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Ultrastructural changes in glomerular epithelial cells in acute puromycin aminonucleoside nephrosis: a study by high-resolution scanning electron microscopy

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Abstract. Ultrastructural changes in the podocytes were studied during the development of, and recovery from, acute puromycin aminonucleoside (PAN) nephrosis using high-resolution scanning electron microscopy (hrSEM) and transmission electron microscopy (TEM). In the process of development of PAN nephrosis, four types of early structural changes were observed before total loss of foot processes: formation of cytoplasmic blebs, masking of filtration clefts, flattening of foot processes, and retraction of foot processes. The masking of filtration clefts visualized by hrSEM corresponded to the multiplication of slit diaphragms and adhesion of foot processes in the TEM findings, and preceded retraction of the foot processes. Changes of podocyte configuration were produced. Recovery from this change of podocyte configuration began as islands of podocyte interdigitation, and was proceeded by expansion of the islands. During recovery, the primary processes were re-established either by retraction or perforation of the thin cytoplasm after the formation of foot processes. We conclude that loss of foot processes begins with the masking of filtration clefts. Recovery from the change in podocyte configuration begins with the formation of new foot processes.

Key words: Podocyte – Foot process – Puromycin aminonucleoside – Scanning electron microscopy

Introduction

Puromycin aminonucleoside (PAN) nephrosis is an experimental model which simulates minimal change disease. Administration of PAN to rats results in severe proteinuria, accompanied by marked morphological changes in podocytes. The nephrotic syndrome in this experimental model appears to result from a direct toxic effect of PAN on the glomerular podocytes (Andrews 1977; Fishman and Karnovsky 1985).

Many investigators have demonstrated alterations of the podocytes in acute PAN nephrosis by transmission (Farquhar and Palade 1961; Venkatachalam et al. 1969; Ryan and Karnovsky 1975; Ryan et al. 1975b; Caulfield et al. 1976) and scanning electron microscopy (Arakawa 1970; Arakawa and Tokunaga 1972; Caroll et al. 1973; Andrews 1977; Messina et al. 1987; Andrews 1988) or freeze fracture techniques (Ryan et al. 1975a). These revealed flattening of foot processes, modification of slit diaphragms, retraction of foot processes and formation of cytoplasmic blebs. However, the reports have not illustrated the chronological changes in podocyte configuration. Furthermore, some investigators have reported that podocyte detachment from the glomerular basement membrane (GBM) was observed in PAN nephrosis,

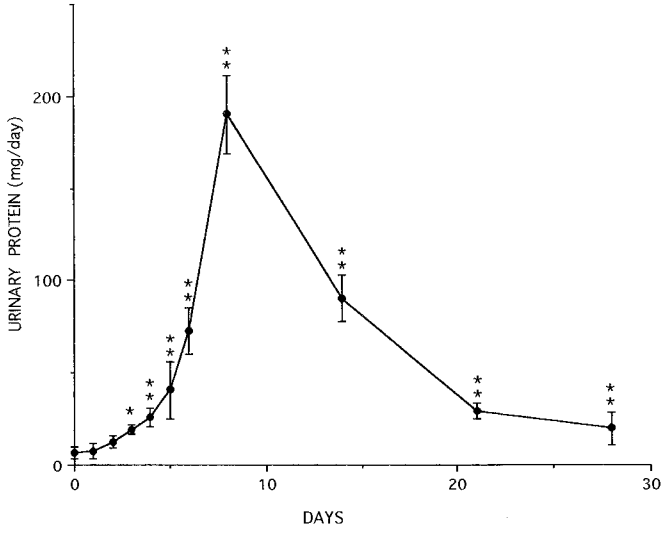

Fig. 1. Urinary protein excretion in puromycin aminonucleoside-induced nephrotic rats. Means ± SD are indicated. $*P<0.05$, $**P<0.01$

