

Immunohistochemical Study of the Expression of α -Smooth Muscle Actin and the Proliferation Marker Ki-67 of Glomerulonephritis

The aim of this study was to determine the relationship of α -smooth muscle actin (ASMA) and the proliferation marker Ki-67 of glomerulonephritis (GN). Immunohistochemical stainings with the usual streptavidin-biotin peroxidase method were performed on 86 renal biopsies using monoclonal 1A4 and Ki-67. The results of the quantitative evaluation of ASMA and Ki-67 were analyzed for the correlation between positive value of ASMA and Ki-67 in different GN. ASMA expressions of glomeruli were highest in acute post-infectious GN [APGN; 16.9 Fraction Volume (FV)%], followed by unclassified proliferative GN (UnGN; 12.5 FV%), membranoproliferative GN (MPGN; 8.5 FV%), lupus nephritis (LupusN; 6.3 FV%), IgA nephritis (IgAN; 5.6 FV%), and normal control (0.1 FV%). The Ki-67 staining was considerably elevated in lupusN (4.3 Ki-67 positives/glomerulus), APGN (2.7), MPGN (2.5), UnGN (1.66), IgAN (0.5), compared with that in normal control group (0.1 Ki-67 positives/glomerulus). Ki-67 value in each category of glomerular diseases was significantly different from that in the control biopsies ($p < 0.004$). The relationship between morphometric results of ASMA and Ki-67 was statistically significant regardless of the diagnosis. ($r_s = 0.425$, $p = 0.000$, $ASMA = 0.1113 + 0.1665 \text{ Ki-67}$). In conclusion, the immunohistochemical assessment of ASMA and Ki-67 expression in GN might be a reliable indicator for the progression of GN. This study indicates that active cellular proliferation is associated with increased actin deposition in glomeruli.

Key Words : Actins; Ki-67 Antigen; Glomerulonephritis; Immunohistochemistry

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INTRODUCTION

Mesangial and glomerular cell proliferations are important histologic findings in renal disease, but their prognostic implications has been difficult to assume due to the histologic variety. The glomerular mesangium, which was originally considered to support the glomerular capillaries, has subsequently been shown to have other important functions in renal diseases, such as phagocytic properties clearing capillary walls (1, 2). More recently, it has been demonstrated that mesangial cells can synthesize and release growth factors, interleukins, and other cytokines. With the demonstration of contractile proteins in mesangial cells (3, 4), their resemblance to smooth muscle cells has been emphasized. Data from experimental glomerulonephritis (5) indicate that mesangial cell proliferation is accompanied by increased α -smooth muscle actin (ASMA). Semi-quantitative studies conducted by Alpers et al. (6) and MacPherson et al. (7) have extended these investigations to human counterparts and found that ASMA is also increased in human glomerular disease. Hewitson and Beaker (8) identified

interstitial and glomerular myofibroblasts by morphology and ASMA immunostaining at light and electron microscopic levels.

The monoclonal antibody Ki-67 reacts with human nuclear cell proliferation associated antigen expressed in all active parts of the cell cycle. The antibodies MIB1-3 against recombinant parts of the Ki-67 antigen are Ki-67 equivalents, as demonstrated by immunostaining of formalin-fixed, paraffin-embedded sections (9). The Ki-67 proliferation marker is another nuclear non-histone protein (10) expressed in proliferating cells throughout all phases except G0 and early G1, with maximum expressions in G2 and M phases. Antibodies against Ki-67 have been used to visualize proliferating cells in normal kidneys as well as in renal biopsy specimens from glomerulopathies (11-13). However no specific attempt has been made to evaluate the relationship between the disease activity and the rate of cell proliferation. The purpose of this study was to determine the quantitative ASMA and the cell proliferation marker Ki-67 in glomerular diseases with an effort to correlate them.

MATERIALS AND METHODS

Tissue sample

This study used renal biopsy specimens obtained from 86 patients over the last 10 yr from the Department of Pathology, Ewha Womans University Hospital, Seoul, Korea. The series included 12 cases of acute post-infectious glomerulonephritis (APGN), 5 cases of membranoproliferative glomerulonephritis (MPGN), 10 cases of lupus nephritis (LupusN), 50 cases of IgA nephropathy (IgAN), and 9 cases of unclassified proliferative glomerulonephritis (UnGN). These cases were diagnosed according to light microscopical, electron microscopical, and immunofluorescence microscopical criteria of the World Health Organization (WHO). Ten renal specimens from surgically removed kidney specimens due to focal neoplasm, which were confirmed as normal renal parenchyme by routine diagnostic procedure, used as the control group.

