The value of proliferating cell nuclear antigen (PCNA)/cyclin in the assessment of cell proliferation in glomerulonephritis

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Summary. Proliferating cell nuclear antigen (PCNA)/ cyclin is an acidic nuclear protein increasing from the late G1 to S phases of the cell cycle and whose detection parallels other standard methods for assessing cell proliferation. The aim of this study was to investigate PCNA expression in normal and diseased human kidneys, in order to clarify cell proliferation in renal tissue and to define a possible correlation of its expression with various types of glomerulonephritis (GN). The immunohistochemical avidin-biotin complex (ABC) method was used for the demonstration of PCNA applying the monoclonal antibody PC-10 to paraffin sections from: 10 normal kidneys, 55 renal biopsies with various types of proliferative GN (PGN), 44 renal biopsies with various types of non proliferative GN (NPGN). In PCNA-positive renal biopsies with GN the antigen showed a heterogeneous nuclear expression in occasional or few mesangial and glomerular epithelial cells as well as in a greater number of tubular epithelial cells. PCNA was expressed in 20% of normal kidneys and in 38% of renal biopsies with GN. The frequency of PCNA expression was significantly increased in the cases of PGN (47%) compared to that observed in the cases of NPGN (27%) (p=0.03). PCNA was detected in 10/24 cases of IgA nephropathy, in 3/4 cases of IgM nephropathy, in 5/14 of other types of primary PGN and in 8/13 of secondary PGN. PCNA expression was not correlated with the degree of mesangial cellularity in PGN. Moreover, there was no significant difference in PCNA expression between primary and secondary PGN. PCNA demonstrated an intense expression in the majority of epithelial cells forming cellular crescents in 8/11 cases of PGN.

In conclusion, PCNA was observed more frequently in diseased than in normal kidneys. The significant increase in the frequency of PCNA intraglomerular expression in PGN suggests that PCNA has a certain value in the assessment of mesangial proliferation. Moreover, the increased PCNA expression in tubular epithelial cells especially in PGN, indicates their proliferative state and may be correlated with their proposed activation and role in the progression of renal injury.

Key words: Proliferating cell nuclear antigen (PCNA), Glomerulonephritis, Cell proliferation

Introduction

Proliferating cell nuclear antigen (PCNA), also known as cyclin, is a 33-36 KDa acidic nuclear protein, the expression of which increases from the late G1 to S phases of the cell cycle followed by a decrease in G2/M (Takasaki et al., 1981; Celis and Celis, 1985; Kurki et al., 1986). The protein was described independently by Bravo and Celis (1980) through two dimensional gel electrophoretic studies of proliferating and quiescent cells and Miyachi et al. (1978) through the use of a nuclear autoantibody occurring in patients with systemic lupus erythematosus.

Recent data suggest that PCNA is an auxiliary protein of DNA polymerase (Bravo et al., 1987; Fairman, 1990) and plays an important role in DNA synthesis during cellular proliferation (Tochi and Bravo, 1988).

PCNA is regulated in a complex manner with the gene being transcribed efficiently in both quiescent and proliferating cells, while PCNA mRNA is normally only accumulated in proliferating cells (Chang et al., 1990). The absence of stable PCNA mRNA in quiescent cells is associated with the presence of intron 4 in the gene, and removal of this intron leads to high levels of accumulation of PCNA mRNA in such cells (Ottavio et al., 1990). PCNA can be identified by monoclonal antibodies applicable to formalin-fixed paraffin

embedded tissues (Garcia et al., 1989; Hall et al., 1990) and parallels other standard methods for assessing cell proliferation in normal tissues and various types of neoplasms (Garcia et al., 1989; Sabin et al., 1991; Takahashi et al., 1991; Woods et al., 1991; Pelosi et al., 1992). Although PCNA cannot be detected by immuno-fluorescence in normal resting cells, like hepatocytes, renal tubular and glomerular cells (Miyachi et al., 1978; Takasaki et al., 1981), recent immunohistochemical studies report a scarce staining for the antigen in normal adult kidney (Hall et al., 1990).

