPN or FSP-1/S100A4 in the same glomerular cells was studied using consecutive sections for confirmation. All specimens were finally reviewed by two pathologists.

Statistical analysis

Statistical analyses were performed using SPSS ver. 12.0 (SPSS Inc., Chicago, IL, USA). The results are expressed as the mean ± standard deviation. The continuous and ordinal variables such as age, GFR, and proteinuria were compared between the groups, with the student t-test. The non-continuous variables including sex, urinalysis results, or staining scores were compared between the groups using the chi-square test. The semi-quantitative staining scores were compared between OPN and FSP-1/S100A4 expression in the glomerular capillary tufts and crescents by way of an ANOVA test. A trend analysis of the non-continuous variables between the groups was performed using the Mantel Haenszel chi-square test. The relationship between the staining scores between OPN and FSP-1/S100A4 expression in the glomerular capillary tufts and crescents were compared by Spearman's correlation coefficient. A p-value of less than 0.05 was considered as statistically significant.

RESULTS

Clinical features of patients with CrGN and NCs

The ages of patients with CrGN ranged from 8 to 79 years

(44.13 ± 24.59 years), while the patients with NCs ranged from 20 to 59 years (41.85 ± 12.09 years). Eight men and eight women were included in the CrGN group, and three men and ten women were included in the NC group. One patient had linear immunoglobulin deposits along the glomerular capillary tufts (type I CrGN) and six patients had granular immunoglobulin deposits along the glomerular capillary tufts or in the mesangium on immunofluorescent study (type II CrGN). The p-ANCA was positive in six patients with CrGN (ANCA-positive pauci-immune [type III] CrGN) and negative for the rest (ANCA-negative pauci-immune [type III] CrGN). The mean serum blood urea nitrogen (38.29 ± 34.53 mg/dL vs 13.92 ± 3.49 mg/dL) and creatinine (2.90 ± 2.46 mg/dL vs 0.76 ± 0.99 mg/dL) levels were higher in the CrGN group than in NC group; the differences between the two groups were statistically significant ($p < 0.05$ and $p < 0.01$, respectively). In the CrGN group, proteinuria was present in fifteen patients ($p < 0.001$) and hematuria was present in all patients ($p < 0.05$). For the NCs, the ten patients had hematuria; however, after several weeks of follow up the hematuria resolved on the following uri-

nalys. The mean 24 hour urine volume was greater in the CrGN group compared to the NC group ($1,534.44 \pm 987.33$ mL vs $1,115.78 \pm 670.44$ mL); in this case, the difference between the two groups was not significant ($p = 0.308$). However, the mean GFR was less in the CrGN group than in the NC group (54.31 ± 53.24 mL/min/ 1.73 m² vs 97.77 ± 17.37 mL/min/ 1.73 m²), and the mean amount of 24 hour protein was greater in the CrGN group than in the NC group ($3,866.93 \pm 1,877.97$ mg/day vs 33.26 ± 31.37 mg/day); and the difference between the two groups was highly significant ($p < 0.001$) (Table 1).

Expression of OPN and FSP-1/S100A4 in patients with CrGN and NCs

OPN expression was localized to the podocytes of patients with CrGN. OPN expression increased in the podocytes from glomerular capillary tufts with or without cellular crescents of patients with CrGN (Fig. 1A) compared to the podocytes of NCs. Without exception, OPN expression was observed in the

Table 1. Clinical data in patients with crescentic glomerulonephritis (CrGN) and normal controls (NCs)

Variable	CrGN	NCs	p-value
No.	16	13	
Age (yr)	44.13 ± 24.59	41.85 ± 12.09	0.740
Sex			
	Males	3	
	Females	10	0.249
IF deposits			
	Linear positive (type I CrGN)	0	
	Granular positive (type II CrGN)	0	
	Negative	13	
ANCA			
	Positive (type III CrGN)	0	
	Negative	13	
Biochemistry			
	BUN (mg/dL)	13.92 ± 3.49	0.013
	sCr (mg/dL)	0.76 ± 0.99	0.003 ^a
Proteinuria			
	Heavy	0	
	Mild to moderate	0	0.000 ^a
	Negative	13	
Hematuria			
	Heavy	8	
	Mild to moderate	2	0.042 ^b
	Negative	3	
24 hr urinalysis			
	Urine volume (mL)	$1,115.78 \pm 670.44$	0.308
	GFR (mL/min/ 1.73 m ²)	97.77 ± 17.37	0.006 ^a
	Protein excretion (mg/day)	33.26 ± 31.37	0.000 ^a

