Transforming growth factor-\(\beta\), endothelin-1, and \(c\)-\(fos\) expression in necrotizing/crescentic IgA glomerulonephritis

Maria Pia Rastaldi, Stefania Tunesi, Franco Ferrario, Antonio Indaco, Hequn Zou, Pietro Napodano and Giuseppe D’Amico

Renal Immunopathology Center, Division of Nephrology, San Carlo Borromeo Hospital, Milano, Italy

Abstract

Background. Among our cases of IgA glomerulonephritis (IgAGN), 10% show necrotizing/extracapillary lesions involving a small percentage of glomeruli and associated with a certain degree of inflammation in absence of glomerular and interstitial scarring. In our experience, also in repeat biopsies, these cases of IgAGN have a worse prognosis probably because necrotizing/extracapillary lesions can repeat and accumulate, leading to the progression of damage. As it is well known that transforming growth factor-\(\beta\) (TGF-\(\beta\)) and endothelin-1 (ET-1) are key-factors in the progression of glomerulonephritis, aim of the study was to examine their expression in renal biopsies of primary IgAGN with necrotizing/crescentic lesions in complete absence of interstitial fibrosis. To obtain information about the mitogenic effect of ET-1, the expression of \(c\)-\(fos\), whose upregulation by ET-1 has been established in culture, was also studied.

Methods. Eighteen renal biopsies of patients with necrotizing/crescentic IgAGN were examined by immunohistochemistry with antibodies against TGF-\(\beta\), ET-1 and \(c\)-\(fos\). The results were compared with those obtained on 22 cases of IgAGN characterized only by pure mesangial proliferation and 25 IgAGN biopsies with advanced, not active, glomerulointerstitial lesions.

Results. In necrotizing/crescentic IgAGN glomerular TGF-\(\beta\) appeared more positive than in cases characterized only by pure mesangial proliferation and was especially expressed on cellular crescents. In the interstitium, TGF-\(\beta\), ET-1 and \(c\)-\(fos\) were expressed by infiltrating leukocytes, tubules, and small vessels. This positivity, although similar as localization, was less diffuse than in biopsies with advanced interstitial damage, but significantly greater than in cases with pure mesangial proliferation.

Conclusions. Positivity of TGF-\(\beta\) on cellular crescents is similar to that observed from other authors in different types of necrotizing/crescentic human glomerulonephritis and supports our hypothesis that this is a peculiar type of IgAGN. Moreover, interstitial expression of TGF-\(\beta\), ET-1 and \(c\)-\(fos\) in biopsies with glomerular active lesions but complete absence of interstitial fibrosis may potentially represent a signal of activation of mechanisms that induce and amplify the damage leading to further progression of the disease.

Key words: \(c\)-\(fos\); ET-1; immunohistochemistry; necrotizing/crescentic IgAGN; TGF-\(\beta\)

Introduction

Among the possible mechanisms involved in the process of glomerular and interstitial scarring, TGF-\(\beta\) is now recognized as crucial, especially because of its ability to promote synthesis and deposition of extracellular matrix [1–4]. Moreover, recent culture studies have shown that TGF-\(\beta\), among its various and complex properties, can also stimulate the synthesis of endothelin-1 (ET-1) [5], a multifunctional peptide potentially implicated in the progression of renal damage by its vasoconstrictive, chemotactic, and mitogenic effects [6–8]. The latter is mainly mediated by activation of phospholipase C and increase in \(c\)-\(fos\) proto-oncogene expression, as demonstrated on renal cultured cells [9,10].

