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An ultrastructural study of the effect of the steroid in puromycin aminonucleoside nephrosis rats

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Summary. In order to investigate the significance of the histological change in glomerular epithelial cells in minimal change nephrotic syndrome in man (MCNS) and to help in clarifing the mechanism of action of a steroid in this disease, methylprednisolone was administered to rats with puromycin aminonucleoside nephrosis (PAN). This is an experimental nephrosis having a close resemblance morphologically and physiologically, to human MCNS. Morphological changes in the glomerulus were observed ultrastructurally. The administration of the steroid to PAN rats showed remarkable changes including, rapid disappearance of proteinuria in PAN rats in a manner similer to that seen in human MCNS, and significantly faster recovery of changes in glomerular epithelial cells when compared with spontaneous recovery. From the present study, it is clear that the steroid is effective in rapidly restoring the normal shape of glomerular epithelial cells in PAN rats. The filtration barrier in the glomerular capillary wall (GCW) is also thought to have recovered and proteinuria is cured. Based on these considerations, it may be suggested that proteinuria in human MCNS is caused by changes in glomerular epithelial cells, and that the clinical treatment of proteinuria in MCNS is effective when glomerular epithelial cells have functionally recovered.

Key words: Puromycin aminonucleoside nephrosis – Minimal change nephrotic syndrome – Corticosteroid – Glomerular epithelial cell

In the clinical treatment of nephrotic syndromes, corticosteroids have been widely utilized as the most effective drugs. Among various nephrotic syndromes, about 80% in children and 30% in adults are minimal change nephrotic syndrome (MCNS). The steroid is highly effective in this disease, and it enables us to induce remission in almost all cases. However, 2 out of 3 patients in remission are strongly dependent on the steroid, and reduc-

tion of the dose or the discontinuation of the steroid results relapse. Even though the steroid is indispensable for the treatment of MCNS, its mechanism of action is not clear. It is also unclear how proteinuria develops in nephrotic syndromes. Even in MCNS, in which morphological changes in the glomerulus are limited to the glomerular epithelial cells and inflammatory changes and immunological abnormalities are relatively minor, the mechanism of proteinuria is not fully understood. In human MCNS, however, the dysfunction of the charge barrier of GCW against serum proteins is thought to be responsible for proteinuria (Blau and Haas 1973; Robson et al. 1974; Carrie et al. 1981). In puromycin aminonucleoside (PAN) rats, the development of proteinuria has been shown to relate closely to morphological changes in glomerular epithelial cells and to the disappearance of the charge barrier (Michael et al. 1970; Andrews 1977; Bretton et al. 1980).

Thus, many characteristics of PAN in rats resemble those of MCNS in human. For the purpose of clarifying the mechanisms of action of the steroid in human MCNS, the steroid was administered to PAN rats, and the effect of the drug on the morphological change of glomerular epithelial cells was ultrastructurally observed in the present study.

Materials and methods

Twentytwo male Sprague-Dawley rats weighing 235–270 g, were used. Five rats were used as normal controls, and puromycin aminonucleoside (PA, Sigma Chemical Co St. Louis, MO, USA) dissolved in physiological saline in a final concentration of 0.5% was subcutaneously injected with a dose of 1.67 mg/100 g body weight to each of 17 rats for 9 consecutive days (Caulfield et al. 1976). Four rats among these 17 rats were sacrificed on the day after the final administration of PA to serve as PAN rat controls. Six rats out of remaining 13 rats (PA injected) were observed without no further treatment, and the rest (7 rats) were treated with a steroid.

Three rats and 2 rats out of untreated 6 PA rats were killed on the 7th and 14th days respectively after the termination of PA administration. (One rat in this group showed systemic deterioration during the course of observation, and was omitted from histological examination.)

Methylprednisolone (Medrol®; UpJohn Co.) was administered every other day with a dose of 3 mg/100 g body weight according to the method of the 'pulse' therapy (Cathcart et al. 1976; Cole et al. 1976). After methylprednisolone was administered 3 times and 7 times respectively, 4 rats were killed on the 7th day remaining 3 rats were killed on the 14th day, after the termination of PA. Urine specimens were semi-quantitatively examined every morning with use of Albustix® (Ames Co.).

All the animals were fixed by means of the perfusion method under Nembutal anesthesia. A vinyl catheter was inserted into the abdominal aorta at the level 1 cm above the iliac bifurcation, and the perfusate was introduced immediately after the aorta was clamped just above both renal arteries. Then both renal veins were cut off, and 100–150 ml of a rinsing solution (cacodylate buffer containing procaine-HCl and heparin, pH = 7.36–7.42, 320–346 mOsm) was injected at a pressure of 90 cm H_2O to wash out the blood from the kidney. The fixing solution consisted of 2.5% glutaraldehyde in cacodylate buffer (pH = 7.32–7.42, 330–353 mOsm) was then introduced for 7–8 min at a pressure of about 90 cm H_2O. When the infusion was carried out satisfactory the surface of the kidney changed in color to light brown. The infused kidney was excised as a whole, placed in the same fixing solution as described above, and cut into small blocks under a stereomicroscope. Tissue blocks were immersed in the fixing solution for additional 2 h, washed in ice-cold cacodylate buffer solution 40 min, then post-fixed with 1% OsO4 solution for 1 h.