Routine stainings

Biopsy specimens were fixed in 4% neutral buffered formaldehyde, embedded in paraffin, sectioned 4 μ m thick, and stained with hematoxylin-eosin (H&E), periodic acid-Schiff (PAS), silver methenamine (GMS), and Masson-trichrome (MT).

Immunohistochemistry

Immunohistochemical staining for ASMA using the avidin-biotinylated horseradish peroxidase complex system was performed by mouse monoclonal antibodies [ASMA Ab-1 (clone 1A4), NeoMarkers, Fremont, CA, U.S.A.]. Formalin fixed paraffin-embedded tissue sections were deparaffinized and endogenous peroxidase activity was blocked with 2% hydrogen peroxide in methanol for 40 min at room temperature. Sections were incubated with 5% fetal calf serum for 20 min at room temperature to block non-specific binding, and then with primary antibodies at a dilution of 1:100 ASMA overnight at 4°C. On the next day, secondary biotinylated mouse antibodies were applied to mouse immunoglobulin (NeoMarkers) at a dilution of 1:100 in fetal calf serum for 30 min at room temperature. Sections were incubated with streptavidin-biotin complex/horseradish peroxidase (NeoMarkers) at a dilution of 1:100 for 30 min at room temperature. After washing in phosphate-buffered saline (PBS) buffer with 0.05 M Tris buffer, the sections were stained using fresh 3.3 diaminobenzidine tetrahydrochloride as a chromogen with 0.03% hydrogen peroxide for 7 min for immunohistochemical demonstration of actin. Finally, the sections were counter-stained with Mayers hematoxylin and mounted in Kaisers glycerol gelatin.

Immunohistochemical staining for Ki-67 using rabbit

antihuman Ki-67 antigen (DAKO Code No. N1574 Via Real, CA, U.S.A.) was performed. Four μ m thick sections were mounted on silanized slides (DAKO Code No. S3003), air-dried, and de-paraffinized through xylene and graded alcohol series. Endogenous peroxidase was inhibited with 0.3% hydrogen peroxide. After washing specimens in PBS, antigen retrieval was performed using wet autoclave pretreatment in 0.01 M/L citrate buffer for 15 min at room temperature and incubated with MIB-1 diluted at 1:50 overnight at 4°C. After rinsing three times in PBS, sections were further incubated for 30 min with bio-tinylated sheep anti-mouse immunoglobulins and streptavidin-biotin-peroxidase complex at room temperature. 3,3-diaminobenzidine was used as a chromogen. Crypt epithelial cells from colonic mucosal biopsy specimens were used as positive controls.

Morphometrics

A quantitative estimate for each of the biopsy specimen was carried out. Fraction volume for ASMA at glomerulus was quantitated by point counting using an eyepiece graticule of 1 cm² with ten equidistant lines at a magnification of $\times 400$, OCM eyepiece Micrometer (OCM 10/10 SQ, Olympus, Japan). Numbers of positive area on micrometer were counted and divided by numbers of total glomerular cross section on micrometer, then multiplied by 100 to calculate fraction volume per cent. The results were expressed as fraction volume (FV) (8) according to the formula.

$$\text{Fraction volume (FV)} = \frac{\text{number of grid intersections with positive staining}}{\text{total number of grid intersections}} \times 100$$

The mean FV in various glomerular diseases was compared by Student t-test. Following immunohistochemical staining for Ki-67 antigen, glomeruli from each biopsy were examined and the number of Ki-67-positive cells per glomerular cross section (Ki-67 positives/glomerulus) was counted. The total number of glomerular cells per glomerular cross section (TGC) was determined by counting the number of cells as one fraction of glomeruli and multiplying the total number of fractions. The cell proliferation index (PI) was calculated as the number of Ki-67-positive nuclei per 1,000 glomerular nuclei. A Student t-test was used to compare means and variations from diseased and control specimens. Pearson's correlation coefficient for ASMA and Ki-67 values was also determined.