Previous experimental tissue culture studies have suggested that mesangial cells show a low proliferative level, when cultured under conditions equivalent to those observed in vivo (Madri and Marx, 1992). In contrast, numerous renal diseases involving the glomerulus are characterized by expansion of the mesangial matrix and an increase in the number of cells located within the glomerulus, including mesangial cells (Davies, 1994; Yoshioka and Maki, 1995). In an attempt to shed light on glomerular diseases, like IgA nephropathy, in which mesangial proliferation is the usual histopathological finding and the cause of renal damage, Yokoyama et al. (1992) have studied the expression of a cellular proliferative antigen (Ki-67) in relation to disease activity.

In order to clarify and further explore cell pro-liferation in normal and diseased human kidneys, we have investigated the presence and distribution of PCNA in non-proliferative glomerulonephritis (NPGN) and in proliferative glomerulonephritis (PGN) as well as any possible correlation between PCNA expression and the degree of mesangial cellularity.

Materials and methods

Histologically normal portions of kidney tissue obtained from 10 patients with renal trauma or renal calculi were used as controls. Percutaneous renal biopsies were obtained from 55 and 44 patients with various types of PGN and NPGN respectively (Tables 2, 3).

The specimens were fixed in buffered formalin and embedded in paraffin. Serial sections were cut at 3 μm and stained with hematoxylin-eosin, PAS, silver-methenamine and trichrome-Masson. In all cases, diagnosis of glomerulonephritis (GN) was based on the characteristic findings and immunofluorescence microscopy.

The immunohistochemical avidin-biotin peroxidase complex (ABC) method (Hsu et al., 1981) was used on other paraffin sections for the detection of PCNA. Deparaffinized and rehydrated paraffin sections were treated with methanol containing 0.3% H_2O_2 in order to block the endogenous peroxidase activity. Afterwards sections were treated with the following antibodies:

a) Normal rabbit serum (1:20, Dakopatts, Denmark) for 20 min.

b) Primary antibody: monoclonal mouse antibody to human PCNA, PC-10 (1:250, Dakopatts, Denmark) with overnight incubation at 4 °C.

c) Biotinylated rabbit< to mouse IgG (1:300, Dakopatts, Denmark) for 30 min.

d) Avidin-biotin peroxidase complex (ABC) (Dakopatts ABC kit, Denmark) for 20 min. Sections were thoroughly rinsed with PBS, pH 7.4, between reaction steps.

In all cases antibody localization was performed with the 3,3'diaminobenzidine (DAB, Sigma Chem. Co) reaction (6 mg DAB in 10 ml PBS at pH 7.4 to which 0.025 ml 30% H_2O_2 was added prior to use). Finally, slides were counterstained with Mayer's haematoxylin.

For each test, negative control studies were carried out in which normal rabbit serum was used instead of the primary antibody. Tissue sections from normal small bowel, tonsils and skin were used as positive controls for PCNA/cyclin. The intensity of positive staining was graded into 0 to +++. Staining for PCNA/cyclin was assessed by counting the number of positively stained nuclei per glomerular section. Expression of PCNA/cyclin in parietal epithelial cells, podocytes and mesangial cells was regarded as positive when at least two cells of each type showed a nuclear staining. The extent of PCNA expression in glomerular cells was classified into three grades: grade 1: when 2 cells were positive, grade 2: when 2-5 cells were positive and grade 3: when more than 5 cells were positive.

For each type of renal tubule (proximal, distal, collecting) PCNA expression was regarded as positive when five or more tubules expressed PCNA in the nuclei of tubular epithelial cells. The extent of PCNA expression in tubular epithelial cells was also classified into three grades: grade 1: less than 5 tubules showed PCNA expression in the majority of epithelial cells, grade 2: 5-10 tubules were PCNA positive and grade 3: more than 10 tubules were PCNA positive in the majority of epithelial cells.

The statistical analysis of our results was performed using Fisher's exact test, one tail.

Results

In all positive cases of renal biopsies with GN, PCNA/cyclin was observed in glomerular and tubular epithelial cells (Figs. 1-3). The antigen was hetero-geneously detected in the glomeruli, showing a nuclear expression in occasional or few mesangial cells, parietal epithelial cells and podocytes. PCNA displayed the same nuclear expression in a greater number of proximal distal and collecting tubular epithelial cells. Collecting tubules whenever included in the renal biopsies, demonstrated PCNA nuclear expression in the majority of tubular epithelial cells.

