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A quantitative immunohistochemical study of the expression of mesangial α-smooth muscle actin and the proliferation marker Ki-67 in glomerulonephritis in man

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Abstract Using a specific marker 1A4 (DAKO), a quantitative evaluation of α-smooth muscle actin (ASMA) in glomeruli has been performed on human renal biopsies from patients suffering from acute, postinfectious, endocapillary glomerulonephritis (GN; 9 biopsies), IgA nephropathy (11 biopsies) and membranoproliferative GN (11 biopsies) and appropriate controls expressing a very weak ASMA reactivity. A significantly increased expression was found in all categories of GN. The glomeruli from IgA nephropathy showed variation of ASMA expression (range 0.1–27.7%) and a pattern of ASMA staining that was mesangial, global and diffuse. This pattern was also seen in cases of IgA nephropathy with focal, segmental, proliferative GN. In all biopsies, the glomerular cell number and proliferation index was determined. All the categories of GN showed significantly increased glomerular cellularity and proliferation index. Among the three types of GN, the glomerular cellularity and proliferation was lowest in IgA nephropathy. The mean number of Ki-67-positive intraglomerular nuclei and the proliferation index were both significantly correlated with the mean number of glomerular cell nuclei. Morphometric estimates demonstrate increased ASMA expression in types of GN with different prognosis. This finding and the lack of correlation with proliferation markers together indicate that the role of ASMA in GN is complex. This method of ASMA estimation may be useful in further studies of its role in disease activity and prognosis.

Key words Mesangial actin · Glomerulonephritis · Proliferation marker Ki-67 · Quantitative immunohistochemistry

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Introduction

The glomerular mesangium, which was originally considered to provide support for the glomerular capillaries [36], has subsequently been shown to have several other important functions in health and disease. Mesangial cells situated in the spongioform structure of basement-membrane-like material have phagocytic properties. They may help to clear the capillary wall and to process macromolecules deposited during filtration and also those deposited in glomerular disease [8, 9, 22]. More recently it has been demonstrated that mesangial cells can synthesize and release growth factors, interleukins and other cytokines, properties that are important for the inflammatory response, regulation of extracellular matrix metabolism, and mesenchymal cell proliferation. With the demonstration of contractile proteins in mesangial cells [2, 3, 28], their resemblance to smooth muscle cells has been emphasized. Data from experimental glomerulonephritis (GN) [20] have indicated that mesangial cell proliferation is accompanied by increased α-smooth muscle actin (ASMA) expression. Semiquantitative studies conducted by Alpers et al. [1] and McPherson et al. [26] have extended these investigations to human renal biopsies. These investigators have found increased ASMA in human glomerular diseases, but the number of biopsies investigated was limited and significantly increased ASMA expression was not demonstrated in the single groups of GN.

Morphometric methods allow an unbiased quantitation of the structures under investigation and are fundamentally superior to subjective scoring. Whereas the outcome of endocapillary glomerulonephritis is good, the prognosis of membranoproliferative glomerulonephritis is poor, and together with IgA nephritis, these types of glomerulonephritis illustrate a spectrum of injury and prognosis. Using a specific quantitation of actin, determination of cell proliferation, and cell numbers, all of which are important factors in the pathogenesis of glomerular injury, we investigated these diseases and anticipate that the variables studied may also provide the basis

for further indices of activity and prognosis of glomerulonephritis.

Materials and methods

Control biopsies were taken from patients prior to treatment with cyclosporin-A for severe, otherwise intractable, psoriasis. Because of the known risk of induction of renal interstitial fibrosis by cyclosporin-A, this treatment was monitored by a protocol including a baseline renal biopsy and yearly follow-up biopsies [35]. These patients had no proteinuria, haematuria or elevation of blood pressure, and they had normal renal function. No light or electron microscopic glomerular abnormalities were found in their biopsies.

