Immunohistochemical Analysis of Cell Cycle for Podocyte

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ABSTRACT. Recently impairment of podocytes has been reported to be continuous with irreversible glomerulosclerosis. We examined the labeling incidences of podocytes (using the puromycin aminonucleoside nephropathy (PAN) and these rats given the fibroblast growth factor 2 (PAN+FGF2) models) and mesangial cells (using the anti-Thy1.1 mesangial proliferatied glomerulonephritis (Thy1.1 GN) model) using two markers (Bromodeoxyuridine (BrdU) and proliferating cell nuclear antigen (PCNA), to clarify differences in the cell cycle of podocytes and mesangial cells under pathological conditions. Our results showed no significant differences in BrdU-labeled and PCNA-positive podocytes were found in the PAN group, but BrdU-labeled mesangial cells to be more numerous than PCNA-positive mesangial cells in the Thy1.1 GN group. However, the incidence of PCNA-positive podocytes was higher than that of BrdU-labeled podocytes in the PAN+FGF2 group. These results suggest that no progression took place in the cell cycle of podocytes and that many podocytes appeared in the late G1 phase. The mechanisms of the inhibition of progression from the G1 phase to the S phase are unclear. These findings have led us to consider that the inhibition of progression to the S phase might be taken as a preventive mechanism of podocyte injuries.

Key words: podocyte — mesangial cell — cell cycle — glomerulosclerosis

Podocytes are unique cells with a complex cellular organization. Cytoarchitecturally, podocytes may be divided into a cell body, major processes and foot processes. They are never directly attached to the glomerular basement membrane by their cell body and major processes. Furthermore, podocytes are the most differentiated cell type in the renal glomerulus. Under pathological conditions, unique changes occur in their cell architecture, such as effacement of the foot process. Among the cells forming the glomerulus, they do not readily proliferate under normal conditions. On the other hand, the epithelial cells of Bowman's capsule (which have the same origin as podocytes during the embryonal period) show a comparatively higher replicative potential.[1-3] In addition, previous studies using rats with diabetic nephropathy,[4] unilateral and subtotal nephrectomy[5,6] or anti-glomerular capillary basement membrane (GBM) nephritis[7] have shown neither evidence of podocyte nuclear division nor of an increase in the number of podocytes per glomerular cross section. However, recent studies showing that podocytes undergo minimal mitosis with an increase in ploidy, suggest there is a failure

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to undergo cytokinesis after mitosis in the various kidney disease.[8,9] We examined the cell cycle analysis of podocytes using Bromodeoxyuridine (BrdU) and proliferating cell nuclear antigen (PCNA), to clarify in the cell cycle of podocytes under pathological conditions. Because of BrdU are cell proliferation markers that mainly recognize the S phase,[10] while PCNA is consistent with expression of DNA polymerase δ from the late G1 phase to the early G2 phase.[11,12] Accordingly, there is a slight difference in the cell cycle recognized by these two markers. Puromycin aminonucleoside nephropathy (PAN) and PAN+fibroblast growth factor 2 (FGF2) model rats were used as podocyte injury models in this study. PAN has been found to be a direct cytotoxic model that injuries podocytes. PAN model is characterized by nephrosis, cytoplasmic vacuolar formation and detachment from the GBM.[13,14] FGF2 has been found to be a proliferative effect on cultured glomerular epithelial cells,[15] furthermore expression of FGF2 and FGF2 mRNA are observed in the cytoplasm of podocytes in the PAN model.[16] In vivo study, the possibility that FGF2 plays a role in proliferous stimuli is suggested by these findings. Furthermore, comparison was made of cell cycle using mesangial cells injury model. As an experimental model of proliferative glomerulonephritis (Thy1.1 GN model) induced by an anti-Thy1.1 antibody.

MATERIALS AND METHODS

Animals

Male Wistar strain rats (Clea Japan Co., Osaka, Japan) weighing 110 g were used. The animals were housed in metabolic cages and given food and water ad *libitum*. All surgery and infusions were perfomed under general anesthesia with ether and additional injections of phenobarbital when necessary. This experiment was approved by the Animal Research Committee of Kawasaki Medical School (No. 97-068, 1997) and conducted according to the "Guide for the Care and Use of Laboratory Animals" of Kawasaki Medical School.

Disease models (Fig 1)

PAN and PAN+FGF2 groups: The PAN nephropathy model was produced by subcutaneous injections of PAN (1.5 mg/100 g body wt/day; Sigma Chemical Co., St. Louis, Mo, USA) into rats for five days.[13,14] After PAN injection, 10 μg of recombinant FGF2 (Intergen Co., NY, USA)[17] (N=4, PAN+FGF2 group) or an equal volume of physiological saline (N=4, PAN group) was injected for four days into the tail vein, and the animals were sacrificed on the day 25 after the start of PAN administration.

