Synaptopodin immunoexpression in steroid-responsive and steroid-resistant minimal change disease and focal segmental glomerulosclerosis

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SUMMARY

Background and objectives: *Synaptopodin is protein of podocytes, and a part of the actin-based contractile apparatus of foot-processes. Recently, proteins expressed by the podocyte were found to be important for the integrity of the glomerular filtration barrier. Podocytes are injured in many forms of glomerulopathies, including minimal change disease (MCD) and focal segmental glomerulosclerosis (FSGS). The study was undertaken to determinate if synaptopodin immunoexpression in renal tissue specimens differs between patients with steroid-responsive MCD, steroid-resistant MCD, and FSGS.* ***Methods:*** *Synaptopodin immunoexpression was evaluated by immunoperioxidase staining with a mouse anti-human monoclonal antibody in 12 renal biopsy specimens in patients with steroid-responsive MCD, 10 renal tissues in steroid-resistant MCD, and in 14 renal biopsy specimens in patients with FSGS. As a control 10 tissue specimens of the kidneys removed because of trauma were used. Synaptopodin expression was quantified as a percentage of glomerular tuft by computerized image analysis system.* ***Results:*** *In normal controls synaptopodin immunoexpression was seen in podocytes along the glomerular basement membrane in a finely linear pattern. No changes were found in synaptopodin immunoexpression in steroid-responsive MCD* versus *controls. In patients with steroid-resistant MCD and FSGS a granular pattern of synaptopodin immunoexpression was seen. Areas of sclerosis in patients with FSGS did not demonstrate synaptopodin expression. Statistical analysis showed significantly diminished synaptopodin immunoexpresion in glomeruli in patients with steroid-resistant MCD and FSGS as compared with steroid-responsive MCD group and controls. Moreover, in renal tissues in patients with FSGS the immunoexpression of synaptopodin was decreased in comparison with renal biopsies in patients with steroid-resistant MCD.* ***In conclusions,*** *our results suggest that abnormal distribution and reduced expression of synaptopodin may be associated with poor response to steroid therapy in MCD and FSGS.*

Key words: ***Synaptopodin. Minimal change disease. Focal segmental glomerulosclerosis.***

INMUNOEXPRESIÓN DE LA SINAPTODINA EN LA ENFERMEDAD DE CAMBIOS MÍNIMOS RESPONDEDORA Y RESISTENTE A ESTEROIDES Y EN LAGLOMERULOSCLEROSIS FOCAL Y SEGMENTARIA

RESUMEN

Antecedentes y objetivos: *la sinaptopodina es una proteína de los podocitos y una parte del aparato contráctil basado en la actina de los procesos pediculados. Recientemente, se encontró que las proteínas expresadas por los podocitos son importantes*

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para la integridad de la barrera de filtración glomerular. Los podocitos pueden verse dañados en muchas formas de glomerulopatías, incluyendo la enfermedad de cambios mínimos (ECM) y la glomerulosclerosis focal y segmentaria (GEFS). Este estudio se realizó para determinar si la inmunoexpresión de sinaptopodina en muestras de tejido renal difiere entre los pacientes con ECM respondedora a esteroides, ECM resistente a esteroides, y la GEFS. ***Métodos:*** *la inmunoexpresión de sinaptopodina se evaluó mediante tinción de inmunoperoxidasa con un anticuerpo monoclonal anti-humano de ratón en 12 muestras de biopsia renal de pacientes con ECM respondedora a esteroides, 10 muestras renales de ECM resistente a esteroides, y en 14 muestras de biopsia renal de pacientes con GEFS. Se tomaron como controles 10 muestras tisulares de riñones extirpados por traumatismo. La expresión de sinaptopodina se cuantificó como el porcentaje de penacho glomerular mediante un sistema de análisi de la imagen computerizada.* ***Resultados:*** *en los contorles normales, la inmunoexpresión de sinaptopodina se vio en los podocitos a lo largo de la membrana basal glomerular en un patrón finalmente granular. No se observaron cambios en la inmunoexpresión de sinaptopodina en la ECM respondedora a esteroides frente a los controles. En pacientes con ECM resistente a esteroides y GEFS, se vio un patrón granular de inmunoexpresión de sinaptopodina. Las áreas de esclerosis en los pacientes con GEFS no mostraron expresión de sinaptopodina. El análisis estadístico mostró una inmunoexpresión de sinaptopodina significativamente disminuida en los glomerulos de pacientes con ECM resistente a esteroides y con GEFS en comparación con los grupos de ECM respondedora a esteroides y control. Además, en los tejidos renales de pacientes con GEFS, la inmunoexpresión de sinaptopodina estaba disminuida en comparación con las biopsias renales de pacientes con ECM resistente a esteroides. En conclusión, nuestros resultados sugieren que la distribución anormal y la expresión reducida de sinaptopodina podrían estar asociadas con una respuesta escasa al tratamiento con corticoides en la ECM y la GEFS.*

