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Mast cells, TGF-β1 and α-SMA expression in IgA nephropathy[1]

G.E.B. Silva[a], R.S. Costa[a,*], R.C. Ravinal[a], M.A. dos Reis[b], M. Dantas[c] and T.M. Coimbra[d]
[a]*Department of Pathology, Faculty of Medicine of Ribeirão Preto, University of São Paulo, Brazil*
[b]*Department of General Pathology, Faculty of Medicine of Triângulo Mineiro, Uberaba, MG, Brazil*
[c]*Division of Nephrology, Faculty of Medicine of Ribeirão Preto, University of São Paulo, Brazil*
[d]*Department of Physiology, Faculty of Medicine of Ribeirão Preto, University of São Paulo, Brazil*

Abstract. IgA nephropathy (IgAN) is a kidney disease with a varying renal prognosis. Recently, many studies have demonstrated that renal α-smooth muscle actin (α-SMA) and transforming growth factor (TGF-β1) expression, as well interstitial mast cell infiltrates could represent a prognostic marker in several renal diseases. The aim of our study was to analyze the prognostic value of mast cell, TGF-β1 and α-SMA expression in IgAN. A survey of the medical records and renal biopsy reports of 62 patients with a diagnosis of IgAN followed-up from 1987 to 2003 was performed. The mean follow-up time was 74.7 $\pm$ 50.0 months. The immunohistochemical studies were performed using a monoclonal antibody anti-human mast cell tryptase, a polyclonal antibody anti-human TGF-β1, and a monoclonal antibody anti-human α-SMA. An unfavorable clinical course of IgAN was related to interstitial mast cell infiltrates and α-SMA expression in the tubulointerstitial area. Expression of glomerular TGF-β1 and α-SMA, and interstitial TGF-β1 is not correlated with clinical course in IgAN. In conclusion, the increased number of mast cells and higher α-SMA expression in the tubulointerstitial area may be predictive factors for the poor prognosis of patients with IgAN.

Keywords: IgA nephropathy, TGF-β1, mast cells, α-SMA, renal fibrosis

1. Introduction

First described by Berger and Hinglais in 1968 [2], IgA nephropathy (IgAN) is now recognized as the most common form of primary glomerulonephritis (GN) worldwide [10]. Once thought to be benign, it causes end-stage renal disease (ESRD) in 5 to 25% of individuals within 10 years of onset [8]. On the other hand, 3.7% of IgAN patients develop long-standing spontaneous clinical remission [9]. On this basis, the possible factors involved in its prognosis have been extensively studied. Previous studies have shown that clinical and histological parameters such as recurring macroscopic hematuria, the absence of hypertension, young age at onset, and a lesser extent of injury to the glomerular, tubulointerstitial and vascular compartments favor a better prognosis for the patient [3,5,11].

The progression of chronic renal failure is characterized by glomerulosclerosis and tubulointerstitial fibrosis. A large number of cytokines, inflammatory mediators and growth factors produced by monocytes/macrophages and other cells such as mast cells (MCs) are probably involved in renal fibrosis [16,31].

In recent years, MCs were shown to be present in the interstitium of human renal biopsy tissue from patients with various renal diseases, such as IgAN, rapidly progressive GN, focal and segmental glomerulosclerosis, and diabetic nephropathy [15,22,32,37]. Furthermore, the number of interstitial MCs in these glomerular diseases correlates well with the degree of interstitial fi-

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*Corresponding author: R.S. Costa, Department of Pathology, Faculty of Medicine of Ribeirão Preto, Av. Bandeirantes 3900, CEP 14049-900, SP, Brazil. Tel.:/Fax: +55 16 3633 1068; E-mail: rscosta@fmrp.usp.br.

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brosis. MCs can produce cytokines and growth factors such as transforming growth factor-β (TGF-β), an important fibrogenic cytokine [20–23,25,32]. Increased expression of TGF-β has been demonstrated with mast cell infiltration in an animal model of progressive renal disease [20,23]. In IgAN, TGF-β expression also results in excessive deposition of extracellular matrix (EMC) proteins, with consequent tissue sclerosis and fibrosis [4,7].

Several studies have demonstrated that progressive glomerulonephritis is associated with phenotypic changes in glomerular and interstitial cells. These phenotypic changes can be recognized by the acquisition of smooth muscle cell type and *de novo* expression of smooth muscle actin (α-SMA) [1]. This transformation is induced by TGF-β [14,38].

The role of tissue MCs in generating α-SMA-positive cells also has been the focus of several reports [12,21].

The aim of the present study was to analyze whether the presence of MCs and the expression of TGF-β1 and α-SMA can predict an unfavorable renal clinical course in patients with IgAN.