After dehydration in cold ethanol series in a conventional manner, and passing through propylene oxide, the sample were embedded in a mixture of Epon and Araldite.

Sectioning of the tissue blocks was carried out by a Poter-Blum MT-1 ultra-microtome with glass knives or a diamond knife. The thin sections with silver to gray interferance color were mounted on copper grids without a supporting film, doubly stained with uranyl acetate and lead citrate, and examined with a Hitachi H-600 or H-500 electron microscope.

Results

Changes in proteinuria. The level of albumin in urine specimens from 5 normal rats was found to be $\pm \sim +$ (30–50 mg/dl) when it was tested with Albustix. In all 17 rats tested, urinary protein on the 9th day after the administration of PA was strongly positive, namely, 4 + by Albustix, which is 1000 mg/dl or more. Four rats out of these 17, which were fixed as the controls of PAN rats, were in an oedematous state. In all of 7 PAN rats which were given steroid, the urinary protein level started decreasing on the second day of steroid administration, and became $\pm - +$ on the 5th day after the first administration of steroid. In 4 rats out of these 7, steroid was administered 3 times every other day and they were then fixed. In all of these rats, the urinary protein was lower than +, and the oedema had subsided. The remaining 3 PAN rats, in whom the steroid was continued, showed their urinary protein level in the normal range even after 7th day from the start of steroid administration. However, 6 rats which received PA only continued to show $3+ - 4+$ urinary protein levels (300–1000 mg/dl or more) even on the 7th day after the administration of PA. Three rats of these 6 were subjected to fixation on the 7th day. At this stage, they showed marked oedema. One rat out of these 6 rats showed systemic deterioration, and was in a dying condition on the 8th day. Therefore, it was not examined histologically. The remaining 2 rats showed. Gradual reduction in urinary proteins, and the levels at the time of fixation (14th day) were + and 2+ respectively.

Histological results. As shown in Fig. 1, the renal glomerulus of a normal rat had orderly arranged endothelial cells, mesangial cells and epithelial cells. The foot processes of epithelial cells aligned regularly, and slit diaphragms were observed between the foot processes. The cytoplasm of epithelial cells is large, and Golgi vesicles and rough endoplasmic reticulum in the intracellular organelle were well developed. The glomerular capillary wall (GCW) was composed of the foot process of epithelial cells including the slit diaphragm, glomerular basement membrane (GBM) and endothelial cells having fenestrae. The lamina rara externa, lamina densa and lamina rara interna of the GBM were all clearly seen and the width of each lamina was constant throughout the entire lamina. Endothelial and mesangial cells had smaller cytoplasm and less developed intracellular organelles, compared with epithelial cells.

Four PAN rats were examined histologically. There was minimal proliferation and swelling of mesangial cells and endothelial cells, while epithelial cells showed a further increase in the size of the cytoplasm, and markedly developed lysosomes of various sizes were observed in the cytoplasm