Thy1.1 GN group: The Thy1.1 GN model was induced by intravenous injection of anti-thymocyte plasma (0.25 mg/100 g body wt; clone: OX-7, Cedarlane Laboratories, Ontario, Canada)[18] into the Wistar rats (N=4, Thy1.1 GN group). The rats of this group were sacrificed on the days 2, 4 and 7 following the induction of disease.

Labeling method

The rats of these groups were injected intraperitoneally with 100 mg/100 g body wt of Bromodeoxyuridine (BrdU) (Sigma, St. Lous, Mo, USA), which

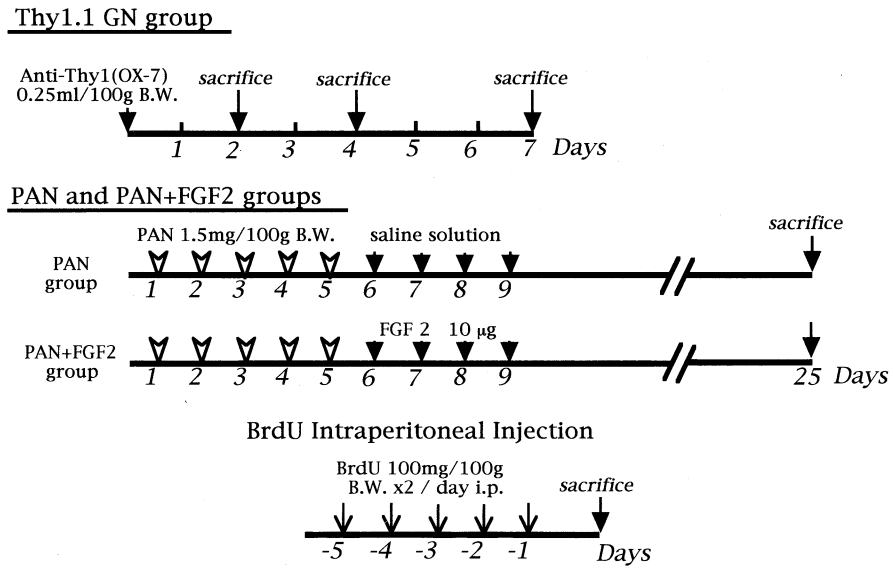


Fig 1. Experimental design. Outline of our experimental design showing various groups of rats studied.

was incorporated into DNA synthesizing nuclei, twice every day for five days before sacrifice in order to obtain high labeling indices.

Renal morphology

Rats were perfused with 4% paraformaldehyde (PFA) via the left ventricle, and both kidneys were fixed. After dehydration, the kidneys were embedded in paraffin and thin sections were prepared. After deparaffinization, hematoxylin eosin (H. E.) and periodic acid-Schiff (PAS) staining were performed on these sections, after which they were examined light microscopically.

Immunohistochemistry of BrdU

After deparaffinization, sections were treated with 0.3% hydrogen peroxidase in methanol for 30 minutes at room temperature to inactivate endogenous peroxidase. Then they were placed flat in moist chambers and flooded with 2 N HCl after incubation at 37°C for 30 minutes. Next, they were immersed in 0.1 M $Na_2B_4O_7$ for 30 minutes to neutralize excess HCl. Then they were washed in 0.02M phosphate-buffered saline (PBS, pH7.2), immersed in non-immunohorse serum for 30 minutes, washed again in 0.02M PBS (pH7.2), and reacted with anti-BrdU antibody (Becton Dickinson, Mountain View, CA, USA) for two hours at room temperature. After washing in PBS, the bound antibody was detected using the ABC kit (Vector Co., Burlingame, CA, USA). The bound antibodies were detected using 3,3-diaminobenzidine tetrahydrochloride (DAB) in Tris-buffered saline containing 0.02% hydrogen peroxide for 5 to 10 minutes. This substrate stained positively labeled cells brown. Following immunostaining, a periodic acid-Schiff (PAS) counterstain was used in combination with hematoxylin to enhance the overall histological appearance and to facilitate the localization of the BrdU-labeled nuclei. Double-immunostaining was also performed to distinguish mesangal cells from

macrophages, using OX-7 (data not shown). The number of BrdU-labeled cells with 50 glomeruli was counted.