2. Subjects and methods

2.1. Patients and clinical evaluation

We studied renal biopsies from 62 patients with IgAN. The biopsies were obtained from the files of the Department of Pathology, Medical School of Ribeirão Preto, Brazil, and were used in accordance with the principles of the Declaration of Helsinki. The procedures involved were also approved by the local University Ethics Committee. It should be pointed out that in our service a renal biopsy is seldom indicated for patients with microscopic hematuria without proteinuria or with asymptomatic proteinuria. On this basis, the real "benign" cases of IgAN are not present in our series. Clinical and laboratory data were obtained from the medical records of the patients using a previously reported protocol [30].

The clinical presentation of IgAN was considered to be the first manifestation of the disease as: (I) nephrotic syndrome when proteinuria was more than 3 g/day, total serum protein was less than 6 g/dL and serum albumin was less than 3 g/dL, or in the presence of a well characterized history of anasarca; (II) non-nephrotic proteinuria when proteinuria was higher than 200 mg/dL and lower than 3 g/dL as determined during clinical evaluation not related to nephrology; (III) macroscopic hematuria defined as the report of a well characterized history of dark urine; asymptomatic microscopic hematuria was defined as the presence of three or more red blood cells per large magnification microscopic field in at least two examinations of urinary sediment at any time during the course of the disease; (IV) acute nephritic syndrome was defined as edema, arterial hypertension, plus microscopic hematuria, plus azotemia (serum creatinine of more than 1.5 mg/dL); (V) arterial hypertension was defined as blood pressure higher than 140/90 mmHg.

The following classification of the outcome of IgAN was considered during clinical follow-up [30]: (i) clinical remission (CR): proteinuria below 200 mg/day with normal renal function; (ii) clinical improvement (CI): regression of nephrotic or nephritic syndrome or reduced proteinuria with stable renal function; (iii) unchanged (UC): unchanged clinical and/or laboratory data during the course of the disease, (iv) clinical worsening (CW): aggravation of nephrotic or nephritic syndrome or increased proteinuria with normal renal function; (v) chronic renal insufficiency (CRI): serum creatinine of 1.5 mg/dL or more and/or creatinine clearance of less than 90 mL/min per 1.73 m^2, or the need for dialysis or renal transplant (ESRD). We considered remission or clinical improvement or unchanged condition to indicate a favorable clinical outcome, and clinical worsening and chronic renal insufficiency to indicate an unfavorable clinical outcome.

Patients with IgAN were considered to have been treated when they received corticosteroids (methylprednisone or prednisone) associated or not with immunosuppressive drugs such as cyclophosphamide, regardless of the schedule used. When biopsy was performed, 49 of 62 patients were on angiotesin-converting enzyme (ACE) inhibitor therapy and 6 of 62 patients had been receiving immunosuppressive treatment.

2.2. Morphological evaluation

Morphological evaluation was performed by renal biopsy using common light microscopy and immunofluorescence microscopy [30]. Tissue obtained by renal biopsy from 62 patients with IgAN and control sections obtained from preserved renal areas from nephrectomized tumors were fixed in Bouin's solution, embedded in paraffin, cut into 4-μm-thick sections, and stained with hematoxylin and eosin, Masson's trichrome and methenamine silver. Interstitial fibrosis was assessed in a semiquantitative manner: ab-

sent, $<$ 10% of the total area; mild, 10 to 25% of the total area; moderate, 26 to 50% of the total area; and severe, $>$ 50% of the total area. Frozen renal tissue specimens were prepared for immunofluorescence microscopy [30].

2.3. Immunohistochemical studies

The immunohistochemical studies were performed using a monoclonal antibody anti-human MCs tryptase (Novocastra Laboratories Ltd, Newcastle upon Tyne, UK), polyclonal antibody anti-human TGF-β1 (Santa Cruz Biotechnology Inc, Santa Cruz, CA, USA), and monoclonal antibody anti-human α-SMA (DAKO Corporation, Glostrup, Denmark). Biopsy tissue was embedded in paraffin and processed by the indirect immunoperoxidase technique as previously described [30]. Serial 4-μm-thick sections were incubated overnight at 4°C with 1:30 mast cell tryptase monoclonal antibody, 1:2500 TGF-β1 polyclonal antibody and 1:1000 α-SMA monoclonal antibody. The sections stained for mast cell tryptase were pretreated with pepsin (1 mg/ml, 0.5 acetic acid) (Sigma Chemical Co, St. Louis, MO, USA) for 30 minutes at 37°C and the sections stained for TGF-β1 were pretreated with 0.01 mol/L citrate buffer, pH 6.0, in a pressure cooker for 40 minutes. The streptavidin-biotin-peroxidase complex procedure (DAKO) was employed as the detection system. Chromogen development was performed with 3.3'-diaminobenzidine (DAB) (Sigma), and the material was counterstained with methyl green, dehydrated, and mounted.