Palabras clave: ***Sinaptopodina. Enfermedad de cambios mínimos. Glomerulosclerosis focal y segmentaria.***

INTRODUCTION

The glomerular podocyte is the cell primarily responsible for the prevention of proteinuria. It is generally accepted that albuminuria is a consequence of changes in podocyte integrity due to cytoskeletal rearrangement causing alteration of slit diaphragm or functional abnormalities of slit pore complex.[1] Podocytes are injured in many forms of glomerulopathies, including minimal change disease (MCD) and focal segmental glomerulosclerosis (FSGS), and both these glomerulopathies are the major cause of idiopathic nephrotic syndrome. Corticosteroids in doses of 60 mg/m^2/day produce remission of nephrotic syndrome by 8 weeks in 90-95% of children with MCD, but the response in adults is slower, and only 75-85% of adults achieve remission by 8 weeks of prednisone therapy.[2,3] By contrast, 75% of FSGS patients are steroid nonresponders. Steroid-resistant patients with FSGS are offered alternative therapy in the form of cyclophosphamide and cyclosporine. Most investigators consider MCD and FSGS to be related entities that lie along a clinical-pathologic continuum.[4] The strongest evidence supporting the relatedness of the two lesions comes from experimental models of MCD and FSGS caused by different severities of injury by the same toxic factors and the identification of a circulating permeability factor in both conditions.[5] MCD is considered the quintessential podocyte disease, because of the presence of severe podocyte abnormalities, and the absence of other glomerular alterations. In FSGS there is usually complete effacement of foot processes, podocyte detachment from the tuft, accompanied by podocyte alterations that include hypertrophy, increased organellar content and focal microvillous transformation. Recently, proteins expressed by the podocyte were found to be important for the integrity of the glomerular filtration barrier.[6-8] Podocyte proteins include nephrin, podocin, podocalyxin, synaptopodin, CD2-associated protein, P-cadherin and α-actinin-4. Synaptopodin is proline-rich 74-kDa protein of mature podocytes, where is a part of the actin-based contractile apparatus of foot-processes.[9] It is postulated that synaptopodin modulate shape and motility of the podocyte foot-processes.[10] In the current study we investigated the pattern of immunoexpression of synaptopodin in renal biopsy specimens in MCD and FSGS and examined

whether synaptopodin immunoexpression differs between patients with steroid-responsive MCD, steroid-resistant MCD, and steroid-resistant FSGS.

PATIENTS AND METHODS

Kidney tissue biopsies were obtained for diagnostic purposes percutaneously from 12 patients (7 males and 5 females, aged 13-35, mean age = 21.5) with steroid-responsive minimal change disease, 10 patients (8 males and 2 females, aged 23-45, mean age = 27.7) with steroid resistant minimal change disease, and 14 patients (8 males and 6 females, aged 26-47, mean age = 35.7) with focal segmental glomerulosclerosis not otherwise specified (table I). No systemic diseases were found in these patients based on clinical and laboratory examinations. All biopsies were taken after institution of therapy. In all patients with FSGS poor response to steroid therapy was documented. The standard definition for response to steroid therapy was used.[11,12] All patients had two years of follow-up from the primary diagnosis to ascertain to response to steroid therapy. Patients with steroid resistant MCD and FSGS had received in addition cyclophosphamide. Laboratory data including urinalysis, 24 h protein excretion and serum creatinine level were collected from each patient. At the time of biopsy all patients presented nephrotic syndrome, and the diffuse podocyte foot process effacement was identified by electron microscopy. Renal function impairment was noted in 2 patients with FSGS. 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. Classification of the histopathological lesions refers to that of the World Health Organization.[13] All biopsy specimens from patients with FSGS contained more than 5 nonsclerosed glomeruli. All biopsy specimens from patients with steroid-responsive as well steroid-resistant MCD contained at least 10 glomeruli. As a control 10 tissue specimens of the kidneys removed because of trauma were used (7 males and 3 females aged 22-49, mean age = 34.5). None of the persons from control group were known to have had previous or actual renal disease. Before the quantitative examination was carried out, all control specimens were examined in microscope 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 monoclonal mouse anti-human synaptopodin antibody (Progen, Biotechnik, GmbH, clone C1D4, dilution 1:50). Afterwards LSAB+/HRP Universal Kit (DakoCytomation) prepared according to the instructions of the manufacturer were used. Visualisation 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

