Histological changes and immunohistochemical markers in the assessment of glomerulosclerosis in patients with glomerulonephritis

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Introduction

The final common pathway in the progression of chronic kidney disease towards end stage renal failure seems to be fibrosis [1]. An important role in this process is played by tubulo-interstitial fibrosis, which was assessed in a previous study [2]. The present study tries to assess the role played by glomerulosclerosis using a histological scoring system, immunohistochemical and biological data. As well as interstitial lesions, glomerular lesions can be studied using standard staining – Hematoxylin and Eosin, periodic acid–Schiff (PAS), Gömöri’s trichrome – in patients with chronic primary and secondary glomerulonephritis. In the present study, we used a simple scoring system, similar to that used by some authors in lupus nephritis and ANCA associated vasculitis. Using this scoring system, we assessed elements of activity-inflammatory lesions, and chronicity-fibrotic/sclerotic lesions. In addition, by using immunohistochemical methods, the presence of growth factors, such as transforming growth factor β (TGF-β) or of microfilaments (α-smooth muscle actin – SMA) can be labeled at the level of different resident glomerular cells. Their presence at the level of the glomerulus should reflect aspects of glomerulosclerosis. However, clinical data should not be neglected in the assessment of glomerular diseases.

Considering this background, we focused on the relationship of histological elements of activity and chronicity, with immunohistochemical markers and with clinico-biological data.

Materials and Methods

Patients

Forty-one patients with chronic glomerulonephritis were studied retrospectively (17 females, 24 males; mean age 45.5±12.9 years, range 18–74 years). From the kidney biopsies that were performed in the Nephrology Department, “Victor Babeș” University of Medicine and Pharmacy, Timisoara, only those cases were included that presented enough paraffin wax...
embedded biopsy material to permit the cutting of additional sections for immunohistochemistry. Cases with fewer than five glomeruli were excluded from the study [3].

The patients had either primary (30 cases) or secondary glomerulonephritis (systemic vasculitis, four cases; infectious, three cases; collagenoses, two cases; neoplasia, two cases). The histopathological diagnoses were: mesangial proliferative glomerulonephritis (12 cases), mesangiocapillary glomerulonephritis (one case), membranous nephropathy (five cases), minimal change disease (five cases), focal and segmental glomerulosclerosis (15 cases), crescentic glomerulonephritis (three cases).

All biopsies were performed after obtaining informed consent from patients regarding the procedure and the possible use of the obtained material for scientific purposes. The present study has the approval of the local ethical committee.

Parameters

Clinical, biological and histological parameters at the time of the biopsy were assessed. In all patients, renal function (serum creatinine and glomerular filtration rate (GFR)), blood pressure, proteinuria and hemoglobin were available. GFR was estimated using the MDRD4 formula [4].

Histology

 Routinely fixed and processed sections of kidney were processed for light microscopy and stained with Hematoxylin and Eosin (HE), periodic acid–Schiff (PAS) and Gömöri’s trichrome techniques using routine methods. All stained slides were assessed separately by two pathologists. In order to better quantify the histological lesions, a scoring system adapted by Neumann I et al. (2005) [5] for ANCA-associated vasculitis, based on the standardized scoring system for activity and chronicity developed for lupus nephritis was employed. We extended this scoring system also to other glomerulopathies because biological processes that take place in vasculitis or lupus nephritis are present also in other types of primary or secondary glomerular diseases. In order to assess the level of glomerular injury glomeruli have been divided into eight segments, every segment has been assessed for active lesions (mesangial cells proliferation) and chronic sclerotic lesions (mesangial matrix increase, glomerulosclerosis). The number of affected segments has been used in order to calculate the percentage of affected glomeruli for each of the mentioned changes. Points have been attributed as follows: 1 point for <20% affection, 2 points for 21–40%, 3 points for 41–60%, 4 points for 61–80% and 5 points for >80%. The results were used to obtain the total activity and total chronicity index [5].