Negative controls consisted of the omission of the primary antibody in the reaction.

MCs quantification was performed by morphometry, and the number of MCs/mm^2 was counted using a light microscope with a 10X magnification objective [30]. The renal biopsy dimension was obtained by morphometric study using a videocamera coupled to a light microscope fitted with a 10X magnification objective. The image obtained on the video monitor was connected to an automatic image analyzer (KS 300 Kontron Imaging System, Kontron Elektronic, Eching bei München, Germany) and the glomerular and tubulointerstitial areas were measured.

Glomerular expression of α-SMA and TGF-β1 was graded semi-quantitatively according to Ravinal et al. [30], modified: 0, no staining; 1, trace mesangial staining; 2, weak segmental mesangial staining, usually involving a small minority of the glomeruli present, and /or weak or moderate expression in podocytes; 3, strong segmental mesangial staining, usually involving a majority of the glomeruli present, and/or strong expression in podocytes; and 4, strong diffuse mesangial staining, usually involving all glomeruli present, and/or very strong expression in podocytes. The α-SMA immunoreaction in the tubulointerstitium and interstitial TGF-β1 of the renal cortex was scored as follows: 0, absent staining; 1, weak staining with focal distribution; 2, moderate staining with focal distribution; 3, strong staining with focal distribution or weak and diffuse, and 4, strong and diffuse.

2.4. Statistical analysis

When data distribution was normal, the values were expressed as mean $\pm$ standard deviation. Proportions were determined using the standard chi-square test (χ^2) with Yates correction or Fisher's exact test. Correlation between non-continuous variables was calculated by Spearman's correlation coefficient. A significant difference was accepted if $p < 0.05$.

Survival analysis was performed to test the association between renal failure and each immunohistochemical parameter (TGF-β, α-SMA and MCs infiltration). Survival times from first clinical assessment to last follow-up were obtained for each patient. Univariate comparisons of renal survival rates made by Kaplan-Meier curves and the log-rank test.

3. Results

Sixty-two patients were studied, 32 men (51.6%) and 30 women (48.4%). Mean age at onset of IgAN was 27.52 $\pm$ 11.19 years. Fifty-two patients were white (83.9%) and ten (16.1%) were nonwhite. Median follow-up time was 74.7 $\pm$ 50.0 months. We did not find a significant correlation between clinical outcome of IgAN and age or race ($p > 0.05$). However, male patients ($p = 0.044$) showed a significantly worse clinical outcome of IgAN compared with female patients.

Histological examination showed the following features: four patients (6.5%) with a mesangiopathic histological picture, six patients (9.7%) with diffuse mesangial hypercellularity, 48 patients (77.4%) with focal and segmental glomerulonephritis (12 of them with focal cellular crescents), and 4 patients (6.4%) with advanced chronic glomerulonephritis. The clinical course of IgAN was distributed as follows: 7 patients (11.2%) with clinical remission, 4 patients (6.5%) with clinical improvement, 17 patients (27.4%) with unchanged

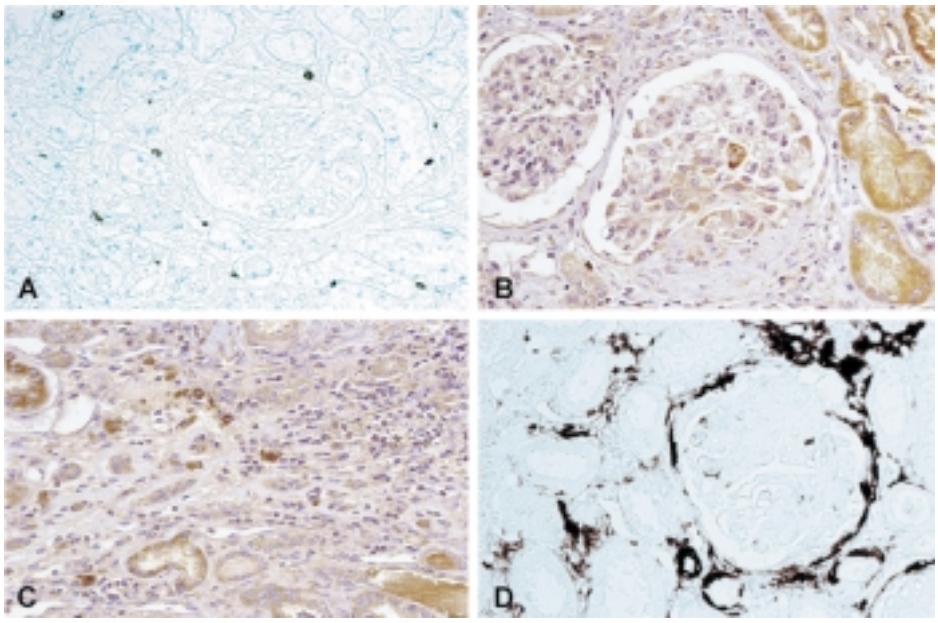

Fig. 1. Immunohistochemistry for MCs, TGF-β1 and α-SMA from patients with IgAN. A: Tryptase-positive interstitial MCs on renal cortex; B: TGF-β1 expression showing a glomerular score of 2; C: TGF-β1 expression showing an interstitial score of 2; D: α-SMA expression showing a glomerular score of 1 and a tubulointerstitial score of 3.

disease, 4 patients (6.5%) with clinical worsening, 12 patients (19.4%) with CRI, and 18 patients (29%) with ESRD.