Cases of acute, postinfectious endocapillary GN, IgA nephropathy and membranoproliferative GN were selected according to the light microscopical and immunofluorescence microscopical criteria of the international WHO classification [6]. Immunofluorescence for immunoglobulins, C3, albumin and fibrin was performed on frozen sections. Electron microscopy was also frequently used for confirmation of the diagnosis. Only biopsies containing more than ten glomeruli in the original sections were included. The slides used for immunohistochemistry for actin and Ki-67 could, however, contain a lesser number owing to deeper cutting into the block.

Eleven patients (6 male, 5 female) had IgA nephropathy. Their age range at biopsy was 20–62 years. Four biopsies showed focal proliferative GN, on light microscopy, while 7 had diffuse mesangial proliferative glomerulonephritis, mostly mild. All biopsies showed mesangial deposits of IgA, which was the inclusion criterion. Some of the biopsies had a slight reaction for C3. No other immunoglobulins were detected. Electron microscopy invariably showed extensive mesangial, finely granular, electron-dense deposits. Nine biopsies from 9 patients were affected by acute postinfectious GN. Light microscopically these biopsies showed endocapillary GN. Eleven biopsies from 11 patients showed membranoproliferative GN type 1 on light- and electron microscopy. The biopsy specimens were fixed in 4% neutral buffered formaldehyde, embedded in paraffin, sectioned 2 μm thick and stained with haematoxylin/eosin, periodic acid–Schiff (PAS), periodic acid–silver methenamine, Masson–trichrome and Congo red.

Two monoclonal anti-actin antibodies were used for immunohistochemical evaluation: the “pan-muscle” HHF-35 (DAKO, Glostrup, Denmark), which is reactive with four actin isoforms [20], alpha and gamma smooth muscle, alpha cardiac and alpha striated muscle actin isoforms, and clone 1A4 anti-ASMA, with specificity for only the alpha smooth muscle actin isoform (DAKO, Glostrup, Denmark). This antibody labels smooth muscle cells, vascular pericytes, myoepithelial cells and myofibroblasts in paraffin-embedded sections [26]. In our quantitative estimation we decided to use the staining with the 1A4 antibody because of its narrower specificity.

The clone MIB-1 anti Ki-67 monoclonal antibody (Immunotech, France), which reacts with the Ki-67 nuclear antigen associated with cell proliferation and which is found throughout the G1, S, G2 and M phases of cell cycle, was used for immunohistochemical evaluation of proliferation in renal biopsy specimens [14, 16, 19, 29]. This antibody detects Ki-67 antigen on paraffin sections after exposure to microwave [5].

For immunohistochemistry, sections were deparaffinized before undergoing microwave processing for antigen retrieval. The duration of this was 5 × 5 min for MIB-1 and 3 × 5 min for ASMA. Endogenous peroxidase activity was blocked with 2% hydrogen peroxide in methanol for 40 min at room temperature.

The sections were incubated with 5% fetal calf serum for 20 min at room temperature to block nonspecific binding, and then with primary antibodies at a dilution of 1:100 (for ASMA and pan-muscle actin antibody) and 1:200 (for MIB-1 antibody) overnight at 4°C. Next day, secondary biotinylated goat antibody to mouse immunoglobulins (DAKO) was applied at a dilution of 1:100 in fetal calf serum for 30 min at room temperature. The sections were incubated with streptavidin–biotin complex/horseradish peroxidase (DAKO; working dilution 1:100) for 30 min at room temperature. Following washing in PBS buffer and 0.05 M Tris buffer the sections were stained using fresh 3,3 diaminobenzidine tetrahydrochloride (KEM-En-Tec; 1 mg/ml) as the chromogen with 0.03% hydrogen peroxide, for 7 min and 10 min for immunohistochemical demonstration of actin and Ki-67 antigen, respectively; finally they were counterstained with Mayer’s haematoxylin and mounted in Kaiser’s glycerol gelatin.

Expression of ASMA in the smooth muscle cells of the renal blood vessels provided an internal control. Lymph node sections were used as positive controls for the reaction with MIB-1.