Primary IgA glomerulonephritis (IgAGN, Berger’s disease) is a very common chronic renal disease [11], which rate of progression to the end-stage kidney is about 30% of cases [12]. By light-microscopy the most common features are mesangial expansion and proliferation, but a great variety of lesions, both glomerular and interstitial, can be observed ranging from pictures of only mild mesangial proliferation to cases with marked glomerular and interstitial damage [12,13]. Whether morphological differences are expression of the time of biopsy intervention or of different types of disease is difficult to establish, also because the disease has generally an indolent course, making it difficult to understand its precise onset [13]. Among the possible pictures, we can also detect, in about 10% of cases of
primary IgAGN, the presence of necrotizing/extracapillary lesions involving generally a small percentage of glomeruli (20–40%) and associated with a certain grade of inflammation in absence of glomerular and interstitial scarring [14]. Many data, also in repeat biopsies, strongly suggest that this form has a worse prognosis, probably because necrotizing/extracapillary lesions can repeat and accumulate, leading to the progression of the disease [15–17].

The aim of our work was to study the expression of TGF-β, ET-1 and c-fos in renal biopsies of primary IgAGN with focal necrotizing/crescentic glomerular damage. To better understand the possible role of these peculiar lesions on the progression of the disease, we compared this group of biopsies to cases characterized only by pure mesangial proliferation and to cases with marked glomerular and interstitial involvement, but absence of active necrotizing/crescentic lesions.

Subjects and methods

Patients

Sixty-five patients (41 males and 24 females), with a mean age of 35.8 ± 14.8 years (ranging from 18 to 80), with immunohistological evidence of IgAGN, were studied. Secondary IgAGNs (i.e. Henoch–Schönlein nephritis, hepatic diseases, coeliac disease, etc.) were excluded on the basis of clinical and laboratory data. The patients, selected on the basis of morphological features, were divided in three groups: group 1 (22 patients) was characterized by the presence of pure mesangial proliferation, without tubulointerstitial lesions. Group 2 (18 patients) showed glomerular necrotizing lesions with cellular extracapillary proliferation. Biopsies of group 3 (25 patients) showed a more advanced glomerular and interstitial damage, in the absence of active necrotizing/crescentic lesions.

Kidney tissue

Kidney tissue was obtained from all patients and, for comparison, from 10 cadaver kidneys which could not be grafted because of vascular abnormalities. Tissue samples for light-microscopy were fixed in Bouin’s fluid, embedded in paraffin and stained according to standard techniques.

For immunofluorescence and immunoperoxidase staining the unfixed renal tissue was embedded in OCT compound (Miles Scientific, Naperville, IL, USA), snap-frozen in a mixture of isopentane and dry ice and stored at −80°C. Subsequently, 5-μm sections were placed on slides and stored at −20°C until immunostained.

Immunoperoxidase labelling

We used an avidin–biotin technique, in which a biotinylated antibody reacts with several peroxidase-conjugated streptavidin molecules. Briefly, after incubation with 0.5% avidin (Sigma Chimica, Gallarate, Milan, Italy) and 0.01% biotin (Sigma), to suppress endogenous avidin-binding activity, tissue sections were incubated with the primary antibody.

We used the following monoclonal antibodies: CD45 (monoclonal mouse anti-human leukocyte common antigen) (Immunotech, Marseille, France), TGF-β (monoclonal mouse anti-human TGF-β1, 2, 3) (Genzyme, Cambridge, MA, USA), ET-1 (monoclonal mouse anti-human endothelin-1) (Serotec, Kidlington-Oxford, UK), c-fos (monoclonal mouse anti-human c-fos gene product) (Medac, Turin, Italy).

After washing, the sections were sequentially fixed in a methanol-H2O2 solution (to block endogenous peroxidase), incubated with the secondary biotinylated antibody (Dako S.p.A., Milan, Italy) and with the peroxidase-labelled streptavidin (Dako). Peroxidase activity was detected with 3,3-diaminobenzidine (DAB) (Dako), then sections were counterstained with Harry’s haematoxylin (BDH, Poole, England), dehydrated and mounted in Entellan (Merck, Darmstadt, Germany).

Specificity of labelling was demonstrated by the lack of staining after substitution of phosphate-buffered saline (PBS) for the primary antibody.