Values are expressed as mean \pm standard deviation. p-values are evaluated by student t-test or by χ^2 test (^a $p < 0.01$; ^b $p < 0.05$).

IF, immunofluorescent; ANCA, antineutrophil cytoplasmic antibody; BUN, blood urea nitrogen; sCr, serum creatinine; GFR, glomerular filtration rate.

podocytes, forming “podocyte bridges” (tuft-to-capsule adhesion). Both synaptopodin and OPN were expressed in the podocytes located in the inner third and outer third of crescents from patients with CrGN (Fig. 1B). OPN expression was scarcely observed in other cells of the glomeruli in the patients with CrGN; except for a few parietal epithelial cells in the cellular crescents or a few distal convoluted tubular epithelial cells adjacent to the glomerular tubular pole. OPN expression was localized to CK-positive parietal epithelial cells adjacent to the tubular pole. Neither synaptopodin-positive podocytes nor CK-positive parietal epithelial cells expressed OPN in the glomeruli from NCs; but OPN expression was noted in the cytoplasm of a few distal convoluted tubular epithelial cells (Fig. 1C).

FSP-1/S100A4 expression was localized to the podocytes of patients with CrGN and increased in the podocytes of glomerular capillary tufts with or without cellular crescents in patients with CrGN (Fig. 1D). Both WT1 and FSP-1/S100A4 were expressed in podocytes located in the inner third of crescents from

patients with CrGN (Fig. 1E). Podocytes that expressed both WT1 and FSP-1/S100A4, changed their morphological phenotype, and formed cellular aggregates on the glomerular capillary tufts and in Bowman’s space, as podocyte casts. FSP-1/S100A4 expression was scarcely observed in other cells of the glomeruli in patients with CrGN; except for the neutrophils and monocytes/macrophages trapped in the glomerular capillary lumen. FSP-1/S100A4 expression was localized to the CK-positive parietal epithelial cells, especially in cells adjacent to the tubular pole. Neither WT1-positive podocytes nor CK-positive parietal epithelial cells expressed FSP-1/S100A4 in the glomeruli from NCs; but FSP-1/S100A4 expression was observed in the nuclei of few distal convoluted tubular epithelial cells adjacent and in the peritubular resident fibroblasts (Fig. 1F).

In NCs, WT1 was expressed in the podocytes forming ‘tuft-to-capsule connections’ adjacent to the vascular pole and in the attenuated parietal epithelial cells along Bowman’s capsule (Fig. 2A). CK was expressed in the cuboidal parietal epithelial cells