Table I. Clinical data as well as synaptopodin immunoexpression in patients with steroid-responsive MCD, steroid-resistant MCD, FSGS and in normal controls

Diagnostic groups	N	Age (years)	Gender m/f	Serum creatinine mg/dl ± SD	Proteinuria g/24 h ± SD	Synaptopodin immunoexpression (%)
Controls	10	22-49 mean age = 34.5	7/3	–	–	39.8 ± 5.9
Steroid-responsive MCD	12	13-35 mean age = 21.5	7/5	1.04 ± 0.15	6.59 ± 3.22	31.1 ± 13.6*
Steroid-resistant MCD	10	23-45 mean age = 27.7	8/2	1.13 ± 0.22	7.13 ± 4.63	18.4 ± 8.7**
FSGS	14	26-47 mean age = 35.7	8/6	1.38 + 0.81	5.27 ± 2.20	10.2 ± 7.9***

Data of the immunoexpression of synaptopodin are expressed as percentage of glomerular tufts ± standard deviation.
* steroid-responsive MCD *vs* steroid-resistant MCD, $P < 0.02$. ** steroid resistant MCD *vs* FSGS, $P < 0.03$. ** steroid-resistant MCD *vs* controls. *** FSGS *vs* steroid-responsive MCD, $P < 0.001$. *** FSGS *vs* controls, $P < 0.001$.

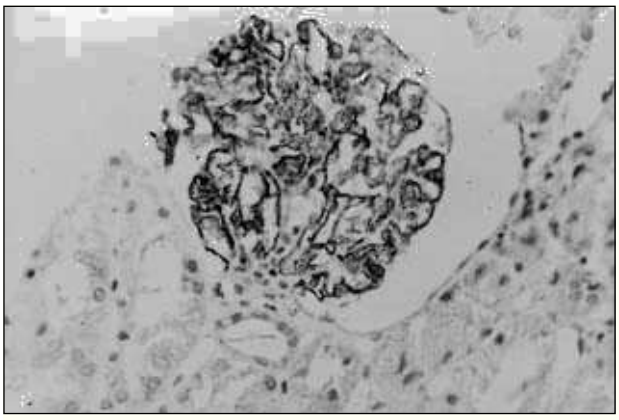

Fig. 1.—Strong linear synaptopodin staining in glomerular podocytes in normal controls. Magn. 400x.

hematoxylin and coverslipped. 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-colour, real-time), produced by Indeo (Taiwan), and colour TV camera Panasonic (Japan) linked to a Carl Zeiss Jenaval microscope (Germany). This system was programmed (program MultiScan 8.08 software by Computer Scanning Systems, Poland) to calculate the surface area of a structure using stereological net (with regulated number of points). The coloured microscopic images were saved serially in the memory of a computer, and then quantitative examinations had been carried out. The immunoexpression of synaptopodin was measured in nonsclerosed glomeruli using point counting method which is an adaptation of the principles of Weibel.[14] The point spacing being 16 μm. Total numbers of the points of a net was 169, and total area was 36,864 staining was an expression of the number sq. mm. The percentage of synaptopodin staining was an expression of the number of points overlying, these structures as a percentage of the total points overlying total tuft 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. Results were considered statistically significant if $p < 0.05$.