Quantitative evaluation

All peroxidase-stained sections had five or more glomeruli and were evaluated blind by two independent observers without any histological or clinical information. Minor differences were subsequently resolved by conference.

Interstitial infiltrating cells were counted in 10 consecutive high-power fields (400 x), avoiding glomeruli and large vessels; the results were expressed as ‘number of positive cells per square millimeter’. Intraglomerular and interstitial staining of TGF-β, ET-1, and c-fos were scored semi-quantitatively, on a 4-point scale: no staining, 0; mild and focal staining, 1; moderate and more diffuse staining, 2; intense and diffuse staining, 3.

Statistical analysis

Results were expressed as mean ± standard deviation. Comparisons were made by χ2 test for categorical variables and by t test for quantitative variables.

Results

Clinicohistological features

Group 1. By definition group 1 renal biopsies showed only mesangial proliferation (+/+/+), without extracapillary lesions, glomerular sclerosis or interstitial fibrosis. Interstitial CD45+ cells were 97.4 ± 42.7/mm2, not differing statistically from normal kidneys (72.1 ± 53.8 cells/mm2, P = 0.15). Mean pre-biopsy follow-up was 64.8 ± 78.2 months. At time of biopsy, mean serum creatinine (s.Creat.) was 88.4 ± 8.8 μmol/l and mean proteinuria was 0.6 ± 0.7 g/day.

Group 2. Necrotizing/extracapillary lesions, as previously stated, characterized biopsies of group 2, involving 19.9 ± 6.9% of glomeruli. Mesangial proliferation did not differ from biopsies of Group 1 (+/+ +) and global glomerular sclerosis was found in few glomeruli (9.7 ± 7.5%). Interstitial fibrosis was absent. Interstitial (CD45+) leukocytes were 183.6 ± 106.1/mm2, with a statistical difference from normals (P = 0.02) and group 1 (P = 0.05). Mean pre-biopsy follow-up was 74.7 ± 107.5 months (P = 0.7 vs group 1). The patients,
at time of renal biopsy, showed a s. Creat. of 150.3 ± 194.5 μmol/l (P=0.1 vs group 1) and a proteinuria of 1.8 ± 2.2 g/day (P=0.01 vs group 1).

**Group 3.** These biopsies were grouped because of the presence of marked glomerular and interstitial lesions. Global glomerular sclerosis was found in 37.9 ± 17.9% of glomeruli, whereas remnant glomeruli showed a mesangial proliferation similar to that found in groups 1 and 2 (+/+ + ). Fibrous crescents were detected in 10.8 ± 15.0% of glomeruli. Interstitial fibrotic and inflammatory lesions were intense: interstitial leukocytes were 348.4 ± 191.7 (CD45 + cells/mm²) (P=0.01 vs normal and group 1, P=0.05 vs group 2). Mean prebiotic follow-up was 71.7 ± 82 months, without a statistical difference from the other groups (P ≥ 0.8 vs group 2 and group 1). Group 3 patients, at time of renal biopsy, had a serum creatinine of 229.8 ± 194.5 μmol/l (P=0.01 vs group 1, P=0.08 vs group 2) and a proteinuria of 2.4 ± 2.1 (P=0.001 vs group 1, P=0.1 vs group 2).

**Immunohistochemical features.** Immunohistochemical results are shown in Table 1.

**Normal kidneys and group 1 biopsies.** The 10 control biopsies and group 1 biopsies showed similar results: TGF-β was positive in rare glomerular cells (Figure 1a) and negative in the interstitium (Figure 2a). ET-1 and c-fos were only observed on rare small vessels (Figure 3a, 4a), whereas glomeruli, tubules, and interstitial cells were negative.

**Group 2.** In necrotizing crescentic IgA GN we observed an intense glomerular positivity of TGF-β (group 2 vs group 1; P=0.004), especially localized on the cellular crescents and expressed by all cell types (Figure 1b,c). Glomerular ET-1 and c-fos were scanty or not expressed (group 2 vs group 1; P=0.2).