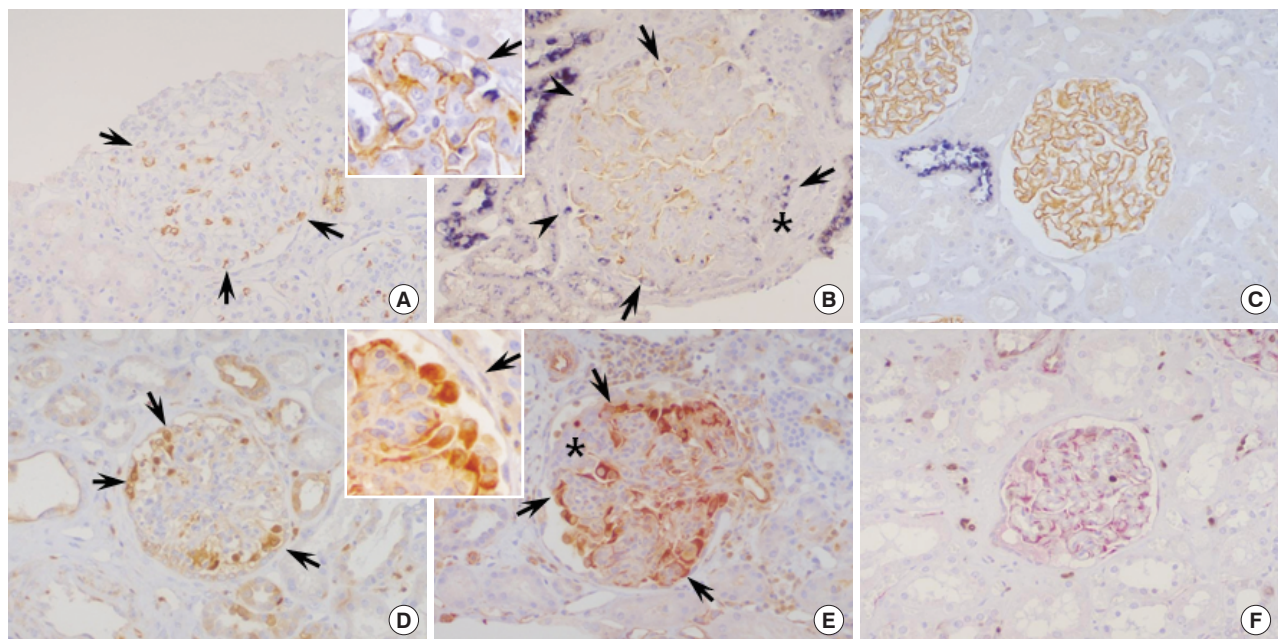


Fig. 1. Osteopontin (OPN) expression in glomerular capillary tufts and cellular crescents from the patients with crescentic glomerulonephritis (CrGN). (A) A normal looking, but hypertrophic glomerulus showing many podocytes with OPN expression (3,3'-diaminobenzidine tetrahydrochloride (DAB) [brown], arrows) in patients with CrGN. (B) A cellular crescent (asterisk) showing podocytes with both synaptopodin (DAB [brown]) and OPN expression (5-bromo-4-chloro-3-indolyl phosphate/nitroblue tetrazolium [purple], arrows), as well as parietal epithelial cells with OPN expression (arrowheads), to form podocyte bridges ('tuft-to-capsule adhesion' in the inset) in patients with CrGN. (C) A normal glomerulus showing a linear synaptopodin-positive podocytes without OPN expression; fibroblast specific protein-1 (FSP-1)/S100A4 expression in glomerular capillary tufts and cellular crescents in patients with CrGN. (D) A normal looking glomerulus showing several podocytes with FSP-1 expression (DAB [brown], arrows), is retracted from the glomerular basement membrane in patients with CrGN. (E) A cellular crescent (asterisk) showing podocytes with both Wilms tumor suppressor protein 1 (WT-1; Permanent Red [red]) and FSP-1/S100A4 expression (DAB [brown], arrows), to form a podocyte bridge ('tuft-to-capsule adhesion' in the inset) from patients with CrGN. (F) A normal glomerulus showing WT1-positive podocytes without FSP-1/S100A4 expression.

adjacent to the tubular pole (Fig. 2B). Though a part of attenuated cells along Bowman's capsule expressed WT1, most did not express WT1 or CK (Fig. 2C).

Analysis of OPN and FSP-1/S100A4 expression scores in patients with CrGN

The podocyte staining scores of OPN and FSP-1/S100A4, in the glomerular capillary tufts from patients with CrGN were significantly higher compared to the NCs (χ^2 test, $p < 0.001$). The podocyte staining scores of OPN were likely to be lower according to the progression of crescents in patients with CrGN; the trend was highly significant (Mantel Haenszel chi-square test; $Q_{MH} = 17.852$, $p < 0.001$; $r = -0.563$). The podocyte stain-

ing scores of FSP-1/S100A4 were likely to be lower according to the progression of crescents in patients with CrGN; however, the trend was statistically not significant (Mantel Haenszel chi-square test; $Q_{MH} = 3.581$, $p = 0.058$; $r = -0.110$) (Table 2).

