Kidney Expression of RhoA, TGF-β1, and Fibronectin in Human IgA Nephropathy

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Key Words
RhoA • Transforming growth factor-beta-1 • Fibronectin • IgA nephropathy • Immunohistochemistry

Abstract
Background: The Rho/transforming growth factor-β (TGF-β) system plays a crucial role in the progression of renal damage due to stimulation of extracellular matrix molecule deposition. In fact, the in vitro TGF-β-mediated production of fibronectin, one of the major TGF-β-regulated extracellular components, has recently been correlated with Rho protein signalling molecules. Although a close relationship between increased renal tissue levels of TGF-β1 and fibronectin has been reported in IgA nephropathy, no data are available on renal tissue expression of Rho proteins. Methods: This study was designed to assess in IgA nephropathy patients the kidney tissue immunohistochemical expression of RhoA, TGF-β1, and fibronectin, and the rate of immunoreactivity for each antigen by image analysis. Results: An increase in RhoA, TGF-β1, and fibronectin expression was detected in tubulointerstitium and in glomeruli of IgA nephropathy compared to normal kidneys; in particular, RhoA was found also in proximal tubules, unlike control kidneys and mainly at the cell boundary level, which is keeping with its activated form. The image analysis confirmed that the kidney tissue levels of RhoA, TGF-β1, and fibronectin were significantly enhanced in the patients. Conclusion: This study suggests that RhoA may represent a key molecule in the signalling transduction pathway of profibrotic signals in IgA nephropathy.

Introduction
IgA nephropathy is the most common primary glomerulonephritis, with about one third of patients progressing to end-stage renal disease after 20 years [1, 2]. Although the pathogenesis of IgA nephropathy is not yet fully understood and its treatment not well defined [3–6], evidence exists that progression of renal failure is significantly related to the extent of glomerular and tubulointerstitial lesions [7–9]. In recent years new insights have been reported on the mechanisms involved in the process of renal scarring, focusing on the role of cytokines and growth factors in the mediation of glomerular injury [10, 11]. Among this group of molecules, transforming growth factor-β (TGF-β) family plays a crucial role in the pathogenesis of renal fibrosis thanks to its prosclerotic activity exerted by inducing cell proliferation and extracellular ...

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matrix (ECM) molecule deposition; in fact, TGF-β and respective receptors are abnormally expressed in experimental and human nephropathies [9, 12, 13]. TGF-β1 has been found to be a TGF-β isoform greatly expressed during the onset and progression of various renal diseases including IgA nephropathy [14–17]; furthermore a close relationship between increased renal tissue levels of TGF-β1 and fibronectin, one of the major TGF-β-regulated extracellular components, has been reported in IgA nephropathy [15].

Cellular action of TGF-β1, as well as of other mitogens involved in the progression of renal damage, can occur through the Ras superfamily of small GTP binding proteins. A link between Ras proteins and progressive human renal disease has been suggested by recent studies concerning the treatment of progressive IgA nephropathies by using HMG-CoA reductase inhibitors [18]. In fact these drugs, apart from their hypocholesterolemic properties, are likely to exert many of their therapeutic effects on progression of renal fibrosis through an inhibitory action on the prenylation/activation of small G proteins [19]. Among the Ras molecules the Rho family may be involved in controlling the activated renal TGF-β1 receptor signals as recently demonstrated in rat mesangial cells [20] or mouse tubulointerstitial fibrosis [21] using specific Rho-protein or Rho-effector inhibitors, respectively. Furthermore, we have recently demonstrated that RhoA, a Rho small G protein, may be involved in renal fibrosclerosis progression in an experimental model of progressive chronic renal failure [22].

On the basis of these considerations and of lacking data about RhoA in human renal progressive diseases, we aimed to evaluate the expression of RhoA, TGF-β1 and fibronectin in human IgA nephropathy biopsies.