In the interstitium TGF-β was positive in about 20% of interstitial infiltrating leukocytes (40.2 ± 10.6 + cells/mm²) (Figure 2b,c) and on some proximal and distal tubules (group 2 vs group 1; P=0.04). ET-1 and c-fos were present on rare tubules (group 2 vs group 1; P>0.05), some small vessels (group 2 vs group 1; P≤0.05) and interstitial cells (ET-1 + cells/mm² = 38.2 ± 26.4; c-fos + cells/mm² = 27.6 ± 11.8) (Figure 3b, 3c, 4b, 4c).

**Group 3.** While global sclerotic glomeruli were negative for every markers studied, the remnant glomeruli in group 3 biopsies showed intense and diffuse positivity for TGF-β (group 3 vs group 1; P=0.001, group 3 vs group 2; P=0.8, Figure 1d), whereas ET-1 and c-fos expression was mild (group 3 vs group 2 and group 1; P>0.05). Interstitial TGF-β was very intense, involving most of interstitial leukocytes (Figure 2d) 297.9 ± 198.6 cells/mm² (group 3 vs group 2; P<0.001) and many proximal and distal tubules (group 3 vs group 2; P=0.02). ET-1 and c-fos were expressed on interstitial leukocytes (ET-1 + cells/mm² = 174.8 ± 96.2; c-fos + cells/mm² = 243.8 ± 125.5; group 3 vs group 2; P<0.01), tubules (group 3 vs group 2; P<0.001) and on many small-sized vessels (group 3 vs group 2; P≤0.01) (Figures 3d, 4d).

**Discussion.**

Our data show a good association between morphological and immunohistochemical features. Biopsies of group 1, with pure mesangial nephropathy, showed in fact a glomerular and interstitial negativity or a very mild positivity for TGF-β, ET-1 and c-fos. On the contrary, group 3 biopsies were characterized not only by marked and diffuse glomerular and interstitial positivity for TGF-β, but also by intense interstitial expression of ET-1 and c-fos. Although a paper has shown a downregulation of TGF-β in IgA nephropathy [18], our results seem to suggest the involvement of these factors in the worsening of IgA GN, in agreement with other recent studies [19–23]. Indeed, TGF-β is a growth factor now widely recognized as crucial in the process of renal scarring, especially by its property of regulating cell turnover and matrix metabolism. In particular, it increases the deposition of extracellular matrix, as shown by the marked intensity of TGF-β expression in group 3 biopsies. Moreover, the expression of ET-1, a proinflammatory cytokine involved in the regulation of cell proliferation and matrix synthesis, was also significantly increased in group 3 biopsies, consistent with the high inflammatory activity observed in this group. These results further support the hypothesis that TGF-β and ET-1 play a pivotal role in the pathogenesis of IgA GN, and suggest the development of targeted therapies to control their expression and prevent renal scarring.

**Table 1.** Glomerular, tubular, and vascular expression of TGF-β, ET-1 and c-fos. Results are expressed as mean values (+ SD) of the scores.