RESULTS

The data of the immunoexpression of synaptopodin are shown in table I. In normal kidneys immunohistochemistry revealed intense linear staining of synaptopodin in podocytes along the capillary walls (fig. 1). The pattern and intensity of immunoexpression of synaptopodin in glomeruli in patients with steroid-responsive MCD were similar that in normal controls ($P = 0.07$, NS) Synaptopodin was detected in podocytes in the granular pattern in glomeruli in patients with steroid-resistant MCD and FSGS (fig. 2 and fig. 3). Areas of glomerular sclerosis in patients with FSGS did not demonstrate synaptopodin expression. The immunostaining of syanaptopodin was significantly decreased in steroid-resistant MCD and FSGS patients as compared with steroid-responsive MCD and controls (steroid-resistant MCD *vs* steroid-responsive MCD, $P < 0.02$; steroid-resistant MCD *vs* controls, $P < 0.001$, FSGS *vs* controls, $P < 0.001$). Moreover, statistical analysis revealed differences in the intensity of synaptopodin immunoexpression between MCD steroid-resistant and FSGS patients ($P < 0.03$).

DISCUSSION

Recently, a great progress has been made in the understanding of the podocyte biology in human diseases and in the molecular structure of the glomerular filtration barrier.[15, 16] It is though that podocytes plays an important role in the maintance of renal glomerular function and in the final pathway to glomerulosclerosis. In the last years, a lot of studies focused on altered expression of podocyte slit diaphragm proteins in several glomerulopathies presented with massive proteinuria;[17-19] whereas observations regarding the immunoexpression of synaptopodin in diseased kidney are rather scanty. To

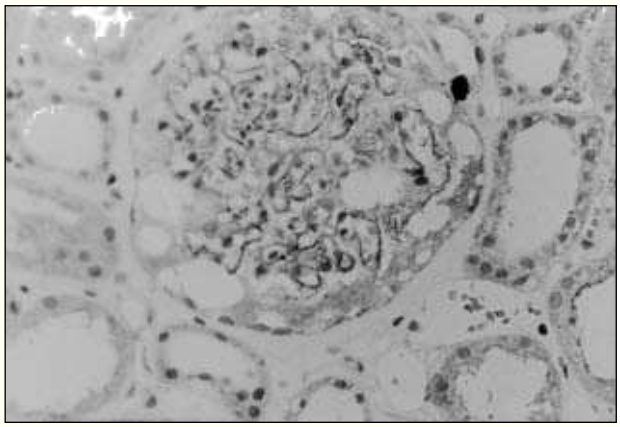

Fig. 2.—Granular staining of synaptopodin in podocytes in renal biopsy in patient with steroid-resistant minimal change disease. Magn. 400x.

our knowledge no data documented synaptopodin expression in older patients. Synaptopodin is the member of a novel class actin-associated proteins expressed in highly dynamic cell compartment such a telencephalic dendrites and foot processes renal podocytes.[10] This protein is believed to play a crucial role in the normal blood-filtering mechanism of the kidney. It is well known that in nephrotic renal glomeruli, concomitant with the loss of podocytic foot processes a reorganization of the podocytic skeleton is observed. Moreover epithelial cell injury and dedifferentiation are important in the pathomechanism of proteinuria in FSGS. All patients in our study presented nephrotic syndrome and in electron microscopy examination diffuse effacement of podocyte processes was seen. It was elucidated that synaptopodin exists in 3 isoforms: neuronal Synpo-short, renal-Synpo long and Synpo-T. All 3 isoforms specifically interact with alpha-actinin and elongate alpha-actinin-induced actin filaments.[20] Taken into consideration the role of synaptopodin in stabilization of podocyte architecture we hypothesized that in MCD and FSGS the distribution of this molecule may be altered. In contrary to our hypothesis we found that the pattern of distribution and intensity of the immunoexpression of synaptopodin on podocytes was similar in steroid-responsive MCD and in normal controls. However, in patients with steroid-resistant MCD and FSGS a distinctly granular pattern to synaptopodin immunoexpression was seen. On the other hand, areas of sclerosis in patients with FSGS did not demonstrate synaptopodin expression. We did observe a significantly decrease in synaptopodin immunoexpresion on podocytes in patients with steroid-resistant MCD and FSGS as compared with steroid-responsive MCD group and controls. We speculate that diminished expression of synaptopodin in steroid-resistant MCD and FSGS may depend on greater degree of podocyte architecture lesions or functional changes in podocyte foot processes in these cases. In study of Asanuma et al.[20] the ultrastructure of podocytes in synaptopodin deficient (synpo-/-) mice was normal, however synpo-/- podocytes showed impaired actin filament formation *in vitro*. In collapsing forms of FSGS including idiopathic FSGS and HIV-associated nephropathy marked reduction of synaptopodin expression was noted.[21] In contrary, Smeets et al.[22] in experimental model of FSGS did not observe major changes in the expression pattern of podocyte-associated and slit pore proteins, including synaptopodin, whilst a reduction in number of podocytic foot processes and significant decrease in width of remaining slit pores were found. In study of Strivastava et al.[23] in idiopathic nephrotic syndrome in children, synaptopodin expression decreased in order from minimal change disease to diffuse mesangial hypercellularity, and to focal segmental glomerulosclerosis, reaching statistical significance between MCD and FSGS. Similar observation was made by Ostalska-Nowicka et al.[24] in nephrotic syndrome glomerulopathies in children. Up to day, the pathomechanism of MCD and FSGS is not fully understood. In pathogenesis of several forms of FSGS genetic mutation are taken into consideration.[25-27] Huber et al.[28] explored whether combinations of heterozygosity for different podocyte-relevant genes would increase the risk for FSGS. These authors found that bigenic heterozygosity of Cd2ap with signaling protein Fyn protooncogene (Fyn) or the long isophorm of actin bundling protein synaptopodin (Synpo) resulted in a significantly increased incidence of proteinuria and pathological changes consisted with FSGS in mice.

