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Originals

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Immunoexpression of Podocyte-Associated Proteins in Acquired Human Glomerulopathies with Nephrotic Syndrome*

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CD2AP, -actinin-4 and podocalyxin are thought to play an important role in the structure and function of glomerular podocytes, therefore we intended to evaluate quantitatively, using computer image analysis system, the immunoexpression of these proteins in renal biopsy specimens in glomerulopathies presented with nephrotic syndrome: minimal change disease (MCD), focal segmental glomerulosclerosis (FSGS) and nephropathy IgA (IgAN). As a control 10 biopsy specimens of the kidneys removed because of trauma were used. In normal kidneys CD2AP, -actinin-4, and podocalyxin showed intense staining in podocytes along the capillary walls of the glomeruli. The intensity of immunoexpression of CD2AP and -actinin-4 in renal tissue in patients with MCD, FSGS, and IgAN was similar to normal controls, but the distribution of these proteins was more granular in glomeruli of diseased kidney. The immunostaining of podocalyxin was weaker in podocytes in patients with FSGS as compared with normal glomeruli. The immunostaining of podocalyxin was not significantly altered in MCD and IgAN. The immunostaining of CD2AP and -actinin-4 did not correlate with the intensity of proteinuria in patients with MCD, FSGS and IgAN, whilst in FSGS patients the significant correlation was found between the glomerular immunostaining of podocalyxin and proteinuria. In conclusion, revealed in our study diminished immunoexpression of podocalyxin and significant correlation with the level of proteinuria in FSGS patients suggests a possible role of this sialoprotein in the alteration of the glomerular filtration barrier in this disease.

Introduction

The nephrotic syndrome is almost always caused by a lesion primary to the kidney or it may be associated with a systemic diseases. The most frequent causes of the nephrotic syndrome are minimal change disease and focal segmental glomerulosclerosis, however approximately 5% to 15% of patients with IgA nephropathy have nephrotic syndrome. The presence of massive proteinuria in glomerular diseases is a marker for a poor prognosis. The manifestation of the nephrotic syndrome includes massive proteinuria, hypoalbuminemia, generalized edema, hyperlipidemia and lipiduria. Certain morphologic changes occur in glomeruli with proteinuria of any cause. These include effacement of the visceral epithelial cell foot processes, microvillous transformation, the appearance of vacuoles and protein resorption droplets in epithelial cells, detachment of podocytes from the glomerular basement membrane (GBM). The glomerular podocyte is the cell primarily responsible for the prevention of proteinuria in health, and podocyte damage and dysfunction underlie proteinuria in disease [1, 2, 6, 17]. Impaired formation of slit diaphragm complex, abnormalities in the GBM or the adhesive interaction between the podocyte and the GBM, and abnormalities of the actin cytoskeleton and alterations in the apical membrane domain of podocytes led to foot process effacement and proteinuria [18, 20, 22, 25, 27, 29]. Podocytes produce a specialized repertoire of proteins, which enable them to assist in establishing and maintaining the glomerular filtration barrier [25]. CD2-associated protein (CD2AP) acts as an adapter molecule to bind nephrin carboxy terminal domain, which serves to link the slit diaphragm to the podocyte cytoskeleton [16, 23]. The podocyte foot processes contain an actin-based cytoskeleton. Binding of -actinin-4 to the cyto-

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plasmic domain of 1-integrin subunit of the 3 1 complex is though to be important in anchoring actin microfilaments of podocyte foot processes to the GBM [19]. The usual pathophysiologic basis of proteinuria lies in loss of the normal negative charge of the glomerular capillary wall. On the luminal side, podocytes are equipped with glycocalyx, which is mostly made of podocalyxin and contributes to negative charge of the filtration barrier [12]. Podocalyxin is of critical importance for the formation and preservation of the characteristic cellular architecture of podocytes [20, 27]. Thus, the aim of the study was to evaluate the immunoexpression of CD2AP, -actinin-4 and podocalyxin in renal biopsy specimens in patients with several acquired glomerulopathies with nephrotic syndrome. We also investigated whether the immunoexpression of the proteins studied were related to clinical parameters.