Relationship between OPN and FSP-1/S100A4 expression in patients with CrGN

By semiquantitative analysis, the podocyte staining scores of OPN and FSP-1/S100A4 in the glomerular capillary tufts from patients with CrGN increased significantly compared to the NCs (2.17 ± 0.89 vs 0.03 ± 0.94 and 1.66 ± 0.69 vs. 0.09 ± 0.13 ; t-test, $p < 0.001$) (Fig. 3). The difference in podocyte staining scores for OPN in the cellular crescents, fibrocellular crescents,

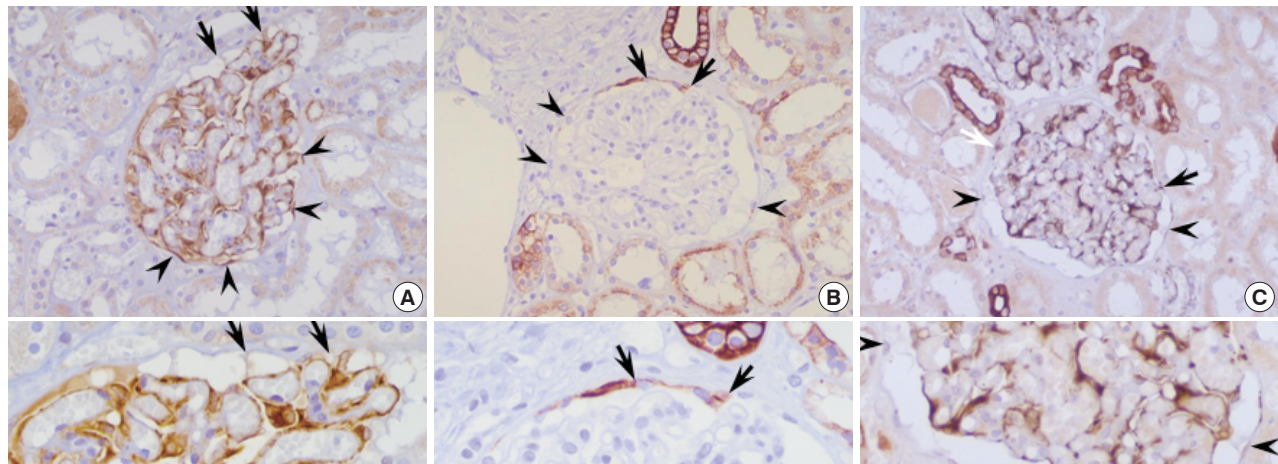


Fig. 2. Normal glomerular structures from the normal controls. (A) A normal glomerulus showing a Wilms tumor suppressor protein 1 (WT1)-positive podocyte 'tuft-to-capsule connection' (3,3'-diaminobenzidine tetrahydrochloride (DAB) [brown], arrows in the inset), and attenuated WT1-positive parietal epithelial cells (DAB [brown], arrowheads) of Bowman's capsule. (B) A normal glomerulus showing cytokeratin (CK)-positive parietal epithelial cell 'capsule-to-tuft reflection' (Nova Red [red], arrows in the inset), and CK-negative parietal epithelial cells (arrowheads), which are probably WT1-positive parietal epithelial cells or both WT1- and CK-negative epithelial cells of Bowman's capsule. (C) A normal glomerulus showing WT1-positive podocytes (DAB-Ni [dark gray]; arrow), CK-positive parietal epithelial cells (DAB [brown]; white arrow), and both WT1- and CK-negative epithelial cell 'capsule-to-tuft reflection' (arrowheads at the inset) of Bowman's capsule.