The interstitial tryptase-positive MCs, the glomerular and interstitial expression of TGF-β, and the glomerular and tubulointerstitial α-SMA expression are shown in Fig. 1. Tryptase-positive MCs could not be detected in renal sections from normal controls or in the glomerular compartment from biopsies of patients with IgAN. However, tryptase-positive MC were detected in the cortical interstitium from biopsies of patients with IgAN. TGF-β1 protein was found in the cytoplasm of tubular epithelial cells of all biopsies from patients with IgAN and normal controls. It was also detected in the glomeruli and interstitium of some biopsies with IgAN. α-SMA expression was detected in the smooth muscle layer of all arteries and arterioles from all patients with IgAN and normal controls. α-SMA expression was also localized in the glomeruli and tubulointerstitial area in some biopsies from patients with IgAN. Only one mesangiopathic histologic picture showed expression of score 1 in the mesangial area and another case showed expression of score 1 in the tubulointerstitial area. On the other hand, markedly increased expression (score 2, 3 or 4) was common in histologic picture with the highest degree of cellular proliferation, glomerulosclerosis and tubulointerstitial fibrosis.

Table 1
Clinical outcome of 62 IgAN patients as a function of α-SMA score in tubulointerstitial area

CLINICAL	$\alpha-$SMA					
OUTCOME	0	1	2	3	4	Total
CR	2	3	0	1	1	7
CI	1	3	0	0	0	4
UC	6	7	3	1	0	17
CW	0	2	2	0	0	4
CRI (ESRD)	1(2)	6(3)	3(7)	1(5)	1(1)	12(18)
TOTAL	12	24	15	8	3	62

Legend: CR, Clinical remission; CI, Clinical improvement; UC, Unchanged; CW, clinical worsening; CRI, Chronic renal insufficiency; ESRD, end-stage renal disease.
Fisher exact test, $p = 0.007$, comparing favorable clinical outcome (CR + CI + UC) to unfavorable clinical outcome (CW + CRI + ESRD) versus 0 + 1, and 2 + 3 + 4, for α-SMA score.

The Spearman correlation coefficient between interstitial tryptase-positive MCs and urinary protein excretion at the time of biopsy and at the end of follow-up, and serum creatinine at the time of biopsy and at the end of follow-up are shown in Fig. 2. A significant correlation was found between interstitial tryptase-positive MCs and serum creatinine at the time of biopsy and serum creatinine at the end of follow-up, ($p = 0.020$ and $p = 0.010$, respectively), but not for urinary protein excretion at the time of biopsy and at the end of follow-up ($p > 0.05$).

The Spearman correlation coefficient between interstitial tryptase-positive MCs and clinical outcome of