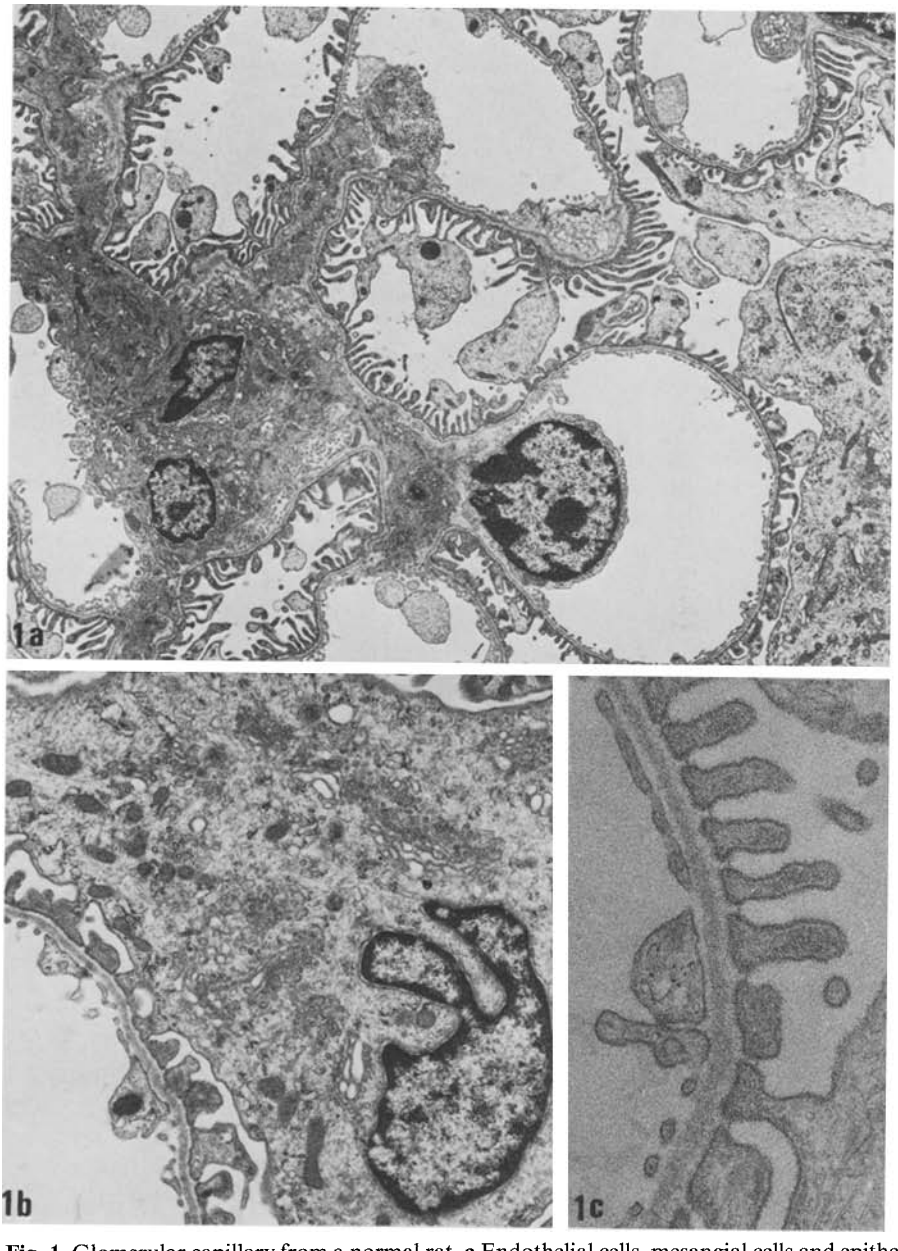

Fig. 1. Glomerular capillary from a normal rat. **a** Endothelial cells, mesangial cells and epithelial cells are arranged in an orderly fashion. The foot processes of the epithelial cells are aligned regularly. ×4,000; **b** The cytoplasm of the epithelial cells is large, and Golgi vesicles and rough ER in the intracellular organelles are well developed. ×10,800; **c** The slit diaphragm is clearly seen. The width of lamina rara externa, lamina densa and lamina rara interna of the GBM is constant throughout the entire lamina. ×29,600

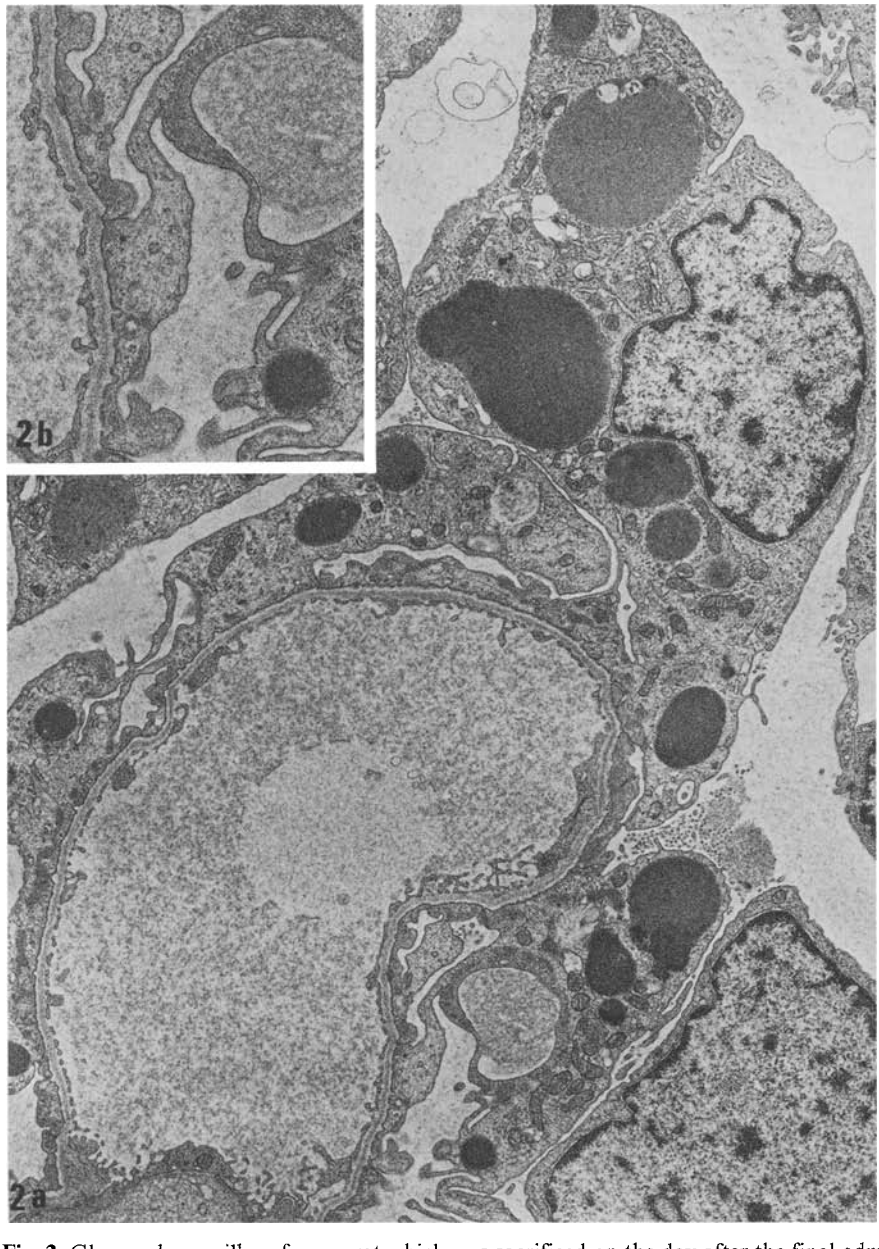

Fig. 2. Glomerular capillary from a rat which was sacrificed on the day after the final administration of PA. **a** The epithelial cells show a further increase in size of cytoplasm, and markedly developed lysosomes with various sizes are observed in the cytoplasm. Not only the slit diaphragm but also the foot processes have disappeared in all regions observed. × 6,800. **b** Pinocytotic vesicles are pronounced. GBM maintains its normal structure. × 11,600