In portions of normal renal tissue, PCNA expression was only observed in the nuclei of tubular epithelial cells.

The incidence of PCNA expression in normal renal tissue, cases of PGN and NPGN is demonstrated in

Table 1. PCNA was observed more frequently in diseased than in normal renal tissue since it was detected in 20% of normal kidneys and in 38% of all cases of GN. The frequency of PCNA expression was significantly increased in the cases of PGN (47%) compared to that observed in the cases of NPGN (27%) (p=0.03). The intensity of positive staining was not correlated with the type of renal injury. The incidence and distribution of PCNA expression in the cases of PGN are shown in Table 2. In all positive cases of PGN, PCNA was detected in both glomerular and tubular epithelial cells, except one case of IgA nephropathy which showed no intraglomerular positivity and one case of IgM nephropathy with no positive tubular epithelial cells. The incidence and the extent of PCNA expression were not correlated with the degree of mesangial cellularity in PGN. Although a trend was observed for a slight increase of PCNA-positive mesangial cells in cases of PGN compared to the one in NPGN, there was no statistically significant difference. Moreover, there was no significant difference in PCNA expression between primary and secondary PGN. The frequency and distribution of PCNA expression in cases of NPGN are demonstrated in Table 3. Although the antigen showed an increased expression in membranous GN, it

Table 1. The incidence of PCNA expression in normal and diseased kidneys.

	No.	POSITIVE CASES*	%
Normal kidneys	10	2	20
PGN	55	26	47
NPGN	44	12	27
TOTAL	109	40	

*: p=0.03 Fisher's exact test.

Table 2. The frequency and distribution of PCNA expression in various types of PGN.

TYPE OF PGN	No.	POSITIVE CASES	MESANGIAL			EPITHELIAL			CONVOLUTED TUBULES		
			1	2	3	1	2	3	1	2	3
Primary PGN											
IgA-GN	24	10*	3	6		5	3		1	4	5
IgM-GN	4	3	1	2		1	2			2	
Other types	14	5	3	2		5			3		2
Secondary PGN	13	8*	3	5		5	2			6	2
TOTAL	55	26	10	15		16	7		4	12	9