Patients and Methods

Patients

Fifteen patients (10 males and 5 females, aged 15–54 years) who had been given a histological diagnosis of IgA nephropathy were considered for the study. Patients previously treated with immunosuppressive and/or steroid therapy were excluded, as well as those with severe or rapidly progressing renal failure. Nephrotic syndrome, diabetes and moderate to severe hypertension were also considered as exclusion criteria. At the time of biopsy no patients was on corticosteroid or immunosuppressant treatment whereas 10 out of 15 patients were on anti-hypertensive, anti-proteinuric treatment with renin-angiotensin system inhibitor drug. In particular, 6 patients were treated with angiotensin-converting enzyme inhibitors and 4 patients with angiotensin 1-receptor blockers.

The diagnosis of IgA nephropathy had been made on the basis of immunofluorescence by anti-IgA immunostaining and light microscopy findings. Morphologically, the patients presented different grades of mesangial expansion/proliferation and/or sclerosis and a variable extent of tubulointerstitial lesions. In particular, with regard to the tubulointerstitial changes, mononuclear infiltrates (lymphocytes and monocytes) (fig. 1), peritubular fibrosis and tubular atrophy/dilatation with flattened epithelium were observed. According to Lee [24], the severity of histological lesions was classified into five (I–V) grades: 5 patients were classified as grade I, 5 patients grade III, 3 patients grade IV and 2 patients were classified as grade V. Some biochemical and clinical data of the studied patients were as follows: creatinine clearance 81 ± 29.25 ml/min 1.73 m²; proteinuria 0.9 ± 0.78 g/24 h; systolic and diastolic blood pressure 130 ± 10.59 and 80 ± 5.95 mmHg, respectively. In all the patients, percutaneous renal biopsy was performed with a 14-gauge disposable needle using a free-hand ultrasound-guided method [23]. Informed consent and the approval of the local ethics committee, according to the terms of the Helsinki declaration, were obtained.

Normal kidney tissue specimens were obtained from 10 patients who had undergone surgical nephrectomy because of renal carcinoma, and were used as controls. These subjects were comparable for age and sex with those affected by IgA nephropathy; none of these patients was affected by proteinuric renal disease, moderate to severe arterial hypertension or diabetes.

Laboratory Procedures

Tissue Sample Preparation

The kidney tissue specimens obtained by means of kidney biopsy, were placed in a 0.9% NaCl solution immediately after withdrawal, snap-frozen by immersion in liquid nitrogen, and stored at −70°C; a number of cryostatic sections had been used for the immunohistochemical diagnosis of IgA nephropathy. Afterwards, the residual renal samples were immediately fixed in cold 10% neutral buffered formalin for 12 h at 4°C. Samples were dehydrated through ethanol, treated with xylene and finally embedded in paraffin at 56°C. 8-μm-thick sections had been processed for either routine histological staining (hematoxylin-eosin, Schiff’s periodic acid, Masson trichrome staining) to diagnose IgA nephropathy or were used in the present immunohistochemical studies.

Immunostaining

The sections were incubated with 3% hydrogen peroxide (H2O2) in distilled water for 5 min and then exposed to a 10-min treatment with proteinase K diluted in phosphate-buffered saline (PBS) (0.01–0.5 mg/ml; Boehringer-Mannheim, Mannheim, Germany) by testing different concentrations to obtain an optimal proteinase K time/concentration in order to avoid tissue disruption. The block of the aspecific antigenic sites was obtained by incubating samples with normal swine serum (1:20; Sigma Chemicals, St. Louis, Mo., USA) for 20 min at 37°C. The slides were then incubated overnight at 4°C with the primary antibody (1:100–1:1,000 in 0.1% bovine serum albumin – PBS). Polyclonal rabbit anti-TGF-β1 (Santa Cruz Biotechnology, Santa Cruz, Calif., USA, cod. no.sc-146), polyclonal rabbit anti-fibronectin (Sigma, cod. no. F3648) and polyclonal rabbit anti-RhoA (Santa Cruz Biotechnology, Santa Cruz, Calif., USA, cod. no. sc-179) immunoglobulins were used. The immunoprecipitates were detected by using an indirect streptavidin-peroxidase method (Dakopatts, Glostrup, Denmark) as previously reported.

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Fig. 1. Hematoxilin and eosin staining in IgA nephropathy renal tissue sample. The peritubular interstitial tissue shows inflammatory infiltrates of mononuclear cells (a) rich in monocytes (b, arrowheads).