Immunohistochemistry of PCNA

After deparaffinization PC10 (a cell proliferation marker) (Dako Co., Denmark), which is a monoclonal antibody to the proliferating cell nuclear antigen (PCNA), was used for immunoperoxidase staining of 4% PFA-fixed paraffin sections. The sections were treated with 0.3% hydrogen peroxidase in methanol for 30 minutes at room temperature to inactivate endogenous peroxidase. Then they were immersed in non-immunohorse serum for 30 minutes, washed in 0.02M PBS (pH7.2), and made to react with primary monoclonal antibodies for two hours at room temperature. After washing in PBS, bound antibodies were detected using the ABC kit (Vector Co., Burlingame, CA, USA), similar to the method used for BrdU. PCNA-positive cells with 50 glomeruli was counted.

Statistical analysis

All values were expressed as the mean$\pm$SD. Comparisons between groups were made using the Student's t-test. A value of 0.05 or less was considered significant.

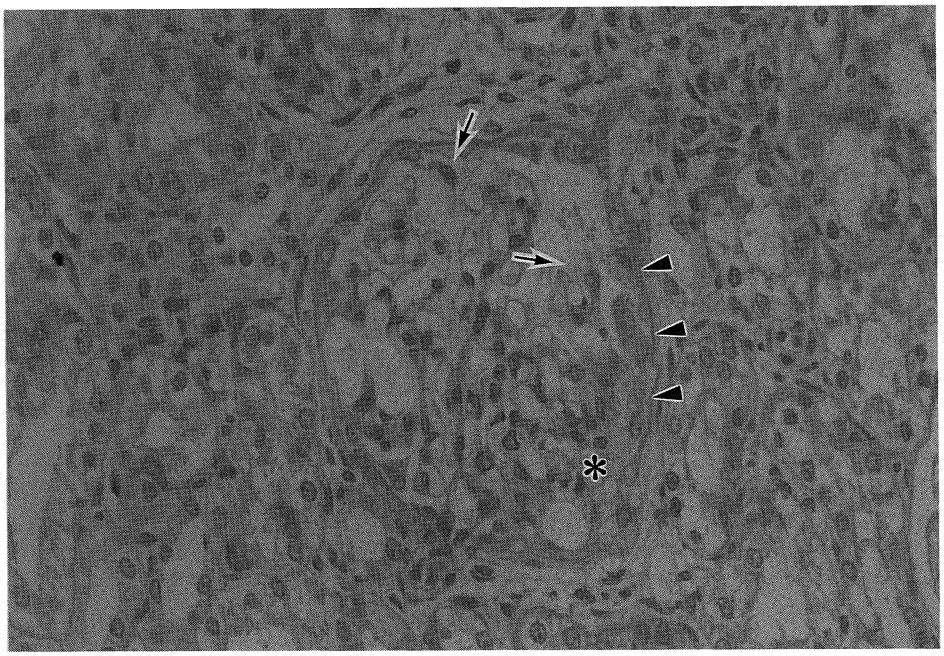
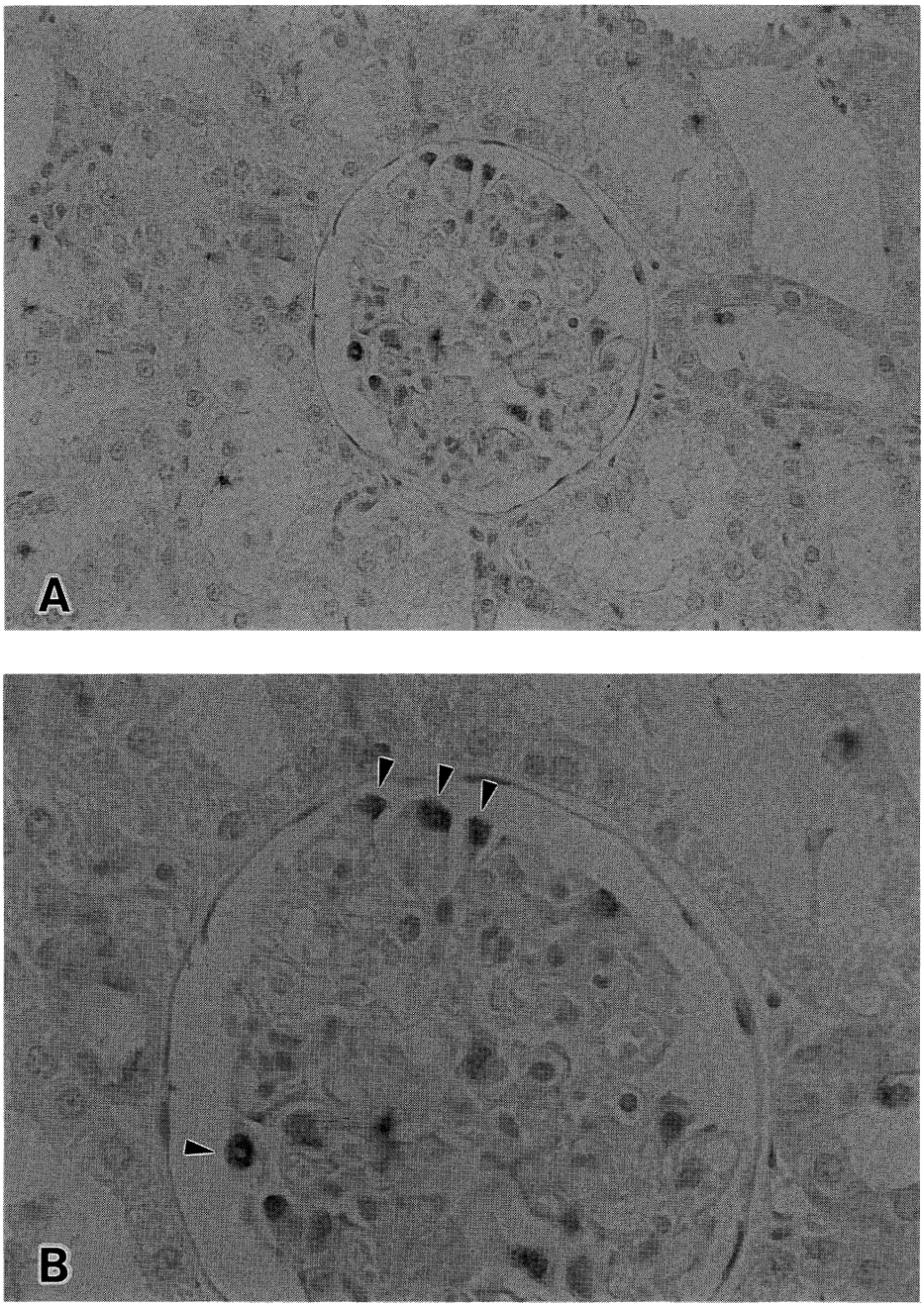