RESULTS

The staining intensities and location within glomeruli of

ASMA were essentially uniform and showed similar patterns, albeit some different staining areas depending on the different glomerular diseases and the disease activity.

All sections from the control and study groups showed intense staining by ASMA in the tunica media of the renal blood vessels and the interstitial tissue surrounding the blood vessels, which served as internal controls (Fig. 2A). Our quantitative data expressed by points are summarized in Table 1. Within the control group, there were approximately 80 examined glomeruli in all biopsies, whereas in the groups with glomerulonephritis the number of examined glomeruli ranged from 45 to 570, according to the disease.

The mesangial actin-positive area by point counting was small in the normal control group (range, 0-0.25 FV% of total glomerular area). The greatest actin-positive areas appeared in 12 cases of APGN (Table 1) with a mean value of 16.9 FV% (range, 2.5-27.1) (Fig. 2B, C), followed by nine cases of UnGN with a mean value of 12.57 FV% (range, 0-39.6), 5 cases of MPGN with 8.58 FV% (range, 1.1-21.9), 10 cases of LupusN with 6.33 FV% (range, 0-13.9), and 50 cases of IgAN with 5.67 FV% (range, 0-33.7) (Fig. 2D). The disease with the least actin-positive areas was IgAN. The ASMA values of all glomerular diseases were significantly different from those in the control group biopsies ($p < 0.011$), confirmed by one-way ANOVA with multiple comparison tests ($p = 0.001$). The most active proliferative disease showed an increased amount of actin deposition, while the least actin deposition was seen in IgAN. It is thus possible that the greater damage and stimulation resulted in the larger areas of actin deposition. APGN and UnGN

had a greater tendency for mesangial fibrosis and crescent formation as well as periglomerular fibrosis, while most of IgAN showed minimal mesangial change by routine histology.

Nuclei stained positive for the Ki-67 antigen were present in all the biopsies, including the control group, where they were few. In the groups with glomerulonephritis, Ki-67 (MIB-1)-positive nuclei were seen in all types of cells, including visceral epithelium and mesangial cells. The mean values of total glomerular cells (TGC), Ki-67-positive cells per glomerular cross section (Ki-67 pos/glo), and proliferation index (PI, Ki-67 pos. cell/1000 glomerular cells) in all the control and diseased groups are summarized in Table 2.

Ki-67 positivity and PI were highest in LupusN followed by APGN (Table 2) (Fig. 3B). Ki-67 positivity were 4.31 in LupusN (Fig. 3A), 2.76 in APGN, 2.5 in MPGN, 1.66 in

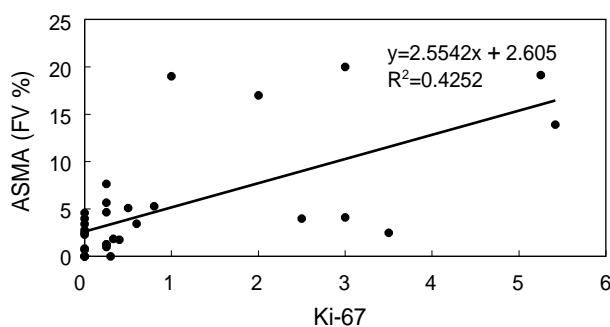


Fig. 1. Correlation of ASMA with Ki-67 positive cells/glomerular section in glomerulitis.

Table 1. Glomerular expression of ASMA in 86 specimens with glomerular diseases

	Normal	IgAN	APGN	MPGN	LupusN	UnGN
No. of cases	10	50	12	5	10	9
No. of glomeruli	80	570	110	45	96	95
Morphometric criteria						
Actin (FV %)	0.11 ± 0.11	5.67 ± 6.96	16.94 ± 8.03	8.58 ± 9.34	6.33 ± 4.78	12.57 ± 13.76
Range	0-0.25	0-33.79	2.5-27.1	1.1-21.9	0-13.9	0-39.6
p value (versus control)	-	0.011	< 0.001	0.001	0.001	0.011

Data of Actin are expressed as mean with standard deviation. FV %, Fraction volume percent; IgAN, IgA nephropathy; APGN, acute post-infectious glomerulonephritis; MPGN, membranoproliferative glomerulonephritis; LupusN, lupus nephritis; UnGN, unclassified proliferative glomerulonephritis