Fig. 2. RhoA kidney immunostaining. The grey and red staining represents RhoA and proximal tubule-megalin immunoreaction, respectively (a, d). a-c Normal human kidney. RhoA is detected in distal tubules and collecting ducts (a original magnification ×400); at higher magnification (b, c original magnification ×1,000) the anti-RhoA immunoprecipitates are observed at the level of the cytoplasm of Bowman's capsule parietal (b, arrow) and tubular (c) cells. d-f IgA nephropathy kidney. An increased amount of RhoA is found to be widespread in tubules (d original magnification ×400); at higher magnification (e, f original magnification ×1,000) the anti-RhoA immunoprecipitates are observed mainly along the plasma membrane of Bowman's capsule parietal cells (e, arrowhead) and tubule cells (f, arrowheads).

Fig. 3. TGF-β1 kidney immunostaining. The grey and red staining represents RhoA and proximal tubule-megalin immunoreaction, respectively. Normal human kidneys (a) are immunostained mainly at the level of the distal tubule and collecting duct epithelial cells; in IgA nephropathy samples (b) a stronger TGF-β1 immunostaining is observed in the proximal tubular epithelial cells. Original magnification ×400.
Briefly, the sections were sequentially exposed to biotinylated immunoglobulins, a peroxidase-labelled streptavidin complex, and to 3,3′-diaminobenzidine tetra-hydrochloride (DAB, 1 mg/ml Tris Buffer) containing 0.02% H₂O₂. This single-immunostaining technique was employed to carry out the image analysis while the double-immunostaining was carried out to identify the renal segments that were immunoreactive for RhoA or TGF-β1. For this purpose, the large membrane-associated protein megalin has been taken into consideration, being widely expressed along the brush border of human renal proximal tubular cells [26]. Briefly, after the development of the immunoreaction for RhoA or TGF-β1 obtained with nickel-enhanced DAB, the sections were treated with the polyclonal rabbit anti-megalin-GST [27] and sequentially exposed to biotinylated immunoglobulins, extravidin-peroxidase reagent and finally 3-amino-9-ethyl-carbazole (Sigma). Finally, the sections were permanently mounted with DPX mountant (Fluka, Buchs, Switzerland).

Negative controls were obtained by substituting the primary antibodies with a non-immune rabbit serum or with PBS + 0.1% bovine serum albumin, or by using primary antibodies pre-absorbed with the original peptide (for RhoA or TGF-β1, Santa Cruz) in a 50- to 100-fold antigen excess. The incubation of the slides with DAB alone or with streptavidin-horseradish peroxidase (HRP) complex alone and DAB, respectively tested the endogenous peroxidases and the avidin-binding activity. All reactions were done in humidified chambers and at room temperature unless otherwise specified.

Image Analysis
The reacting surfaces of the sections were quantified with a ‘Quantimet +’ image analysis system. Twenty microscope fields representative of the observed staining pattern were chosen from each biopsy specimen and captured at ×200 magnification. After storage, the medium grey threshold (i.e. the level above which the pictures were considered to be reacting) was evaluated on negative controls. Image analysis was performed taking into account the percentage of the reacting surface and the respective level of grey intensity per field. The reactivity degree, final rate of immunoreactivity of each sample, was calculated as mean of the products between the % of positive surface and the medium grey level per field, by analyzing 20 fields.

The analyzed biopsies contained more than five glomeruli and corresponding interstitial regions.

Statistical Analysis
The results were expressed as mean ± SD. Comparisons between groups were made using the Mann-Whitney U test. Relationships among the studied parameters were evaluated by Pearson’s correlation test. p < 0.05 was accepted to denote statistical significance.