Material and Methods

Patients

Kidney tissue biopsies were obtained for diagnostic purposes percutaneously from 12 patients (9 males and 3 females, aged 4–26, mean age=6) with minimal change disease (MCD), 14 patients (8 males and 6 females, aged 9–39, mean age=26) with focal segmental glomerulosclerosis (FSGS), and 11 patients (7 males and 4 females, aged 17–37, mean age=24) with IgA nephropathy (IgAN). Laboratory data including urinalysis, 24h-protein excretion and serum creatinine level were collected from each patient. At the time of biopsy all patients presented nephrotic syndrome. Renal function impairment was noted in one patient with FSGS and in one patient with IgAN. In all cases diagnosis of glomerulonephritis was based on characteristic findings by light microscopy (sections stained with hematoxylin and eosin, Masson trichrome, Jones' silver impregnation and periodic acid-Schiff followed by alcian blue), immunofluorescence and electron microscopy using standard protocols. In all patients with glomerulopathies foot process effacement was identified by electron microscopy. Classification of the histopathological lesions refers to that of the World Health Organization [4]. As a control 10 biopsy specimens of the kidneys removed because of trauma were used. None of the persons from control group was known to have had previous or actual renal disease. Before the quantitative examination was carried out, all control specimens had been histologically examined by an experienced nephropathologists and found to be a normal renal tissue.

Immunohistochemistry

Paraffin sections were mounted onto superfrost slides, deparaffinized, then treated in a microwave oven in a solution of citrate buffer, pH 6.0 for 20 min and transferred to distilled water. Endogenous peroxidase activity was blocked by 3% hydrogen peroxide in distilled water for 5 min, and then sections were rinsed in Tris-buffered saline (TBS, DakoCytomation) and incubated overnight with polyclonal goat anti-human CD2AP antibody (dilution 1:150, Santa Cruz Biotechnology Inc), monoclonal mouse anti-human -actinin-4 antibody (dilution 1:100, Chemicon Int.), monoclonal mouse anti-human podocalyxin antibody (dilution 1:40, Chemicon Int.). Afterwards LSAB+/HRP Universal Kit (DakoCytomation) was used prepared according to the instructions of the manufacturer. Visualization was performed by incubating the sections in a solution of 0.5 mg 3,3'-diaminobenzidine (DakoCytomation) per ml Tris-HCl buffer (DakoCytomation), pH 7.6, containing 0.02% hydrogen peroxide, for 10 min. After washing, the sections were counter-stained with hematoxylin and coversliped. Negative controls were carried out by incubation in the absence of the primary antibody and always yielded negative results.

Morphometry

Histological morphometry was performed by means of image analysis system consisting of a IBM-compatible computer equipped with an optical mouse, Indeo Fast card (frame grabber, true-color, real-time), produced by Indeo (Taiwan), and color TV camera Panasonic (Japan) linked to a Carl Zeiss Jenaval microscope (Germany). This system was programmed (program MultiScan 8.08, produced by Computer Scanning Systems, Poland) to calculate the surface area of a structure using stereological net (with regulated number of points). The colored microscopic images were saved serially in the memory of a computer, and then quantitative examinations were carried out. The glomerular immunoexpression of CD2AP, podocalyxin and -actinin- were measured using point counting method, which is an adaptation of the principles of Weibel [28]. The point spacing was 16 m. Total numbers of the points of a net was 169, and total area was 36864 sq. m. The percentage of CD2AP, podocalyxin and -actinin-4 staining was an expression of the number of points overlying these structures as a percentage of the total points overlying total glomerular area.

Statistical methods

Differences between groups were tested using unpaired Student's t-test preceded by evaluation of normality and Levene's test. The Mann-Whitney U test was used where appropriate. Correlation coefficients were calculated using Spearman's method. Results were considered statistically significant if $p<0.05$.