Immunohistochemistry

The detection of cytoskeletal proteins (α-smooth muscle actin – SMA, transforming growth factor beta – TGF-β) was performed 4 μm-thick formalin-fixed, paraffin was-embedded sections using a horseradish peroxidase labeled Streptavidin–Biotin (LSAB2-HRP) method (a system intended for use with primary antibodies for the qualitative identification of antigens in paraffin-embedded tissues).

The primary antibodies used were: ready-to-use monoclonal mouse anti-smooth muscle actin (clone 1A4, DAKO), and concentrated monoclonal mouse anti-TGF-β (MCA 797, Serotec).

Sections were firstly deparaffinized and rehydrated by routine protocol, then incubated with 3% hydrogen peroxide in distilled water for 5 minutes and afterwards rinsed with distilled water and placed in Tris-buffered saline (TBS) for 5 minutes. The next step was incubation with primary antibody, diluted 1/75, for 10–30 minutes, followed by sequential 10 minutes incubations with a biotinylated link antibody and peroxidase-labeled Streptavidin (both purchased ready-to-use, DAKO). Labeling was completed by incubation with chromogenic substrate solution 3,3’-diaminobenzidine (DAB) (liquid DAB substrate-chromogen system, prepared according to manufacturer’s instructions, DAKO), for 10 minutes. After this, a counterstain with Hematoxylin Mayer for one minute was performed, followed by differentiation with tap water, then dehydration with successive alcohol (70, 80, 96, 100 degrees each for 3 minutes), Clearing was performed twice with toluene and was followed by mounting using Eukit.

The glomerular labeling of TGF-β and α-smooth muscle actin (SMA), immunoreactivity was graded for statistical evaluation using a semi-quantitative intensity scale from 0 to 3, where 0 – no labeling (negative), 3 – the most intense labeling and 1 and 2 are labeling of an intermediate degree, as used by Alexopoulos E et al. [6].

Statistical analysis

Data were recorded on a file created in Microsoft Excel, organized and managed as a database. Correlations between histological and immunohistochemical parameters were performed using the non-parametric Spearman’s rank order test, while correlations between clinical, biological data and immunohistochemistry were performed using parametric Pearson’s test. Correlation coefficients of linear regression analysis are presented in relation with p-values. The significance of the correlation coefficient (r) is as follows: r=0–0.25 indicates little or no correlation; r=0.25–0.5 indicates a fair degree of relationship; r=0.5–0.75 indicates moderate to good correlation; r=0.75–1 indicates very good to excellent correlation [7]. In order to perform these test, we used WinStat for Microsoft Excel and Epi 3.2.2.

Results

The glomerular lesions were studied on standard stains in light microscopy (HE, PAS and Gömöri’s trichrome) using the scoring system adapted from Neumann I et al. (2005) [5]. As already mentioned in the “Materials and Methods” section, the following glomerular lesions were assessed semi-quantitatively: active/inflammatory lesions (mesangial proliferation) and sclerotic/fibrotic lesions (mesangial matrix increase,
glomerulosclerosis) and the obtained scores were related to the studied clinical parameters. Concerning the immunohistochemical parameters studied, a semi-quantitative evaluation scale was used.

Glomerular TGF-β immunostaining was present at the level of endothelial cells in 31 cases (in three cases the endothelial immunolabeling was only present at the vascular pole of the glomerulus), mesangial cells in five cases and epithelial cells in 10 cases, while in nine cases glomerular expression of TGF-β was totally absent (Figures 1 and 2).

**Figure 1 – TGF-β immunolabeling in mesangial cells. TGF-β LSAB2-DAB stain, ×200.**

**Figure 2 – TGF-β immunolabeling in the endothelium of glomerular capillaries, negative immunolabeling in the sclerosed glomerulus. TGF-β LSAB2-DAB stain, ×100.**

TGF-β expression in glomerular endothelial cells correlated with mesangial matrix increase ($r=0.28$, $p<0.05$), total activity index ($r=0.29$, $p<0.05$) and total chronicity index ($r=0.34$, $p<0.05$). Mesangial TGF-β immunolabeling showed no correlation with the histological parameters studied. Glomerular epithelial cell TGF-β correlates with mesangial proliferation ($r=0.29$, $p<0.05$), mesangial matrix increase ($r=0.4$, $p<0.01$) and total activity index ($r=0.28$, $p<0.05$) (Table 1).