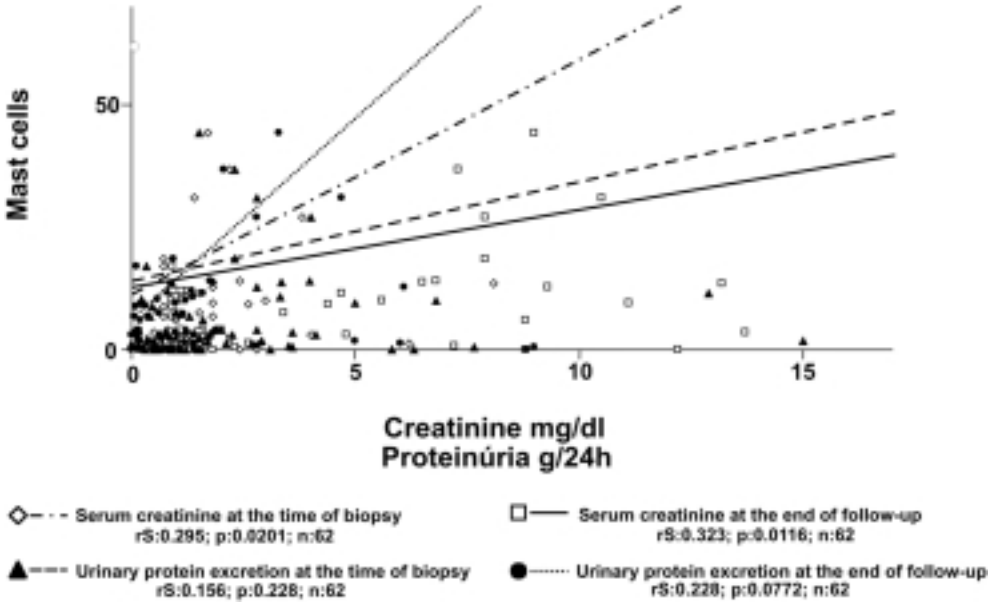

Fig. 2. Spearman's correlation coefficient (rS) between interstitial tryptase–positive MCs and urinary protein excretion at the time of biopsy, urinary protein excretion at the end of follow-up, serum creatinine at the time of biopsy, and serum creatinine at the end of follow-up.

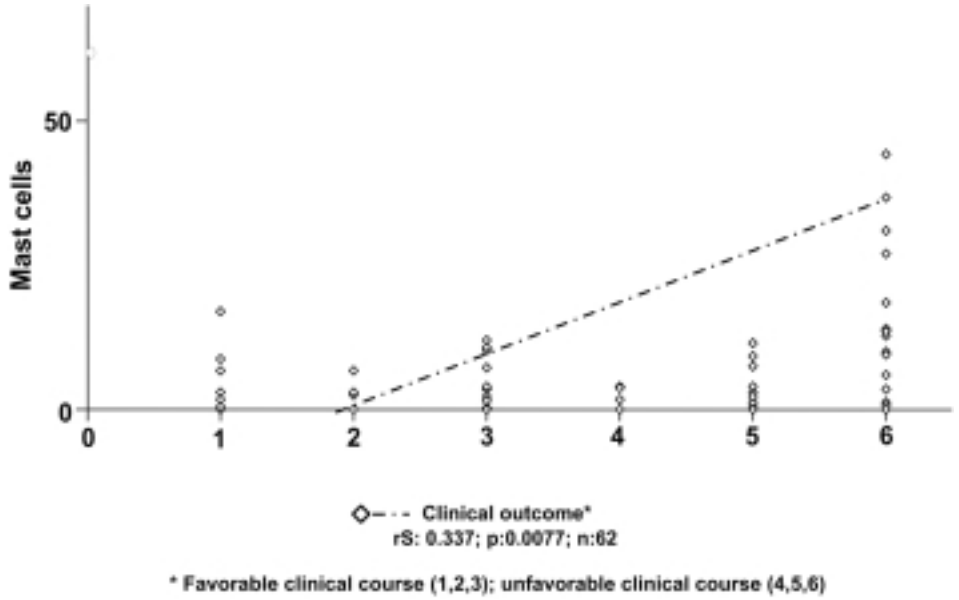

Fig. 3. Spearman's correlation coefficient (rS) between interstitial tryptase–positive MCs and clinical outcome.

IgAN is shown in Fig. 3. The correlation between these parameters was significant ($p = 0.0077$). The Spearman correlation coefficients between interstitial tryptase-positive MCs and interstitial and glomerular TGF-β1, and glomerular and tubulointerstitial α-SMA are shown in Fig. 4. The correlation between these parameters was not significant ($p > 0.05$).

Table 1 shows the relationship between histological scores for α-SMA expression in the tubulointerstitial area and clinical course of 62 NIgA patients. When comparing a favorable clinical course (CR + CI + UC) and an unfavorable clinical course (CW + CRI + ESRD) with a low α-SMA score (0 + 1) and a high α-SMA score (2 + 3 + 4), a significant correlation

Table 2
Clinical outcome of 62 1gAN patients as a function of α-SMA score in glomeruli and TGF-β1 score in interstitial and glomeruli

SCORE	$\alpha-$SMA – glomeruli						TGF-β1 – glomeruli						TGF-β1 – interstitial					
CLINICAL OUTCOME	0	1	2	3	4	Total	0	1	2	3	4	Total	0	1	2	3	4	Total
CR	3	3	0	1	0	7	6	0	0	1	0	7	7	0	0	0	0	7
CI	3	1	0	0	0	4	4	0	0	0	0	4	4	0	0	0	0	4
UC	6	7	3	1	0	17	16	0	1	0	0	17	16	0	1	0	0	17
CW	2	0	2	0	0	4	3	1	0	0	0	4	3	0	1	0	0	4
CRI(ESRD)	2(4)	6(5)	3(8)	1(1)	0(0)	12(18)	11(16)	1(2)	0(0)	0(0)	0(0)	12(18)	11(16)	1(2)	0(0)	0(0)	0(0)	12(18)
TOTAL	20	22	16	4	0	62	56	4	1	1	0	62	57	3	2	0	0	62

Legend: CR, clinical remission; CI, clinical improvement; UC, unchanged; CW, clinical worsening; CRI, chronic renal insufficiency; ESRD, end-stage renal disease.
Fisher exact test, $p = 0.054$, $p = 0.366$, $p = 0.245$, comparing favorable clinical outcome (CR + CI + UC) to unfavorable clinical outcome (CW + CRI + ESRD) versus $0 + 1$, and $2 + 3 + 4$, for the α-SMA score for glomeruli and the TGF-β1 score for interstitial cells and glomeruli, respectively.