Results
RhoA
Normal kidneys reacted towards anti-RhoA immunoglobulins at the level of distal tubules and collecting ducts (fig. 2a) while glomeruli showed immunoreactive products restricted to Bowman’s capsule parietal cells (fig. 2b). With respect to the normal samples, the IgA nephropathy biopsies showed an enhanced diffused RhoA immunoreaction especially marked at the level of proximal tubules (fig. 2d) and glomeruli, which appeared entirely reactive mainly at the Bowman’s capsule parietal cell level (fig. 2e). The image analysis confirmed the increased RhoA expression in IgA nephropathy (reactivity degree: 1.578.32 ± 478.23 in IgA nephropathies vs. 385.23 ± 89.19 in normal kidneys; p < 0.0001; fig. 5a). High magnifications were employed to evaluate the RhoA subcellular localization that corresponds to its metabolic state: in fact, cytosol RhoA represents the inactive (GDP-bound)-form whilst membrane-linked RhoA corresponds to the active (GTP-bound)-form [28-30]. In this view, normal kidneys showed a prevalent cytoplasmic, finely granular anti-RhoA immunoreaction distributed in the epithelial cells of parietal Bowman’s capsule and distal tubules (fig. 2b, c) consistent with the presence of predominantly inactive RhoA in normal conditions. Different subcellular localization patterns were observed in pathological samples: RhoA could be detected in cytoplasm but also and mainly at the plasma membrane level of Bowman’s capsule parietal and tubule cells (fig. 2e, f) as an activated form.

An inverse relationship was found between creatinine clearance and RhoA reactivity degree (z = -0.51; p < 0.05).

TGF-β1
In normal kidney tissue, TGF-β1 was detected in the cytoplasm of epithelial cells prevalently in distal tubules and collecting ducts (fig. 3a). The distribution of TGF-β1 was similar in IgA nephropathy samples but larger amounts of immunoprecipitate were found in the cytoplasm of proximal tubular cells of pathological kidneys (fig. 3b) compared to normal ones. In fact, TGF-β1 reactivity degree, evaluated by image analysis, was significantly higher in the IgA nephropathy (1,224.92 ± 203.37) than in normal kidney samples (904.96 ± 90.19; p < 0.0001) (fig. 5b).

Fibronectin
In normal kidney tissue a large number of specific immunoprecipitates were detected in the glomeruli at the level of the glomerular capillary loops and vascular pole, and in the peritubular interstitial tissue (fig. 4a). IgA nephropathy specimens displayed a stronger immunoreaction signal (fig. 4b) compared to normal specimens although the distribution pattern was approximately the
same. The image analysis showed a significant increase in fibronectin reactivity degree in pathological (1.278.58 ± 373.82) compared to normal kidneys (811.01 ± 189.60; p = 0.0003) (fig. 5c).

**Discussion**

In the recent years a large number of mitogens, including platelet-derived growth factor, epidermal growth factor, TGF-α, and TGF-β, have been reported to be involved in proliferation of renal epithelial and fibroblast cells, and in induction and progression of fibrosis through the deposition of extracellular matrix molecules [12, 13, 31].

The intracellular signals activated in kidney by these mitogens are mediated, in many cases, through the Ras superfamily of small monomeric GTPases [32], suggesting a role for Ras molecules in the pathogenesis of renal fibrosis. Therefore, targeting of Ras proteins is an emerging attractive strategy in a range of proliferative renal diseases to overcome the limits of treatments directed towards single cytokines [33]. In this view, recent experimental and clinical data suggest that inhibitors of HMG-CoA reductase, such as lovastatin and fluvastatin, may
display protective effects in experimental renal diseases [20, 34] and in patients with IgA nephropathy [18] probably by inhibiting Ras proteins. In fact, although the exact mechanism involved in the pharmacological effect of statins at the cellular and molecular level is not known, it appears that they decrease lipiddation and thus activity of Ras proteins, because this proteins must be bound to membrane, through lipid molecules, to become functional. Ras inhibition could lead to suppression of TGF-β1 expression and increase in ECM-degrading enzymes, reducing accumulation of ECM proteins [20]. Among more than 50 Ras-related small GTPases, Rho proteins are recently known to play an important role in the development of tissue fibrosis in progressive renal disease. Rho proteins include at least 20 members (e.g. RhoA, RhoB, RhoC, Cdc42) [35] and participate in various cellular functions including cell cycle progression, exocytosis, endocytosis, and actin cytoskeletal reorganization that results in changes in morphology, motility, adhesion [29, 36, 37], and malignant transformation [38]. Rho family members are controlled by a great number of regulating proteins; with regard to kidney, recent experimental data have demonstrated that mice lacking Rho GD1α, a regulator of Rho proteins, were affected by kidney morphological and functional abnormalities: glomerular sclerosis and interstitial infiltration of inflammatory cells are the renal morphological consequence of RhoA hyperactivation [39], indicating a key role of Rho GD1α in the development as well as maintenance of normal renal tissue morphology. Concerning Rho effectors, investigations on ROCK, a specific target which activated-Rho binds to, demonstrated that the Rho-ROCK system may play an important role in the progression of renal tissue fibrosis and represents a new therapeutic target for preventing interstitial fibrosis in progressive renal diseases [21].