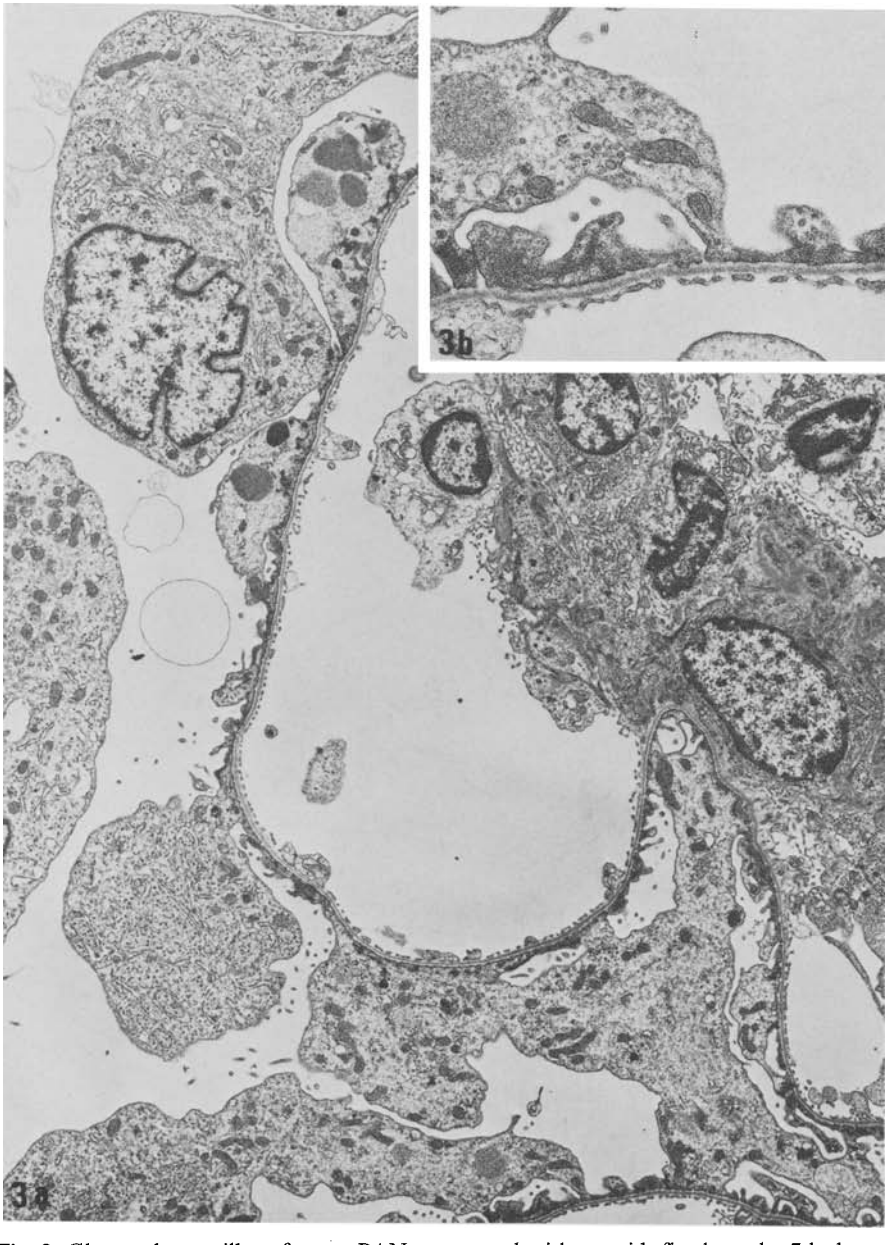

Fig. 3. Glomerular capillary from a PAN rat treated with steroid, fixed on the 7th day after the start of steroid administration. **a** Residual bodies are still present in some regions, but lysosomes are markedly decreased in number, and rough ER and Golgi vesicles increased. ×5,000. **b** The foot processes of the epithelial cells are reformed, though each of them is coarse and irregular, and there is a marked accumulation of microfilaments at such a site. ×11,400