All biopsies were stained for both ASMA and Ki-67 antigen. A few cases had to be excluded owing to technical difficulties. After cutting and immunostaining only two glomeruli were present in two biopsies, which were therefore omitted. In 3 biopsies, ASMA staining showed very low activity in the internal controls (vessel walls) and in 6 biopsies the staining for Ki-67 was totally negative, also in tubules. These cases were also excluded. The number of the remaining, successful stainings is shown in Tables 1 and 2. The quantitative estimation of the biopsies was carried out as a blind study.

Point counting was used to estimate the volume fraction of actin positive areas in the glomeruli [15]. The ratio between points used for counting on glomeruli and on ASMA-positive areas was 1:16. The use of this ratio made the counting fast but also ensured that a sufficient number of points were counted. The slide was projected on the screen of an Amiga Commodore 2000 connected with an Amiga Commodore computer and a microscope Olympus Vanex-S equipped with an objective Olympus APO 40/075. The grid was shown on the screen with two sets of points (ratio 1:16). The number of points hitting the glomerular tuft was counted in each glomerulus, and with the tighter grid the points hitting the actin-positive areas were counted.

Following immunohistochemical staining for Ki-67 antigen all glomeruli from each biopsy were examined and the number of Ki-67-positive cells per glomerular cross section was counted. The total number of glomerular cells per glomerular cross section was determined by counting the number of cells in a certain fraction of the glomeruli and multiplying that number by the fraction. The counting was done in an unbiased counting frame [15].

Table 1 Glomerular expression of alpha-smooth muscle actin

	Normal controls	Acute postinfectious glomerulonephritis	Membranoproliferative glomerulonephritis	IgA nephropathy
No. (cases)	14	9	11	11
No. (glomeruli)	227	94	81	168
Morphometry				
Actin area%	1.9	22.0	16.5	12.4
Range	0.2–4.4	7.3–41.3	3.0–30.7	0.1–27.7
SD	1.38	12.8	8.4	9.5
Difference from control		$P = 0.0001$	$P = 0.0001$	$P = 0.0004$

Table 2 Glomerular cell number and Ki-67 proliferation marker

	Normal controls	Acute postinfectious Glomerulonephritis	Membranoproliferative glomerulonephr.	IgA Nephropathy
No. (cases)	12	6	11	11
No. (glomeruli)	197	68	81	168
Total number of cells/glomerulus	74	139	108	89
SD	14	36	21	20
Difference from controls		$P = 0.0010$	$P = 0.0010$	$P = 0.0046$
Ki-67 pos. cells/glomerulus	0.31	5.43	2.31	1.27
SD	0.24	2.22	2.10	1.17
Difference from controls		$P = 0.0012$	$P = 0.0033$	$P = 0.011$
Index[a]	4.4	41.2	21.6	13.4
SD	4.1	33.5	20.5	12.2
Difference from controls		$P = 0.0011$	$P = 0.0092$	$P = 0.026$

[a] Proliferation index = number of Ki-67 positive cells per 1000 glomerular cells

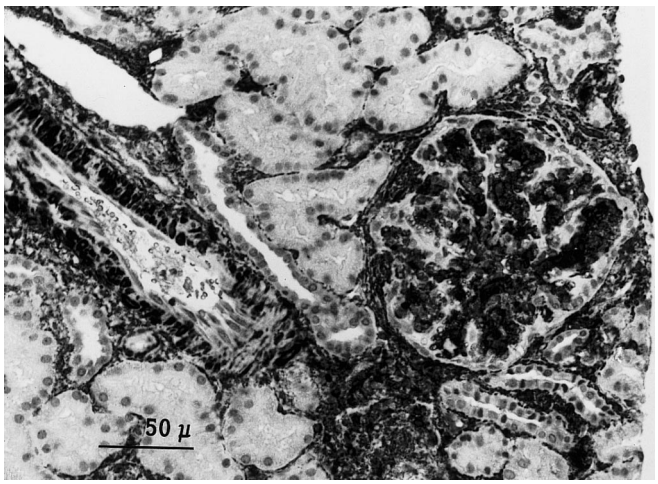

Fig. 1 Alpha smooth muscle actin in a case of membranoproliferative glomerulonephritis. Internal control in muscular coat of an artery