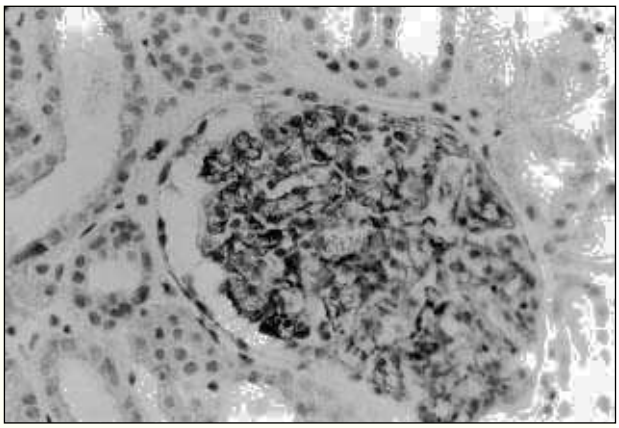

Fig. 3.—Week and granular staining of synaptopodin in podocytes in renal biopsy specimen in patient with focal segmental glomerulosclerosis. Magn. 400x.

In MCD and FSGS patients, besides of the lot clinical and histological parameters studied, it is not possible to predict a response to administrated steroids. Srivastava et al.[23] found that greater synaptopodin expression in podocytes was associated with a significantly better response to steroid therapy in idiopathic nephritic syndrome of childhood. Ostalska-Nowicka et al.[24] observed diminished synaptopodin expression in steroid-resistant diffuse mesangial proliferation and focal segmental sclerosis in children. Our results are in concordance with these observations, but it must be taken into consideration that mentioned above findings regards to nephrotic syndrome glomerulopathies in children. Our study groups consisted essentially of young adults, and the oldest patient in steroid-responsive MCD was 35 years, in steroid-resistant MCD -45 years and 47 years in FSGS. It is though that, the long-term prognosis for children with steroid-responsive MCD is very good, however relapses may occur for many years, commonly until puberty.[11] Although most adults with MCD respond to steroid therapy, the response rate appears to be lower and remission slower to develop than in chil-

dren.[2,29] FSGS is poorly responsive to prednisone therapy; however the prognosis and response to therapy of FSGS are similar in children and adults.[11] Although our results suggest relationship between synaptopodin immunoexpression on podocytes and the response to steroid therapy in MCD and FSGS, it needs further investigations on more numerous patients groups. It is possible that in steroid-resistant MCD and FSGS structural podocyte lesions are irreversible in contrary to podocyte injury noted in steroid-responsive MCD.

In conclusions, our results revealed diminished immunoexpression of synaptopodin in steroid-resistant MCD and FSGS as compared with steroid-responsive MCD and controls. We suggest that abnormal distribution and reduced expression of synaptopodin may be associated with poor response to steroid therapy in MCD and FSGS.

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