Fig 2. Morphological changes in the PAN+FGF2 rat glomerulus. Cytoplasmic vacuolar formation, accumulation of PAS-positive materials and detachment from the glomerular capillary basement membrane are present in podocytes (arrows); hypertrophic and proliferative epithelial cells of Bowman's capsule (arrowheads) with adhesive lesions (asterisks) can be seen in the PAN+FGF2 group rats. (PAS X400).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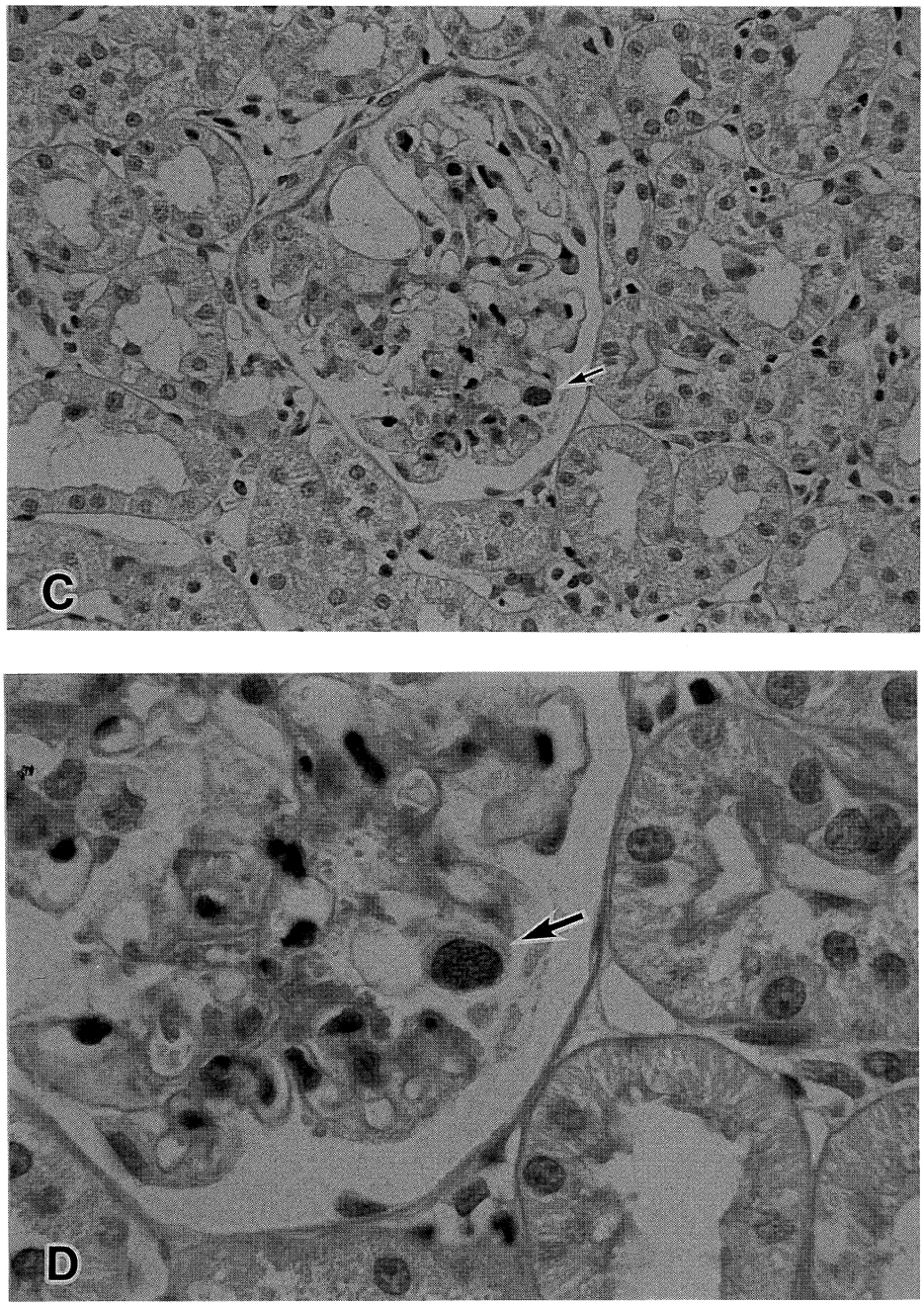
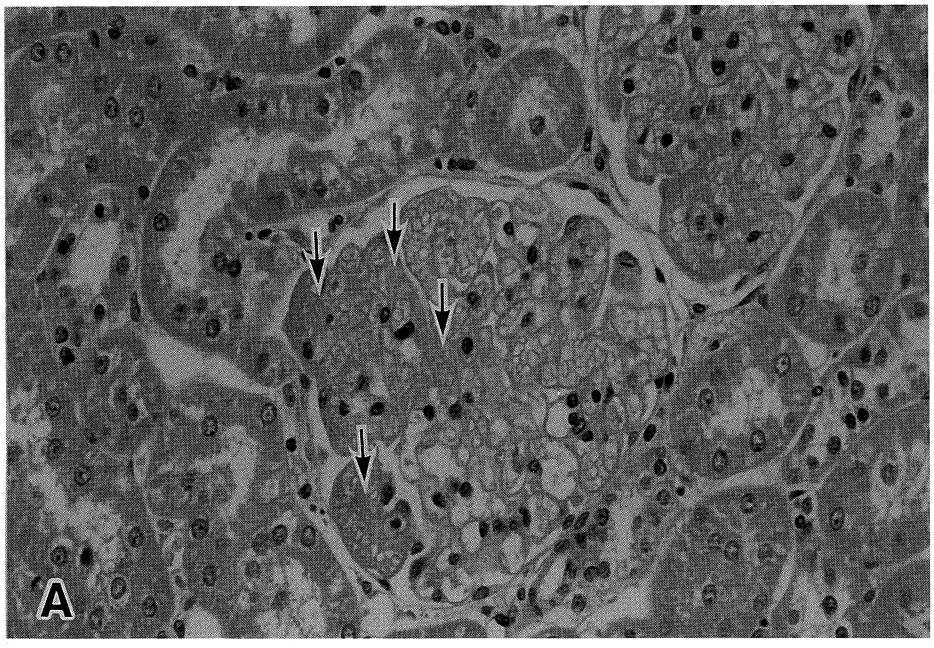