*: p=NS.
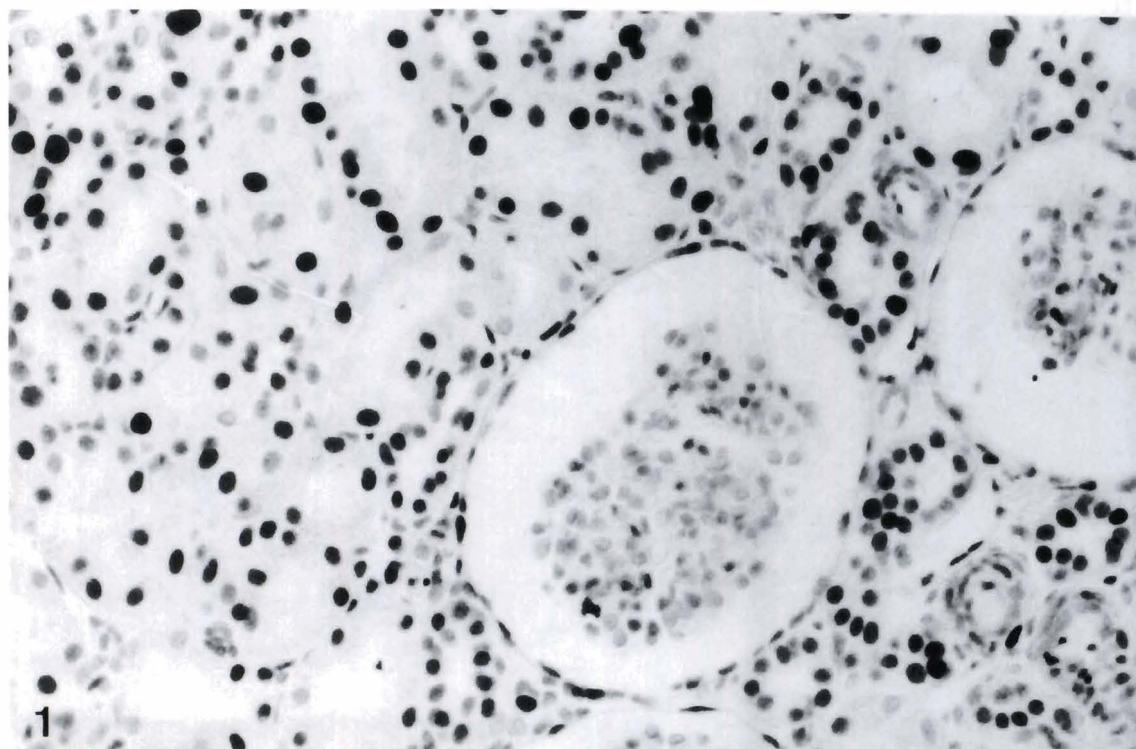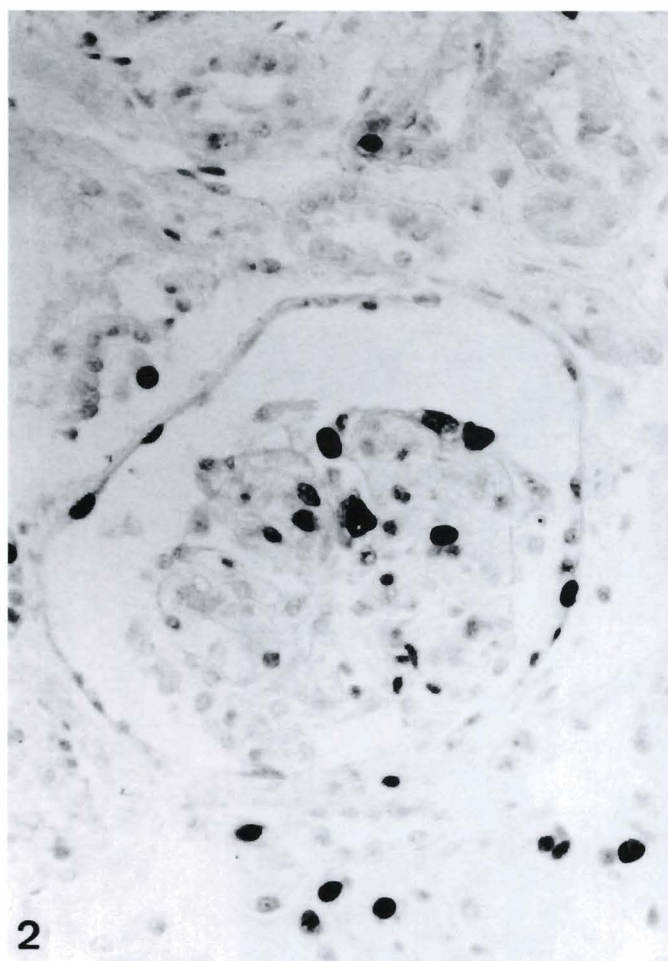

Fig. 1. PCNA nuclear expression in the majority of tubular epithelial cells. ABC, x 300

Table 3. The frequency and distribution of PCNA expression in various types of NPGN.

TYPE OF NPGN	No.	POSITIVE CASES	MESANGIAL			EPITHELIAL			CONVOLUTED TUBULES		
			1	2	3	1	2	3	1	2	3
Membranous GN	23	8	5	2		4	3		2	6	
FSG	16	4	2	2		2	2		2	2	
Minimal change disease	5	0									
TOTAL	44	12	7	4		6	5		4	8	

was not detected at all in cases of minimal change disease. In one case of membranous GN, PCNA was detected only in tubular epithelial cells. PCNA demonstrated an intense expression in cellular crescents observed in 6/9 cases of secondary PGN and in 2/3 cases of primary PGN (Fig. 4), while it was not detected at all in fibrocellular crescents. It is interesting that PCNA was expressed in a variable number of infiltrating mononuclear cells in the interstitium especially in the cases of PGN.

