Marian Danilewicz, Małgorzata Wągrowska-Danilewicz

Immunohistochemical Analysis of Transforming Growth Factor Beta–1 in AA and AL Renal Amyloidosis

Department of Nephropathology, Medical University of Łódź

It has been found that the prognosis of both AA and AL renal amyloidosis is significantly worse in cases in which the renal cortical interstitium exhibits fibrosis at the time of the biopsy than in those in which it is normal. However, the fibrogenic mechanisms operating locally in the kidney are not well understood. Transforming growth factor beta (TGF-β) has been recognized as a key mediator of renal fibrogenesis. Therefore, the present study on AA and AL renal amyloidosis was undertaken to ascertain if potential pathway towards renal tubulointerstitial fibrosis involves TGF-β and to examine the possible relationship between the immunoexpression of TGF-β and interstitial α-smooth muscle actin (α-SMA) expression as well as interstitial infiltrates. The mean values of the immunoexpression of TGF-β–1, interstitial CD3+ cells, α-SMA expression as well as interstitial area were in AA and AL groups significantly increased in comparison with controls. The mean values of the interstitial CD68+ cells were in both AA and AL groups increased in comparison with controls however in AL amyloidosis this difference was not significant. Moreover, all investigated parameters were significantly increased in AA group as compared to AL cases. In both AA and AL groups there were significant positive correlations between immunostaining of TGFβ-1 and α-SMA as well as immunostaining of TGF-β-1 and interstitial volume. In the AA group, a significant negative correlation existed between immunostaining of TGF-β–1 and CD 3+ cells. In the AL group, this correlation tended to be negative, however it did not reach statistical significance. In both AA and AL groups we did not find significant relationship between TGF-β–1 and interstitial monocytes/macrophages. In conclusion, our study suggests a role of transforming growth factor β–1 in interstitial fibrotic changes in renal AA and AL amyloidosis and we hypothesize that myofibroblast pathway may be important in this process.

Introduction

Amyloid is a substance that appears to be homogenous and amorphous under the light microscope and the spectrum of renal symptoms in amyloidosis is variable, including isolated proteinuria, nephritic syndrome, hypertension as well as renal insufficiency [23]. Amyloid deposition occurs in a wide variety of conditions. Reactive systemic amyloidosis (AA) occurs when there is an imbalance in the production and degradation of acute-phase inflammatory proteins, such as serum amyloid, and deposition of insoluble fibrils in body organs. Secondary amyloidosis is seen almost exclusively in three groups of conditions: chronic inflammatory diseases, chronic infections and heredofamilial disorders, such as familial Mediterranean fever [13]. Primary amyloidosis with deposition of AL fibrils (amyloid light-chain) may be associated with almost any dyscrasia of the B lymphocyte lineage, ranging from frank malignancy of plasma cells (multiple myeloma) to "benign" monoclonal gammopathy, in which the only demonstrable abnormality may be the overproduction of monoclonal light chains [25]. Almost all patients with amyloidosis have renal disease and most of those who die because of renal failure, areAL type [21].

Renal interstitial fibrosis is the final common pathway leading to end-stage disease in various nephropathies including renal amyloidosis [27], however the fibrogenic mechanisms operating locally in the kidney are not well understood at the present time [6]. Transforming growth factor beta (TGF-β) has been recognized as a key mediator of renal fibrogenesis [9]. The amount of extracellular matrix in the interstitium reflects the balance between its production and degradation by proteases. TGF-β contributes to fibrogenesis by acting through both pathways. It directly enhances the synthesis of all major matrix proteins, such fibronectin, proteoglycans, and collagens. On the other hand, TGF-β inhibits

matrix degradation by enhancing the production of plasminogen activator inhibitors and enhancing the activity of tissue inhibitors of metalloproteinases [2]. It is noteworthy that myofibroblasts, T lymphocytes and monocytes/macrophages which play a crucial role in renal injury, also seem to be related to TGF-β, however these relationships, up to date, are not clearly elucidated [9, 12, 18, 20, 24]. Although the recent studies have focused on transforming growth factor beta independent pathways as mechanisms of tubulointerstitial fibrosis, TGF-β is invariably believed to be a critical fibrogenic factor [15].

Therefore, the present study on AA and AL renal amyloidosis was undertaken to ascertain if potential pathway towards renal tubulointerstitial fibrosis involves TGF-β and to examine the possible relationship between immunoexpression of TGF-β and interstitial α-smooth muscle actin (α-SMA) expression as well as interstitial infiltrates.

