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ORIGINALES

A quantitative study of the interstitial expression of α-smooth muscle actin (α-SMA) in IgA-nephropathy and proliferative mesangial (non IgA) glomerulonephritis

M. Danilewicz*, L. Antoszczyk and M. Wagrowska-Danilewicz***

*Department of Pathology (Morphometry Division). **Department of Legal Medicine. Medical University of Łódź, ul. Czechoslowacka 8/10 Poland.

SUMMARY

Eleven renal biopsy specimens from patients with idiopathic diffuse IgA nephropathy (IgAN) and 11 from patients with primary proliferative mesangial non IgA glomerulonephritis (MPG) for whom light, electron and immunofluorescence microscopy and full clinical data were available were examined and compared quantitatively. Morphometric investigations were undertaken to compare the expression of α-SMA in IgAN and MPG, and to find whether this parameter correlated, with interstitial fibrosis. Another purpose of this study was to see if the expression of α-SMA correlated with the intensity of the interstitial leukocyte infiltrates in these glomerulopathies. The results showed, that the mean values of the interstitial expresión of α-SMA, interstitial volume, CD 68+, CD 45RB+, CD 43+ and CD20+ cells were significantly increased in both IgAN and MPG in comparison with control patients. Moreover, the interstitial expression of α-SMA in IgAN patients was significantly higher than in the MPG groups, but the mean values of the interstitial volume and interstitial infiltrates did not differ significantly in these glomerulopathies. In both IgAN and MPG group there were significant positive correlations between interstitial expression of α-SMA and interstitial volume as well as CD 68+ and CD 45RB+ cells.

In conclusion, our study suggests that interstitial α-SMA positive cells play a role in the development of interstitial fibrosis in both IgAN and MPG groups, hence, increased interstitial α-SMA staining may have useful prognostic implications in these glomerulopathies. We postulate that interstitial monocytes/macrophages play an important role in inducing the myofibroblast phenotype in resting fibroblasts.

Key words: ***IgA nephropathy. Mesangial proliferative glomerulonephritis. Myofibroblasts.***

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Correspondencia: Dra. Marian Danilewicz MD, PhD
ul. Zamenhofa 5 m. 4
90-431 Łódź, Poland

ESTUDIO CUANTITATIVO DE LA EXPRESION INTERSTICIAL DE LA ACTINA DE MUSCULO LISO-x (x-SMA) EN LA NEFROLOGIA IGA Y EN LA GLOMERULONEFRITIS PROLIFERATIVA MESANGIAL (NO IgA)

RESUMEN

Se examinaron 11 biopsias renales de enfermos con nefropatía IgA difusa idiopática (IgA N) y 11 de enfermos con glomerulonefritis proliferativa mesangial primaria no IgA (MPG) con técnicas de microscopía óptica electrónica y de inmunofluorescencia comparándose entre sí, junto con los datos clínicos correspondientes.

Se llevaron a cabo investigaciones morfométricas para comparar, la expresión de x-SMA y IgAN y MPG y para determinar si este parámetro se correlaciona o no con el grado de fibrosis intersticial. Otro de los objetivos de este estudio fue verificar si la expresión de x-SMA se correlaciona con la intensidad de los infiltrados de leucocitos intersticiales en este tipo de glomerulopatías. Los resultados mostraron que los valores medios de la expresión intersticial de x-SMA, volumen intersticial, Cd 68+, CD 45 RB+, CD 43+ y CD 20+ estaban significativamente elevados en relación con los enfermos control, tanto en las IgAN como en las IgMP? Además, la expresión intersticial de x-SMA en los enfermos con IgAN fue significativamente superior que la encontrada en el grupo MPG, mientras que los valores medios del volumen intersticial y de los infiltrados intersticiales no difirieron significativamente en estas glomerulopatías. Tanto en el grupo IgN como el grupo MPG se encontraron correlaciones positivas significativas entre la presión intersticial de x-SMA y el volumen intersticial, así como entre las células CD 68+ y CD 45 RB+.