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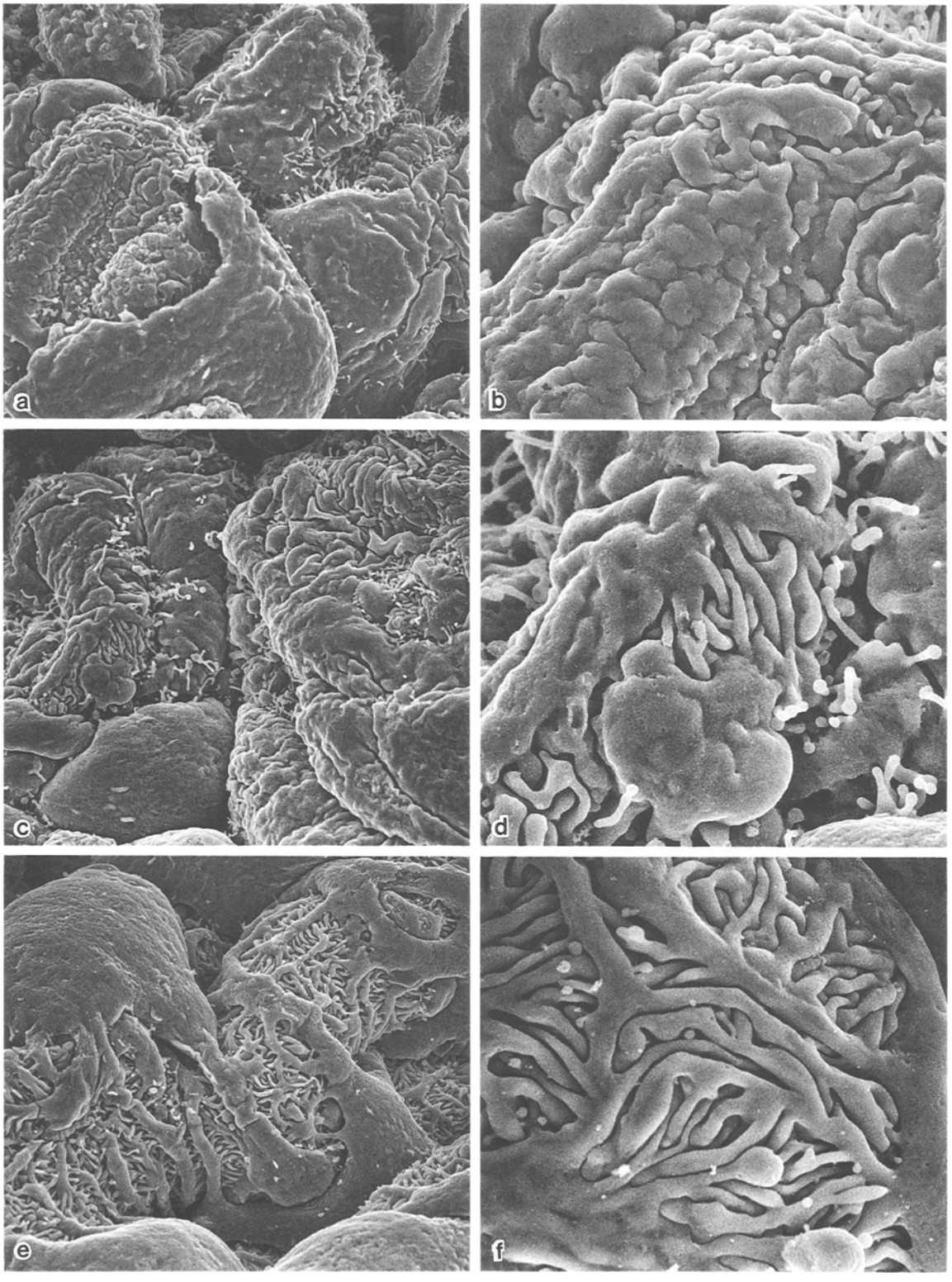

Fig. 2. Scanning electron micrograph of glomeruli in nephrotic rat on day 8 (**a, b**), 14 (**c, d**) and 28 (**e, f**), showing the urinary surface of podocytes. **a, b** The glomerular surface on day 8 is covered by irregular shaped podocytes which lost interdigitating primary and foot processes. The cell body area with relatively smooth surface and peripheral area with ragged surface can be distinguished. However, the cell border is difficult to distinguish from cell folds on the ragged surface; **a** × 3,000; **b** × 10,000. **c, d** On the glomerular surface on day 14, islands of interdigitating fields are occasionally found, whereas the other area appears similar to that on day 8. In the interdigitating field slender foot processes are separated from each other by deep filtration clefts, **c** × 3,000; **d** × 10,000. **e, f** The podocytes on day 28 restored primary processes as well as interdigitating foot processes almost totally. The foot processes are slender and high, but show occasional irregular thickening. The filtration clefts are deep. The primary processes are occasionally fused with each other to form a loop; **e** × 3,000; **f** × 10,000

