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Immunoexpression of alpha-SMA and CD68 in native kidney biopsies

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Abstract

Introduction: The role for inflammation and fibrosis as predictive histopathological markers for renal function has been discussed in several studies. Aim of our investigation was to evaluate the clinico-pathological correlation of myofibroblasts expression as markers for initial development of fibrotic processes and macrophagic infiltration in a population with impaired renal function, in order to better understand their value in diagnostic biopsies. *Materials, Methods and Results*: We evaluated 20 consecutive native kidney biopsies performed for diagnostic purposes. Material remaining after routine light microscopy and immunofluorescence, was stained for α-SMA as myofibroblast marker and CD68 as macrophage infiltration marker. Quantitative evaluation was conducted by electronic image analysis on consecutive low power fields, avoiding glomeruli, and estimated as percentage of the total area or as number of positive cells/field for macrophage infiltration. The renal biopsies were also evaluated for histological characteristics such as percentual area of inflammation infiltration and fibrosis. Clinical and laboratory data were recorded at biopsy moment and followed-up on a period of 17±11 months after the renal biopsy. Interstitial α-SMA immunoexpression proved to be related with interstitial fibrosis (r=-0.47, p<0.001) and macrophage infiltration (r=0.21, p=0.03). Higher immunoexpression of α-SMA was related with renal function assessed by creatinine level at biopsy moment (r=0.32, p=0.002). *Conclusions*: In this study, detection of myofibroblast infiltration using α-smooth-muscle actin (α-SMA) proved to be a good marker in describing the initial phases of interstitial fibrosis development in early stages of chronic kidney dysfunction.

Keywords: myofibroblast, fibrosis, macrophage infiltration, chronic kidney disease.

Introduction

Chronic kidney disease is clinically characterized by a progressive deterioration in glomerular filtration rate and the histopathological findings of progressive interstitial fibrosis as well as tubular damage in the progression of renal impairment, regardless of the initiating cause for the renal pathology [1, 2].

The differentiation into myofibroblasts is considered one of the essential early cellular events that initiate the development of organ fibrosis [3, 4].

Myofibroblasts are considered to play an important role in tissue repair and in production of extracellular matrix, they are characterized by the presence of cytoplasmic microfilament bundles expressing α-smooth-muscle actin (α-SMA), the actin isoform characteristic for vascular smooth-muscle cells [5, 6]. Also, myofibroblasts are observed to be important elements in organ remodeling through their function of extracellular matrix organization, due to these properties myofibroblasts presence is described also in pathological processes as hypertrophic scars as well as fibrosis processes in kidney and heart tissue [7].

In an experimental model of obstructive nephropathy induced by unilateral ureteral obstruction has been observed the presence of multiple cells that showed co-expression of both α-SMA and tubular markers, indicating that these cells are in a transitional stage between epithelium and mesenchyma [8, 9].

Fibroblasts renal origin remains controversial, currently the most common theory prioritizes local interstitial cells but other authors claim that migrated leukocytes derived from local fibroblasts may be responsible for the fibroblasts expression and extracellular matrix deposition [10]. Nevertheless, processes as infiltration of blood mononuclear cells and macrophage recruitment, proliferation of interstitial mesenchymal cells and apoptosis of tubular epithelial cells represent important steps in renal fibrosis development [11].

The aim of this study was to evaluate the immunoexpression of myofibroblasts and macrophage infiltration in early stages of fibrosis development and their prognostic value for the progression of chronic kidney disease.

Materials and Methods

The study was performed in kidney tissues obtained from 20 patients that underwent a biopsy procedure during 2009–2011. The protocol was approved by the Ethic Committees of "Carol Davila" Nephrology Hospital and University of Medicine and Pharmacy of Craiova, and was conducted according to the ethical principles of the Helsinki Convention.

Medical history, physical examination and laboratory data recorded during the hospitalization period were retrieved from the patient files and the informed consent

for using confidential data was obtained from each patient. The biopsies were performed according to the approved local procedure, with a 16 Gauge needle and under ultrasound control. The biopsies were performed on various clinical indications, which were as follows: isolated proteinuria, isolated reduced renal function (RF) assessed by increased serum creatinine >25% compared with previous presentations, association of both proteinuria and reduced RF.

Estimated glomerular filtration rate (eGFR) was determined using the Modification of Diet in Renal Disease (MDRD) formula [12].

Chronic kidney disease stages were defined as the reduction in glomerular filtration rate calculated with the MDRD formula, according to the current diagnosis procedures [13].

The exclusion criteria represented biopsies with acute injury or active proliferative lesions such as: proliferative glomerulonephritis, interstitial nephritis, acute tubular necrosis, thrombotic microangiopathies or vasculitis.