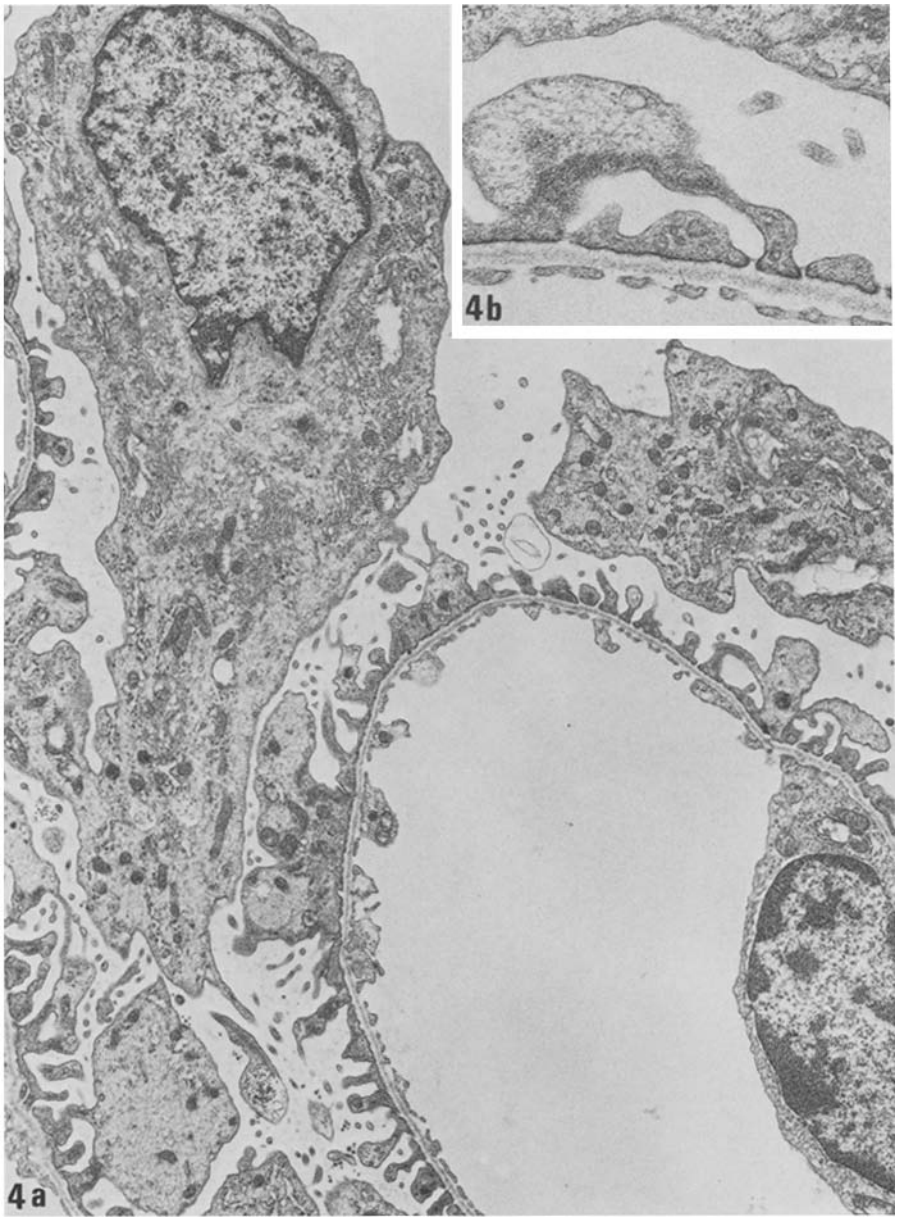

Fig. 4. Glomerular capillary from a PAN rat treated with the steroid, fixed on the 14th day after the start of steroid administration. **a** The foot processes are restored in almost all regions. Intracellular organelles in the epithelial cells also organized in a manner similar to those in the normal cells. ×6,800. **b** The slit diaphragm has also reappeared and GBM does not show morphological changes at this stage. ×14,500