Fig 3. Immunohistochemical staining for PCNA and BrdU in the PAN+FGF2 rat glomerulus. A. B. The PCNA-positive glomerular cells are mainly podocytes. (arrowheads, A X400, B X800). C. D. The Brdu-labeled cell is a podocyte. (arrow, C X400, D X1000).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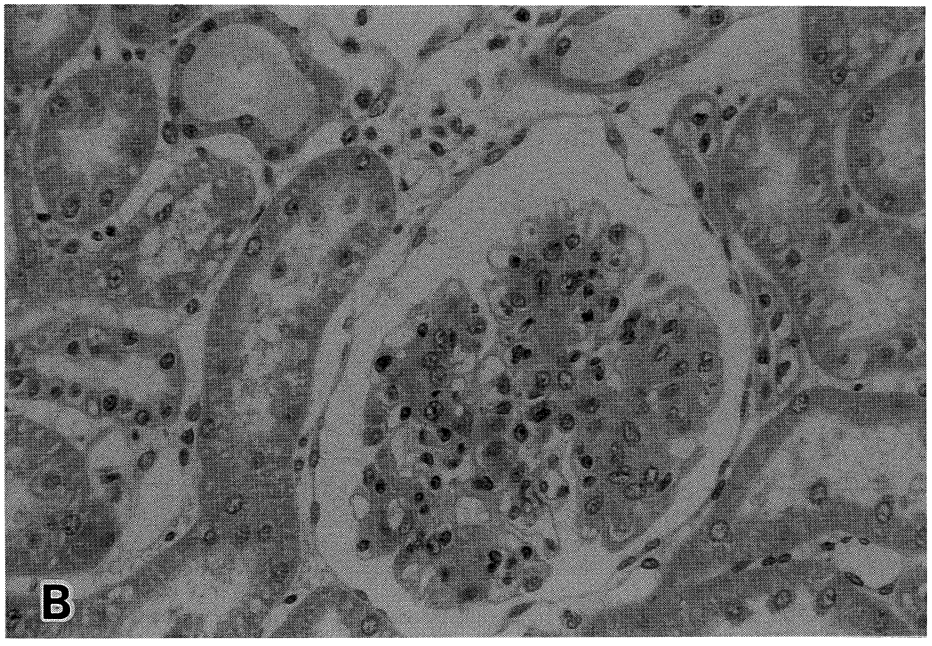
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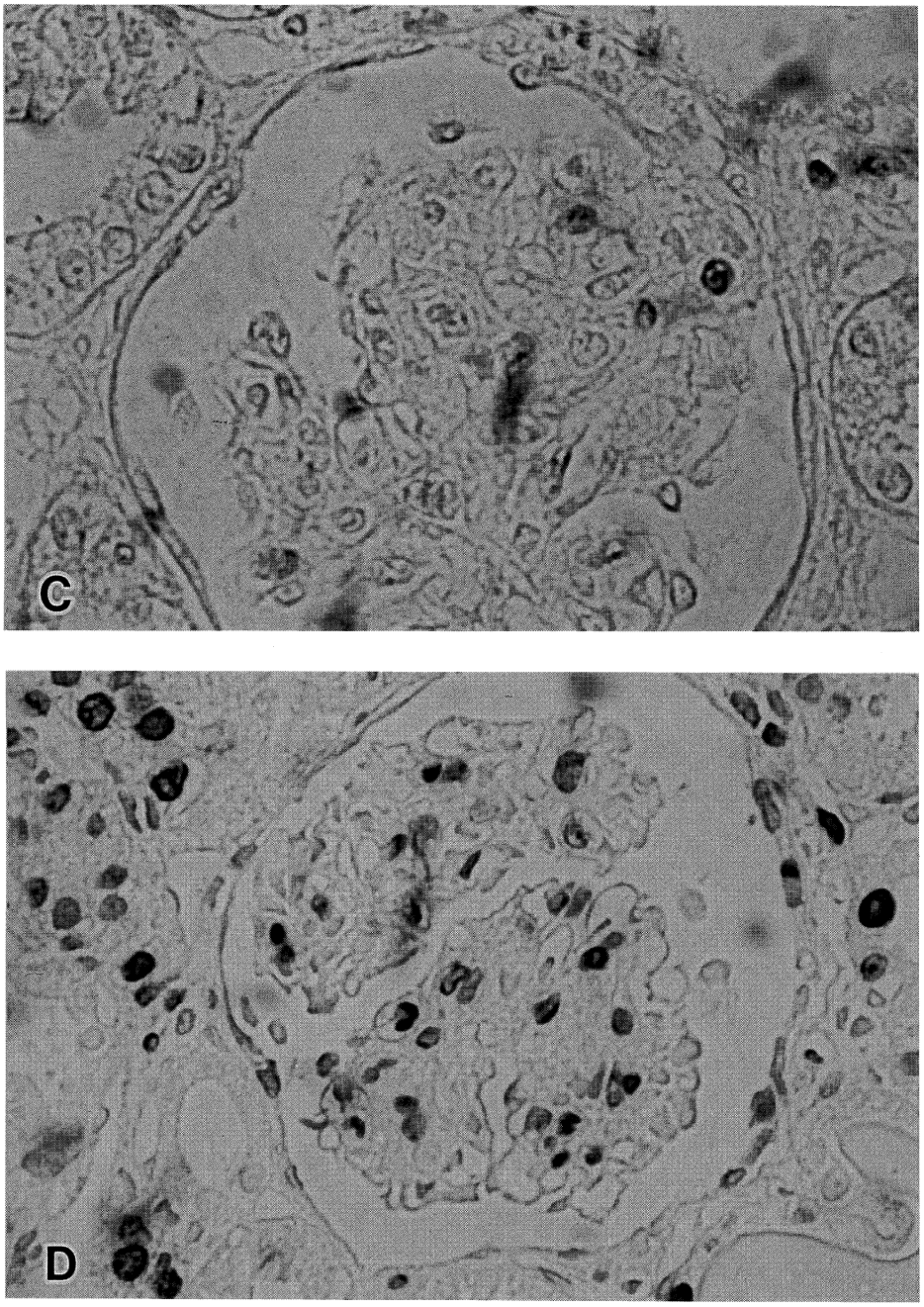