Tissue samples, fixed in 4% buffered paraformaldehyde and embedded in paraffin, were processed following the international guidelines and examined by light microscopy and immunostaining.

Material remaining after routine light microscopy was stained for α-SMA as myofibroblast marker and CD68 as macrophage infiltration marker.

The immunohistochemical processing was made on sections using the LSAB+ System-HRP (Dako). The antibodies used, clone, dilution and antigen retrieval are shown in Table 1.

Table 1 – ***Panel of antibodies used for immunostaining***

Antibody	Clone	Dilution	Antigen retrieval
α-SMA	1A4	1:50	Citrate buffer, pH 6
CD68	PM-1K	1:50	

Images were acquired by a Nikon Eclipse (Nikon, Apidrag, Romania) microscope, equipped with a 5-megapixels CCD digital videocamera.

Consecutive images, avoiding glomeruli, were recorded from the whole renal biopsy tissue at ×400 magnification. Quantification was estimated on captured high quality images by considering the percentage of stained areas in the total histological fields using the Image Pro Plus 4.5.1.

Results were expressed as percentage of surface density or as number of macrophages/fields.

Statistical analysis was performed using the SPSS 10 software. In all statistical analysis significance was set for p-values <0.05. Continuous variables were expressed as average values ± SD. Differences among percentages were determined by χ^2 test and Fisher exact test.

Results

In this study there were investigated the clinical and pathological characteristics of 20 patients that underwent a kidney biopsy procedure and that were followed up on a period of 17±11 months after the renal biopsy.

The clinical and laboratory data at the biopsy moment showed the prevalence of nephrotic syndrome (69%) as clinical diagnose defined by the following criteria: proteinuria >3.5 g/24 hrs., edema and/or hypoalbuminemia (serum albumin<3.5g/dL), without acute kidney injury and with or without hematuria.

The most common indication for renal biopsy was represented of association of both proteinuria and reduced RF (49%), followed by isolated proteinuria (30%) and decreased RF assessed by increased serum creatinine >25% compared with previous presentations (21%).

Glomerular sclerosis, tubular atrophy and interstitial fibrosis were assessed in classical histological staining as Hematoxylin–Eosin and trichromic (Goldner–Szekely) according to the following criteria: glomerular sclerosis was evaluated as the percentage of sclerotic glomeruli in each sample, conversely, tubular atrophy and interstitial fibrosis were qualitatively graded using a scale of 0–3 (0 – no pathology; 1 – <25% involvement, mild; 2 – 25–50% involvement, moderate; and 3 – >50% involvement, severe).

Tubular atrophy in a severe degree and interstitial fibrosis were observed in 21% and 37% of biopsy specimens, respectively. Interestingly, we found also the association of inflammation infiltrates associated with mild fibrosis expression in 15% of the cases.

Primary and secondary forms of focal segmental glomerulisclerosis was the most common histopathological diagnosis (n=7, 35%), followed by diabetic nephropathy and membranous nephropathy each in 15% of the cases, minimal change disease and nephroangiosclerosis each in 10% of the cases and amyloidosis/monoclonal immunoglobulin deposition disease each in 5% of the cases.

Renal function assessed at biopsy moment and on follow-up moments after the renal biopsy, moment registered a decrease of proteinuria/24 hrs. measured in different time points after the renal biopsy. There was not observed any differentiation in histopathological findings regarding the diagnostic stages of chronic kidney disease.

In the present study, α-SMA was not found in epithelial tubular cells but essentially in the interstitial area, particularly in areas with tubulointerstitial injury and adjacent to areas with interstitial fibrosis organization (Figure 1, A and B).

An important observation was the immunoexpression of α-SMA in periglomerular fibrotic areas in cases with nephroangiosclerosis, as well as the absence of myofibroblasts immunostaining in the complete sclerotic glomeruli (Figure 2, A and B).

In the entire group of patients, α-SMA staining ranged from 0.9 to 20.5% (mean 6.1±2.3%) for the interstitial area. Comparing the histological and clinical parameters for patients having a degree of myofibroblast interstitial infiltration less or higher than the immunostaining mean expression, it was demonstrated that higher expression of α-SMA related only with creatinine level at follow-up (Table 2).