En conclusión, nuestro estudio sugiere que la presencia de células intersticiales x-SMA positivas juega algún papel en el desarrollo de la fibrosis intersticial, tanto en el grupo IgN como en el MPG. Por consiguiente el hallazgo de una tinción positiva de x-SMA en el intersticio puede tener implicaciones pronósticas de utilidad en estas glomerulonefritis. En base a estos hallazgos, sugerimos que los monocitos/macrófagos intersticiales desempeñan un papel importante en la inducción del fenotipo de miofibroblasto en éstas células.

Palabras clave: ***Nefropatía IgA. Glomerulonefritis proliferativa mesangial. Miofibroblastos.***

INTRODUCTION

IgA nephropathy (IgAN) is considered the most common glomerular disease worldwide[1]. The most prominent clinical features of IgAN patients are recurrent hematuria or proteinuria[2,3]. By immunofluorescence IgA deposits more or less confined to the mesangium are by definition invariably present[4]. On electron microscopy in IgAN cases mesangial hypercellularity and diffuse, discrete glomerular mesangial electron-dense deposits are demonstrated[5]. Similar morphological changes on renal biopsy as well as sometimes similar clinical features are noted in proliferative mesangial glomerulonephritis without predominant IgA depositions (MPG). Especially, on electron microscopy the content, character and distribution of glomerular deposits in IgAN are indistinguishable from patients with MPG[5]. Approximately 40% of IgAN patients have interstitial fibrosis, mostly focal and mild to moderate in severity[6,7]. Moreover, quantitative analysis revealed that cortical interstitial volume in IgAN and MPG cases did not differ significantly[8]. It was well documented that irrespective of the nature of primary disease the decline in renal function correlates better with the degree of tubulointerstitial injury (as measured by the relative intyerstitial volume) than with the glomerular changes[9-13]. So far, however, the precise cellular

mediators involved in these fibrotic process remain unknown[14]. Recent evidence suggest a role of myofibroblasts in the pathogenesis of renal scarring in experimental animals[15,16] as well as in humans[17,18]. The myofibroblast is a cell phenotype with features of both fibroblast and smooth muscle cells[19]. The hallamark of the myofibroblastic phenotype is the expression of α-smooth muscle actin (α-SMA)[20].

Therefore, the present investigations were undertaken to compare the expression of α-SMA in IgAN and MPG, as well as to find whether this parameter could correlate with the interstitial fibrosis and serum creatinine. Another purpose of this study was to verify if the expression of α-SMA could correlate with intensity of the interstitial leukocyte infiltrates in these glomerulopathies.

MATERIAL AND METHODS

Patients

Eleven renal biopsy specimens from patients with diffuse IgAN and 11 with diffuse MPG were examined by percutaneous renal biopsy. As a control 10 biopsy specimens of the kidney from patients with minimal change disease (MCD) were used in view of commonly known fact that tubular atrophy, interstitial inflammation and fibrosis are usually absent in this glomerulopathy[21]. Morphological diagnosis of IgAN, MPG and MCD was established independently by two experienced nephropathologists according to WHO criteria[4] and based on light microscopy, immunofluorescence and electron microscopy. None of these patients had been treated with immunosuppressive drugs or hypotensive durgs from ACE inhibitor group prior to renal biopsy. The mean duration of the disease prior to biopsy was 6.2 months ± 1.7 in IgAN 5.6 months ± 1.5 in MPG and 5.5 months ± 1.9 in MCD.

Light microscopy

Tissue specimens were embedded in paraffin, sections cut precisely at 4 μm, and stained by hematoxylin and eosin, periodic acid-Shiff (PAS)-alcian blue, trichrome light green (Masson), and by silver impregnation (Jones). Thickness of each section was controlled according to the method described by Weibel[22].

Immunofluorescence microscopy

Tissue was snap frozen, sectioned at 5 μm and fixed in 95% alcohol for 10 min. Sections incubated with FITC-antisera (Hoechst) to human IgG, IgA, IgM and complement (C3) were viewed on Carl Zeiss (Jena) NU-2 microscope, using and HBO 200 lamp and proper filters.