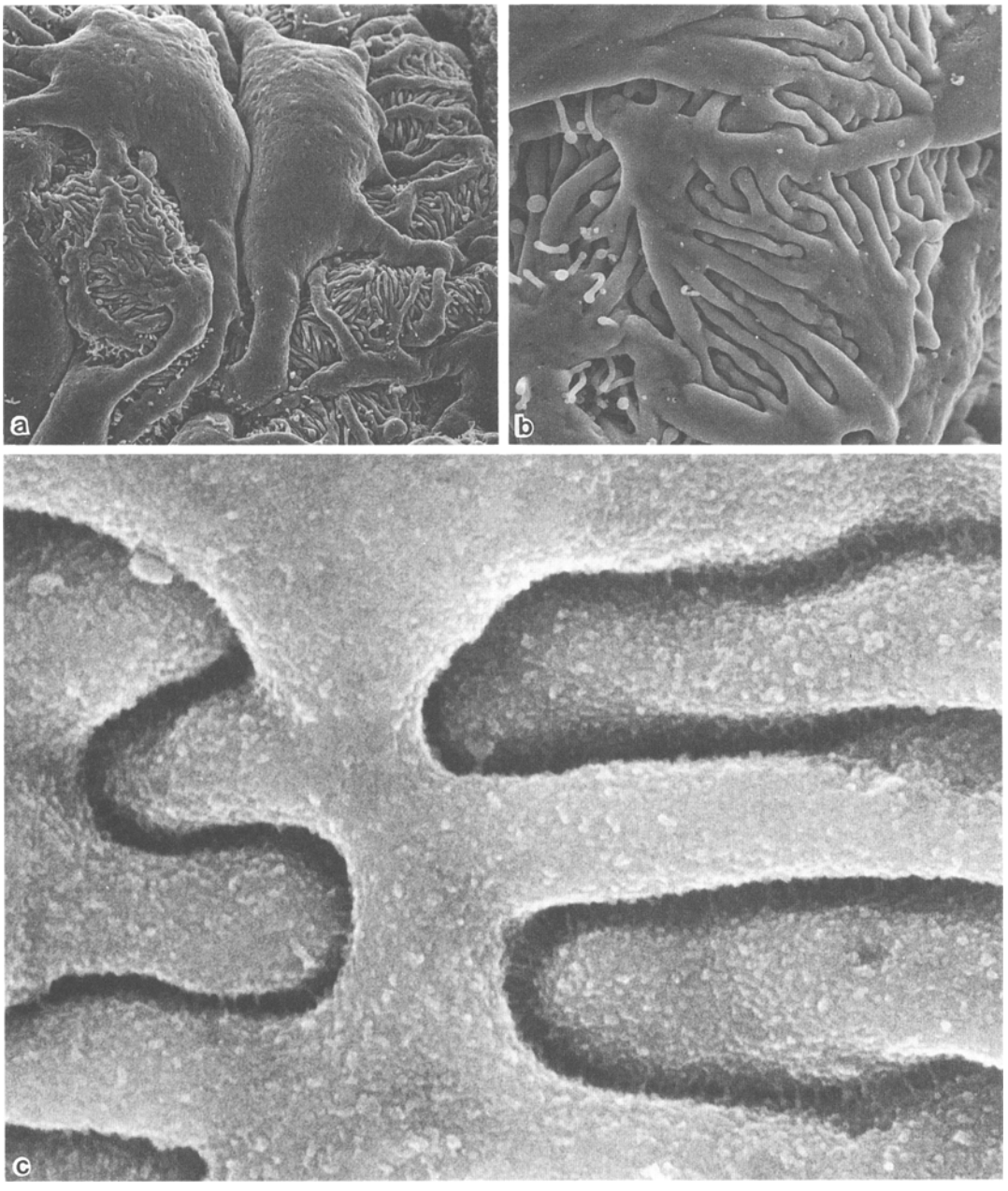

Fig. 3a–c. Scanning electron micrographs of glomeruli in the control rats, showing the urinary surface. **a, b** Podocytes send numerous foot processes which interdigitate with those of neighbouring podocytes **a** × 3,000; **b** × 10,000; **c** The filtration clefts between foot processes are deep and regular in width. Fine fibrillar structures are regularly bridged in the filtration clefts, × 100,000

causing massive proteinuria (Ryan and Karnovsky 1975; Messina et al. 1987; Whiteside et al. 1989). The chronology of foot process changes resulting in podocyte detachment from the GBM has not been described.

In the present study, we investigated the nature and time course of the glomerular podocyte structural change associated with acute PAN nephrosis using high-resolution scanning electron microscopy (hrSEM) and compared the findings with those of transmission electron microscopy (TEM).

Materials and methods

Twenty male Spraque-Dawley rats weighing about 200 g each (Charles River Japan, Kanagawa, Japan) were used. Rats were placed in metabolic cages for acclimatization 5 days before the experiments started. They had free access to standard chow and water. Urine samples were collected every 24 h, and urinary protein excretion was measured daily by Coomassie brilliant blue G 250 (Tonein TP kit; Otsuka Pharmacy, Tokyo, Japan).

Eighteen rats were made nephrotic by a single intraperitoneal injection (15 mg/100 g of body weight) of PAN (Sigma, St. Louis, Mo). Two control rats were given saline. PAN nephrotic rats were sacrificed on days 2, 4, 8, 14, 21 and 28. The two control rats were sacrificed on the 6th day.