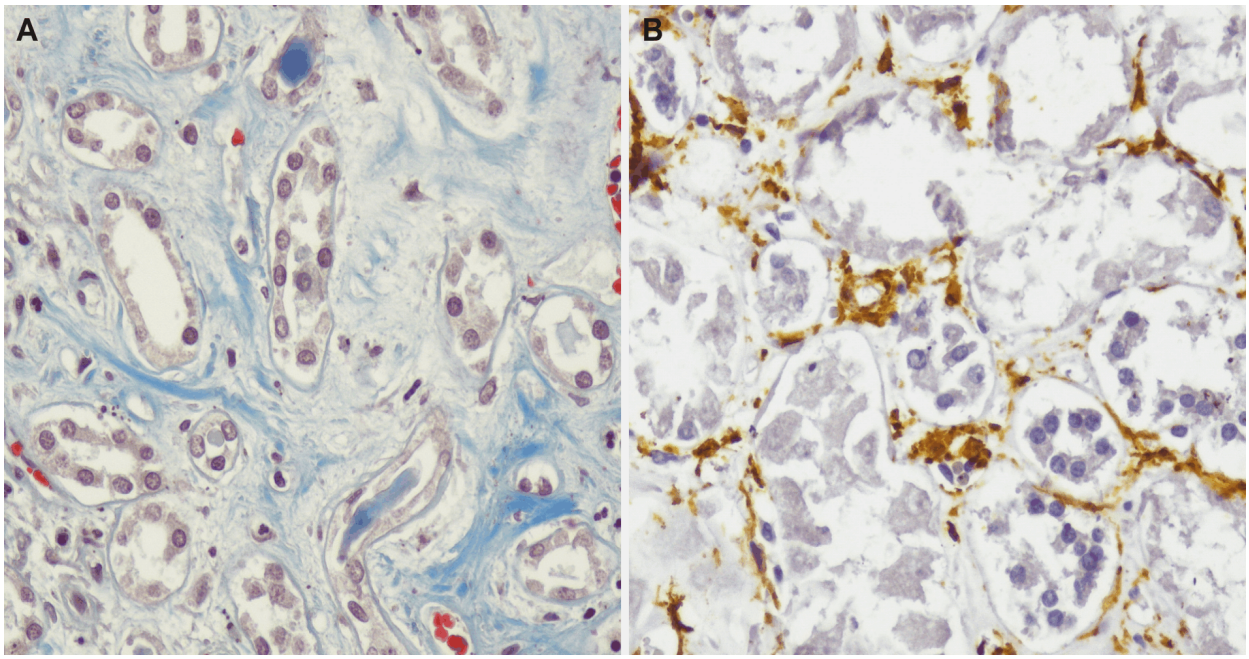

Figure 1 – ***Morphological and immunohistochemical expression of interstitial fibrosis area: (A) Interstitial fibrosis organized in fine bundles present peritubular (trichromic GS, ×200); (B) Myofibroblasts immunoexpression in fibrotic interstitial area (α-SMA immunostain, ×200).***