**Table 1 – Correlations found between immunohistochemical scores (TGF-β, SMA – α-smooth muscle actin) and histological scores**

<table>
<thead>
<tr>
<th>Spearman rank order test</th>
<th>Mesangial proliferation</th>
<th>Total Al</th>
<th>Mesangial matrix increase</th>
<th>Glomerulosclerosis</th>
<th>Total Cl</th>
</tr>
</thead>
<tbody>
<tr>
<td>Endothelial cells TGF-β</td>
<td>NS</td>
<td>$r=0.29$</td>
<td>$p&lt;0.05$</td>
<td>$r=0.28$</td>
<td>$p&lt;0.05$</td>
</tr>
<tr>
<td>Mesangial cells TGF-β</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>Epithelial cells TGF-β</td>
<td>$r=0.29$</td>
<td>$p&lt;0.05$</td>
<td>$r=0.40$</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>Endothelial cells SMA</td>
<td>$r=0.96$</td>
<td>$p&lt;0.001$</td>
<td>NS</td>
<td>$r=0.35$</td>
<td>$p&lt;0.005$</td>
</tr>
<tr>
<td>Mesangial cells SMA</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>Epithelial cells SMA</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
</tr>
</tbody>
</table>

NS – not significant.

Concerning biological data, the only statistically significant correlations that could be proven were between glomerular epithelial cells TGF-β expression and serum creatinine ($r=0.26$, $p<0.05$) and an indirect correlation between glomerular endothelial cells TGF-β expression and GFR ($r=-0.3$, $p<0.05$).

Glomerular SMA immunostaining was present in mesangial cells (11 cases), epithelial cells (three cases) and endothelial cells (11 cases). In two of the cases, mesangial expression had a focal character, while in one case SMA was present at the level of extracapillary proliferation.

We observed a strong correlation between endothelial immunolabeling of SMA and the mesangial proliferation score ($r=0.96$, $p<0.005$) and also an indirect correlation with the glomerulosclerosis score ($r=-0.35$, $p<0.05$) and the total chronicity index ($r=-0.39$, $p<0.05$). Mesangial and epithelial cells SMA labeling showed no correlation with histological data.

Concerning biological data, there was a correlation between mesangial SMA expression and serum creatinine ($r=0.60$, $p<0.001$) and an indirect correlation with GFR ($r=0.37$, $p<0.05$). There was also a direct correlation between epithelial SMA immunolabeling and systolic blood pressure ($r=0.44$, $p<0.05$) (Table 2).

No statistically significant correlation could be found between SMA and TGF-β.

The glomerulosclerosis score correlated with serum creatinine ($r=0.4$, $p<0.05$) and GFR ($r=-0.4$, $p<0.05$). No correlation was present between the other histological parameters (mesangial matrix increase, mesangial proliferation) and the clinical data.

The other clinical parameters (proteinuria, blood pressure and hemoglobin) that were performed during this study showed no correlation with the histological scores that were assessed.
TGF-β plays a critical role in the pathogenesis of renal injury by stimulating production of extracellular matrix components, proliferation of mesangial cells, and inducing epithelial-mesenchymal transformation in renal tissue [8].

We observed a positive immunostaining for TGF-β in the resident glomerular cells (mesangial, epithelial and endothelial) of the cases studied by us; however, this staining was not uniformly distributed among the cases.

As already mentioned, TGF-β is involved in glomerulosclerosis primarily by mediating mesangial matrix accumulation: stimulating the synthesis of extracellular matrix components and decreasing collagenase production [8]. It directly stimulates the transcription of many extracellular matrix genes in renal cells including mesangial and endothelial cells. Deposition of extracellular matrix components, including fibronectin and collagen types I, III and IV, is an important component of the scarring observed during the progression of glomerulosclerosis [9, 10]. TGF-β leads to mesangial matrix accumulation not only by inducing synthesis, but also by decreasing matrix degrading enzymes such as plasmin and by stimulating the synthesis of protease inhibitors, such as “plasminogen activator inhibitor” (PAI-1) [11]. TGF-β1 and PAI-1 together constitute a positive feedback loop in the development of renal fibrosis [12].