Electron microscopy

Tissue was fixed in glutaraldehyde, post-fixed in 1% osmium tetroxide, embedded in epon and sectioned on a LKB ultratome. Sections were stained by lead citrate and uranyl acetate, and viewed in a JEM 100B electron microscope.

Immunohistochemistry

The preparation and staining of tissue sections for immunohistochemistry (an indirect StreptABComplex/HRP technique) was carried our as follows: paraffin-embedded tissues, after deparaffinization and rehydratation were reacted for 5 minutes with 3% hydrogen peroxide in distilled water. If necessary, pretreated with trypsin (DAKO) for 30 minutes at 37 °C was performed (monoclonal antibody-MoAb anti-CD68). After blocking by normal rabbit serum (DAKO) during 20 minutes (dilution 1:5), the sections were incubated with the following solutions, each followed by TBS washing: appropriately diluted mouse antihuman monoclonal antibodies (in a moist chamber for 30 minutes at room temperature), biotinylated rabbit anti-mouse immunoglobulin (DAKO) diluted 1:600 in TBS for 20 minutes and, StreptABComplex/HRP (DAKO) for 30 minutes prepater according to the instruction. The final reaction was achieved by incubating the sections with 3.3'-diaminobenzidine (DAB tablets DAKO) 0,5 mg/ml Tris-HCL buffer, pH 7.6, containing 0.02% hydrogen peroxide, for 10 minutes. After washing, sections were counter-stained with hematoxylin and coversliped.

The monoclonal antibodies (DAKO) employed, their specific reactivities and dilutions are listed in table I.

Table I. Monoclonal antibodies employed and their specificity

Monoclonal antibody	Specifity	Source	Dilution
α-SMA	Myofibroblasts, smooth muscles	Dako	1:50
CD 45RB	All leukocytes	Dako	1:100
CD 43	Pan - T cells	Dako	1:100
CD 20	Pan - B cells	Dako	1:100
CD 68	Monocytes/macrophages	Dako	1:100

Tissue control for immunostaining

For each MoAb and for each sample a positive control (paraffin-embedded sections of surgically removed lymph node for interstitial infiltrates and vascular smooth muscle cells of the renal biopsy species for myofibroblasts) and negative control were processed. The following negative controls were used: 1) omission of primary antibody, 2) incubation with appropriately diluted mouse IgG (DAKO) as a first layer. The samples were prepared by the same method as described above. Specificity of labeling was shown by lack of staining in these samples.

Morphometry

Histological morphometry was performed by means of image analysis system consisting of a IBM-compatible computer equipped with an optical mouse, AVer 2000 card (frame grabber, true-color, real-time), produced by ADDA Technologies (Taiwan), and color TV camera Panasonic (Japan) linked to a Carl Zeiss Jenaval microscope (Germany). This system was programmed (program MultiScan, produced by CSS-Poland) to calculate:

– the surface area of a structure using stereological net (with regulated number of points).

– the number of objects (semiautomatic function).

Interstitial myofibroblasts were identified by their morphology and positive staining with anti-α-SMA. The interstitial immunoperoxidase staining for α-SMA was measured using point counting method which is an adaptation of the principles of Weibel[22]. The point spacing being 16 μm. Total number of the points of a net was 169, and total area was 36,864 sq μm. Under the net described above, 8-10 randomly selected adjacent fields of the renal cortex were investigated. Glomeruli and large blood vessels were neglected. As most of the α-SMA positivity was within cytoplasmic processes, these structures were included in calculation. The percentage of α-SMA positive staining was an expression of the number of points overlying α-SMA positive areas as a percentage of the total points counted. The same method was used to estimate interstitial volume in sections stained with Masson trichrome. The percentage interstitial volume was an expression of the number of points overlying renal cortical interstitium as a percentage of the total points counted.

The immunophenotype of interstitial infiltration was determined by counting all positive cells for each monoclonal antibody (semiautomatic function) in a sequence of ten consecutive computer images of 400 x high power fields - 0.0047 mm^2 each. Only immunoreactive cells with the clear identifiable nucleus were counted. Cells were scored positive when displayed a distinctly brown membrane. The only adjustments of field were made to avoid glomeruli and large vessles. The results were expressed as a mean number of immunopositive cells per mm^2.