Table 2. Scores of OPN and FSP-1/S100A4 positive podocytes in patients with CrGN and NCs

Score	OPN					Score	FSP-1/S100A4				
	NCs ^a	CrGN					NCs ^a	CrGN			
		GCT ^b	CCr	FCCr	FCr			GCT ^b	CCr	FCCr	FCr
4	0	22	5	1	0	4	0	2	1	0	0
3	0	31	8	1	0	3	0	13	3	0	0
2	0	37	17	0	0	2	1	72	19	5	0
1	4	63	16	8	1	1	2	79	48	10	0
0	208	8	0	3	5	0	165	10	1	0	2
Total	212	161 ^{b,c}	46 ^b	13	6	Total	168	176 ^{b,c}	72	15	2

^ap-values were evaluated by χ^2 test ($p = 0.000$); ^bCorrelation between the podocyte staining scores of OPN and FSP-1/S100A4 in GCT ($r = 0.607$, $p = 0.048$);

^cThose of OPN in GCT and CCr ($r = 0.591$, $p = 0.016$).

OPN, osteopontin; FSP-1, fibroblast specific protein-1; CrGN, crescentic glomerulonephritis; NC, normal controls; GCT, glomerular capillary tufts; CCr, cellular crescents; FCCr, fibrocellular crescents; FCr, fibrous crescents.

and fibrous crescents from patients with CrGN were highly significant (2.25 ± 1.08 , 1.23 ± 1.02 , and 0.25 ± 0.50 , respectively; ANOVA test, $p < 0.001$) (Fig. 4A). In contrast, the difference in podocyte staining scores of FSP-1/S100A4 in the cellular crescents, fibrocellular crescents, and fibrous crescents from patients with CrGN were not significant (1.61 ± 0.85 , 1.38 ± 0.49 , and 0.00 ± 0.00 , respectively; ANOVA test, $p = 0.137$) (Fig. 4B). The podocyte staining scores of OPN in the glomerular capillary tufts were significantly correlated with those in the cellular crescents of patients with CrGN ($r = 0.607$, $p < 0.05$) (Table 2). In addition, the podocyte staining scores of OPN in the glomerular capillary tufts were significantly correlated with those of FSP-1/S100A4 in the glomerular capillary tufts of patients with CrGN (Spearman's correlation coefficient = 0.591, $p < 0.05$) (Table 2).

DISCUSSION

The podocyte phenotype markers, WT1 and synaptopodin were rarely observed in cellular crescents;¹ hence, podocytes have traditionally been excluded as components of crescents. However, it has been reported that podocytes form bridges between the glomerular capillary tufts and Bowman's capsule during the early phase of disease in a mouse model of anti-GBM nephritis.² Le Hir *et al.*² observed "podocyte bridges" in almost one half of the glomeruli, even without crescents and in more than 90% of the glomeruli with crescents. These features, previously described as 'tuft-to-capsule adhesion' by Gibson *et al.*,¹⁷ give us a clue to suggest that podocytes play an initiating key role in the cellular crescent formation by cell-cell or cell-matrix adhesion.

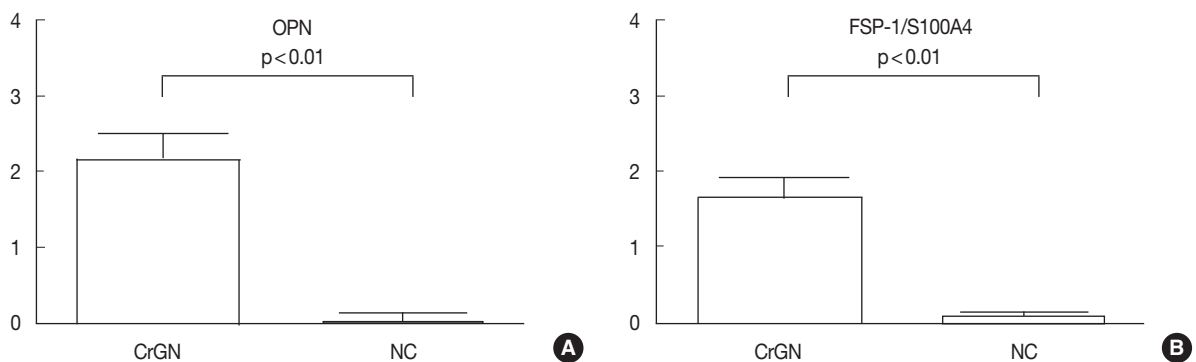


Fig. 3. Semiquantitative analysis of osteopontin (OPN) and fibroblast specific protein-1 (FSP-1)/S100A4 expression within the glomerular capillary tufts of 16 patients with crescentic glomerulonephritis (CrGN) and 13 normal controls (NCs). (A) OPN, each bar represents the mean \pm standard deviation (SD) (2.17 ± 0.89 vs 0.03 ± 0.94 ; t-test, $p < 0.001$). (B) FSP-1/S100A4, each bar represents the mean \pm SD (1.66 ± 0.69 vs 0.09 ± 0.13 ; t-test, $p < 0.001$).