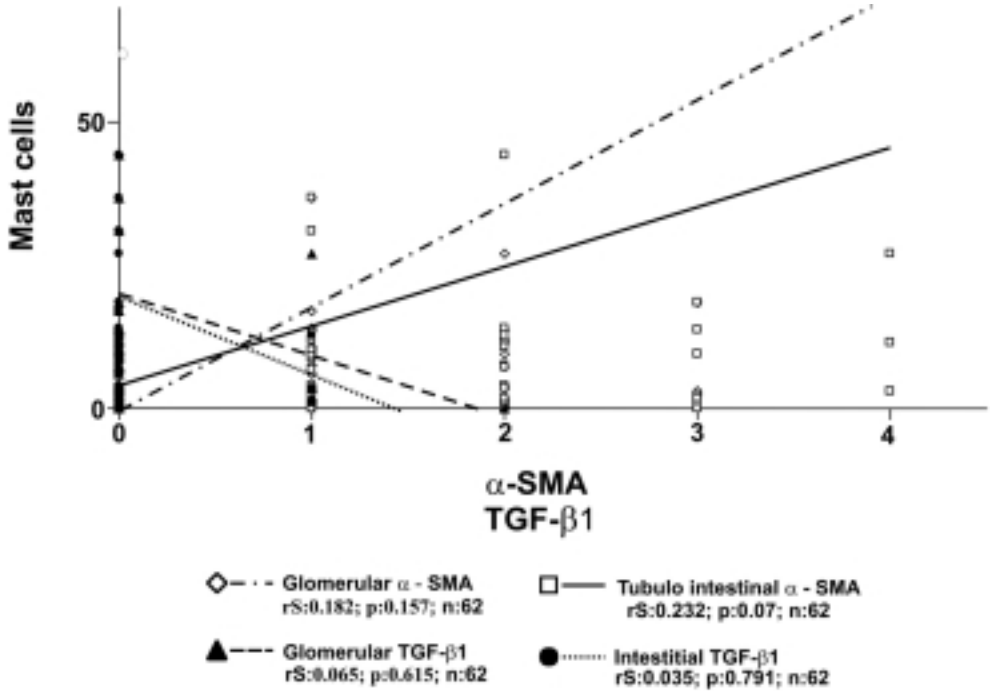

Fig. 4. Spearman's correlation coefficient (rS) between interstitial tryptase–positive MCs and glomerular and tubulointerstitial α-SMA, and glomerular and interstitial TGF-β1.

was observed ($p = 0.007$). When α-SMA expression in glomeruli and TGF-β1 expression in glomeruli and interstitial area were compared with the clinical course, no significant correlation was observed ($p = 0.054$, $p = 0.366$, and $p = 0.245$, respectively). (Table 2).

A significant correlation was found between histological score for glomerular and interstitial TGF-β1 and urinary protein excretion at the end of follow-up ($p = 0.006$ and $p = 0.02$, respectively), and serum creatinine at the end of follow-up ($p = 0.009$ and $p = 0.02$, respectively), but not for urinary protein excretion and serum creatinine at the time of biopsy ($p > 0.05$ both).

Tubulointerstitial α-SMA expression was correlated with serum creatinine at the time of biopsy and the end of follow-up ($p < 0.01$ both), and urinary protein excretion at the time of biopsy and the end of follow-up ($p < 0.01$ both). Glomerular α-SMA expression showed correlation with serum creatinine at the time of biopsy and the end of follow-up ($p = 0.03$ and $p = 0.02$, respectively), and urinary protein excretion at the time of biopsy and the end of follow-up ($p = 0.03$ and $p < 0.01$, respectively).