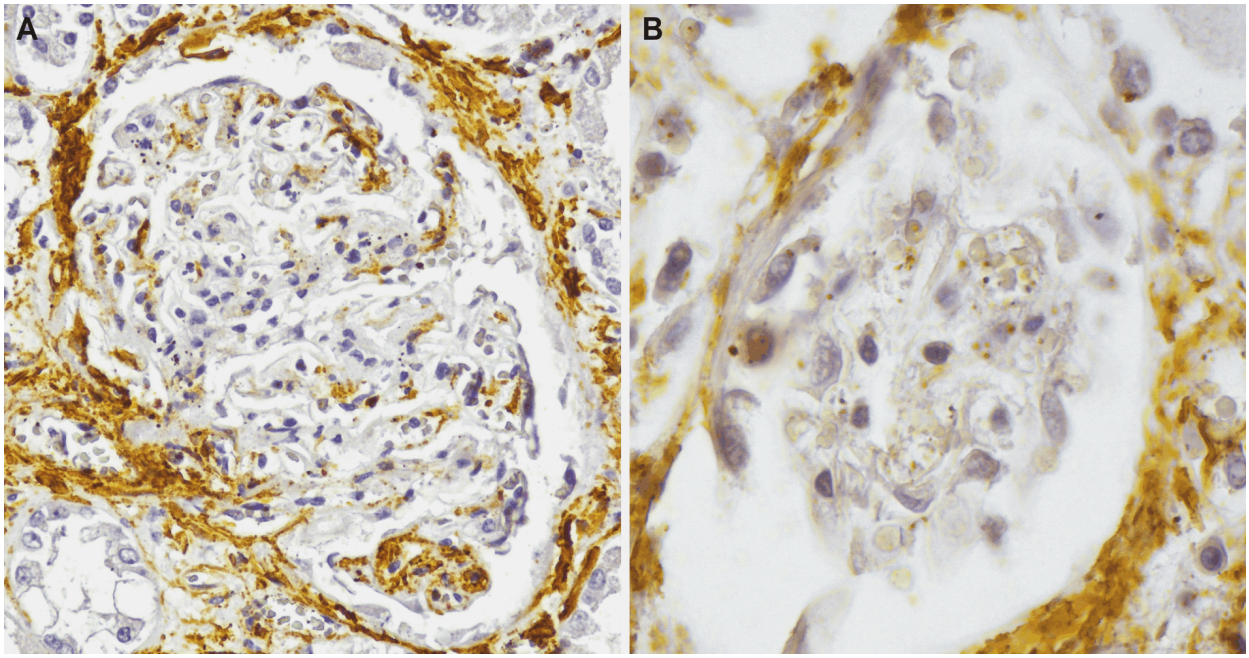

Figure 2 – ***Myofibroblast expression in glomerular area: (A) Periglomerular expression of myofibroblasts presence in fibrotic periglomerular area and also capilary lumens (α-SMA immunostain, ×200); (B) Myofibroblasts presence only in the periglomerular area in a case with complete glomerulosclerosis (α-SMA immunostain, ×400).***

Table 2 – ***Comparison of clinical, histological parameters and interstitial α-SMA immunostaining***

Group	α-SMA <6% *(n=8)*[1]	α-SMA >6% *(n=12)*[1]
Interstitial fibrosis grade 1 [%]	19	25
Interstitial fibrosis grade 3 [%]	11	12
Creatinine [mg/dL]	2.1±1.2	2.9±2.3
Creatinine at follow-up [mg/dL]	2.7±2.3	3.4±1.1
eGFR [mL/min./1.73m^2]	39±14	37±21
Proteinuria/24 hrs. [g/24 hrs.]	3.1±2.3	3.4±3.2
Steroid therapy [%]	21	17
Antihypertensive therapy [%]	56	43

[1]Some values are expressed as mean ± standard deviation.

Interstitial α-SMA immunoexpression proved to be related with lesser scores of interstitial fibrosis (r=-0.47, p<0.001) in the entire group. At univariate analysis, α-SMA showed a statistically significant relationship with creatinine (p=0.002) and eGFR (p=0.007) at biopsy moment.

Macrophage infiltration assessed by CD68 immunostaining ranged from 0.4 to 11.2 positive cells/field for the interstitial area. It was observed in this study the particular immunoexpression of CD68 both in the peripheral area of inflammatory infiltration and also in the interstitial fibrotic areas (Figure 3, A and B).