Results

The data of the immunoexpression of CD2AP, -actinin-4, and podocalyxin are shown in Table 1. In normal kidneys CD2AP, -actinin-4, and podocalyxin showed intense staining in podocytes along the capillary walls of the glomeruli (Fig. 1). The intensity of immunoexpression of CD2AP and -actinin-4 in renal tissue in patients in the glomerulopathies studied were similar to the normal controls, but the distribution of these proteins was more granular and dispersed in glomeruli of diseased kidney. Statistical analysis did not reveal significant differences between the glomerular immunostaining of CD2AP and -actinin-4 in renal tissue in patients with MCD, FSGS and IgAN and in normal controls. The immunostaining of podocalyxin (Fig. 2) was weaker in

TABLE 1
The immunoexpression of CD2AP, -actinin-4 and podocalyxin in renal tissue in patients with MCD, FSGS, IgAN presented with nephrotic syndrome and in normal controls

	CD2AP	-actinin-4	Podocalyxin
MCD (n=12)	24.05±11.83	24.33±11.07	9.51±5.04
FSGS (n=14)	25.21±13.72	22.67±9.08	6.05±4.87*
IgAN (n=11)	30.25±12.23	30.40±10.40	11.37±6.91
Controls (n=10)	22.59±9.77	28.14±11.12	12.06±8.16

*p<0.04
Data are expressed as percentage of glomerular area ± standard deviation

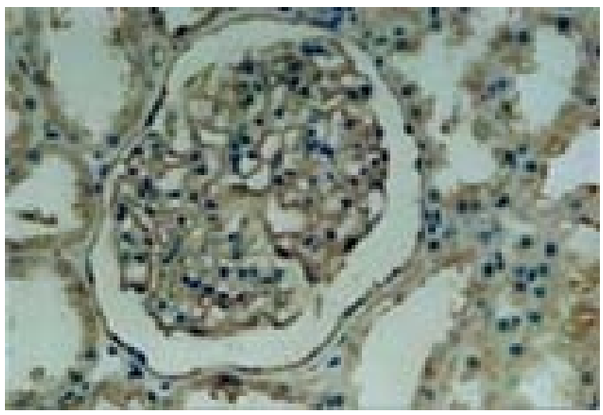

Fig. 1. The immunostaining of podocalyxin in normal glomerulus. Magn. 400×.

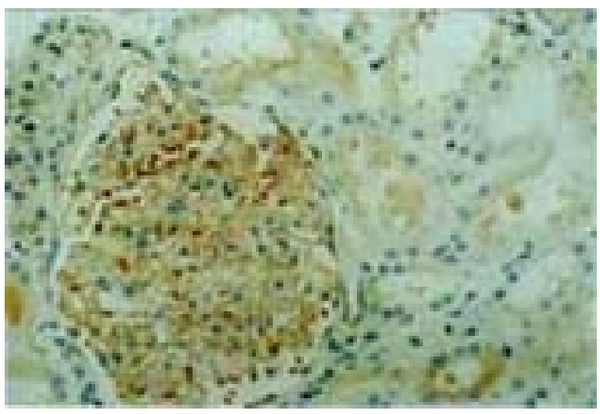

Fig. 2. Weak and granular immunoexpression of podocalyxin in glomerulus in patient with FSGS. Magn. 400×.

TABLE 2
The correlations between the glomerular immunostaining of CD2AP, -actinin-4, podocalyxin and the serum creatinine levels in patients with MCD, FSGS and IgAN presented with nephrotic syndrome

Correlations between:	MCD	FSGS	IgAN
CD2AP and serum creatinine	r=0.14, p=0.66, (NS)	r=-0.06, p=0.83, (NS)	r=0.31, p=0.35, (NS)
-actinin-4 and serum creatinine	r=0.51, p=0.09, (NS)	r=0.25, p=0.35, (NS)	r=0.17, p=0.61, (NS)
Podocalyxin and serum creatinine	r=0.11, p=0.73, (NS)	r=0.19, p=0.51, (NS)	r=0.09, p=0.79, (NS)

TABLE 3
The correlations between the glomerular immunoexpression of CD2AP, -actinin-4, podocalyxin and proteinuria in patients with MCD, FSGS and IgAN presented with nephrotic syndrome