In accordance with the evidence for the involvement of the Rho system in renal fibrosis progression, we have recently found that RhoA, a specific form of Rho, is overexpressed in kidney tissue of rats affected by an experimental model of progressive chronic renal failure [22]. However, up to now, no evidence exists about the involvement and/or expression of RhoA in IgA nephropathy. Therefore, on the basis of these considerations, the aim of the present investigation was to immunolocalize and quantify RhoA and two molecules involved in renal fibrosis such as TGF-β1 and fibronectin in kidney tissue from IgA nephropathy patients.

Increased RhoA tissue levels were observed in IgA renal specimens compared with normal ones: in particular the expression of RhoA was enhanced in all the tubular segments (proximal, distal and collecting tubules) as well as in glomeruli of IgA nephropathy patients. The strict correlation between Rho protein function and their cellular localization prompted us to investigate also the subcellular localization of RhoA. In fact Rho proteins cycle between the GTP-bound active form and the GDP-bound inactive form [28, 29] localizing in the plasma-membrane or cytosol fraction, respectively [30] as further confirmed in our laboratories: the RhoA sublocalization carried out by microscopic analysis really correlates to its metabolic form [40]. Accordingly, our previous studies on renal cancer cells demonstrated a RhoA subcellular translocation under different stimuli [41]. The IgA nephropathy samples expressed large amount of cell-boundary RhoA positive staining in the tubular and capsule glomerular cells: this finding suggests that, at least in the examined IgA nephropathy tissue samples, a lot of RhoA is activated at the level of tubule cells and this activated signalling molecule may take a role in the pathogenesis and progression of IgA nephropathy as recently demonstrated for experimental kidney pathologies and/or abnormalities [39]. This could be in keeping with the inverse relationship we found between residual renal function and RhoA tissue expression. This was the only statistical correlation we observed, though the small number of patients prevents us from drawing definite conclusions.

The finding that RhoA is expressed, at higher levels and mainly in its active form, in all the renal tubule segments compared with control kidneys that showed RhoA in its inactive form only in distal tubular cells, suggests that the proximal tubules deserve to be taken into consideration within the histological alterations in IgA nephropathy. Furthermore, we found that proximal tubules express higher levels of TGF-β1 and fibronectin and are surrounded by inflammatory interstitial infiltrates rich in monocytes. Altogether, these observations give evidence for a role of proximal tubules in the activation of the fibrogenic events leading to the kidney impairment of IgA nephropathy in agreement with literature. In fact, in vitro evidence reported in the last years demonstrates the effective contribution of proximal tubular cells to the pathogenesis of renal fibrosis, directly by the alterations in the production of components of extracellular matrix molecules, and indirectly by the production of pro-fibrotic cytokines such as TGF-β1 [42, 43] that mediates collagen synthesis in proximal tubule cells [42]. These proximal tubule-mediated fibrogenic processes might be controlled, at least in part, by monocytes since they can directly in-
interact with proximal tubule cells and stimulate their TGF-β1 transcription and protein synthesis as recently demonstrated in vitro [44].

Thus, the high levels of activated RhoA, TGF-β1 and fibronectin found in proximal renal tubules might be induced by the presence of monocytes that are able to activate these epithelial cells by direct cell contact. In spite of this, it can’t be ruled out the possibility that the other renal tubular segments, distal and collecting tubules that are found to express active RhoA, are involved in the cellular pathogenetic dynamics of renal fibrosis in IgA nephropathy. Further studies are needed to better define the correlation among RhoA, TGF-β1 and fibronectin expressed by the proximal renal tubules in the different stages of IgA nephropathy.

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References