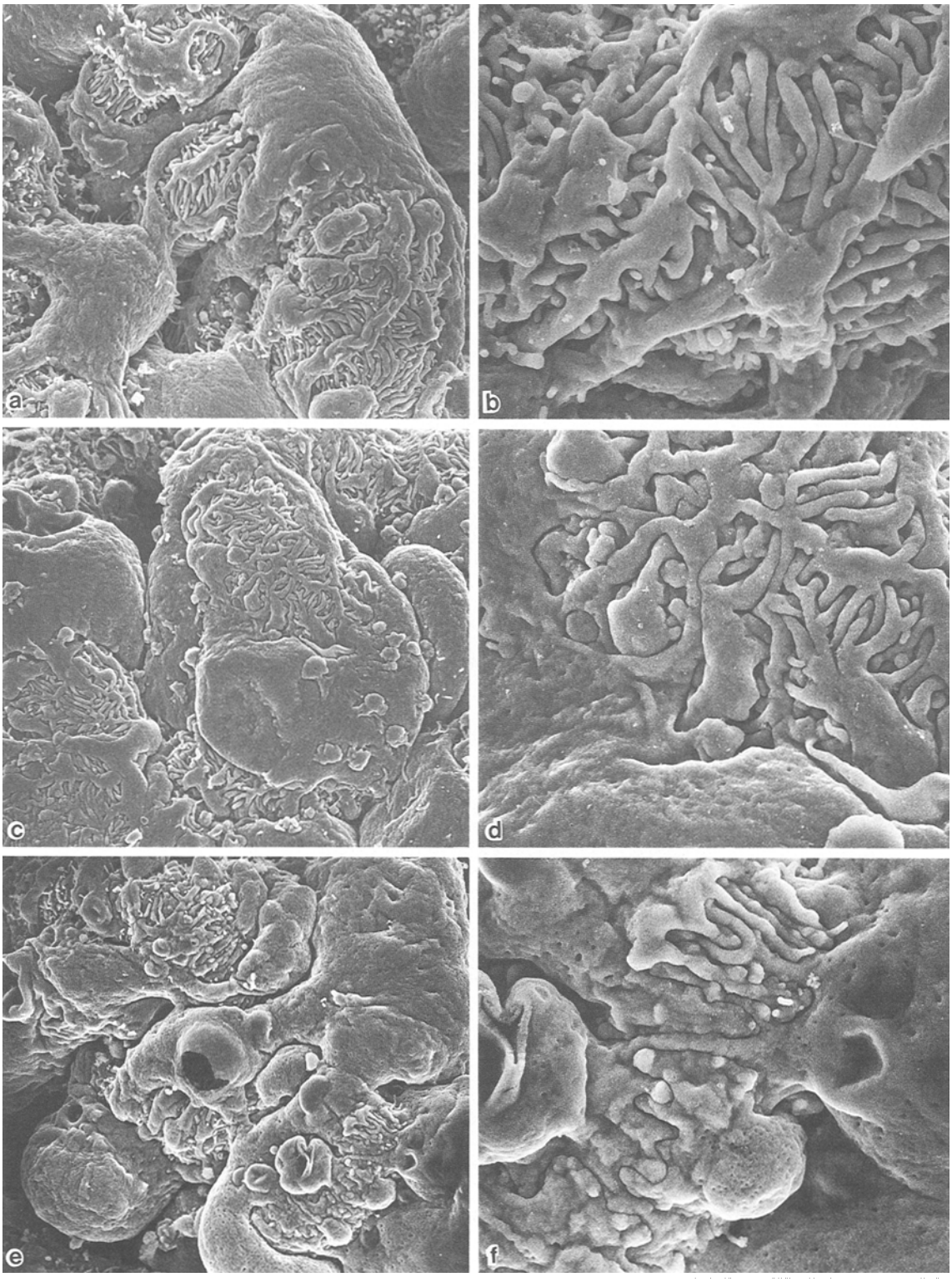

Fig. 4. Scanning electron micrographs of glomeruli in puromycin aminonucleoside-induced nephrotic rats on day 2 (**a, b**) day 3 (**c, d**), and day 4 (**e, f**), showing the urinary surface of podocytes. **a, b** The podocytes on day 2 possess some oval or round cytoplasmic blebs of various sizes on the primary processes. The foot processes occasionally show irregular thickening; **a** × 3,000; **b** × 10,000. **c, d** Foot processes of the podocytes on day 3 become slightly flattened. A number of cytoplasmic blebs are found on the podocytes; **c** × 3,000; **d** × 10,000. **e, f** Podocyte cell bodies and processes on day 4 possess more numerous blebs than in **c.** Parts of cytoplasmic blebs are broken. The foot processes are flattened and retracted to some degree, resulting a decreased interdigitation; **e** × 3,000; **f** × 10,000