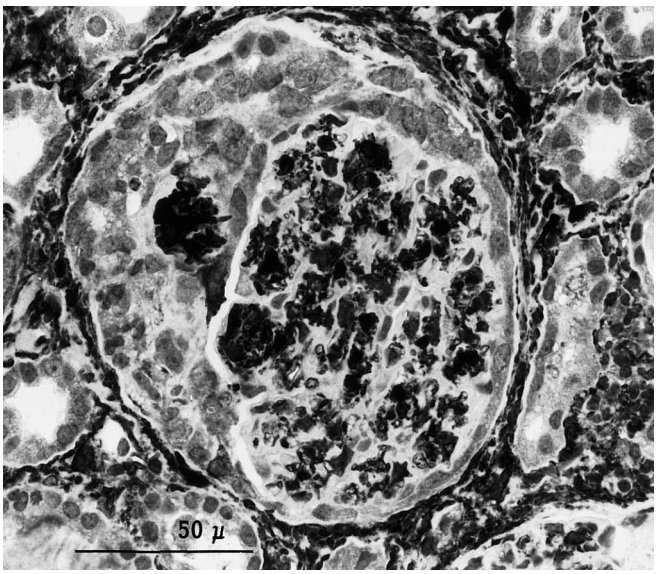

Fig. 2 Focal, segmental proliferative glomerulonephritis (IgA nephropathy). Strong mesangial expression of alpha smooth muscle actin. The segmental epithelial proliferation is negative

The cell proliferation index was calculated as the number of Ki-67 positive nuclei per 1000 glomerular nuclei.

Student's unpaired *t*-test was used for pairwise comparisons of group means. Linear regression analysis and Spearman's correlation coefficient were used for the comparison between proliferation markers (Ki-67 and glomerular cell numbers) and expression of ASMA.

Results

The staining intensities and location within the glomeruli with the 1A4 and HHF-35 antibodies were essentially uniform and showed the same pattern. The glomerular activity was predominantly mesangial (Figs. 1, 2) with very few stained cells outside the mesangial areas. The mesangial staining in cases of IgA nephropathy was always diffuse, even in cases with only focal GN. Epithelial or fibrous crescents were few and occurred only in IgA nephropathy. Staining of these structures was almost nil (Fig. 2). The mesangial staining included areas with peripheral mesangial interposition in cases of membranoproliferative GN.

All sections from controls and study groups showed intense staining for ASMA in the tunica media of the renal blood vessels (Fig. 1). Interstitial tissue surrounding the blood vessels and tubules showed moderate staining, which was increased in some cases of GN associated with interstitial fibrosis and tubular atrophy

Data from quantitative examinations based on point counting are summarized in Table 1. In the control group the number of examined glomeruli in each biopsy varied from 4 to 43 (mean: 16). In the groups with glomerulonephritis the number of examined glomeruli varied from 8 to 28 (mean: 15), from 4 to 12 (mean: 7) and from 3 to 20

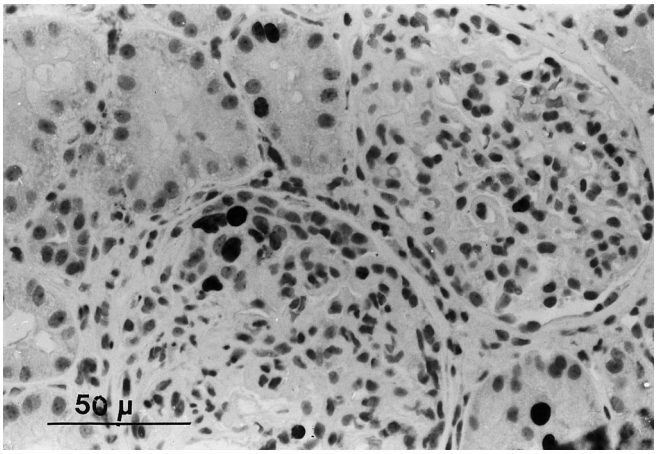

Fig. 3 Glomerulus showing many Ki-67-positive cells stained with the MIB-1 antibody in membranoproliferative glomerulonephritis. Some Ki-67 positively stained tubular cells also present