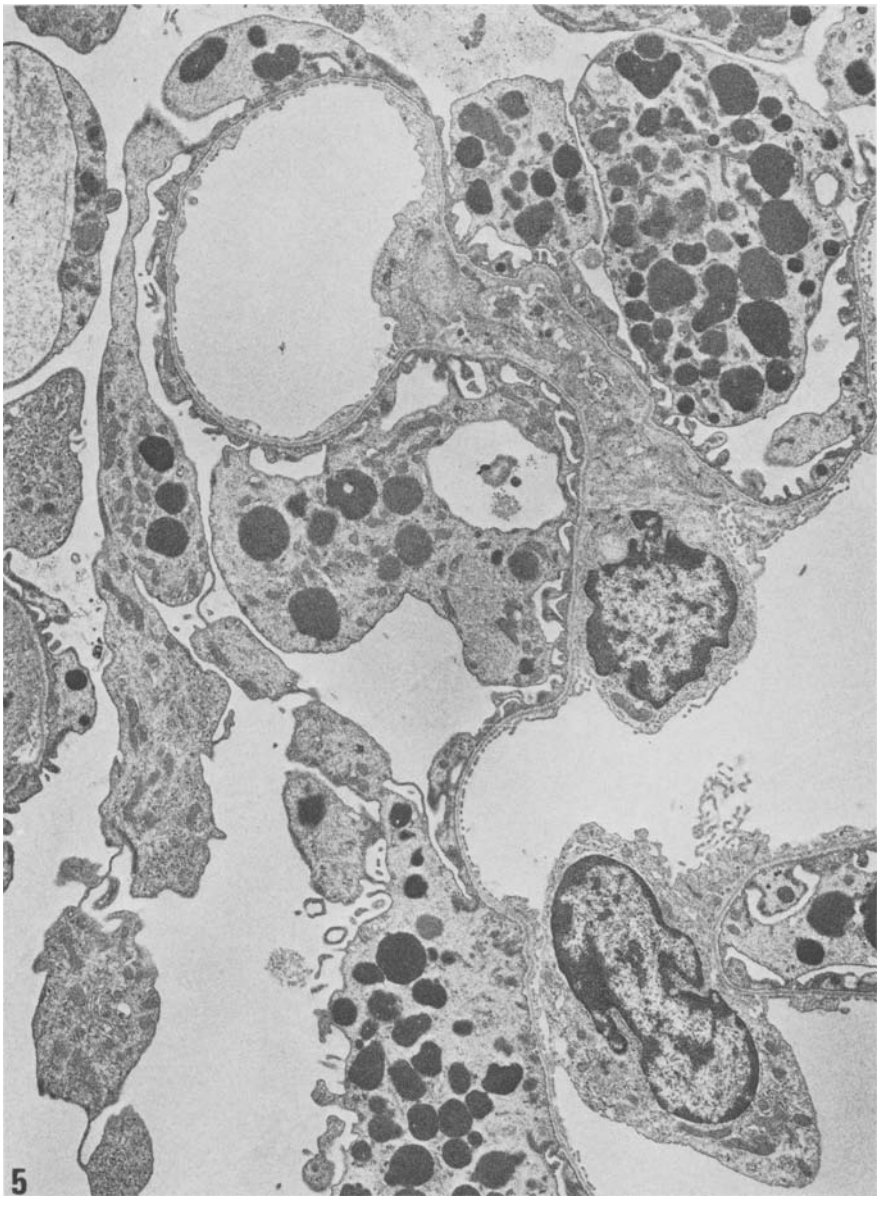

Fig. 5. Glomerular capillary from a PAN rat to which no steroid was given for 7 days after PA administration. The epithelial cells still contain many lysosomal systems and vacuolizations. The majority of the foot processes of the epithelial cells remained fused. × 5,700

(Fig. 2). Vacuolization and lysosomal cupture (in some regions) were also observed. Not only the slit diaphragm but also the foot process had disappeared in all regions observed. At the site where the foot processes was absent and the GBM was adjacent, a microflament-like substance had accumulated, the electron density being high in this region. Pinocytotic vesicles-