Table 2. Total glomerular cells, Ki-67 proliferation marker, and proliferation index in 86 specimens with glomerular diseases

	Normal	IgAN	APGN	MPGN	LupusN	UnGN
No. of cases	10	50	12	5	10	9
No. of glomeruli	80	570	110	45	96	85
Total glomerular cells	76.90 ± 4.58	137.52 ± 28.64	181.08 ± 42.89	140.58 ± 52.36	179.62 ± 61.79	162.71 ± 41.19
p value (versus control)	-	< 0.001	< 0.001	0.002	0.001	0.001
Ki-67 pos/glo	0.11 ± 0.17	0.56 ± 0.98	2.76 ± 1.29	2.50 ± 0.70	4.31 ± 2.63	1.66 ± 1.44
p value (versus control)	-	0.003	< 0.001	< 0.001	< 0.001	0.004
Proliferation index	1.35 ± 2.04	4.30 ± 7.09	14.41 ± 6.88	13.60 ± 0.42	19.62 ± 8.75	9.16 ± 7.38
p value (versus control)	-	0.018	< 0.001	< 0.001	< 0.001	0.007

Data are expressed as mean with standard deviation. Ki-67 pos/glo, Ki-67 positive cells per glomerular cross section

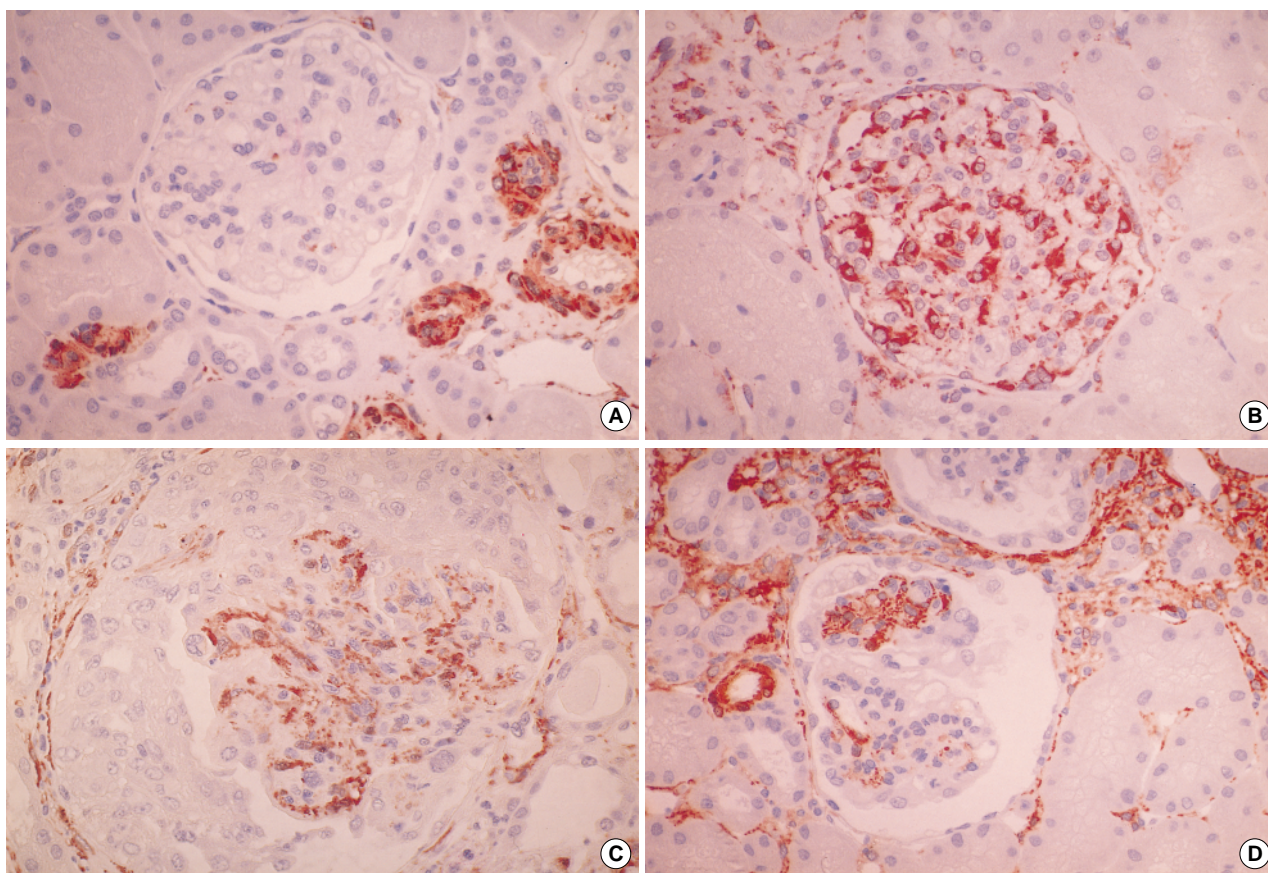


Fig. 2. Expression of ASMA in normal and diseased glomeruli: (A) ASMA in control cases: negative in glomerular tuft and positive in muscular coat of arterioles ($\times 200$). (B) ASMA in mesangial proliferative APGN: diffuse positive in mesangium ($\times 200$). (C) ASMA in crescentic APGN: mainly positive in mesangium ($\times 400$). (D) ASMA in IgA Nephropathy; segmental mesangial positive expression ($\times 200$).

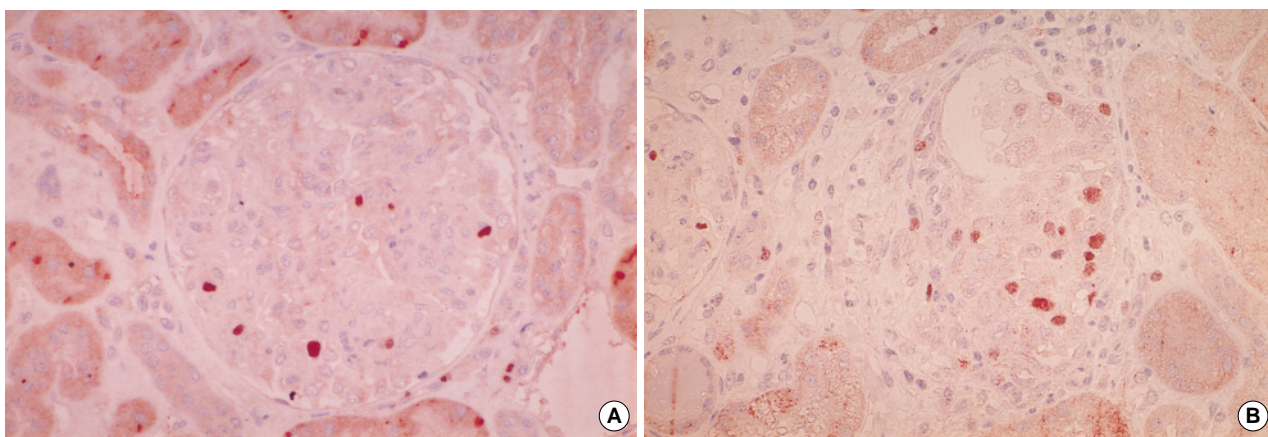


Fig. 3. Expression of Ki-67 proliferation marker in diseased glomeruli. (A) Ki-67 positive cells in endocapillary and mesangium in a case with LupusN ($\times 400$). (B) Ki-67 positive cells in endocapillary and crescentic epithelium in a case of APGN ($\times 400$).

UnGN, and 0.56 in IgAN. All values of Ki-67 were significantly different from the value of the control group ($p < 0.004$), and Ki-67 values in individual groups were also significantly different from each other by one-way ANOVA ($p < 0.01$). The PI were 19.62 for LupusN, 14.41 for APGN,

13.6 for MPGN, 9.16 for UnGN, and 4.30 for IgAN. Each of the PI values in disease groups were significantly different from that of the control group ($p < 0.018$).