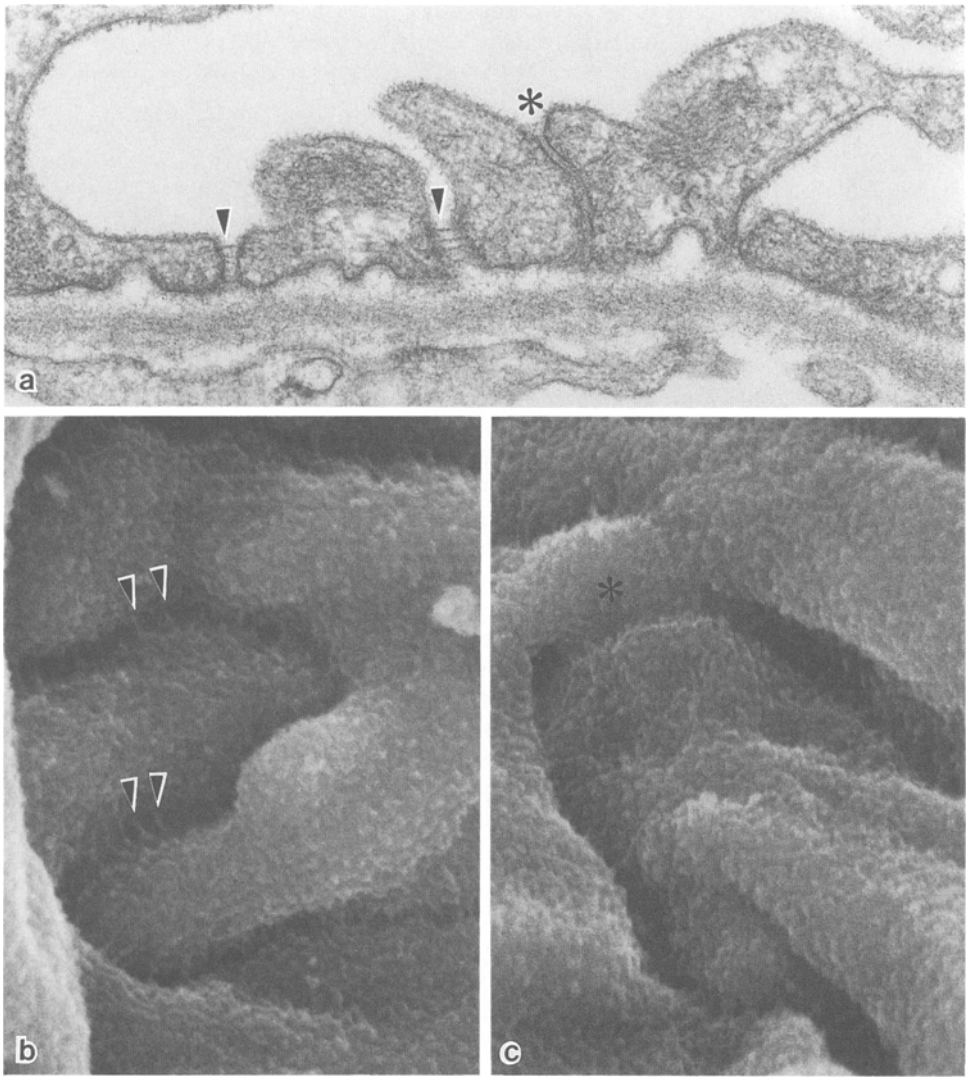

Fig. 5. a Transmission electron micrograph of the glomerular filtration barrier showing podocyte foot processes and filtration slits in puromycin aminonucleoside-induced (PAN) nephrosis on day 4. The filtration slits may be bridged by a multilayered filtration diaphragm *(arrowheads)* or closed by adhesion of neighbouring foot processes *(asterisk)*; × 100,000. **b, c** Scanning electron micrographs of the filtration clefts in PAN nephrosis on day 4. The filtration clefts are so shallow that their floor is clearly visible. The clefts are covered either by a series of fibrillar structures about 7 nm thick and 30 nm long *(arrowheads)*, obviously representing the multilayered diaphragms, or by a continuous structure *(asterisk)*, clearly representing the adhesion of foot processes; each × 100,000

After anaesthesia with an intraperitoneal injection of sodium pentobarbital (Nembutal, Abbott Laboratory, Ill.; 25 mg/kg body weight), renal perfusion fixation was performed through a retrograde cannula inserted into the abdominal aorta using a perfusion fixation apparatus, VPF–1 (Nisshin Electron Microscopy, Osaka, Japan). The perfusion pressure was 200 mmHg. The kidneys were flushed briefly with Hanks' balanced salt solution, pH 7.3, at room temperature, then fixed with 1% glutaraldehyde in 0.1 M phosphate buffer for 10 min. Cortical tissue was excised with razor blades into 1 × 1 × 2 mm pieces and fixed in the same fixative at 4° C for an additional 2 h. After washing overnight with 0.1 M phosphate buffer at 4° C, the tissue was processed for TEM and SEM.

For SEM, specimens were processed by conductive staining, which increases the electron conductivity of specimens, by immersion three times in 1% osmium tetroxide (O_sO_4) for 2 h and interposed by 1% tannic acid for 2 h. The specimens were then dehydrated in a graded series of ethanols, substituted with *t*-butyl alcohol, dried at −20° C in a vacuum, and then observed with a Hitachi S–900 hrSEM (Hitachi, Tokyo, Japan) equipped with a field emission cathode and an objective lens with a very short focal length, at an accelerating voltage of 5 kV.

For TEM, specimens were postfixed with 2% O_sO_4 in 0.1 M phosphate buffer for 1 h at 4° C and dehydrated through a graded series of ethanols and embedded in Epok 812 (Oken, Tokyo, Japan). Thin sections were stained with uranyl acetate and lead citrate, and examined in an H–700H electron microscope (Hitachi).

Results

The PAN-induced nephrotic rats showed severe proteinuria (Fig. 1). The urinary protein excretion was increased above the control level on day 3 and later in the experimental period. The proteinuria reached the maximum on day 8 (190.3 ± 21.4 mg/24 h) and declined gradually thereafter. The podocytes had lost their normal structure with interdigitating foot processes almost totally by day 8 (Fig. 2a, b). The interdigitation of foot processes was recovered almost completely by day 28 (Fig. 2e, f).

In normal glomeruli, the filtration clefts between foot processes were so deep that their floor was hardly visible. The floor of the filtration clefts was regularly bridged by fine fibrillar structures, which were about 45 nm in length (Fig. 3).