Material and Methods

Patients

On the basis of immunohistochemical studies two groups were distinguished: the first composed of patients with AA amyloidosis (18 cases, 12 females and 6 males aged from 25 to 63 years, mean age: 50,3) and the second, comprising patients with AL amyloidosis (7 females and 4 males aged from 22 to 55 years, mean age: 48,7).

The mean duration of the renal manifestations prior to biopsy was 4.5 month in AA-type amyloidosis group and 6 month in AL-type group. In all cases data concerning light microscopy, immunofluorescence and full clinical data were also available. As a control, 10 biopsy specimens of the kidneys removed because of trauma were used (the male to female ratio was 7:3, the mean age was 38.1±7.2). None of the persons from whom the control renal tissue originated were known to have had previous or actual renal disease. Before the semiquantitative and quantitative studies were carried out, all control specimens were histologically examined by a nephropathologist and found to be normal renal tissue.

Light microscopy

Tissue specimens were embedded in paraffin, sections cut precisely at 4 μm, and stained with hematoxylin and eosin, periodic acid-Shiff (PAS)-alcian blue, trichrome light green (Masson), silver impregnation (Jones) and crystal violet. In addition, Congo red staining was performed and examined by ordinary and polarized light microscopy. Thickness of each section was controlled according to the method described by Weibel [29].

Immunofluorescence microscopy

Tissue was snap frozen, sectioned at 5 μm and fixed in 95% alcohol for 10 min. Sections incubated with FITC-conjugated antisera (DakoCytomation, Denmark) against human IgG, IgA, IgM and complement (C3) were viewed on BX 41 Olympus microscope using proper filters.

Immunohistochemistry

Paraffin sections were mounted onto superfrost slides, deparaffinized, then (for TGF-β–1, α-SMA and CD68 only) treated in a microwave oven in a solution of citrate buffer, pH 6.0 for 20 min and transferred to distilled water. Endogenous peroxidase activity was blocked by 3% hydrogen peroxide in distilled water for 5 min, and then sections were rinsed with Tris-buffered saline (TBS, DakoCytomation, Denmark) and incubated with: monoclonal mouse anti-human amyloid A component (DakoCytomation, Denmark, dilution 1:75), mouse anti-human Lambda light chains (DakoCytomation, Denmark, dilution 1:100), mouse anti-human Kappa light chains (DakoCytomation, Denmark, dilution 1:100), polyclonal goat-anti-human TGF-β–1 antibody (Santa Cruz Lab., dilution 1:200), α-SMA (clone P1b5, DakoCytomation, Denmark, dilution 1:50), monoclonal mouse anti-human CD3 T cell antibody (Clone PC3/188A, DakoCytomation, Denmark, dilution 1:50) and monoclonal mouse anti-human CD68 antibody (DakoCytomation, Denmark, dilution 1:100). Afterwards LSAB+/HRP Universal kit (DakoCytomation, Denmark) prepared according to the instruction of the manufacturer was used. Visualisation was performed by incubating the sections in a solution of 0.5 mg/ml 3, 3'-diaminobenzidine (DakoCytomation, Denmark), in Tris-HCl buffer, pH 7.6, containing 0.02% hydrogen peroxide, for 10 min. After washing, the sections were counter-stained with hematoxylin and coverslipped. For each antibody and for each sample a negative control was processed in parallel by incubation in the absence of the primary antibody and always yielded negative results. In each specimen staining intensity of TGF-β–1 in renal tubules was recorded semiquantitatively by two independent observers in 10 adjacent high power fields and graded from 0 (staining not detectable), 1 (minimal immunostaining in some cells), 2 (weak immunostaining intensity in all cells) and 3 (strong staining in all cells). The mean grade was calculated by averaging grades assigned by the two observers and rounding up to the arithmetical mean to the nearest unity.

Morphometry

Morphometry was performed by means of image analysis system consisting of a PC computer equipped with a Pentagram graphic tablet, Indeo Fast card (frame grabber, true-color, real-time), produced by Indeo (Taiwan), and color TV camera Panasonic (Japan) coupled with Carl Zeiss micro-

scope (Germany). This system was programmed (MultiScan 8.08 software, produced by Computer Scanning Systems, Poland) to calculate the number of objects (semiautomatic function) and the surface area of a structure using stereological net (with regulated number of points).