As for the correlation between ASMA and Ki-67, a statistical significance was noted. Regardless of the diagnosis, the

correlation coefficient (Pearson correlation) and regression coefficient were significant between ASMA and Ki-67 ($r_s=0.425$, $p=0.000$, $ASMA=0.1113+0.1665$ Ki-67; Fig. 1). Therefore, the increase of actin deposition was accompanied by an increase of glomerular cellularity in most cases. There was a positively linear relationship between glomerular ASMA expression and glomerular cell proliferation. Accordingly, the greatest cell proliferation was concentrated in those glomerulopathies with the highest expression of ASMA, as in APGN and proliferative lupusN.

DISCUSSION

Actin is found in eukaryotic cells and constitutes one of the most abundant cellular proteins available. At least six isoforms exist, among which ASMA is a good marker for smooth muscle differentiation and is considered almost specific for smooth muscle tissue (14, 15). Because of the well documented resemblance of mesangial cells to smooth muscle cells and their contractile properties (3, 4), ASMA might be expressed in mesangial cells. It is therefore surprising that ASMA is not expressed at all in the glomerulus of the normal rats (5, 16, 17) and that mesangial cells are only faintly stained for ASMA in normal human glomeruli (6, 7). However, it has been reported that ASMA is expressed in mesangial cell cultures and in several conditions with mesangial cell activation in experimental models (5, 17-20).

Using subjective semi-quantitative scoring, Alpers et al. and MacPherson et al. found an increased ASMA in mesangial cells in many human glomerular diseases. Among the various categories of glomerular diseases, MPGN and IgAN showed increased staining in series on semi-quantitative evaluation, but the number of cases in each group was too small (7). The total series of biopsies with glomerular diseases showed an increased expression of ASMA in the mesangial region, which was statistically significant ($p<0.015$), but except for lupusN, the number of cases in each disease category was too small to allow statistical evaluation: in the series all of the eight cases of IgAN, three cases of APGN, and three cases of MPGN showed increased scores for ASMA expression (6). Also it was confirmed that increased interstitial myofibroblasts in IgAN by morphology and ASMA immunostaining (8).

In the present investigation, the greatest actin deposition was found in patients with APGN (16.9 FV%), followed by UnGN, MPGN, LupusN, and IgAN. The disease with the least expression of actin was IgAN (5.67 FV%). In our cases of APGN, mostly from adult patients with an average age of 22 yr. Among 12 cases of APGN, the histology shows crescent formation in 6 cases, diffuse hypercellularity in 4, and mesangial fibrosis as well as periglomerular and interstitial fibrosis in 2.

All ASMA values for glomerular diseases were signifi-

cantly different from that in the control biopsies ($p<0.011$). It was reported that IgAN showed a variation of ASMA expression (range 0.1-27.7 FV%), which was also observed in our study (9). It is likely that the staining of actin is due to its expression in mesangial cells. Other cells, however, such as macrophages, might have emigrated into the mesangium and expressed actin. The interstitial cells expressing ASMA could be interpreted as interstitial myofibroblasts, pericytes or perivascular adventitial cells (21). Increased ASMA immunoreactivity revealed in fibrotic areas could be due to the presence of these cells. However inflammatory reaction as well as interstitial fibrosis are minimal and seen in only a few cases.

There are many categories of glomerular diseases with hypercellular mesangium. Quantitative methods (5) have improved the quality of data formerly obtained exclusively by subjective evaluation. In APGN, both endocapillary and mesangial hypercellularity have been reported (23). Mesangial hyperplasia has also been reported in focal proliferative glomerulonephritis, most likely in the cases of IgAN. However it was also reported that the mesangial cell proliferation in normal glomeruli was found (24, 25).

In all of our biopsy specimens, total glomerular cells, PI, and proliferation markers Ki-67 were analyzed. The greatest Total glomerular cell value was observed in APGN (181/glomerulus) followed by LupusN (179), while the least value was in IgAN (137). The greatest value of PI was LupusN (19.6), followed by APGN (14, 4), while the least value of PI was in IgAN (4.3). The greatest value of Ki-67 was noted in LupusN (4.3/glomerulus), followed by APGN (2.76), while the least was in IgAN (0.56). There was no statistically significant correlation between groups of Ki-67, TGC, and PI by Pearson correlation in our series, while it was reported that Ki-67 and PI were significantly correlated (9). The Ki-67 antigen is expressed in the cell nucleus in all stages of the cell cycle except G0 (26, 27). The anti-Ki-67 antibody works on cryostat section only, but a monoclonal antibody to the antigen MIB-1, which is generated using recombinant parts of the antigen, immunostains formalin-fixed paraffin-embedded tissue following microwave antigen retrieval (28). The Ki-67 antigen expression measured by this technique correlates well with the mitotic figure index (29) and is considered a reliable tool to measure cell proliferation.