Observation of glomeruli in the early experimental stage of PAN nephrosis by hrSEM revealed structural changes in podocytes that preceded total loss of the interdigitating pattern. These early structural changes included formation of cytoplasmic blebs, masking of filtration clefts, flattening of foot processes, and retrac-

tion of foot processes (Figs. 4, 5). Foot processes were finally lost on day 8 (Fig. 2a, b) and podocytes were partially detached from the GBM in the areas showing loss of foot processes on day 8 by TEM (Fig. 6d), although we could not find the detached area by SEM.

The cytoplasmic blebs appeared on day 2 (Fig. 4a) in PAN-induced nephrotic rats and increased both in size and number on day 3 (Fig. 4c) and day 4 (Fig. 4e, f). The blebs were found on the foot processes, primary processes and cell body. Some large blebs were collapsed on day 4 (Fig. 4e, f), indicating that the blebs contained a vacuole (Fig. 4e).

The filtration diaphragm on day 2 was occasionally laminated in TEM (Fig. 6b), although filtration clefts on day 2 were as deep as in normal glomeruli and we could not find them by SEM. On day 3, adhesion of neighbouring foot processes was found in addition to the laminated slit diaphragm, using TEM (Fig. 6c). These structures increased on day 4. The floor of filtration clefts in nephrotic rats was partially masked by bridging structures on day 3 and extensively masked on day 4 (Fig. 5b, c). The bridging structures were either fibrillary or membranous in the high-power SEM view (Fig. 5b, c). From a comparison of SEM and TEM images on day 4, it was obvious that the fibrillary bridging structures represented the laminated filtration diaphragm and that the membranous structures represented adhesion of neighbouring foot processes. On day 4, neighbouring foot processes were frequently either bridged by multiple layers of filtration diaphragm or made contact with each other so that filtration slits lost the normal pattern of a single layer of diaphragm (Fig. 5a).

The low-power view revealed that the foot processes slightly flattened on day 3 (Fig. 4c, d), compared with those in the control (Fig. 2a, b) or on day 2 (Fig. 4a, b). The foot processes were remarkably flattened on day 4 (Fig. 4f). Interdigitation of the foot processes was largely unchanged on day 3 (Fig. 4c, d). However, the grade of interdigitation decreased gradually due to considerable retraction of foot processes on day 4 (Fig. 4e, f) and total retraction by day 8 (Fig. 2a, b). On day 4, the meandering course of the filtration clefts was somewhat straightened, but was still obvious. On day 8, the borders between neighbouring podocytes could be seen as almost straight lines.

The interdigitation of foot processes did not recover homogeneously on the glomerular surface, but reappeared first as islands of interdigitating fields in PAN rats on day 14 (Fig. 2c, d). In the interdigitations, the foot processes were extensively interdigitated and the filtration clefts were so deep that their floor was hardly visible with SEM. In the later experimental stage, the interdigitation field expanded gradually until it occupied large areas of the glomerular surface on day 28.

During expansion of the interdigitation, the primary processes of the podocytes were reestablished. On day 21