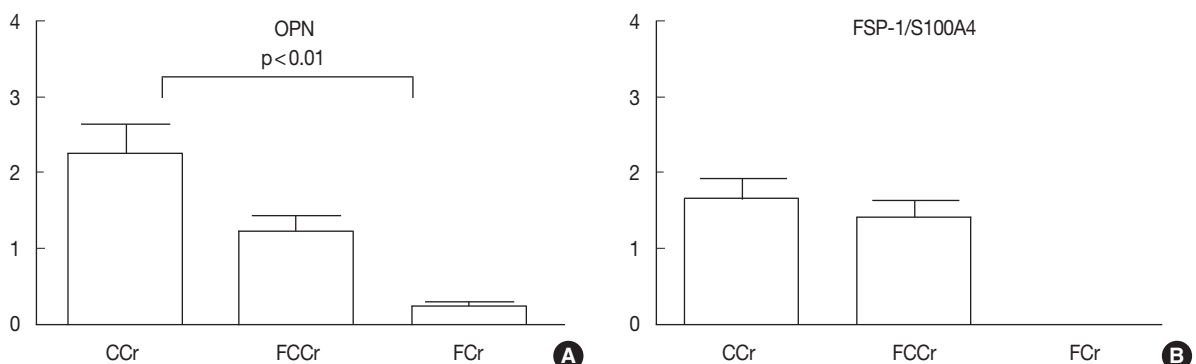


Fig. 4. Semiquantitative analysis of osteopontin (OPN) and fibroblast specific protein-1 (FSP-1)/S100A4 expression within the crescents in 16 patients with crescentic glomerulonephritis. (A) OPN, each bar represents the mean \pm standard deviation (SD) (2.25 ± 1.08 , 1.23 ± 1.02 , and 0.25 ± 0.50 , respectively; ANOVA test, $p = 0.009$). (B) FSP-1/S100A4, each bar represents the mean \pm SD (1.61 ± 0.85 , 1.38 ± 0.49 , and 0.00 ± 0.00 , respectively; ANOVA test, $p = 0.137$). CCr, cellular crescents; FCCr, fibrocellular crescents; FCr, fibrous crescents.

In the present study, cytoplasmic N-terminal truncated WT1 positive podocytes were connected to Bowman's capsule (tuft-to-capsule [podocyte-to-parietal epithelial cell] connections), and the parietal epithelial cells were connected to the podocytes (capsule-to-tuft reflections) in normal glomeruli of NCs. This "long slender spike-like" podocyte connection (tuft-to-capsule) to Bowman's capsule was frequently observed around the glomerular vascular pole. Otherwise, the "broad" parietal epithelial cell cytoplasmic reflection (capsule-to-tuft) onto the podocyte surface was seen especially around the glomerular tubular pole, as previously described in normal human glomeruli.¹⁷ Previous studies have not determined whether podocyte connections or parietal epithelial cell reflections are permanent or not. Similarly, the significance of these connections in normal glomeruli was not yet defined.¹⁸ In the present study, OPN and FSP-1/S100A4 expression at the podocyte connections and parietal epithelial cell reflections were not observed in the normal glomeruli of NCs. According to the results of the present study, the podocyte 'tuft-to-capsule connections' or parietal epithelial cell 'capsule-to-tuft reflections' appeared to be natural differentiated structures, due to both cell having the same embryological origin, that were negative for markers of an adhesive or migratory phenotype, for both OPN and FSP-1/S100A4.