Fig 4. Morphological changes and immunohistochemical staining for PCNA and BrdU in the Thy1.1 GN rat glomerulus. A. Mesangiolytic lesions are shown on day 2. (arrows, H.E. X400). B. Glomerular cell (mainly mesangial cell) proliferation is shown on day 7. (H.E. X400). C. The PCNA-positive glomerular cells are mainly mesangial cells on day 7. (X600). D. The BrdU-labeled glomerular cells are mainly mesangial cells on day 7. (X600).

RESULTS

Renal morphology and immunohistochemistry :

PAN and PAN+FGF2 groups : Morphological changes in podocytes (such as cytoplasmic vacuolar formation and accumulation of PAS-positive materials) were detected in both groups, but more frequently in the PAN+FGF2 group. Other morphological changes in the glomeruli consisted of hypertrophy and proliferation of the epithelial cells of Bowman's capsule with adhesive lesions (Fig 2). PCNA-positive and BrdU-labeled podocytes were not detected in the normal rats (data not shown). However, PCNA-positive and BrdU-labeled glomerular cells were mainly podocytes in the PAN and PAN+FGF2 groups (Fig 3A, B, C, D). In the PAN+FGF2 group, PCNA-positive podocytes were easily detectable than those of the PAN group.

Thy1.1 GN group : Administration of the Thy1 antibody caused early mesangiolysis on days 1 and 2 (Fig 4A). This was associated with a reduction in the number of mesangial cells. Significant glomerular cell proliferation was observed between days 2 and 7. By day 7, the number of mesangial cells had maximally increased (Fig 4B), and PCNA-positive and BrdU-labeled glomerular cells were mainly mesangial cells on day 7 (Fig 4C, D).

Comparison of BrdU-labeled and PCNA-positive cells :

There were no significant differences between the numbers of BrdU-labeled and PCNA-positive podocytes in the PAN group. However, in the PAN+FGF2 group, there was a significant increase in PCNA-positive cells than those of the PAN group (Fig 5). Furthermore, PCNA-positive podocytes were more numerous than BrdU-labeled podocytes in the PAN+FGF2 group. BrdU-labeled mesangial cells were more numerous than PCNA-positive mesangial cells in the Thy1.1 GN group. (Fig 6).

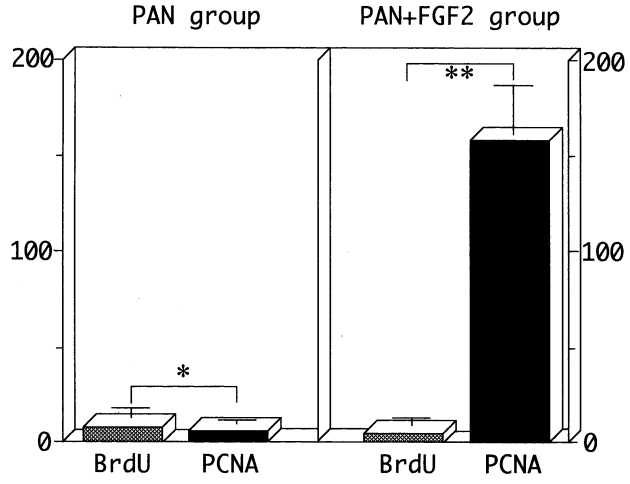

Fig 5. The number of BrdU-labeled and PCNA-positive glomerular cells per 50 glomeruli in the PAN and PAN+FGF2 groups. There was no significant difference between the incidence of BrdU-labeled podocytes and that of PCNA-positive podocytes in the PAN group. However, PCNA-positive podocytes were more numerous than BrdU-labeled podocytes in the PAN+FGF2 group. Data are the mean±SD. *not significant **$P<0.001$