The color microscopic images were saved serially in the memory of the computer, and then quantitative examinations were carried out. Interstitial myofibroblasts were identified by their morphology and positive immunostaining with anti-α-SMA. The expression of α-SMA was measured as a surface fraction using point counting method according to Weibel [29] (point spacing 16 μm, total number of the points of net 169, total area 36864 μm^2). Under the net described above 8–10 randomly selected adjacent fields of the renal cortex were investigated. Glomeruli and large blood vessels were neglected. As most of the α-SMA immunostaining was within cytoplasmic processes, these structures were included in calculation. The α-SMA-positive staining was expressed as the percentage of points overlying α-SMA-positive areas. The same method was used to estimate interstitial volume in sections stained with Masson trichrome: it was expressed as the percentage of points overlying renal cortical interstitium.

Interstitial T lymphocytes and monocytes/macrophages were determined by counting CD3+ as well as CD68+cells (semiautomatic function) in a sequence of ten consecutive computer images of 400 x high power fields – 0.0047 mm^2 each. The only adjustments of the field were made to avoid glomeruli and large vessels. The results were expressed as the mean number of CD3 and CD68 immunopositive cells per mm^2.

Statistical methods

All values were expressed as the mean ± SD (standard deviation). The differences between groups were tested using Student t-test for independent samples preceded by evaluation of normality and homogenity of variances with Levene's test. Additionally the Mann-Whitney U test was used where appropriate. Correlation coefficients were calculated using Spearman's method. Results were considered statistically significant at $P<0.05$.

Results

In renal biopsy specimens obtained from patients with AA and AL amyloidosis, TGF-β-1 was detected in the renal tubular epithelial cells (Fig. 1, Fig. 2). In some sections, weak immunoexpression of TGF-β-1 was detected in isolated cells in the interstitial inflammatory infiltrate. These cells were excluded from quantitative analysis. In the controls, only slight focal expression of TGF-β-1 in tubular epithelial cells was seen (Fig. 3). In both AA and AL groups as well as in the controls TGF-β-1 expression was absent from glomerular areas.

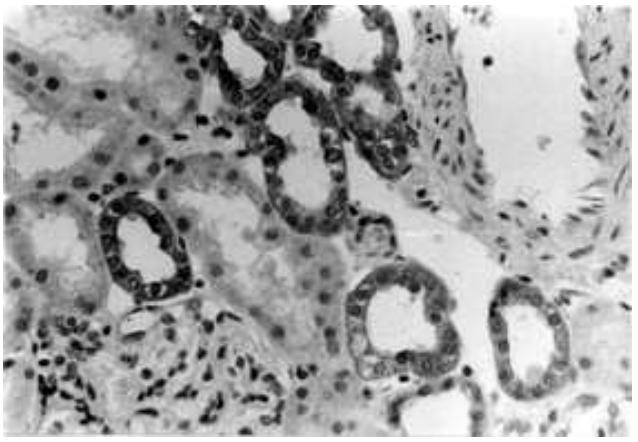

Fig. 1. AA renal amyloidosis. Strong focal immunoexpression of TGF-β-1 in tubular epithelial cells. Magn.× 400.

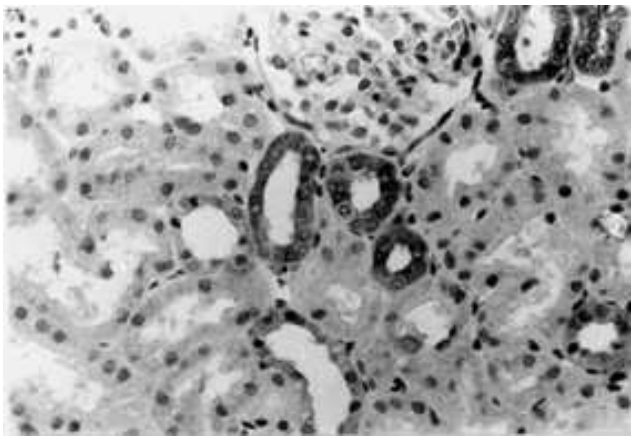

Fig. 2. AL renal amyloidosis. Moderate immunoexpression of TGF-β-1 in tubular epithelial cells. Magn. × 400.

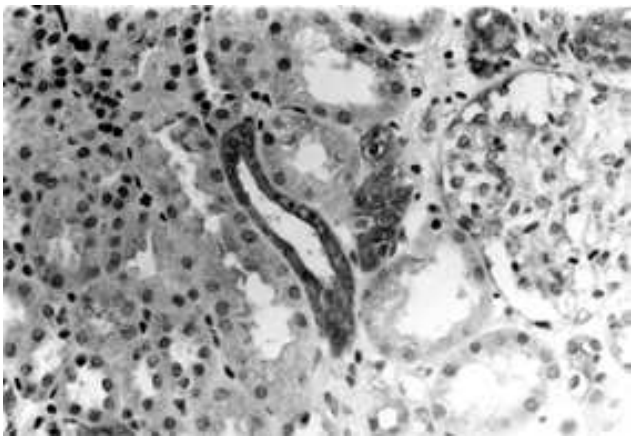

Fig. 3. Control case. Only slight focal expression of TGF-β-1 in tubular epithelial cells was seen. Magn. × 400.