Correlations between:	MCD	FSGS	IgAN
CD2AP and proteinuria	r=-0.03, P=0.96, (NS)	r=0.24, P=0.41, (NS)	r=0.07, P=0.84, (NS)
-actinin-4 and proteinuria	r=0.21, P=0.51, (NS)	r=0.42, P=0.13, (NS)	r=0.04, P=0.90, (NS)
Podocalyxin and proteinuria	r=-0.12, P=0.71, (NS)	r=-0.58, P<0.03	r=0.22, P=0.51, (NS)

podocytes in renal biopsy specimens in patients with FSGS as compared with normal glomeruli ($p<0.04$). Moreover, in FSGS podocalyxin protein was detected in podocytes in the granular pattern. The immunostaining of podocalyxin was not significantly altered in MCD and IgAN as compared with controls. There were no correlations between glomerular immunoexpression of all the proteins studied and serum creatinine levels (Table 2). The immunostaining of CD2AP and -actinin-4 did not correlate with the intensity of proteinuria in patients with MCD, FSGS and IgAN. In FSGS patients the significant correlation was found between the glomerular immunostaining of podocalyxin and proteinuria ($p<0.03$), whereas the immunoexpression of podocalyxin was not related to the intensity of proteinuria in MCD and IgAN patients (Table 3).

Discussion

In last years a lot of studies concerned the slit diaphragm proteins: nephrin and podocin in glomerulopathies presented with nephrotic syndrome, thus in the present study we focused on the evaluation of the immunoexpression of podocyte-associated molecules: CD2AP, -actinin-4 and podocalyxin in normal and diseased glomeruli. It is well known that podocalyxin, the major sialoprotein of glomerular epithelial cells, helps maintain the characteristic architecture of the foot processes and the patency of filtration slits [27]. Our study revealed a remarkable reduction in podocalyxin immunostaining in the renal tissue in FSGS patients, moreover the immunoexpression of podocalyxin in FSGS patients correlated with the level of proteinuria. On the other hand, we failed to find any differences in the intensity of podocalyxin immunostaining in MCD, IgAN patients and controls. The immunoexpression of podocalyxin was not related to the level of proteinuria in these patients. However, it is noteworthy that we observed the granular distribution pattern of podocalyxin within glomeruli in diseased kidneys. In concordance to our findings, Koop et al. [14] observed reduced glomerular podocalyxin expression in most proteinuric diseases as compared with controls. These authors suggest that uncoupling of podocalyxin from the actinin cytoskeleton is a result of rearrangements of the cytoskeleton [14]. In contrary to our study Ostalska-Nowicka et al. [21] did not found statistical differences in the expression of podocalyxin in patients with submicroscopic glomerulonephritis, FSGS, diffuse mesangial proliferation and controls. They concluded that in primary glomerulopathies in children the abnormal distribution of sialoprotein within the podocyte coat is not the main pathological sign. However, experimental study revealed that in puromycin aminonucleoside nephrosis, loss of the foot process organization is associated with reduction in the sialic acid content of podocalyxin to one-third of normal [13]. Recently, it has been found that deletion of the podocalyxin gene in the mouse results in neonatal lethality and failure to form foot processes in the homozygote [5], similarly, mice lacking CD2AP exhibit a nephrotic syndrome that resembles the congenital form of nephrosis due to nephrin mutations and die of renal failure within 2 months from birth [24]. The cytoplasmic domain of podocalyxin is linked to the actin cytoskeleton by ezrin, which is concentrated on the cytoplasmic side of the apical domain of the plasma membrane along the cell bodies and foot processes of glomerular epithelial cells [20]. In our study all patients with glomerulopathies presented with nephrotic syndrome and foot process effacement was identified by electron microscopy, meanwhile the glomerular immunoexpression of podocyte-associated proteins: CD2AP and -actinin-4 in diseased kidneys did not differ in comparison to controls. Moreover, the immunoexpression of CD2AP and -actinin-4 did not correlate with the level of proteinuria. In all the groups studied there were no correlations between glomerular immunoexpression of podocyte-associated proteins and serum creatinine levels. In concordance to our results, Gagliardini et al. [7] found that the staining of CD2AP did not differ among patients with MCD, FSGS, IgAN and controls. Similarly to us, Koop et al. [14] did not show differences in CD2AP protein expression between patients groups and controls. It is suggested that upon damage to the podocyte, the binding of CD2AP with nephrin and podocin is disconnected, whereas CD2AP itself remains attached to the actin cytoskeleton [30]. In nephrotic renal glomeruli, concomitant with the loss of podocytic foot processes a reorganization of the podocytic skeleton and the concentration of some of its elements into thick uniform bands was observed and actinin and -actinin-4 were revealed in these bands [15]. -actinins are actin-binding proteins and can attach the actin bundle to the podocyte membrane [3]. -actinin is localized mainly at the podocyte in the kidney, especially in the foot process [15]. In humans, ACTN4 mutations cause a form of FSGS characterized by a dominant pattern of inheritance with focal podocyte abnormalities, non-nephrotic levels of proteinuria and slowly progressive adult-onset disease leading to significant renal failure at adulthood [11]. To now there is not clear if alteration of -actinin-4 expression or conformation is a cause or mediator of secondary forms of kidney disease, however in experimental study injection of puromycin aminonucleoside resulted in marked induction of glomerular -actinin increase, which preceded development of podocyte foot pro-