As we have expected, the highest Ki-67 expression was observed in cases of LupusN, followed by APGN, MPGN, UnGN, and IgAN. The present data show that the diffuse involvement of mesangium in the activation and injury processes can take place even in apparently normal appearing glomeruli. IgAN is of great practical value and may predict the progression of the disease. It could also be assumed that the outcome of the earliest lesion visible at the light microscopical level. It has been reported that the assessment of proliferation activity in IgAN by immunohistology provid-

ed an additional information on the disease activity and prognosis (30). The importance of mesangial activation and proliferation for the progression of glomerulopathies is currently under investigation (16, 17, 20). It is highly probable that the mesangial cells are involved in the disease process by regulating glomerular hemodynamics and synthesis of mesangial matrix, and as a consequence, by the development of glomerular sclerosis (31). In glomerulonephritis, Ki-67-positive cells might be either mesangial cells or monocytes/macrophages infiltrating capillary loops. Some inflammatory cells infiltrating the renal interstitium were also positively stained with the MIB-1 antibody.

Studies on the correlation between morphometrically determined glomerular ASMA expression and the proliferation marker Ki-67, on one hand, and functional data of long-term follow up, on the other, should be performed in order to assess whether these parameters are useful in determining the disease activity and prognosis. The present data may provide an important basis for such studies. Regardless of the diagnosis, however, the correlation coefficient and regression coefficient were statistically significant between ASMA and Ki-67.

There was positive and linear relationship showing that the highest cell proliferation was concentrated in those glomerulopathies with the highest expression of ASMA, such as APGN and LupusN. An increase of cellular proliferation rate represents a kind of cardinal pathological event in a variety of kidney diseases. To evaluate proliferative activity, immunohistological labeling of PCNA and Ki-67 nuclear markers were used in both snap-frozen (10) and formalin-fixed paraffin-embedded renal biopsy specimens (12, 13, 32, 33). The morphometric estimates demonstrated that different ASMA expression in types of GN affected the prognosis. The finding of other reports, along with the lack of correlation between ASMA expression and proliferation markers indicates that the role of ASMA in GN is complex (9). In another recent study, it was mentioned a rough correlation between the interstitial count of proliferating cells and the proportion of biopsy specimens from patients with increased serum creatinine concentration (13).

In a relatively large homogeneous series, it was documented a correlation of the proliferation rate in the kidney as assessed by the Ki-67 antigen expression with a variety of clinical and histological indicators of disease activity and with prognosis of the disease. The data suggest that quantitative immuno-visualization of proliferating renal cells, primarily in the interstitial compartment, may provide clinically relevant information complementary to that obtained by conventional pathological techniques (30). Radford et al. (1997) postulated that characterization of proliferative activity using antibodies against the Ki-67 antigen might be useful as an additional marker for predicting the likelihood of progressive renal failure in IgAN (33).

As seen in Table 1, the enhanced expression may be seen

in any form of glomerular injury, and marked increases are common in proliferative GN, such as APGN and diffuse proliferative LupusN. This finding is in close line with those findings in studies of immune complex-mediated mesangial injuries in rats, where marked expression of ASMA by mesangial cells was revealed to correlate with the onset of cellular proliferation (22).

It was determined that there is a strong association between ASMA expression and cellular proliferation in the glomerulus. Therefore, our observations indicate that enhanced ASMA expression in human glomeruli may be best interpreted as an indication of mesangial cell proliferation and activation.

In summary, this study demonstrates that the enhanced expression of ASMA is a sensitive but non-specific marker of mesangial injury detectable in fixed tissue sections of human renal biopsies. In many cases, this enhanced expression is also a marker for cellular proliferation within glomeruli. Identification of the expression of ASMA and Ki-67 in renal biopsies may eventually prove to have prognostic significance in the assessment of human glomerular diseases. Therefore this study indicates that active cellular proliferation associated with increased actin deposition in glomeruli leads to the progression of the renal disease.

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