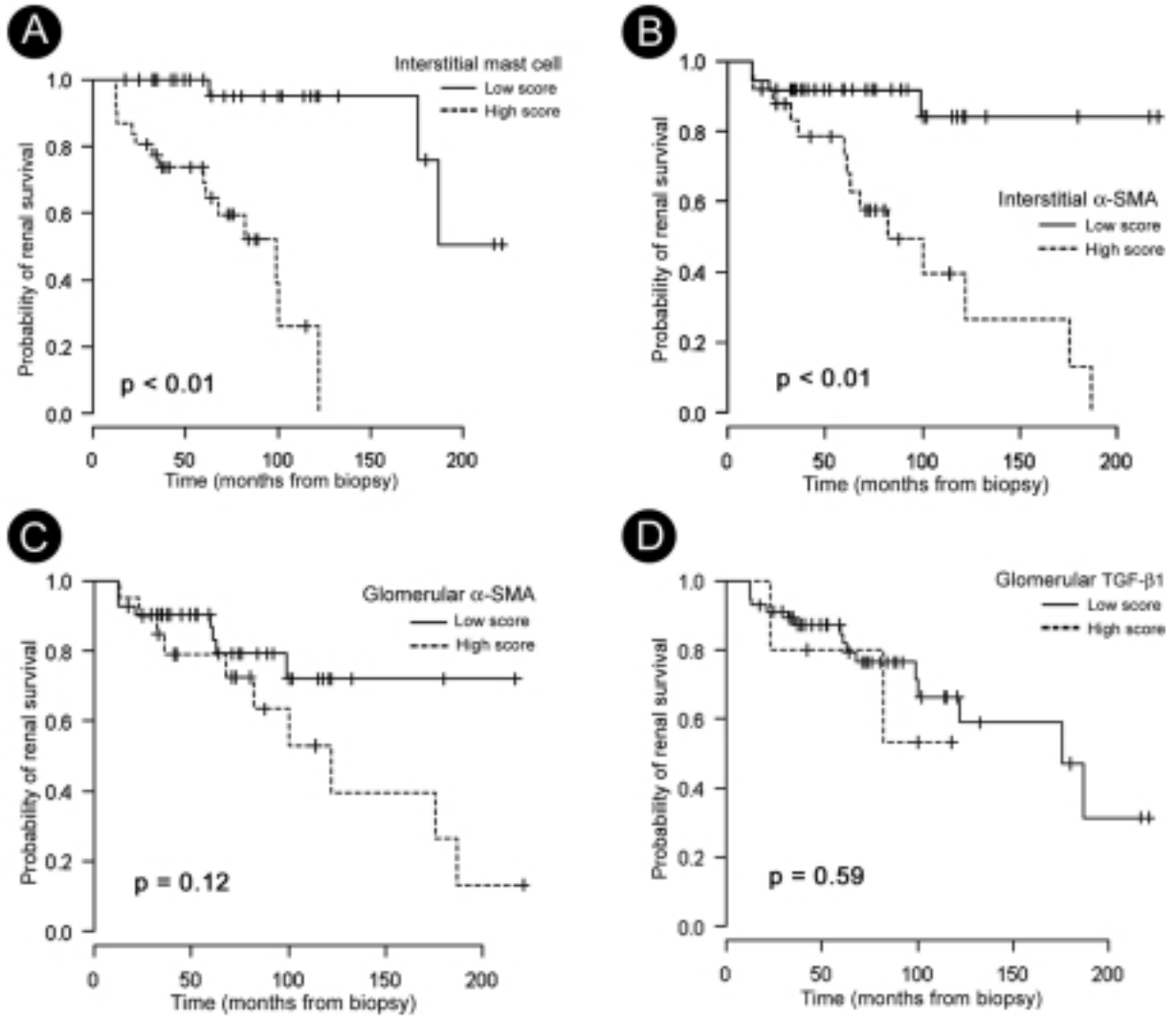

Fig. 5. Renal survival of patients with IgAN ($n = 62$) as a function of immunohistochemical score. For TGF-β1 and α-SMA, a score of 0 or 1 is considered to be low, and a score of 2, 3 or 4 is considered to be high. For MCs, a low score corresponded to those cases in which the number of cells was below the median of 3.5 cells/mm^2 and a high score corresponded to a number of cells above the median. Interstitial TGF-β1 immunohistochemistry showed low labeling and for this reason the graph was no included in the above scheme. The end point for renal survival is defined as ESRD requiring dialysis or transplant. Vertical hatched marks on the survival curves indicate the end of follow-up for patients who did not develop ESRD.

In Fig. 5, these same scores were plotted on a Kaplan-Meier graph correlating them to the survival function over subsequent months. The univariate determinants that predicted worse renal survival were a higher score for infiltrating MCs and interstitial α-SMA ($p < 0.01$).

Compared to patients with absent or mild interstitial fibrosis, tubulointerstitial α-SMA expression and number of interstitial MCs were strikingly increased in patients with moderate or severe interstitial fibrosis ($p < 0.01$ both). Interstitial TGF-β1 expression was not correlated with the degree of fibrosis ($p > 0.05$).

4. Discussion

The study of the prognosis of IgAN involves demographic, clinical and histological parameters which may contribute to the outcome. In the present study, male gender, the absence of macroscopic hematuria, arterial hypertension, high levels of serum creatinine, severe proteinuria, and elevated glomerular or tubulointerstitial score of the injury were significantly associated with an unfavorable clinical course. These findings agree with the observations of Bogenschütz et al. [3]

and D'Amico [10,11]. We could not confirm the worse prognosis for older age at onset and the presence of vascular damage, as found by these authors. In a review of the literature, D'Amico [10] showed that the last two variables were considered to be weak predictors, while the first seven were strong predictors. This fact can explain our findings.