There are also studies performed on human renal biopsies that indicate the involvement of TGF-β in mesangial matrix accumulation. Yamamoto T et al. showed that normal kidneys, as well as diseases with a decreased mesangial matrix production (minimal change disease) show a decreased expression of TGF-β at the glomerular level, compared to other diseases with increased mesangial matrix deposition (IgA nephropathy, focal segmental glomerulosclerosis, crescentic glomerulonephritis, lupus nephropathy and diabetic nephropathy) [13]. TGF-β can be involved together with Smad 2 in the excessive deposition of extracellular matrix, playing an important role in glomerulosclerosis [14]. Liu J et al. showed that abnormally increased expression of TGF-β could be an important factor associated with glomerulosclerosis through increased amount and abnormal distribution of collagen I, III and IV [15].

In the cases studied by us, we found a correlation between the mesangial matrix increase score and TGF-β immunolabeling at the level of epithelial cells ($r=0.44$, $p<0.05$) and a weaker one at the level of glomerular endothelial cells ($r=0.28$, $p<0.05$).

We found out that glomerular epithelial cell TGF-β immunostaining correlates with the mesangial proliferation score ($r=0.29$, $p<0.05$). This leads us to the role played by TGF-β in mesangial proliferation, a role that is still controversial: Isaka Y et al. observed glomerular hyper-cellularity, while Kitamura M et al. showed inhibition of glomerular proliferation due to TGF-β [16, 17].

But, despite the role played in mesangial matrix accumulation and possibly in mesangial proliferation, we found that mesangial TGF-β immunostaining was present in just five cases, while the epithelial immunostaining was present in 10 cases.

Lee HS and Sony CY have explained the differences that occur in TGF-β immunostaining in the two types of resident cells: mesangial and epithelial. Despite the fact that mesangial cells secrete TGF-β in response to fibrogenic stimuli, mesangial immunostaining for active TGF-β1 in chronic glomerular disease is almost negligible, while podocytes covering the sclerotic glomerular segments exhibit increased TGF-β1 protein expression. TGF-β secreted as latent complexes by mesangial cells is stored in the mesangial matrix, from which soluble forms of latent TGF-β is released and localized to the podocyte surface in chronic glomerular disease [18]. The presence of TGF-β in glomerular epithelial cells has been proven by Kim JH et al. in biopsies of patients with focal segmental glomerulosclerosis. TGF-β was absent in non-sclerosed glomeruli while in sclerosed areas epithelial cells showed an overexpression of this growth factor [19].

Another role attributed to TGF-β is the induction of epithelial-mesenchymal transition. The consequences of epithelial-mesenchymal transition in chronic fibrosis could be on the one hand, the supplementing of new mesenchymal cells, responsible for the matrix accumulation and on the other hand, it could cause loss of epithelial cells, thus, contributing to the parenchyma destruction seen in advanced fibrosis [20].

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The role played by TGF-β in epithelial-mesenchymal transition has been mainly shown related to tubular epithelial cells. In addition to tubular epithelial cells, recent studies indicate that endothelial cells and glomerular podocytes may also undergo transition after injury [21].

Podocytes are recognized to play a key role in the development of kidney fibrosis. Changes in the structure and function of podocytes, such as podocyte-induced epithelial-mesenchymal transition, are involved in the

### Table 2 – Correlations found between histological scores found on standard stains, immunohistochemistry scores and clinical data in the 41 studied patients