Discussion

Irrespective of the mechanism, an imbalance in the control of glomerular especially mesangial cell proliferation may play an early and perhaps crucial role in the genesis of progressive glomerular injury through excess production of extracellular matrix resulting in glomerulosclerosis (Foegle et al., 1991a,b; Sterzel et al., 1993; Davies, 1994; Eng et al., 1994).

In an effort to elucidate cell proliferation in human renal disease, we have immunohistochemically investigated the expression of PCNA, an index of cell

Fig. 2. PCNA nuclear expression in few mesangial, visceral, and parietal glomerular epithelial cells in an NPGN. PNCA nuclear expression in few proximal tubular epithelial cells as well. ABC, x 300
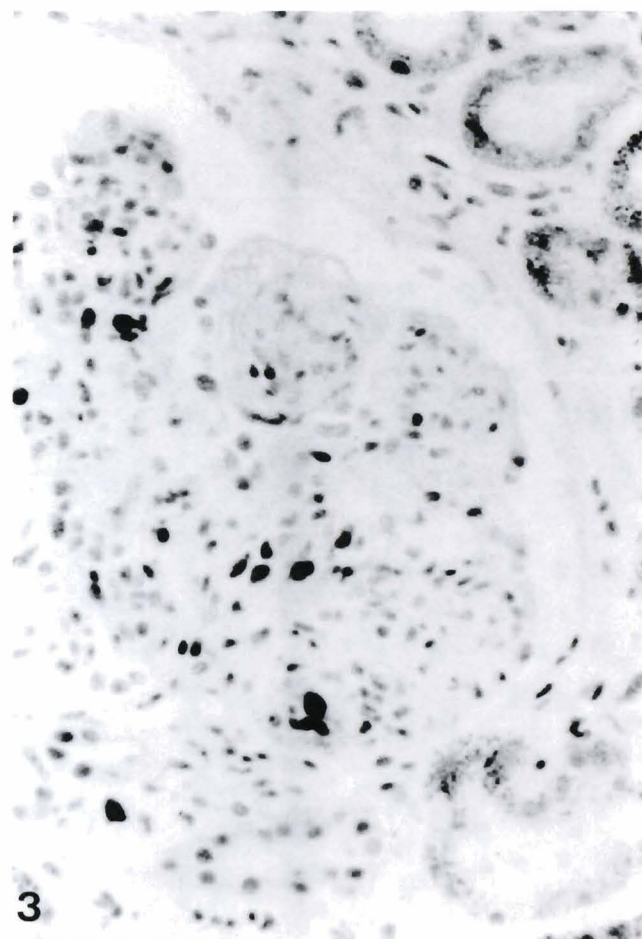
Fig. 3. PCNA nuclear expression in few mesangial cells of PGN. ABC, x 300

proliferation, in normal and diseased human kidneys. PCNA showed a higher incidence of expression in diseased than in normal human kidneys since it was expressed in 20% of normal human kidney tissue and in 38% of renal biopsies with various types of GN. In our series PCNA expression in normal human kidney tissues was restricted to the nuclei of tubular epithelial cells. According to the literature, glomerular and tubular epithelial cells in normal renal tissue are resting cells lacking the expression of cell proliferation antigens like Ki-67 and PCNA, when immunohistochemistry or immunofluorescence are employed for their detection (Miyachi et al., 1978; Takasaki et al., 1981; Yokoyama et al., 1992). The above findings are further supported by animal experimental studies demonstrating the non-proliferative state of mesangial cells in vivo (Pabst and Sterzel, 1993). The scarce expression of PCNA in tubular epithelial cells in our cases of normal renal tissue and in those of Hall et al. (1990) may be due to the different methods and reagents employed in comparison to previous studies, as well as to the renal tissues used.
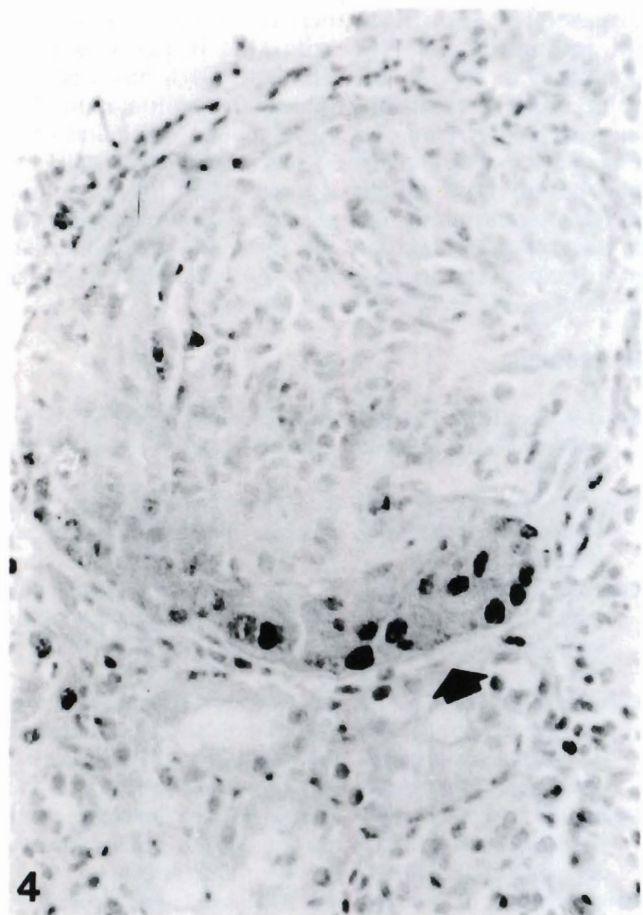