Experimental and human GN studies have demonstrated a strong correlation between the number of interstitial MCs and the severity of interstitial injury in renal diseases [15,16,37]. Recently, mitogenic basic fibroblast growth factor was detected in renal MCs cytoplasm in biopsy specimens from patients with IgAN, suggesting the potential fibrogenic role of MCs in IgAN [15]. Kurusu et al. studied 45 patients with IgAN, and found a significant positive correlation between interstitial tryptase-positive MCs and degree of tubulointerstitial fibrosis and renal function [26]. Hiromura et al. studied patients with primary and secondary GN (42 of them with IgAN) and showed that the number of mast cell appeared to be lower in diseases in which the function improved or rarely deteriorated [22]. In contrast, the number of MCs appeared to be higher in disease that had the potential to progress to deterioration of renal function [22]. However, recent experiments did not confirm this view but proposed a protective role of MCs in renal fibrosis [24,30,36]. Our data showed a significant correlation between the number of interstitial MCs and serum creatinine at onset and follow-up, the degree of interstitial fibrosis, as well as clinical outcome.

MCs may potentiate fibrosis in a number of ways. They are capable of synthesizing several fibrogenic cytokines and may also contribute to matrix remodeling and fibrosis (activation of matrix metalloproteinases) [21]. Tryptase, histamine and heparin can stimulate collagen synthesis and induce chemotaxis [21]. Nevertheless, the question remains as to how these cells are involved in the interstitial fibrosis of the kidney. Kurusu et al. showed a relationship between infiltration of MCs in the tubulointerstitial area and urinary protein excretion. This may suggest some preceding mechanism of chemotaxis for MCs in the tubulointerstitium. In our study, there was not correlation between MC infiltration and urinary protein excretion, as also observed in other human and animal studies [22,36]. Although MCs had no effect on proteinuria, an increase in MCs was associated with higher serum creatinine levels. These results are supported by the fact that a more rapid progression to ESRD occurred in cases with high MCs and α-SMA scores (Fig. 5).

More recently, Grandaliano et al. suggested that protease-activated receptor-2 (PAR-2) expressed by renal resident cells and activated by MCs tryptase may induce ECM deposition resulting in TGF-β expression [19]. We did not find a significant correlation between interstitial tryptase-positive MCs and interstitial TGF-β1 expression. In recent years, biochemical and pathological studies have revealed that the expression of TGF-β1 in the kidney is also involved in the progression of renal injury in NIgA [20,35]. Our data showed that there was no relationship between the interstitial scores for TGF-β1 and the degree of interstitial fibrosis, proteinuria and creatinine at onset, as well as clinical outcome; but only for proteinuria and creatinine at the end of follow-up. This can be explained because in our patients, there were a very low percentage of cases expressing TGF-β1. The immunostaining is not a very sensitive technique for assessing TGF-β1 activity in the kidney [18]. On the other hand, ACE inhibitor therapy reduced renal TGF-β1 gene expression in patients with IgAN [34]. In our patients, 49 (79%) were on ACE inhibitor treatment at the time of biopsy.

In vitro and vivo analyses demonstrated that MCs products such as heparin and TNF-α influence α-SMA expression [6,13]. Ruger et al. observed periglomerular accumulation of MCs, type VIII collagen, and α-SMA-positive cells in diabetic nephropathy, suggesting that MC may up-regulate renal fibrosis and α-SMA expression [33]. In the present study, no significant correlation between the interstitial tryptase-positive MCs and glomerular and tubulointerstitial α-SMA expression was observed. Although previous study with 43 patients in our laboratory have not found correlation between α-SMA and renal damage [28], several authors have shown that α-SMA expression is increased in patients with IgAN who have moderate or severe renal tubulointerstitial injury compared to patients with discrete injury [7,29]. These data are similar to our results, now with 62 patients, which showed a positive correlation between α-SMA expression and the degree of interstitial fibrosis, serum creatinine, as well as clinical outcome. We also have found correlation between α-SMA and proteinuria.

Similarly, recent studies from our laboratory (69 patients with lupus nephritis) showed no correlation between MCs and TGF-β1 or α-SMA, nor any correlation between TGF-β1 and clinical outcome [30]. However, further studies are necessary to define the correlations between MCs, TGF-β1 and α-SMA in IgAN.

In conclusion, the increased number of MCs and the higher α-SMA expression in the tubulointerstitial area may be predictive factors for the poor prognosis of patients with IgAN. Our results also indicate that MCs within the kidney do not affect proteinuria but do result in increased serum creatinine levels.

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