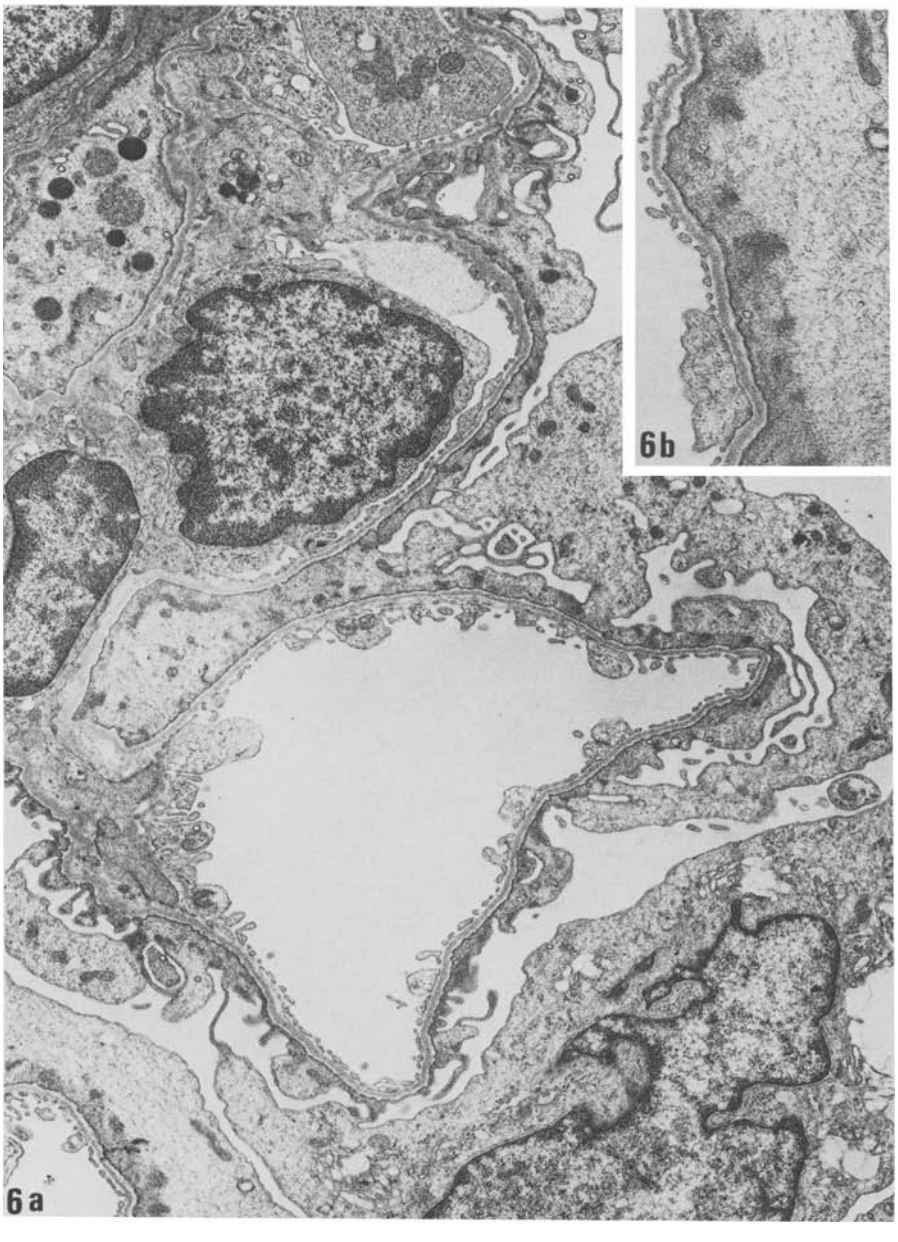

Fig. 6. Glomerular capillary from a PAN rat without steroid administration, fixed on the 14th day after PA injections. **a** Epithelial cells with and without foot processes are found to co-exist. Lysosomes and residual bodies are sporadically observed in the cytoplasm, and Golgi vesicles have reappeared. ×6,600. **b** The region where no foot processes are present is associated with marked accumulation of microfilaments, and the electron density is high in a band shape along GBM. ×11,400

like concavities were pronounced also in this region. Detachment of epithelial cells from the GBM was not observed. GBM maintained its normal structure and changes such as winding, splitting, thinning, thickening, lamination, and the deposit of abnormal substances in GBM were not observed.

In the PAN rats treated with the steroid, such changes in epithelial cells as those in PAN rats had markedly improved on the 7th day after the start of steroid administration as exemplified in Fig. 3. Residual bodies were still present in some regions, but lysosomes markedly decreased in number, and rough ER and Golgi vesicles increased. The foot process of epithelial cells reformed, though each of them was coarse and irregular, and there was a marked accumulation of microfilaments at such a site. Endothelial cells, mesangial cells and GBM were normal.

As shown in Fig. 4, on the 14th day after the start of steroid administration, the foot process was restored in almost all regions, and the slit diaphragm also reappeared. Intracellular organelles in the epithelial cells also regained an appearance similar to those in the normal cells. Endothelial and mesangial cells and GBM did not show morphological changes at this stage.

In the PAN rats to which no steroid was given for 7 days after PA administration, there was no marked change in the endothelial and mesangial cells of the glomerulus, while the epithelial cells still contained many lysosomal systems and vacuolizations (Fig. 5). In some of the regions where foot processes were absent and GBM was adjacent, microfilaments had accumulated showing high electron densities. The basic structure of GBM was not disturbed.

On the 14th day after PA administration, where no steroid had been given, the slit diaphragm was not formed, but epithelial cells with and without foot processes were found to co-exist (Fig. 6). The region where no foot process was present was associated with marked accumulation of microfilaments, and the electron density was high in a band shape along GBM. Lysosomes and residual bodies were sporadically observed in the cytoplasm, and Golgi vesicles, which were not observed in PAN rats on the next day after PA administration or in PAN rats on the 7th day after PA administration without giving the steroid, were observed in the cytoplasm. Even at this stage, there was no marked change in the endothelial and mesangial cells or GBM.

Discussion

The development of proteinuria in the nephrotic syndromes has been thought to result from the destruction of the filtration barrier of GCW to serum proteins. This filtration barrier of GCW is composed of two barrier systems, namely the size barrier whose function is determined by the effective pore size of GCW and the molecular radius of serum albumin, and the charge barrier in which the electrical repulsion between the anionic sites of GCW and negative charges on serum albumin is crucial. Since the effective pore size of the size barrier in GCW is larger than the molecular radius

of serum albumin, the electrical repulsion between negatively charged serum albumin and negatively charged GCW has attracted attention (Chang et al. 1975; Bennet et al. 1976; Seiler et al. 1977). Reduced electrical charges in GCW have also been reported in nephrotic patients and the rat (Deen et al. 1980).

The anionic sites of the charge barrier are composed primarily of sialic acid and glycosaminoglycans (GAGs) (Kanwar 1979; Kanwar 1980a), the former is distributed in the cell coat and slit diaphragm of epithelial cells and in the lamina rara externa of GBM, and the latter is in the lamina rara externa and interna of GBM.

Sialic acid has a strong negative charge due to the polarity of a carboxylic group, and is also one of the constituents of the epithelial cell coat which is thought to be necessary for the fixation of epithelial cells to GBM and for the maintenance of special structures such as the foot process of epithelial cells (Kanwar et al. 1980a). The function of GCW as the charge barrier is thought to be largely dependent upon this polyanion (Latta et al. 1975), since the treatment of sialic acid with neuraminidase induces the reduction of anionic sites with massive proteinuria (Andrews 1979; Kanwar et al. 1980b). However, the foot process and slit diaphragm of epithelial cells were not morphologically changed by treating GAGs with GAGs-degrading enzymes such as heparinase (Rosenzweig et al. 1982). Therefore, it is difficult to consider the decrease of GAGs as the major cause of nephrosis when the importance of epithelial cells is taken into consideration as described later.

The PAN rat is the experimental nephrosis model first reported by Frenk (1955). It was confirmed that systemically administered PA acts directly on the kidney within a short period of time (Hoyer et al. 1972). Morphological changes in renal tissues in PAN rats are limited to the epithelial cell but biochemical changes in the composition of epithelial cell membranes are known to occur before morphological changes and appearance of proteinuria (Bhuyan et al. 1980). The blockage by PA of protein synthesis in the epithelial cell is thought to be responsible for such biochemical changes (Studzinski et al. 1980).