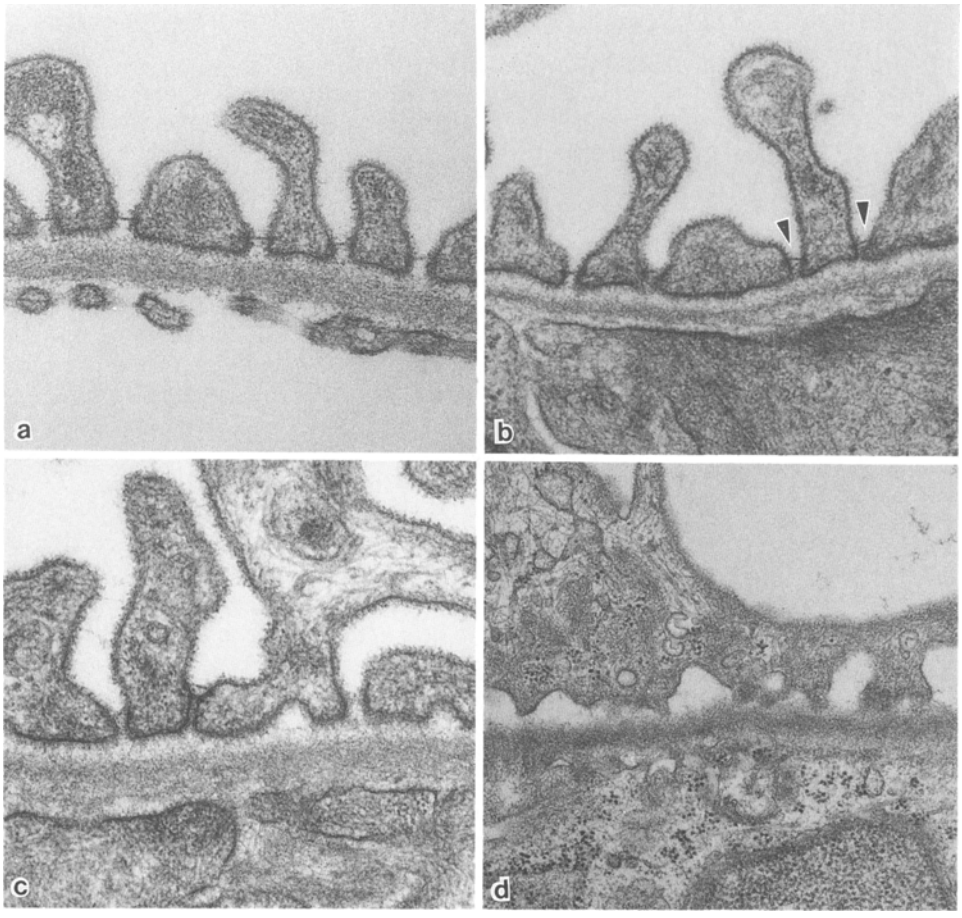

Fig. 6. Transmission electron micrographs of foot processes in a control rat (**a**), and puromycin aminonucleoside-induced nephrotic rats (**b–d**). **a** A single layered slit diaphragm is bridged between foot processes in a control rat, × 75,000. **b** Slit diaphragms are laminated *(arrowheads)* on day 2, × 75,000. **c** Neighbouring foot processes are occasionally adhesive on day 3, × 75,000. **d** Foot processes are lost and the wide cytoplasm of the podocytes is partially detached from the glomerular basement membrane, × 30,000

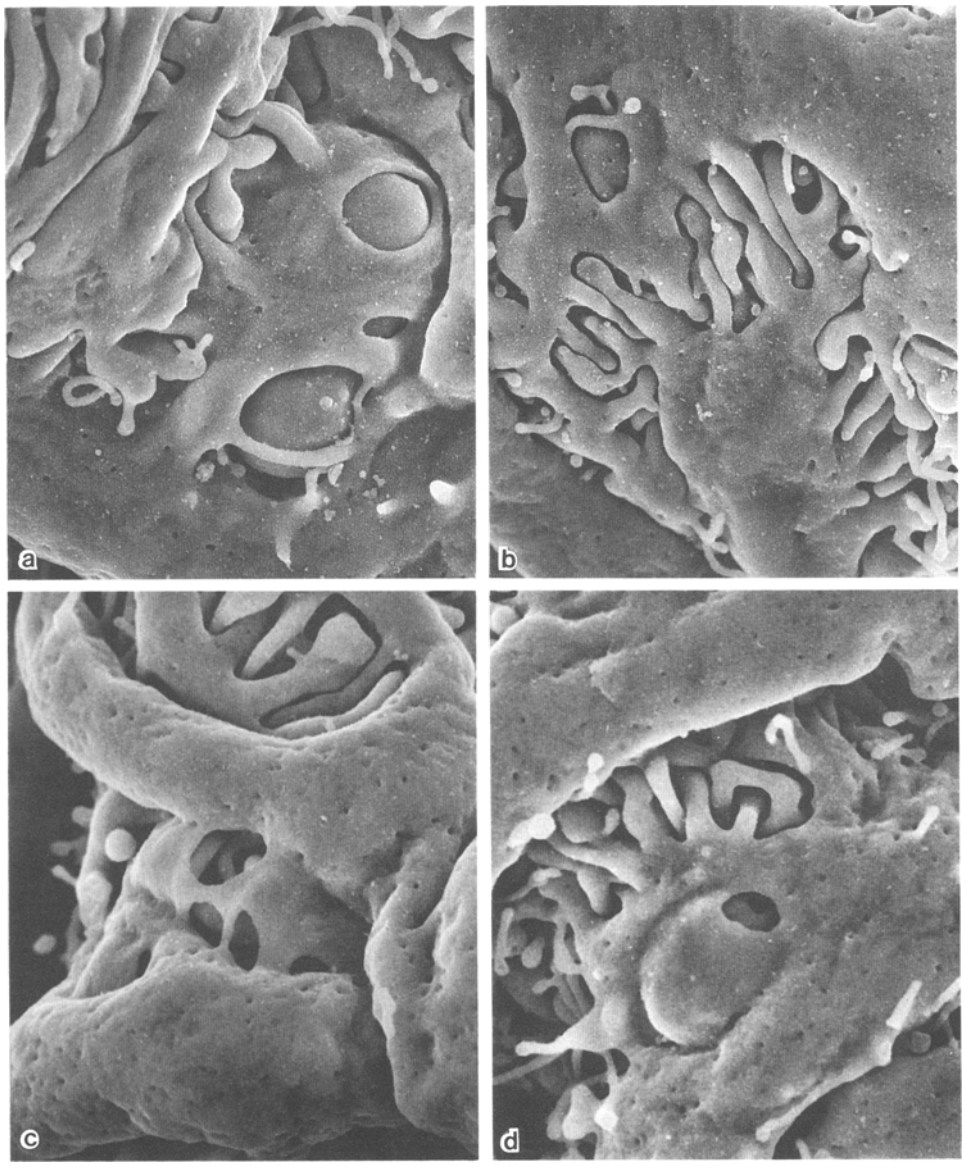

Fig. 7a–e. Scanning electron micrograph of podocytes in puromycin aminonucleoside-induced nephrotic rat on day 21, showing recovery of primary processes. The primary processes are connected by membrane-like cytoplasm, which is occasionally perforated by round or oval pores of various sizes; each × 15,000

the primary processes were joined frequently by membranous extensions of cytoplasm which were perforated by round pores (Fig. 7). This suggested that the primary processes were reestablished not only by the retraction of cell parts between the processes but also by perforation of these parts. On day 28, small processes joined together to encircle round pores, probably representing the enlargement of cytoplasmic pores between the primary processes (Fig. 2e).