The semiquantitative data concerning the immunoexpression of TGF-β-1 in renal tubules and morphometric data

TABLE 1
Clinical and laboratory findings at the time of biopsy in cases with AA and AL renal amyloidosis

Number of cases	Micro-hematuria	Proteinuria <1g/24h	1–2 g/24h	2–3,5g/24h	Nephrotic syndrome	Renal function impairment[1)]	Hypertension (>90/160)
AA (n=18)	4	1	-	6	11	5	10
AL (n=11)	2	-	1	4	6	3	2

[1)] Serum creatinine > 1.5 mg/dl

TABLE 2
Tubular immunoexpression of TGF-β-1, α-SMA and analysis of interstitial volume, CD3+ and CD68+cells in AA and AL amyloidosis as well as in controls

Number of cases	TGF-β-1 (mean score)	α-SMA (%)	Interstitial volume (%)	CD3+ (cells / 1mm^2)	CD68+ (cells / 1mm^2)
Controls (n=10)	0.21±0.31	0.52±0.26	10.08±1.25	54.66±19.72	33.96±17.91
AA (n=18) AL (n=11)	1.73±0.9 0.98±0.74	8.23±3.25 5.27±2.85	22.6±4.43 17.15±3.18	182.46±79.12 122.26±39.18	65.22±23.06 45.36±15.87
P value	<0.001 [1)] <0.007[2)] <0.03[3)]	<0.001 [1)] <0.001[2)] <0.02[3)]	<0.001[1)] <0.001 [2)] <0.002[3)]	<0.001 [1)] <0.001[2)] <0.03[3)]	<0.001[1)] =0.3 (NS) [2)] <0.02[3)]

1) between AA and controls, 2) between AL and controls, 3) between AA and AL, NS – not significant

TABLE 3
The correlations between tubular immunoexpression of TGF-β-1 and interstitial parameters in AA and AL amyloidosis

Correlation between:	AA (n=18)	AL (n=11)
interstitial expression of TGF-β-1 and α-SMA	r=0.58, p<0.02	r=0.74, p<0.01
interstitial expression of TGF-β-1 and interstitial volume	r=0.69, p<0.002	r=0.62, p<0.04
interstitial expression of TGF-β-1and CD3+ cells	r=-0.51, p<0.04	r=-0.44, p=0.17 (NS)
interstitial expression of TGF-β-1+ and CD68+ cells	r=0.28, p=0.26 (NS)	r=0.35 p=0.29 (NS)

on the interstitial CD3+ cells, CD68+ cells, α-SMA and interstitial area are presented in Table 2. The mean values of the immunoexpression of TGF-β-1, number of interstitial CD3+ cells, α-SMA expression as well as interstitial area were significantly increased in AA and AL groups in comparison with controls. The mean values of the interstitial CD68+ cells were increased in both AA and AL groups in comparison with controls however in AL amyloidosis this difference was not significant. Moreover, all the investigated parameters were significantly increased in AA group as compared to AL cases. The correlations between the tubular immunoexpression of TGF-β-1 and immunostaining of α-SMA, interstitial volume as well as the number of interstitial CD3+ cells and CD68+ cells are shown in Table 3. In both AA and AL groups, there were significant positive correlations between TGF-β-1 immunostaining and α-SMA as well as interstitial volume. In AA group, a significant negative correlation existed between TGF-β-1 immunostaining and the number of CD 3+ cells. In the AL group this correlation tended to be negative, however it did not reach statistical significance. In both AA and AL groups we did not find significant relationship between TGF-β-1 and interstitial monocytes/macrophages. In the controls, all these correlations were not significant.

Discussion

The prognosis of both AA and AL renal amyloidosis is significantly worse in cases in which the renal cortical interstitium exhibits fibrosis at the time of the biopsy than in those in which it is normal [3, 21]. These findings suggest that in renal amyloidosis interstitial fibrosis plays a crucial role in the pathogenesis of chronic renal failure. The role of TGF-β-1 as a major profibrotic cytokine in various glomerulopathies [12] chronic allograft nephropathy [1, 4, 5, 28] and in acute renal

transplant rejections [7] has been well established. The finding that TGF-β-1 is involved in the pathogenesis of dialysis-related (end product-modified beta2-microglobulin) amyloidosis is also well-known [16, 17]. To our knowledge, however no data have documented TGF-β-1-dependent pathway as a mechanism of tubulointerstitial fibrosis in AA and AL renal amyloidosis. TGF-β consists of three isoforms (β1, β2 and β3) and is produced by monocytes and resident cells. TGF-β binds three specific cell-surface receptors, and all resident cells express at least one of the receptors and respond to TGF-β. The most important isoform in humans is TGF-β-1 [12].