Statistical methods

Differences between groups were tested using analysis of variance (One-way ANOVA) using LSD test to adjust for multiple comparisons. 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

Clinical features of the patients with IgAN, MPG and MCD at the time of biopsy are given in table II.

Table II. Clinical and laboratory findings at the time of biopsy in cases with IgAN, MPG and MCD

Number of cases	Sex (M/F)	Microhematuria	Gross hematuria	Proteinuria			Nephrotic syndrome	Renal function impairment[1]	Hypertension (> 90/160)
				> 1 g/24 h	1-2 g/24 h	2-3,5 g/24 h			
MCD (n = 10)	6/4	1	0	0	0	0	10	0	1
IgAN (n = 11)	5/6	1	10	4	2	0	0	4	3
MPG (n = 11)	7/4	5	3	1	2	4	4	3	2

[1]Serum creatinine > 1.5 mg/dl.

Most of our patients were young adults: the mean age was 34.6 in IgAN group, 27.4 in MPG patients and 28.9 in controls. At the time of renal biopsy, hematuria was present in all patients with IgAN and MPG. All patients with MPG showed proteinuria or nephrotic syndrome. Mild proteinuria was also present in 6 cases from IgAN group. Clinical renal impairment (serum creatinine greater than 1.5 mg/dl) was noted in 4 IgAN patients and in 3 patients with MPG. Elevated blood pressure was observed in 3 IgAN cases, in 4 MPG patients and in 1 patient from MCD group.

The morphometric data of the interstitial expression of α-SMA, interstitial fibrosis and interstitial infiltrates appear from table III. The mean values of the interstitial expression of α-SMA, interstitial volume, CD 68+, CD 45 RB+, CD 43+ and CD 20+ cells were in both IgAN and MPG groups significantly increased in comparison with MCD patients (figs. 1, 2 and 3). Moreover, the interstitial expresion of α-SMA in IgAN patients was significantly higher as compared with MPG group, meanwhile the mean values of the interstitial volume and interstitial infiltrates did not differ significantly in these glomerulopathies.

The correlations between interstitial expression of α-SMA and interstitial volume, serum creatinine as well as interstitial infriltrates are shown in table IV. In both IgAN and MPG groups there were significant positive correlations between interstitial expression of α-SMA and interstitial volume, serum creatine as well as CD 68+ and CD 45RB+cells. The correlations between interstitial expression α-SMA and CD 43+ and CD 20+ cells were positive, but they have not reach statistical significance. Similarly, correlations between all these parameters in MCD were weak and not significant.

DISSCUSSION

Although in 1968 Berger and Hinglais described IgAN as focal segmental glomerulonephritic changes by light microscopy[23], the most authors have indicated that in IgAN the glomeruli usually are diffusely affected[24, 25]. In the present study all IgAN and MPG cases showed by light microscopy glomerular changes defined as diffuse glomerulonephritis.

Our morphometric study showed that cytoplasmic interstitial expression of α-SMA was in both IgAN and MPG patients significantly increased as compared with MCD group. We observed interstitial staining for α-SMA in a distribution comparable to that of connective interstitial tissue. In IgAN at first glance our observations seem to correspond with the ear-

Table III. Interstitial expresion of α-SMA and analysis of interstitial infiltrates in IgAN, MPG and MCD

			Number of immunopositive cells per 1 mm²			
Number of cases	**α-SMA (%)**	**Interstitial volume (%)**	**CD68+**	**CD45RB+**	**CD43+**	**CD20+**
MCD (n = 10)	0.62 ± 0.29	10.42 ± 1.42	42.12 ± 14.32	69.82 ± 16.44	34.39 ± 12.62	0.66 ± 0.65
IgAN (n =11)	6.09 ± 3.3	23.21 ± 7.47	102.23 ± 48.08	171.02 ± 68.86	101.6 ± 38.16	8.8 ± 3.71
MPG (n = 11)	2.66 ± 1.41	17.85 ± 5.57	89.83 ± 39.25	176.6 ± 41.84	113.0 ± 21.44	11.06 ± 7.89
P value	< 0.001[1]	< 0.001[1]	< 0.001[1]	< 0.001[1]	< 0.02[1]	< 0.001[1]
	< 0.04[2]	< 0.02[2]	< 0.01[2]	< 0.001[2]	< 0.002[2]	< 0.001[2]
	< 0.001[3]	0.6 (NS)[3]	0.4 (NS)[3]	0.7 (NS)[3]	0.3 (NS)[3]	0.5 (NS)[3]