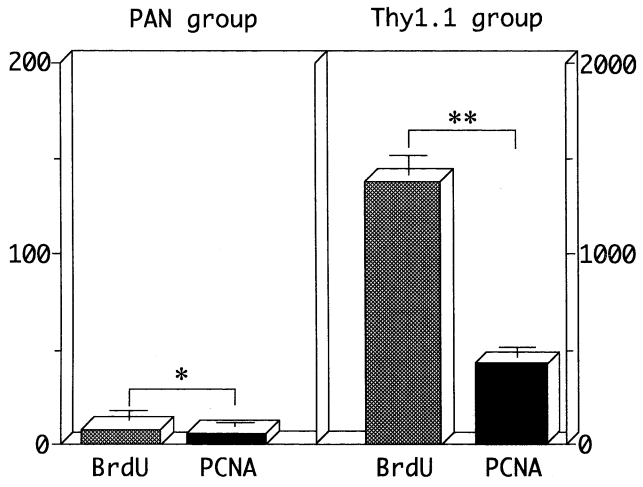

Fig 6. The number of BrdU-labeled and PCNA-positive glomerular cells per 50 glomeruli in the PAN and Thy1.1 GN groups. BrdU-labeled mesangial cells are more numerous than PCNA-positive mesangial cells in the Thy1.1 GN group. However, there was no significant difference between the incidence of BrdU-labeled podocytes and that of PCNA-positive podocytes in the PAN group. Data are the mean±SD. *not significant **$P<0.01$

DISCUSSION

In this study, the expression of each cell proliferation marker in PAN, PAN+FGF2 and Thy1.1 GN groups was studied. There were few BrdU-labeled podocytes in the PAN and PAN+FGF2 groups. However, there was a significant increase in PCNA-positive podocytes in the PAN+FGF2 group. These results may indicate that FGF2 has a proliferative effect on podocytes. The results of a recent study indicated a similar effect on cultured glomerular epithelial cells.[15] In contrast, there was a significant increase in the number of BrdU-labeled mesangial cells over that of PCNA-positive cells during the mesangial proliferative phase in the Thy1.1 GN group. Thymidine and BrdU are cell proliferation markers that mainly recognize the S phase,[10] while PCNA is consistent with expression of DNA polymerase δ from the late G1 phase to the early G2 phase.[11,12] Accordingly, there is a slight difference in the cell cycle recognized by these two markers. These results showed that the number of podocytes in late G1 phase increased, and that podocytes were few in the S phase. It is, therefore, suggested that podocytes, unlike mesangial cells, can enter the cell cycle with proliferative stimulation but cannot easily proceed from the G1 phase to S phase under pathological conditions.

Podocytes are highly differentiated and maintained during the G0 or G1 phase.[2] The mechanisms of the inhibition of progression from the G1 phase to S phase are unclear. In their study using an FGF2 long-term administration model, Kriz *et al*[8] reported that an increase in multinuclear podocytes aggravated the prognosis of glomerular impairment. With these findings considered together, the inhibition of progression to the S phase may be taken as one of the preventived mechanisms of podocyte injuries.

Progression through the cell cycle is controlled by a group of intricately linked nuclear proteins, and is dependent on phase specific cell cycle regulatory

protein interactions. A central role is played in regulation of the cell cycle by positive regulatory cell cycle proteins; i.e., cyclins and cyclin-dependent kinases (CDK), and by negative regulatory cell cycle proteins; i.e., cyclin kinase inhibitors (CKI). Furthermore, two families have also been confirmed; the CKI, CIP/KIP family (comprised of $p21^{SDI1/CIP1/WAF1}$, $p27^{KIP1}$ and $p57^{KIP2}$) and the INK4 family (comprised of $p16^{INK4a}$, $p15^{INK4b}$, $p18^{INK4c}$ and $p19^{INK4d}$). The CIP/KIP family has an inhibitory effect on transition from the G1 phase to the S phase.[19] Therefore, it would be necessary to examine the relationship between the cell cycle of podocyte and the CIP/KIP family.

In conclusion, the results of this study suggest that many podocytes appeared in the late G1 phase under pathological conditions. Furthermore, it is necessary to clarify the factors which inhibit progression of podocytes to the S stage.

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