(mean: 11) in the sections obtained from patients with IgA nephropathy, membranoproliferative GN and endocapillary GN, respectively.

The mesangial actin-positive area found by point counting was small in normal controls (range 0.20–4.4% of the total glomerular area). The greatest actin-positive areas appeared in patients with endocapillary GN, with a mean value of 22.0% (range 7.3–41.3%). In the other disease groups, actin-positive areas were smaller than in endocapillary GN, but still remained significantly different from the control group.

Nuclei stained for Ki-67 antigen were present in all biopsies, including the control biopsies (Fig. 3), in which they were few in number and almost only tubular epithelial nuclei were stained. The MIB-1 antibody also stained the brush border of proximal tubules in some biopsies.

In the groups with GN, MIB-1-positive nuclei were seen in all types of cells, including parietal epithelium and crescents. In biopsies with interstitial inflammatory infiltrates, these included MIB-1 positive cells.

The means and standard deviations of the three major variables estimated in the present study are summarized in Table 2.

The greatest glomerular cellularity and proliferation index was observed in endocapillary GN, where the total number of cells per glomerular cross section, the cell proliferation index and the number of Ki-67-positive cells per glomerulus varied from 100 to 189, 12.8 to 97.8 and 1.80 to 14.85, respectively.

No significant correlations were found between the quantitative determinations of ASMA and the mean number of glomerular cells per glomerular cross section, the number of Ki-67-positive nuclei per glomerular cross section, and the proliferation index when the whole material was taken into consideration (Spearman's rho between 0.14 and 0.27; *r*-values between 0.12 and 0.17). There were also no significant correlations when each category of GN was considered alone.

The mean number of Ki-67-positive intraglomerular nuclei and the proliferation index were both significantly correlated with the mean number of glomerular cell nuclei (Ki-67-positive nuclei: Spearman's rho=0.60; r=0.67; P=0.0001; proliferation index: rho=0.49; r=0.49; P=0.0001).

Discussion

Actin is ubiquitous in eukaryotic cells and constitutes one of the most abundant cellular proteins. At least six isoforms exist, among them ASMA, which is a good marker of smooth muscle differentiation and has been considered to be almost specific for smooth muscle cells [30, 31]. Because of the well-documented resemblance of mesangial cells to smooth muscle cells and their contractile properties [2, 3, 28], this isotype might be expected to be expressed in mesangial cells. It is therefore surprising that ASMA is not expressed at all in the glomerulus of the normal rat [11–13, 20] and that mesangial cells are only faintly stained in man [1, 26]. ASMA is, however, expressed in mesangial cell cultures and in several conditions with mesangial cell activation in experimental models [7, 10–13, 20].

Using subjective semiquantitative scoring, Alpers et al. [1] and McPherson et al. [26] found marked up-regulation of ASMA in mesangial cells in many glomerular diseases in man. These authors compared glomerular diseases with control biopsies from macroscopically normal renal parenchyma from kidneys excised because of a localized neoplasm and from tubulointerstitial conditions not thought to include glomerular lesions. Among various categories of glomerular disease, mesangioproliferative GN and IgA glomerulopathy showed increased staining in McPherson et al.'s series on semiquantitative evaluation, but the number of cases in each group was small (3 biopsies in each). The total series of biopsies with glomerular diseases in the series of Alpers et al. showed increased expression of the mean ASMA score in mesangial regions, which was statistically significant (P<0.015), but (with the possible exception of lupus nephritis) the individual disease categories were too small to allow statistical evaluation. Eight cases of IgA nephropathy, three cases of acute post-infectious GN and three cases of membranoproliferative GN all had increased scores for ASMA expression. In the present investigation these three categories of GN all had highly significantly increased mesangial ASMA expression in the morphometric evaluation (Table 1). Endocapillary glomerulonephritis generally has a good prognosis. The large amount of actin in this type of GN indicates that other factors than actin expression determine the prognosis.