Fig. 4. PCNA nuclear expression in epithelial cells of cellular crescents (arrow) in a PGN. ABC, x 400

In this respect, the presence of scarce PCNA-positive tubular cells in our cases of normal renal tissue may be correlated with the probability of tubular regeneration, since normal portions of kidney tissue were obtained from nephrectomy specimens with renal trauma or renal calculi.

The frequency of PCNA expression in our material demonstrated a statistically significant increase in the cases of PGN (47%) compared to the one observed in NPGN (27%). Previous immunohistochemical studies applying Ki-67 as a cell proliferation marker to a small number of biopsies with various types of GN have shown that Ki-67 expression in glomerular tufts was rare, except those that demonstrated mesangial proliferation, where occasional cells stained (Hall et al., 1988). Experimental studies in animal models have indicated PCNA expression in proliferating mesangial cells in cases of mesangioproliferative GN induced by anti-Thy-1 antibody (Floege et al., 1991a), antimesangial antibodies (Johnson et al., 1990) or streptozotocin (Pesce et al., 1991).

Although PCNA showed a heterogeneous expression in occasional or few mesangial cells in our cases of PGN, the present study is the first to emphasize the significantly increased frequency of PCNA glomerular expression in cases of PGN in human. Recently, Thom et al. (1994) have employed PCNA on routinely processed biopsies of renal grafts and demonstrated its expression in infiltrating inflammatory cells in cortical interstitium but not in glomerular or tubular epithelial cells.

It is interesting that the frequency of PCNA expression was increased in our cases of IgA nephropathy (41.7%).

Yokoyama et al. (1992) have also observed an increased Ki-67 expression (38%) in their cases of IgA nephropathy, which showed a significant correlation with HLA-DQ glomerular expression, increased serum γ-interferon levels and acute clinical exacerbation but not with the degree of mesangial proliferation. According to our results, there was no obvious correlation between PCNA expression and the degree of mesangial cellularity in cases of PGN. Moreover, there was no significant difference in PCNA expression between cases of primary and secondary PGN. Our findings in combination with the bibliographic data (Hall et al., 1988; Yokoyama et al., 1992) suggest that only a small percentage of intraglomerular cells proliferate or activate under normal conditions. The increased frequency of PCNA expression only in few mesangial cells in the cases of PGN without a substantial correlation with the degree of mesangial cellularity suggest that the increase in the number of mesangial cells in these conditions may occur over a longer period of time. Therefore, it might be expected that only a small percentage of mesangial cells might be proliferating at any given time, thus there being very few PCNA-positive nuclei. On the other hand, cellular crescents observed in 6 cases of secondary PGN and in two cases of primary PGN demonstrated an intense PCNA expression in the majority of crescenting