Data are expressed as mean ± standard deviation.
[1]IgAN *versus* MCD, [2]MPG *versus* MCD, [3]IgAN *versus* MPG.

Table IV. The correlations between interstitial expresion of a-SMA and interstitial volume as well as interstitial infiltrates in IgAN, MPG and MCD

Correlation between	**IgAN**	**MPG**	**MCD**
Interstitial expression of α-SMA and interstitial volume	r = 0.72; p < 0.02	r = 0.78, p < 0.005	r = 0.52; p = 0.1 (NS)
Interstitial expression of α-SMA and serum creatinine	r = 0.79; p < 0,005	r = 0.64, p < 0,04	r = 0.29; p = 0.42 (NS)
Interstitial expression of α-SMA and CD 68+ cells	r = 0.68; p < 0.03	r = 0.65; p < 0.03	r = 0.22; p = 0.4 (NS)
Interstitial expression of α-SMA and CD 45RB+ cells	r = 0.65; p < 0.03	r = 0,74; p < 0.01	r = 0,46; p = 0.2 (NS)
Interstitial expression of α-SMA and CD 43+ cells	r = 0.6; p = 0.06 (NS)	r = 0.6; p = 0.06 (NS)	r = 0.41; p = 0.2 (NS)
Interstitial expression of α-SMA and CD 20+ cells	r = 0.11; p = 0.72 (NS)	r = 0.21; p = 0.52 (NS)	r = 0.44; p = 0.2 (NS)

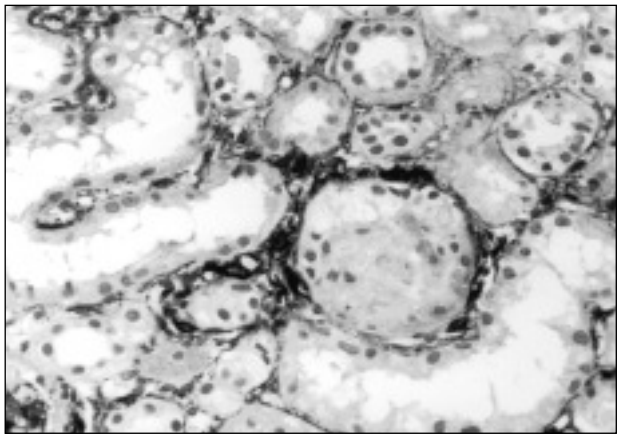

Fig. 1.—MCD. Peritubular expression of α-SMA. Magn. 400x.

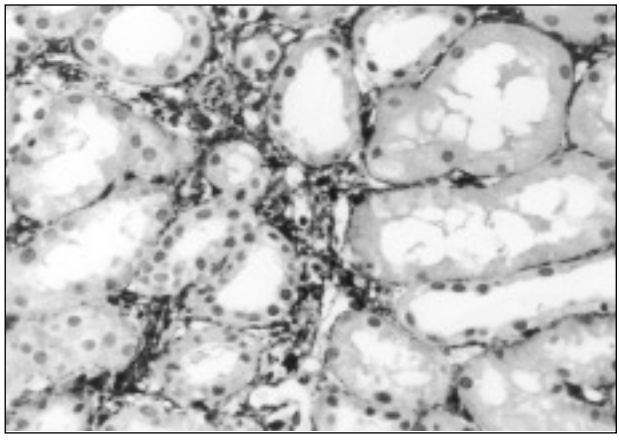

Fig. 2.—MPG. Moderate interstitial expression of α-SMA. Magn. 400x.