Previous studies have suggested that 'tuft-to-capsule connections' described in the normal glomeruli are likely precursors of 'tuft-to-capsule adhesions' in glomerular hilar sclerotic lesions during hemodynamic stress or 'capsule-to-tuft adhesions' in glomerular tip lesions associated with massive proteinuria;¹⁷ however, their role in human CrGN remains unclear. Podocyte 'tuft-to-capsule adhesions' in human glomerular diseases, so called "podocyte bridges," have been reported in an anti-GBM nephritis mouse model.² In the present study, podocyte bridges were also observed in glomeruli without crescents from patients with CrGN, despite variable preservation status of each renal biopsy specimen. The morphologic features of these adhesions were more likely to be "broad" pathologic adhesions, which were different from the "long slender spike-like" normal connections between podocyte and parietal epithelial cells as originally described by Gibson *et al.*¹⁷

WT1 controls adherence junction proteins and the CD2 associated protein/nephrin/podocin complex via WT1-interacting proteins (WTIPs). Signaling associated with podocyte injury initiates WTIP nuclear translocation, and the concomitant loss of WTIP from the adherence junction proteins, which changes the dynamic assembly of actin.¹⁹ That is, the initial podocyte phenotype changes during early crescent formation may regu-

late the nuclear WT1 transcription to induce the process leading to the loss of other proteins, such as synaptopodin, podocin, and nephrin. However, even in the pathologic status, podocytes may constantly express N-terminal truncated WT1, but not nuclear translocated C-terminal motif. In the present study, the OPN or FSP-1/S100A4 positive cells, expressing cytoplasmic synaptopodin and N-terminal truncated WT1, were observed in the glomerular capillary tufts or in the inner or outer parts of cellular crescents. In addition, OPN or FSP-1/S100A4 positive cells never expressed CK in the cellular crescents of human CrGN. These findings suggest that WT1-positive well-differentiated tuft podocytes contribute to early podocyte bridges and cellular crescent formation in human CrGN, as both WT1 and CK negative renal progenitor cells do.^{4,12}

OPN, as a secreted cytokine, is efficiently cleaved by thrombin and regulates cell-matrix adhesion by attenuating OPN-integrin $\alpha\beta 3$ interaction, in a RGD dependent manner.⁵ Podocytes adhere via integrin $\alpha\beta 3$ to the Lys-Arg-Gly-Asp-Ser (KRGDS) motif of $\alpha 3$ type IV collagen on the GBM in physiologic states. However, the KRGDS motif is phosphorylated, and its interaction with integrin $\alpha\beta 3$ may play a critical role in intracellular signaling in pathologic states.²⁰ In addition, as a matrix component, OPN is capable of binding to the CD44 heparan sulfate proteoglycan, but competes with HA, in a RGD sequence independent manner.⁶ A previous study using a mouse model showed that CD44s expression in podocytes contribute to early cellular crescent formation,²¹ but not in human CrGN. However, Nakamura *et al.*²² has defined CD44s expression in the cellular components of crescents in human CrGN. Neither CD44v6 nor CD44v7 expression was determined in CrGN. Therefore, OPN expression in podocytes may initially induce CD44s in an autocrine and paracrine manner, and CD44s contributes to podocyte adhesion to Bowman's capsule and to renal progenitor cell aggregation via cell-matrix adhesion. In the present study, OPN positive podocytes in glomerular capillary tufts were frequently found to permeate into Bowman's capsule (especially on 'tuft-to-capsule adhesions' or 'podocyte bridges') and cellular crescents. However, OPN positive podocytes were not found in the normal glomeruli of NCs. These findings suggest that the expression of OPN in both tuft podocytes initiated the alteration of the podocyte phenotype, which contributed to the formation of the podocyte bridge and cellular crescent formation via its interaction with integrin $\alpha\beta 3$ and CD44s, in patients with CrGN.