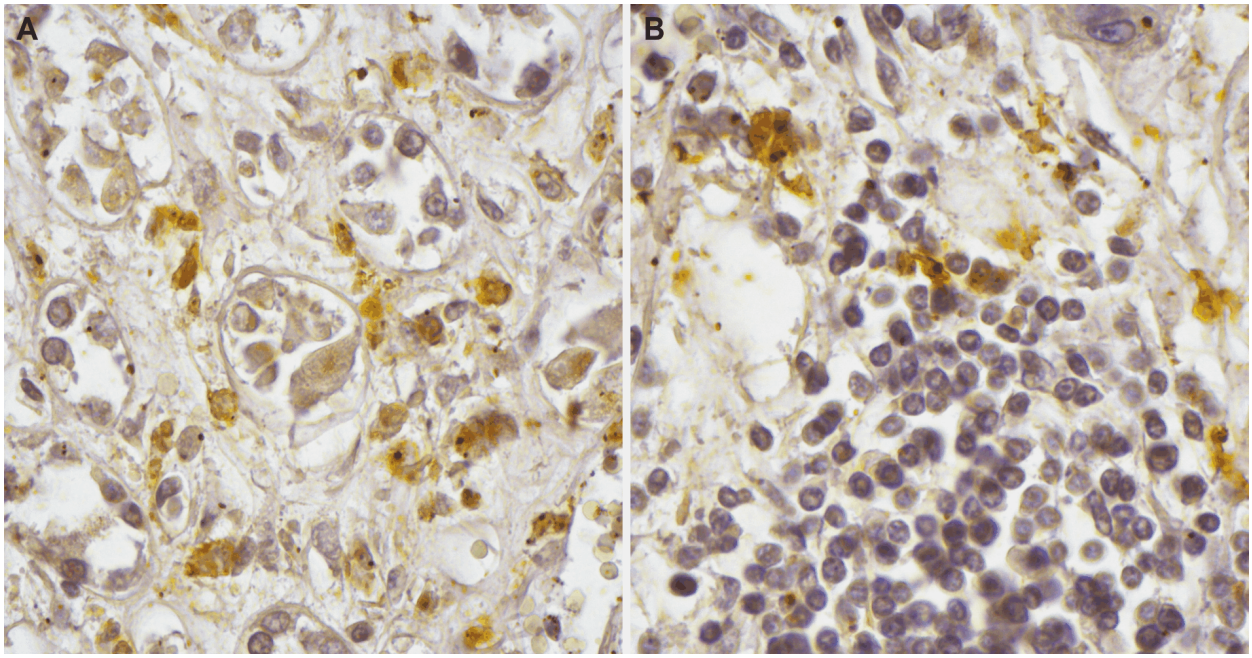

Figure 3 – ***Immunoexpression of macrophage infiltration in interstitial area: (A) Immunoexpression of CD68 in the interstitial area in a case with fibrosis score <3 (CD68 immunostain, ×400); (B) Interstitial inflammatory infiltrate with CD68 expression in the peripheral areas (CD68 immunostain, ×400).***

Discussion

Fibrosis development has been studied extensively, being proposed several mechanisms that trigger this process. In native kidneys, the fibrotic stages have been described as showing important deposition of extracellular matrix, myofibroblasts accumulation, glomerulosclerosis, tubular atrophy and peritubular capillary reduction [14, 15].

The presence of epithelial to mesenchymal transition (EMT) as initial process in fibrosis development has been supported in various studies [16, 17]. Rastaldi MP *et al.* demonstrated in an extensive study on native kidney biopsies the importance of EMT characteristics in interstitial damage and also EMT expression was related with renal function [18].

EMT represents a process regulated by several genes and growth factors as TGF-β1 that is the main regulator factor of tissue healing following the injury induced by various stimuli [19]. In proteinuric glomerulopathies direct damage of tubular cells and tubular ischemia represent usual processes that result in expression of adhesion molecules with proinflammatory characteristics at tubular surface that can influence the initiation of EMT process [20, 21].

The presence of interstitial expression of myofibroblasts has been also demonstrated as a predictor of outcome in renal graft in earlier renal transplantation studies [22, 23].

In the present study, α-SMA expression was not found in epithelial tubular cells, only in the interstitial compartment and in areas with a higher degree of tubulo-interstitial damage. These observations are in agreement with other studies that describe myofibrolasts infiltration as an important process in the early stages of tubular damage and interstitial fibrosis development [24, 25]. This study had not evaluated the origin of interstitial myofibroblasts, whether they originate from resident fibroblasts or from EMT.

In the present study we described inflammation infiltration associated with early stages of interstitial fibrosis, these results are supported by various studies that suggest that any degree of inflammation of any etiology perhaps leads to progressive renal impairment as processes often described in the progression of chronic allograft dysfunction [22, 26]. In this study, isolated mild fibrosis, without inflammation or glomerulosclerosis, was not associated with an increased risk of renal function impairment over the observation time.

Various studies describe that interstitial and glomerular renal diseases are characterized by macrophage accumulation and sustained macrophage infiltration may result in continuous production of various growth factors similar to wound-healing processes [27, 28]. This initial process of wound healing becomes pathological, resulting in irreversible fibrosis, tissue destruction and progressive chronic kidney disease.

In a model of obstructive nephropathy, Nishida M *et al.* showed that angiotensin II receptor type 1-expressing macrophages had a protective effect in development of fibrotic injury [29].

Macrophages demonstrate cell plasticity and have the ability to undergo cell–cell fusion with themselves or other cell types, particularly in response to inflammatory stimuli [30]. In our study, macropahgic infiltration was described both in interstitial areas with inflammatory infiltrates and fibrosis development, this findings being in agreement with previous studies that underline the importance of macrophages implication in renal injury in models of renal disease including glomerulonephrites and interstitial fibrosis in renal allograft [31, 32].

Mature blood monocytes and inflammatory macrophages have been shown to transform into vascular elements including endothelial cells, myofibroblasts, and smooth muscle cells in addition to neuronal and liver cells [33, 34].

In the present study, we confirmed the association between immunoexpression of both myofibroblasts and macrophages markers in the interstitial fibrotic areas.

The present study has some limitations. The small number of patients included in the study, as well as the inclusion of patients in different time points, this study being a retrospective study, it would be interesting to investigate in future studies the presence of α-SMA and CD68 in sequential biopsies and to analyze whether these markers could be used to predict renal function impairment.

Conclusions

This study demonstrated that introduction of myofibroblast and macrophages evaluation is important in describing the interstitial fibrosis process in native kidney biopsies. Even in early stages of chronic kidney dysfunction myofibroblasts are important markers in the tubulo-interstitial damage and renal function evaluation.

Acknowledgments

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