The foot processes which had recovered by day 28 were different from normal in that they were irregular in thickness. Many were partially thickened by round or spindle-shaped swellings, and some were quite thin. Otherwise they appeared normal by day 28, showing extensive interdigitation and separation by deep filtration clefts (Fig. 2f).

Discussion

We have followed successive structural changes in podocytes of rats after PAN administration using SEM and TEM. We used a new-generation hrSEM equipped with a field emission cathode and objective lens with very short focal length, and observed the kidney tissue after conductive staining without metal coating. The hrSEM observation of conductively stained material, which permits high resolution of biological structures up to 0.5 nm, was successfully applied to visualization of the ultrastructure of the GBM by Shirato and coworkers (1991).

Four types of structural changes preceded total loss of foot processes after PAN administration: formation of cytoplasmic blebs, masking of filtration clefts, flattening of foot processes and retraction of foot processes. These structural changes occurred successively, and in part simultaneously, during the change of podocyte configuration.

Podocytes were progressively detached from the GBM in the areas showing loss of foot processes. It is suggested that loss of foot processes leads to podocyte detachment from the GBM. Many investigators have reported that focal detachment from the GBM in the podocytes leads to massive proteinuria in PAN nephrosis (Ryan and Karnovsky 1975; Messina et al. 1987; Whiteside et al. 1989) and it is thus important to elucidate the course of foot process loss in detail. Andrews (1977) reported that loss of podocyte pedicels involves a gradual decrease in pedicel height beginning at the pedicel tip and progressing down the arm, the formation of nub-like protrusions and interpedicel microbridges along the base, the merging of microbridges to form more extensive regions of interpedicel contact, and a gradual broadening and retraction. Although the present study has similar findings to Andrews in the point that flattening of foot processes preceded retraction of foot processes, we did not find the formation of nub-like protrusions at all. The merging of microbridges may correspond to masking of filtration clefts and we found that masking of filtration clefts was initiated prior to flattening of foot processes.

TEM studies on acute PAN-induced nephrosis have described the multiplication of slit diaphragms (Ryan et al. 1975b; Caulfield et al. 1976) and adhesion of neighbouring foot processes (Farquhar and Palade 1961; Venkatachalam 1969; Ryan et al. 1975b; Caulfield et al. 1976). Ryan and coworkers (1975b) discussed the multiplication of slit diaphragms in acute PAN nephrosis and suggested that this change may be brought about by the shortening of filtration clefts due to the retraction of foot processes. We do not support their speculation since the multiplication of slit diaphragms clearly preceded retraction of the foot processes.

The change in foot processes begins by alteration of filtration clefts. This may mean changes in the podocyte surface containing slit diaphragms; neutralization of negative surface charge by infusion of polycation or the removal of the sialic acid surface coat by neuraminidase have been reported to cause multiplication of slit diaphragm and adhesion of foot processes, flattening and retraction of foot processes (Seilar et al. 1975, 1977; Kerjaschki 1978; Andrews 1979).

The present observations suggest that loss of foot processes by PAN administration is caused in part by reduction of the negative surface charge and leads to the podocyte detachment from the GBM. PAN administration was reported to decrease the negative surface charge by reducing the sialilation of podocalyxin, the major epithelial polyanion of podocytes (Michael et al. 1970; Kerjaschki et al. 1985). However, other factors may also promote the loss of foot processes. Masking of filtration clefts was preceded by the formation of cytoplasmic blebs, which was obviously independent of the flattening and retraction of foot processes.

Loss of foot processes has been observed in the glomeruli of nephrotic patients with various types of glomerulonephritis. Loss of foot processes in human nephrotic syndrome may also result from the same morphological changes including masking of filtration clefts, flattening of foot processes, and retraction of foot processes, as in PAN nephrosis.

The process of recovery from structural alterations of podocytes in acute PAN nephrosis was analysed in detail. Recovery of podocytes began as islands of interdigitation and proceeded by their expansion, during which the primary processes were re-established either by retraction or perforation of the cell parts between them. The foot processes were re-established prior to the primary processes.

The process in which podocytes aquire their interdigitating foot and primary processes has been studied in the differentiation of immature podocytes from newborn rats by SEM and TEM (Miyoshi et al. 1971; Reeves et al. 1978). Since the development of glomeruli is heterogeneous in the kidney, precise analysis of the chronological sequence of podocyte maturation is difficult. The recovery of podocytes in acute PAN nephrosis may provide a useful model with which to analyse the maturation of podocytes in development.

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