<table>
<thead>
<tr>
<th>Pearson correlation</th>
<th>Mesangial proliferation</th>
<th>Mesangial matrix increase</th>
<th>Glomerulosclerosis</th>
<th>Endothelial TGF-β</th>
<th>Mesangial TGF-β</th>
<th>Epithelial TGF-β</th>
<th>SMA</th>
<th>Mesangial SMA</th>
<th>Epithelial SMA</th>
</tr>
</thead>
<tbody>
<tr>
<td>SC</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>$p$</td>
<td>$&lt;0.05$</td>
<td>$&lt;0.05$</td>
<td>$&lt;0.05$</td>
<td>$&lt;0.05$</td>
<td>$&lt;0.05$</td>
<td>$&lt;0.05$</td>
<td>$&lt;0.05$</td>
<td>$=0.3$</td>
<td>$=0.3$</td>
</tr>
<tr>
<td>GFR</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>$r$</td>
<td>$=0.24$</td>
<td>$=0.29$</td>
<td>$&lt;0.05$</td>
<td>$&lt;0.05$</td>
<td>$&lt;0.05$</td>
<td>$&lt;0.05$</td>
<td>$&lt;0.05$</td>
<td>$=0.37$</td>
<td>$=0.37$</td>
</tr>
</tbody>
</table>

SC – serum creatinine, GFR – estimated glomerular filtration rate, NS – not significant.
development and progression of kidney disease [22]. Li Y et al. showed using cell cultures, that also podocytes may undergo epithelial-to-mesenchymal transition after injury [23].

One of the markers of epithelium undergoing epithelial-mesenchymal transition is de novo expression of SMA. In three of the cases studied by us, we found SMA immunostaining at the level of the glomerular epithelial cells, indicating a possible mesenchymal transformation of these cells.

As mentioned above, not only epithelial cells can undergo the process of transformation into mesenchymal cells. Increasing evidence suggests that endothelial cells may undergo endothelial-myofibroblast transition under physiological and pathophysiological circumstances. In our study, we found a strong correlation between endothelial SMA expression and mesangial proliferation score, as well as an indirect correlation with the glomerulosclerosis score. Studies performed by Zeisberg EM et al. and Li J et al. on different types of animal models showed that endothelial mesenchymal transition contributes to the accumulation of activated fibroblasts and myofibroblasts in kidney fibrosis [24, 25].

The endothelial TGF-β immunostaining was present in 31 cases; in 17 out of them, it was not associated with mesangial or epithelial immunolabeling. Moreover, in three cases TGF-β was only present at the level of the vascular pole of the glomerulus.

An important role in the pathogenesis of renal injury is played by the glomerular endothelium. Loss of GFR and proteinuria can result from glomerular endothelial cell injury [26]. Lee JK et al. showed on an experimental model of subtotal nephrectomy the fact that endothelial cells express angiotensinogen and TGF-β at the level of dilated capillaries from the vascular pole at a moment when sclerosis was not present yet. After a while, TGF-β extended on the other glomerular endothelial cells and on mesangial cells, being more intense in the areas adjacent to the glomerulosclerosis zone. Thus, it seems that due to hemodynamic changes the endothelium is involved in TGF-β production, the result being extra-cellular matrix production with sclerosis lesions [27]. Wolf G et al. showed the proliferation of rat endothelial cell cultures and TGF-β expression at this level using leptin [28].

But, the histological and immunohistochemical data showed also some correlations with some of the clinical data studied. The glomerulosclerosis score correlated with renal function at the moment of the biopsy, fact that could indicate that not only tubulo-interstitial lesions correlate with the severity of the disease as it was mentioned in different studies [29–31].

Renal function at the time of the biopsy showed a correlation with epithelial TGF-β (whose role as determinant of the severity of the disease has already been mentioned) and also with mesangial SMA expression. These results are similar to those reported by Utsunomiya Y et al., who showed in a group of patients with IgA nephropathy that mesangial SMA expression was higher in patients that showed an evolution towards renal failure, compared with the group with stable renal function [32]. El-Koraie AF et al. showed also that glomerular SMA expression was significantly higher in patients with glomerular affection compared to the control group [33].

Conclusions

We conclude that TGF-β has a key role in determining glomerulosclerosis especially through mesangial matrix increase, but possibly also through mesangial cell proliferation. Another role of this growth factor is related to transdifferentiation, not only epithelial-mesenchymal, but also endothelial-mesenchymal. The immunohistochemical data obtained have to be related to histological data and also to clinical data in order to assess glomerular lesions.

References


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