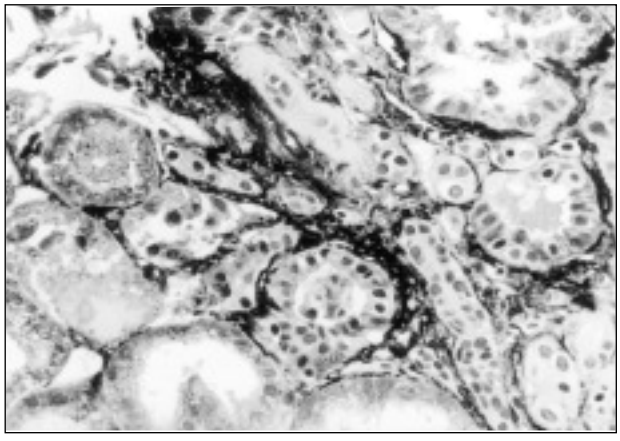

Fig. 3.—IgAN. Tubular atrophy and enhanced interstitial expression of α-SMA. Magn. 400x.

lier results of Goumenos y cols. and Hewitson y cols.[18,19]. It is worthy of note, however, that in these reports expression of α-SMA was higher than in our study (6.09% *versus* 12.9% and 17.2%, respectively). The possible explanation of these divergences is an relatively early stage of the disease in our IgAN cases and differences in methodology. To our knowledge no data have documented quantitatively interstitial staining for α-SMA in MPG. Interestingly, althouth morphological similarity between IgAN and MPG was suggested[8], the present study revealed that interstitial staining for α-SMA was in IgAN patients significantly greater that in MPG cases. As interstitial α-SMA staining was reported to be a reliable predictor of outcome[18], is not surprising that in our study significant positive correlation existed in IgAN and MPG patients between α-SMA staining and serum creatinine. Moreover, we found strong positive correlation between interstitial expression of α-SMA and interstitial volume in both igAN and MPG groups. These findings suggest that interstitial α-SMA positive cells play a role in the development of interstitial fibrosis in these glomerulopathies, as they may act as active, extracellular matrix producing cells[26]. It is worthy of note, however, that in study of Roberts y cols.[27] interstitial volume did not correlate significantly with the number of myofibroblasts in cases with membranous glomerulopathy. These conflicting results depend probably on the different method of counting of α-SMA positive cells. Counts were expressed by these authors as mean number of α-SMA positive cells/high power field.

However, when very large numbers of α-SMA positive cells were present, the counts became unrealiable and these cases were then expressed only as > 10 cells/field.

Although in our study groups the mean values of the interstitial leukocytes did not differ significantly, we found in both IgAN as well as MPG patients strong positive correlations between interstitial expression of α-SMA and interstitial CD 68+ and CD 45RB+ cells. These results correspond with findings of Goumenous y cols.[18] and Hewitson y cols.[19]. As the correlations between α-SMA and interstitial CD 43+ and CD 20+ cells were in our study weak and not significant it may be speculated that correlation between interstitial expression of α-SMA and all leukocytes depends solely on CD68+ cells. Although α-SMA positive cells are thought to be derived from the cortical arterial and arteriolar walls and diffuse into the interstitium[18], the phenotypic changes within renal interstitial fibroblasts leading to their expression of α-SMA and the acquisition of contractile properties cannot be excluded[28]. It has been demonstrated that cytokines such as transforming

growth factor β (TGF-β) released by tubular cells and macrophages[27], which plays a key role in the induction of fibrosis, may induce the myofibroblast phenotype in resting fibroblasts[29, 30]. Although our results support these observations, since in the present study significant positive correlation existed between interstitial expression of α-SMA and interstitial monocytes/macrophages, we are aware that they do not prove a cause-effect relationship.

In conclusion, our study suggest that interstitial α-SMA positive cells play a role in the development of interstitial fibrosis in both IgAN and MPG groups. The role of interstitial monocytes/macrophages as possible mediators in the process of inducing the myofibroblasts phenotype in resting fibroblasts remains to be shown.

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