cesses effacement and proteinuria [26]. Guan et al. [9] revealed that -actinin intensity increased and its distribution changed when proteinuria disappeared, so they concluded that the delayed -actinin induction might be a reparative response. In concordance to our study, Goode et al. [8] observed similar expression of -actinin-4 in MCD and normal glomeruli. Similarly to us, Guan et al. [10] failed to reveal significant differences in the expression of -actinin-4 among patients with MCNS, IgAN, mesangial proliferative glomerulonephritis presented with nephrotic syndrome, hematuria and control group however, in patients with nephrotic syndrome abnormal glomerular distribution of podocyte-associated proteins was observed.

In conclusion, diminished immunoexpression of podocalyxin and the significant correlation with the level of proteinuria in FSGS patients revealed in our study suggest a possible role of this sialoprotein in the alteration of the glomerular filtration barrier in this disease. Abnormal distribution of podocyte-associated proteins observed in all patients with glomerulopathies presented with nephrotic syndrome may be related to the structural changes in the glomerular podocyte foot processes.

References

1. *Asanuma K, Mundel P:* The role of podocytes in glomerular pathology. Clin Exp Nephrol 2003, 7(4), 255–259.
2. *Barisoni L, Mundel P:* Podocyte biology and the emerging understanding of podocyte diseases. Am J Nephrol 2003, 23, 353–360.
3. *Blanchard A, Ohanian V, Crithley D:* The structure and function of alpha-actinin. J Muscle Res Cell Motil, 1989, 10, 280–289.
4. *Churg J, Bernstein J, Glassock RJ:* Renal Disease. Classification and Atlas of Glomerular Diseases. Igaku-Shoin, New York, Tokyo 1995, pp45–51, 181–182.
5. *Doyonnas R, Kerhaw DB, Duhme C, Merkens H, Chelliah S, Graf T, McNagny KM:* Anuria, omphalocele, and perinatal lethality in mice lacking the CD34-related protein podocalyxin. J Exp Med 2001, 194, 13–27.
6. *Endlich K, Kriz W, Witzgall R:* Update in podocyte biology. Curr Op Nephrol Hypertens 2001, 10, 331–340.
7. *Gagliardini E, Benigni A, Tomasoni S, Abbate M, Kalluri R, Remuzzi G:* Target downregulation of extracellular nephrin in human IgA nephropathy. Am J Nephrol 2003, 23, 277–286.
8. *Goode NP, Shires M, Khan TN, Mooney AF:* Expression of -actinin-4 in acquired human nephrotic syndrome: a quantitative immunoelectron microscopy study. Nephrol Dial Transplant 2004, 19, 844–851.
9. *Guan N, Ding J, Deng J, Zhang J, Yang J:* Key molecular events in puromycin aminonucleoside nephrosis rats. Pathol Int 2004, 54(9), 703–711.
10. *Guan N, Ding J, Zhang J, Yang J:* Expression of nephrin, podocin, alpha-actinin, and WT1 in children with nephritic syndrome. Pediatr Nephrol 2003, 18(11), 1122–1127.
11. *Kaplan JM, Kim SH, North KN, Rennke H, Correia LA, Tong HQ, Mathis BJ, Rodriguez-Perez JC, Allen PG, Beggs AH, Pollak MR:* Mutations in ACTN4, encoding alpha-actinin-4, cause familial focal segmental glomerulosclerosis. Nat Genet 2000, 24, 251–256.
12. *Kerjaschki D, Sharkey DJ, Farquhar MG:* Identification and characterization of podocalyxin – the major sialoglycoprotein of the renal glomerular epithelial cell. J Cell Biol 1984, 98, 1591–1596.