The permeability of GCW to anionic horse raddish peroxidase (HRP), an enzyme having a slightly smaller molecular radius than serum albumin (36 Å), is extremely low in normal condition, while it increases 18.5 times in PAN rats. The effective pore size for neutral dextran with a molecular radius smaller than 36 Å, however, is known to be decreased in PAN rats (Olson et al. 1981). The charge of GCW in normal is 120–170 mEq/L, while it is 100 mEq/L in PAN rats (Deen et al. 1980). It is conceivable, therefore, that the anionic sites are disturbed by abnormal changes in epithelial cells in PAN rats, and such changes lead to marked leakage of serum albumin.

In human MCNS, morphological changes in the glomerulus are limited only to epithelial cells, and inflammatory findings such as cell proliferation and infiltrating changes are rare. In these points, human MCNS is clearly distinct from nephrotic syndromes associated with nephritis or systemic diseases. As for the filtration barrier of GCW to serum albumin, the effective

pore size for serum albumin or other substance having a molecular radius smaller than that of serum albumin was reported to be decreased in human MCNS (Robson et al. 1974). In spite of such a small pore size, a large amount of albumin is leaked into the urine. This is due to the reduction of electrostatic interaction to serum albumin, namely the decrease of sialic acid in the cell coat and slit diaphragm of epithelial cells is thought to be responsible for the leakage of albumin (Carrie et al. 1981). In the normal human, the charge of GCW is 140–220 mEq/L, while it is confirmed to be decreased to be 60–90 mEq/L, in human MCNS (Bridges et al. 1982).

As described above, the PAN rat shares many morphological and physiological characteristics with human MCNS.

Since glomerular epithelial cells do not show morpholoical changes in proteinuria induced by hyperproteinaemia, the epithelial abnormalities in nephrotic syndromes associated with morphological changes in the epithelial cell are thought to be a primary change (Andrews 1977). As the epithelial cell has a characteristic and highly active structure (Haynes 1981), it may be thought to be easily affected by drugs such as PA. As stated above, cytochemical changes in the composition of epithelial cell membranes precede either morphological changes or the development of proteinuria. Moreover, it has also been observed that morphological changes in the rough endoplasmic reticulum of the epithelial cell also precede the onset of proteinuria, and proteinuria disappears after the morphological changes are returned to normal (Bretton et al. 1979). Dysfunction of the epithelial cell results in failure of not only the morphological maintenance of the cell itself but also the functional maintenance of the filtration barrier to serum albumin, which is present in the cell membrane and GBM. Nephrotic syndromes are thought to be developed by these process. It is also conceivable that proteinuria is improved only after the filtration barrier in GCW is restored to be normal. Similar phenomena may be also observed in human MCNS and the role of epithelial cells in steroid therapy is also highly significant.

It is known that the administration of a steroid to PAN rats causes a change in the content of sugars in GBM and the permeability of GCW to proteins is consequently reduced (Misra et al. 1972). However, as far as I am aware, there is no report in which the process of steroid induced recovery of morphological changes in PAN rats is described. 'Pulse' therapy with steroids has been widely utilized for treating various nephrotic syndromes (Cole et al. 1975; Webel et al. 1972). Particularly in the case of human MCNS, the effect of the steroid appears within several days after its administration. In the present study, a steroid was administered to PAN rats by 'pulse' therapy in order to prevent side effects due to long-term administration of steroid.

Although various changes observed in PAN rats in the present study were reversible, proteinuria persisted for more than 3 weeks, and morphological changes lasted for more than 28 days, when PAN rats were left untreated. On the 7th day after PA administration, proteinuria was still at an advanced stage, glomerular epithelial cells had no foot processes,

and well developed lysosomes filled the cytoplasm. Proteinuria did not disappear even on the 14th day after PA administration when foot processes were partly restored, but the majority of histological changes were still unimproved. On the other hand, proteinuria subsided on the 5th day after the administration of the steroid to PAN rats. Histological observations on the 7th day showed that the numbers of Golgi vesicles and rough ER in the epithelial cytoplasm were increased, and also that previously abolished foot processes were on the way to re-formation, though they were still in imperfect shapes. In such regions, microfilaments were densely accumulated. On the 14th day after the commencement of steroid administration, some foot processes were still lacking, but almost all epithelial cells were restored to normal.

These results indicate that the steroid is as effective in PAN in rats as in human MCNS. It has been confirmed that the steroid not only rapidly cured proteinuria but also quickly normalized morphological changes in the epithelial cell in PAN rats. Since only the morphological changes of epithelial cells were improved during a recovery period, and since the GBM maintained its basic normal structure throughout the observation period, the importance of the epithelial cell in the repair mechanism for PAN in the rat was re-confirmed in the present study. In other words, changes in the slit diaphragm and the foot process of the epithelial cell were thought to be more closely related to the pathogenesis of proteinuria than the morphological changes in the GBM. Based on the results reported by other investigators, this may be through to be synonymous with the reduction of anionic sites (Sialic acid) in epithelial cell membranes, particularly in the foot process adjacent to GBM and in the slit diaphragm. It may be inferred that the steroid improves proteinuria in PAN rats by restoring the chemical properties and the morphological structure of epithelial cell membranes. Considering the close resemblance between PAN rats and human MCNS, the high clinical efficacy of the steroid in human MCNS may also be thought to be mediated by the functional recovery of the glomerular epithelial cell which is responsible for the maintenance of the function of the filtration barrier of GCW.

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