<table>
<thead>
<tr>
<th></th>
<th>Glom TGF-β</th>
<th>Glom ET-1</th>
<th>Glom c-fos</th>
<th>Tub TGF-β</th>
<th>Tub ET-1</th>
<th>Tub c-fos</th>
<th>Vasc ET-1</th>
<th>Vasc c-fos</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal</td>
<td>0.5 ± 0.1</td>
<td>0.04 ± 0.01</td>
<td>0.02 ± 0.001</td>
<td>0.02 ± 0.02</td>
<td>0.03 ± 0.01</td>
<td>0.01 ± 0.004</td>
<td>0.6 ± 0.2</td>
<td>0.7 ± 0.1</td>
</tr>
<tr>
<td>Group 1</td>
<td>0.5 ± 0.2</td>
<td>0.03 ± 0.001</td>
<td>0.02 ± 0.01</td>
<td>0.03 ± 0.02</td>
<td>0.02 ± 0.02</td>
<td>0.01 ± 0.005</td>
<td>0.65 ± 0.7</td>
<td>0.69 ± 0.1</td>
</tr>
<tr>
<td>Group 2</td>
<td>1.8 ± 0.9</td>
<td>0.4 ± 0.1</td>
<td>0.5 ± 0.2</td>
<td>0.8 ± 0.3</td>
<td>0.5 ± 0.3</td>
<td>0.5 ± 0.2</td>
<td>1.3 ± 0.2</td>
<td>1.8 ± 0.6</td>
</tr>
<tr>
<td>Group 3</td>
<td>1.9 ± 1.1</td>
<td>1.0 ± 0.3</td>
<td>0.8 ± 0.3</td>
<td>2.0 ± 0.9</td>
<td>1.8 ± 0.8</td>
<td>2.2 ± 1.0</td>
<td>2.8 ± 0.4</td>
<td>2.6 ± 1.1</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>P values</th>
<th>Glom TGF-β</th>
<th>Glom ET-1</th>
<th>Glom c-fos</th>
<th>Tub TGF-β</th>
<th>Tub ET-1</th>
<th>Tub c-fos</th>
<th>Vasc ET-1</th>
<th>Vasc c-fos</th>
</tr>
</thead>
<tbody>
<tr>
<td>Group 2 vs Normal</td>
<td>0.004</td>
<td>0.2</td>
<td>0.2</td>
<td>0.04</td>
<td>0.1</td>
<td>0.08</td>
<td>0.05</td>
<td>0.008</td>
</tr>
<tr>
<td>Group 2 vs group 3</td>
<td>0.8</td>
<td>0.1</td>
<td>0.3</td>
<td>0.02 &lt;0.001</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
<td>0.008</td>
<td>0.01</td>
</tr>
<tr>
<td>Group 3 vs Normal + group 1</td>
<td>0.001</td>
<td>0.08</td>
<td>0.1</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
<td>0.002</td>
<td>0.004</td>
</tr>
</tbody>
</table>
Fig. 1 a–d. TGF-β: glomerular staining. (a) Scattered glomerular cells are positive in a biopsy of group 1. (b,c) Two small cellular crescents are labelled in glomeruli of group 2, and (d) a diffuse positivity characterizes a remnant glomerulus of a group 3 biopsy (immunoperoxidase ×200).

Fig. 2 a–d. Interstitial TGF-β positivity. (a) A group 1 biopsy shows complete absence of interstitial staining. (b,c) Some interstitial infiltrating leukocytes are positive in group 2 biopsies. (d) The great majority of cells are stained in a large leukocyte cluster of a group 3 biopsy (immunoperoxidase ×100, ×200).
Fig. 3 a–d. Interstitial ET-1 expression. (a) Small vessel positivity is evident in a group 1 specimen. Some (b) tubules and (c) interstitial cells are stained in a group 2 biopsy. (d) Intense interstitial positivity involving small vessels, tubules, and infiltrating leukocytes is present in a biopsy of group 3 (immunoperoxidase ×100, ×200).

Fig. 4 a–d. c-fos expression. (a) Only a small vessel is positive in a group 1 biopsy. Some small vessels, tubules and interstitial leukocytes are positively stained in two biopsies of group 2. (b,c) Two glomeruli are also evident with very mild/negative staining. (d) Intense interstitial positivity involving tubules, small vessels, and interstitial leukocytes, is shown by a group 3 biopsy (immunoperoxidase ×100, ×200).
matrix either stimulating new matrix synthesis or inhibiting matrix-degrading enzymes [1–4].