13. *Kerjaschki D, Vernillo AT, Farquhar MG:* Reduced sialylation of podocalyxin – the major sialoprotein of the rat kidney glomerulus – in aminonucleoside nephrosis. Am J Pathol 1985, 118, 343–349.
14. *Koop K, Eikmans M, Baelde HJ, Kawachi H, de Heer E, Paul LC, Bruijn JA:* Expression of podocyte associated molecules in acquired human kidney diseases. J Am Soc Nephrol 2003, 14, 2063–2071.
15. *Lachapelle M, Bendayan M:* Contractile proteins in podocytes: immunohistochemical localization of actin and alpha-actinin in normal and nephritic rat kidneys. Virchows Arch B Cell Pathol Incl Mol Pathol 1991, 60(2), 105–111.
16. *Li C, Ruotsalainen V, Tryggvason K, Shaw AS, Miner JH:* CD2AP is expressed with nephrin in developing podocytes and is found widely in mature kidney and elsewhere. Am J Physiol Renal Physiol 2000, 279, F785–F792.
17. *Mathieson PW:* The cellular basis of proteinuria. Clin Sci 2004, 107, 533–538.
18. *Morello R, Lee B:* Insight into podocyte differentiation from the study of human genetic disease: nail-patella syndrome and transcriptional regulation in podocytes. Pediatr Res 2002, 51, 551–558.
19. *Otey CA, Vasquez GB, Burridge K, Erickson BW:* Mapping of the alpha-actinin binding site within the beta-1-integrin cytoplasmic domain. J Biol Chem 1993, 268, 21193–21197.
20. *Orlando RA, Takeda T, Zak B, Schmieder S, Benoit VM, McQuistan T, Furthmayr H, Farquhar MG:* The glomerular epithelial cell antiadhesin podocalyxin associates with the actin cytoskeleton through interactions with ezrin. J Am Soc Nephrol 2002, 12, 1589–1598.
21. *Ostalska-Nowicka D, Zachwieja J, Nowicki M, Kaczmarek E, SiwinskaA , Witt A:* Vascular and endothelial growth factor (VEGF-C1)-dependent inflammatory response of podocytes in nephritic syndrome glomerulopathies in children: an immunohistochemical approach. Histopathology 2005, 46, 176–183.
22. *Raats CJ, van den Born J, Bakker MA, Oppers-Walgreen B, Pisa BJ, Dijkman HB, Assmann KJ, Berden JH:* Expression of agrin, dystroglycan and utrophin in normal renal tissue and in experimental glomerulopathies. Am J Pathol 2000, 156, 1749–1765.
23. *Shih NY, Li J, Cotran R, Mundel P, Miner JH:* CD2AP localizes to the slit diaphragm and binds to nephrin via a novel C-terminal domain. Am J Pathol 2001, 159(6), 2303–2308.
24. *Shih NY, Li J, Karpitskii V, Nguyen A, Dustin ML, Kanagava O, Miner JH, Shaw A:* Congenital nephritic syndrome in mice lacking CD2-associated protein. Science 1999, 286, 312–315.
25. *Smoyer WE, Mundel P:* Regulation of podocyte structure during the development of nephrotic syndrome. J Mol Med 1998, 76, 172–183.
26. *Smoyer WE, Mundel P, Gupta A, Welsh MJ:* Podocyte alpha-actinin induction precedes foot processes effacement in experimental nephritic syndrome. Am J Physiol 1997, 273 (1Pt 2), F150–F157.
27. *Takeda T, McQuistan T, Orlando RA, Farquhar MG:* Loss of glomerular foot processes is associated with uncoupling of podocalyxin from the actin cytoskeleton. J Clin Invest 2001, 108, 289–301.
28. *Weibel ER:* Stereological Methods. Vol 1. Practical methods for biological morphometry. Academic Press, London, New York, Toronto, Sydney, San Francisco 1979, pp100–161.
29. *Wolf G, Stahl RA:* CD2-associated protein and glomerular disease. Lancet 2003, 362(9397), 1746–1748.

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