It is likely that the staining of actin is due to its expression in mesangial cells. Other cells, for example macrophages, might, however, have emigrated into the mesangium and expressed actin. The interstitial cells expressing ASMA could be interpreted as interstitial myo-

fibroblasts, pericytes or perivascular adventitial cells [32]. Increased ASMA immunoreactivity revealed in fibrotic areas could reasonably be assumed to be related to the presence of these cells.

There are many categories of glomerular disease in which mesangial hypercellularity is present. Quantitative methods [17, 18] have improved data formerly obtained exclusively by subjective evaluation. In postinfectious acute diffuse proliferative GN, both endothelial and mesangial hypercellularity have been reported [23, 33]. In focal proliferative GN (which most likely have been cases of IgA nephritis although no documentation was possible at that time) Kawano et al. [21] and Wehner [33] also found mesangial cell proliferation in glomeruli that were light microscopically normal.

Using an antibody against the proliferation antigen PCNA, Alpers et al. [1] showed correlation of the expression of ASMA with glomerular cell proliferation. There are problems with the interpretation of this antigen [19, 25, 29], and we have preferred to use the expression of Ki-67 to assess proliferation. The Ki-67 antigen is expressed in the cell nucleus in all stages of the cell cycle except G0 [4, 24]. The anti-Ki-67 antibody works on cryostat sections only, but a monoclonal antibody to this antigen, MIB-1, which is generated using recombinant parts of the antigen, immunostains formalin-fixed, paraffin-embedded tissue following microwave antigen retrieval [5]. The Ki-67 antigen expression measured with this technique correllates well with the mitotic figure index [34] and is considered a reliable measure of cell proliferation. As we expected, the highest Ki-67 expression was observed in cases of acute postinfectious endocapillary GN.

The present data showing that the diffuse involvement of mesangium in the activation/injury process can take place even in the apparently normal glomeruli in cases of IgA nephropathy are of great practical value and may predict the progression of disease. It could be assumed to be the earliest lesion visible at the light microscopical level. The importance of mesangial activation and proliferation for progression of glomerulopathies is currently under intense investigation [11–13] and it is clear from experimental studies that the co-operation of a great number of cytokines and growth factors is involved. It is highly probable that the mesangial cell is involved in such important activities as regulation of glomerular haemodynamics and synthesis of mesangial matrix and, as a consequence, the development of glomerular sclerosis [27].

In GN the Ki-67-positive cells might be either monocytes/macrophages infiltrating capillary loops, or mesangial cells. Some inflammatory cells found to infiltrate the renal interstitium were also positively stained with the MIB-1 antibody.

There was a strong correlation between the number of glomerular cells in a biopsy and the proliferation parameters. ASMA expression was *not*, however, correlated with proliferation or cell number. The reason may be that ASMA expression is present exclusively in some stages of mesangial cell proliferation and the material was composed of cases of GN with widely varying (and often unknown) duration. Another explanation could be that ASMA expression is not directly related to mesangial cell proliferation, both these being independent consequences of activation of these cells. Finally, the possibility also exists that influx of non-actin-bearing but proliferating cells may lead to a poor correlation.

Studies of correlation between morphometrically determinated glomerular ASMA expression and proliferation markers on the one hand and functional data and long-term follow-up on the other should be performed in order to assess whether these parameters are useful in determination of disease activity and prognosis. The present data may provide an important basis for such studies.

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