Although culture studies have demonstrated that TGF-β, added to mesangial cells in culture, can markedly increase their production of ET-1 and their proliferating activity [5,9], we observed, in comparison with the diffuse staining for TGF-β, only a mild expression of glomerular ET-1 in remnant glomeruli of group 3 biopsies. Considering also that TGF-β is not the unique factor involved in ET-1 regulation, this feature was consistent with the morphological finding of a moderate mesangial proliferation and with the mild glomerular immunostaining for c-fos shown by these cases.

In fact, among its various and potentially damaging properties, ET-1 is also able to induce mitogenesis [9], effect that appear mediated by activation of phospholipase C and enhanced expression of some proto-oncogenes, mainly c-fos [10]. We actually observed in all biopsies examined a strict correlation between the immunohistochemical positivity of ET-1 and that of c-fos, both at glomerular and at interstitial levels. Given the complexity of the in vivo situation, we do not want to assert that all the c-fos expression recognizes ET-1 as unique stimulus, but our results seem to support in human IgAGN the data obtained in culture about at least one of the pathways through which ET-1 exerts its mitogenic effect.

About 10% of cases of IgAGN show a picture of focal and segmental necrotizing/crescentic glomerulonephritis. We have recently described in 26 biopsies with these lesions the presence of peculiar immunohistochemical features, such as the expression of intraglomerular VCAM-1 (vascular cell adhesion molecule-1) and an intense intraglomerular, periglomerular and interstitial leukocyte infiltration [24]. By immunofluorescence, in addition to the obvious presence of IgA, we found fibrinogen to be strongly positive in segmental areas of glomerular necrosis, the same areas found also positive for VCAM-1. Both morphological and immunohistochemical features were similar to those found for example in renal vasculitis [25] or in necrotizing forms of Henoch–Schönlein nephritis [15], suggesting that also in Berger’s disease there is a form of capillaritis associated to the mesangial lesions [14,15].

The glomerular staining for TGF-β in these biopsies (group 2) was as intense as that found in remnant glomeruli of group 3. However, where in group 3 the positivity was diffuse, in group 2 TGF-β was especially positive on cellular crescents. The role of TGF-β in these lesions is not easy to explain. In fact, it is known that complex processes, not completely understood in vivo, are involved in TGF-β regulation and activation [26]. Moreover, TGF-β has both proliferative and antiproliferative properties and multiple immunomodulating effects [1], and obviously is not the unique factor involved in necrotizing crescentic/lesions. It is worth stressing in our opinion that the same type of glomerular positivity for TGF-β was found in a recent study on other types of necrotizing/crescentic human glomerulonephritis [27], supporting our hypothesis that necrotizing/crescentic IgAGN is comparable not only morphologically, but possibly also pathogenetically, to other necrotizing/crescentic glomerular diseases.

Interestingly, an increased interstitial expression of TGF-β, ET-1 and c-fos was shown by group 2 biopsies. All the factors stained positively about 20% of interstitial inflammatory cells and several tubules and ET-1 and c-fos were also expressed by some small vessels. Since it is well known that tubulointerstitial damage plays a key-role in the progression of glomerulonephritis [28], the tubulointerstitial positivity of TGF-β, ET-1, and c-fos in these biopsies, in which interstitial fibrosis is totally absent, could be a signal of activation of potential mechanisms of worsening. It is worth stressing that patients selected for this study had a comparable pre-biopsy follow-up, suggesting that morphological and immunohistochemical differences are not a consequence of early or late biopsy time, but probably are related to different courses of the disease.

Taken together, our data seem to suggest that necrotizing/crescentic IgAGN is a particular type of Berger’s disease, in which TGF-β and ET-1 may play an important role. Moreover, ET-1 has not only mitogenic, chemotactic, and vasoconstrictive properties, but it can also promote the expression of transcripts for several growth factors [29,30], and among them, for instance, TGF-β [9], therefore inducing a vicious cycle that might amplify the damage.

References


Received for publication: 7.7.97
Accepted in revised form: 5.3.98