cells. The above observation, which is compatible with those reported by Hall et al. (1988), reflects an acute proliferation and is analogous to PCNA expression in proximal and distal tubular epithelial cells in our series. If the epithelial nature of cellular crescents is taken into account, the common embryologic origin of glomerular and convoluted tubular epithelial cells might justify the similar PCNA expression during renal impairment. Recent immunohistochemical studies on fetal kidneys demonstrated an increased PCNA expression in epithelial cells of both poles of the S-renal vesicle, a property lost during differentiation (Chandler et al., 1994). Thus, under certain conditions like renal injury epithelial cells forming crescents and tubular epithelial cells may be recapturing their fetal proliferation rates. PCNA was detected in a greater number of tubular epithelial cells compared to glomerular cells, showing an intense nuclear expression especially in the cases of PGN. Our observation indicates the proliferating state of tubular epithelial cells in GN and may be correlated with the activation of tubular epithelial cells in renal injury, which is denoted by upregulation of major-histo-compatibility complex (MHC) class II and ICAM-1 production of platelet-derived growth-factor (PDGF) and granulocyte-macrophage-colony stimulating factor (GM-CSF) and is induced by cytokines like interferon-γ, IL-1 and tumor necrosis factor-α (TNFα) (Wuthrich et al., 1990; Markovic-Lipkovski et al., 1991; Frank et al., 1993). Furthermore, the expression of PCNA in a variable number of interstitial mononuclear cells in our material, especially in renal biopsies with PGN, indicates activation in at least part of the infiltrates. This finding is compatible with previous reports that only activated peripheral mononuclear cells and IL-2 stimulated T lymphocytes express PCNA (Moore et al., 1987; Hall et al., 1990), while a recent study indicates that PCNA expression in mononuclear infiltrates of renal grafts can be a reliable index of acute rejection (Thom et al., 1994).

Our data concerning proliferation of tubular epithelial cells and interstitial infiltrates may be correlated with the proposed role of tubulointerstitial processes in progression of renal disease probably through production of cytokines (Strutz and Muller, 1994; Strutz and Nielsen, 1994). Recent culture data, experimental findings in animal models and in renal biopsies of patients with PGN support the hypothesis that cytokines like PDGF, IL-6, and insulin-growth factor (IGF) exert a potent mitogen activity of mesangial cells in a paracrine or autocrine fashion through specific receptors (Gesualdo et al., 1991; Iida et al., 1991; Johnson et al., 1992; Sedor et al., 1993). It is also well known that stability and accumulation of PCNA mRNA and consequently increased synthesis of the protein are stimulated by growth factors, notably PDGF, without a necessary association with DNA synthesis (Hall et al., 1990). Thus, PCNA expression in mesangial cells in PGN may also reflect an autocrine or paracrine regulation mediated through growth factors like PDGF,

which are believed to participate in mesangial proliferation and can be produced by tubulointerstitial infiltrates, tubular epithelial cells or even mesangial cells.

As far as PCNA expression in NPGN is concerned, the frequency of PCNA expression in MGN was 34% being detected in occasional or few glomerular epithelial cells and in rare mesangial cells compared to the cases of PGN. Our observations are consistent with recent experimental studies in the passive Heymann nephritis rat model of membranous GN indicating PCNA expression in proliferating visceral glomerular epithelial cells which synthesize PDGF-ß-chain (Floege et al., 1993). On the other hand, previous studies employing Ki-67 as a cell proliferation marker have failed to demonstrate Ki-67 expression in cases of membranous GN (Hall et al., 1988). The discrepancy of the results may be due to the different methods and reagents used and particularly to the small number of cases studied by the last mentioned authors.

In conclusion, PCNA expression was observed more frequently in diseased than in normal human kidneys probably reflecting the upregulation of proliferating rates of glomerular and tubular epithelial cells in renal disease. The significant increase in the frequency of intraglomerular PCNA expression in cases of PGN compared to NPGN suggests that PCNA has a certain value in the assessment of mesangial proliferation. The increased PCNA expression in tubular epithelial cells especially in PGN indicates their proliferative state and may be correlated with their proposed activation and role in the progression of renal disease. Further investigation is necessary to elucidate the rates of intraglomerular proliferation and to investigate its correlation with the promoting effects of various cytokines.

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