In the present study, the immunoexpression of TGF-β-1 and interstitial volumes were significantly increased in both AA and AL renal amyloidosis groups in comparison with controls. Moreover, in AA amyloidosis these parameters were significantly increased as compared with the AL group. It must be noted, however, that a major finding in this study was the demonstration that in both AA and AL groups there were significant positive correlations between the immunoexpression of TGF-β-1 and interstitial volume suggests that TGF-β-1 is actively involved in the pathogenesis of renal scarring in these nephropathies. Similarly, the study of Palomar et al. [22] on 53 early renal transplant biopsies showed that TGF-β-1 is expressed in early stages and seems to be important for the development of interstitial allograft fibrosis, whereas Ishimura et al. [14] showed that strong TGF-β-1 expression in grafts observed 100 days after transplantation tends to be associated with increased fibrosis at 3 years. Our results support earlier observations that this pathway is probably common to various renal diseases as the severity of TGF-β-1 immunoexpression is related rather to the degree of renal damage than to the type of renal injury [12].

We also revealed that in both AA and AL groups interstitial immunoexpression of α-SMA was significantly increased as compared with the controls and that this immunoexpression was significantly greater in AA than in AL patients. Interestingly, in both types of amyloidosis investigated, tubulointerstitial expression of TGF-β-1 significantly positively correlated with α-SMA. This result confirmed earlier observation of Goumenos et al. [12] who found strong positive correlation between the expression of TGF-β-1 in the tubulointerstitial area and interstitial α-SMA+ cells in selected primary and secondary glomerulopathies. The process of scar tissue remodeling includes tissue retraction. This retractile feature is the result of the action of retractile microfilaments which can be found in myofibroblasts [20]. The hallmark of the myofibroblastic phenotype is the expression of α-SMA. The origin of interstitial myofibroblasts remains a subject of speculation as they are thought to derive from either fibroblasts, pericytes or vascular smooth muscle cells [11, 12]. Alternatively, they could be derived from tubular cells through a process of transdifferentiation [8, 20, 26]. Our present finding indicates that in both types of renal amyloidosis, similarly to various primary and secondary glomerulopathies [12], TGF-β-1 produced in tubular epithelial cells probably can transform quiescent renal fibroblasts into activated myofibroblasts and/or induce epithelial-mesenchymal transformation [10, 18]. As TGF-β-1 may also stimulate these cells to the synthesis of various components of the extracellular matrix, this pathway leading to the interstitial fibrosis in AA and AL renal amyloidosis should be taken into consideration.

Although in AA amyloidosis interstitial CD3+ cells were significantly more numerous than those in AL cases, in both investigated types of renal amyloidosis negative correlations existed between TGF-β-1 immunostaining and CD 3+ cells. It is noteworthy, however, that probably due to smaller number of cases in the AL group, only in AA patients this correlation was statistically significant. This is in concordance with findings that TGF-β inhibits T-cell proliferation, and this biological effect may be of relevance in limiting the acute inflammatory response [20]. However, TGF-β has probably variable effects on the immune system both inhibiting cellular proliferation and promoting T-cell memory and cytotoxic function [5]. Therefore, the relationship between TGF-β and T lymphocytes in renal amyloidosis is up to now not fully elucidated.

We also revealed in AA patients significant increase in interstitial monocytes/macrophages as compared with AL patients. On the other hand, we did not find any significant correlation between TGF-β-1 and interstitial monocytes/macrophages. Macrophages produce numerous profibrotic factors, which increase matrix synthesis by resident parenchymal cells. In mice in the absence of TGF-β signalling, macrophages may be ineffectual in promoting fibrosis [9, 19]. In human renal amyloidosis however, this relationship is less evident.

In conclusion, our study suggests a role of transforming growth factor β-1 in interstitial fibrotic changes in renal AA and AL amyloidosis and we hypothesize that myofibroblast pathway may be important in this process.

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Address for correspondence and reprint requests to:
Marian Danilewicz M.D, Ph.D.
ul. Zamenhofa 5 m. 4
90–431 Łódź
E-mail: hobo@csk.umed.lodz.pl