OPN expression increased in the atrophic or attenuated distal tubules in patients with CrGN compared to the NCs. Previous

studies have reported OPN expression in the distal tubules and thick loops of Henle, in the normal animal and human adult kidneys.²³ OPN expression in tubular epithelial cells is due in part to hypoxic injury associated with peritubular capillary damage induced by glomerular loss;²⁴ as well as the adaptive process contributed to the increased intraluminal hydrodynamic pressure associated with proteinuria.²⁵ An increase in hydrodynamic pressure has been shown to affect intracellular Ca^{2+} influx, reorganize the actin cytoskeleton and change the cellular phenotype of the distal tubular epithelial cells.²⁵ Enhanced OPN expression, by both causes, may reinforce the cellular attachment and survival of the tubular epithelial cells via the interaction with integrin $\alpha\beta3$ and CD44s in human CrGN.^{8,22}

FSP-1/S100A4 has been independently cloned by several groups, using various names, including S100A4, metastasin 1, and calvasulin. The FSP-1 protein is part of the S100 family of Ca^{2+} binding proteins. Many studies have focused on the role of FSP-1/S100A4 in the alteration of the cytoskeletal rearrangement and increased cell motility *in vitro*.¹³ With respect to the autocrine effects, most of the established binding partners of FSP-1/S100A4 are cytoskeletal target proteins, such as non-muscle myosin heavy chain IIA (NMMHCIIA). FSP-1/S100A4 is known to regulate the dynamic cytoskeletal assembly by the regulation of NMMHCIIA phosphorylation of protein kinase C.²⁶ However, the mechanism of secretion and the receptors of FSP-1/S100A4 remain largely unknown. Several paracrine effects of extracellular FSP-1/S100A4 have been divided into three categories, including increase of cell motility, promotion of cell growth, and inhibition of cell death, by interacting with the surface proteoglycans, CD44, and are dependent on the activation of the nuclear factor- κB transcription pathway.²⁷

FSP-1/S100A4 expression increased in the nuclei or cytoplasm of tubular epithelial cells, interstitial mononuclear inflammatory cells, and myofibroblasts in patients with CrGN compared to patients with NCs. Rossini *et al.*¹⁵ reported that FSP-1/S100A4 was expressed in a few podocytes in the glomeruli with cellular crescents from patients with lupus nephropathy. The results of another study suggested that viable, FSP-1/S100A4 positive podocytes were detected in the urinary sediment of patients with minimal change diseases; although, FSP-1/S100A4 positive podocytes were not seen in glomerular capillary tufts *per se*.²⁸ That is, the FSP-1/S100A4 positive podocytes changed their phenotype to retract their foot processes, which were detached from the GBM or permeated into the cellular crescents. Podocytes are terminally differentiated cells with both epithelial and mesenchymal characteristics. Le Hir *et*

*al.*¹⁴ reported that FSP-1/S100A4 is not a “fibroblast-specific protein” as suggested by its name, but actually a hallmark of ‘epithelial-to-mesenchymal transition’ in kidney disease. Though FSP-1/S100A4 is a more potent inducer of cell motility and phenotype change, as well as a better inhibitor of cell death than OPN, the role of OPN in the regulation of FSP-1/S100A4 expression is still unclear. The FSP-1/S100A4 promoter is up-regulated by the CD44-Src-integrin signaling axis, by engagement of the OPN RGD residues.^{6,29} The genetic OPN depletion decreased FSP-1/S100A4 expression and increased apoptosis of the tubular epithelial cells in mouse models of unilateral obstructive nephropathy.³⁰

In summary, OPN and FSP-1/S100A4 were strongly expressed in podocytes of glomeruli, with or without cellular crescents, in patients with CrGN, compared to the NCs. There was a significant correlation between the expression of OPN in glomerular capillary tufts and cellular crescents, as well as between the expression of OPN and FSP-1/S100A4 in glomerular capillary tufts. These results suggest that OPN contributes to the early podocyte bridge formation via mediating cell-matrix adhesion and delaying cell survival. Further, FSP-1/S100A4 plays a key role in podocyte contribution to the formation of cellular crescents, by inducing cell migration/growth and inhibiting cell death. In addition, OPN appears to potentiate the effects of forming cellular crescents by increasing the expression of FSP-1/S100A4.

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