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# Ultrastructure of matriceal changes in chronic phase of Masugi nephritis by quick-freezing and deep-etching method

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**Summary.** The three-dimensional ultrastructure of glomerular sclerosis in the chronic phase of Masugi nephritis was investigated using a quick-freezing and deep-etching method. Newly formed mesangial matrix, which was increased in the axial portions, was composed of fine fibrillar networks similar to those in the lamina densa of the basement membrane. These fibrils were 10–20 nm in diameter and directly attached to the cell membranes of mesangial cells, endothelial cells and podocytes by connecting fibrils. Moreover, thicker fibrils with diameters of 20–30 nm were present in the networks and were connected with cross-bridges. A newly formed matrix of fine fibrillar networks was also seen in the areas of mesangial interposition in the glomerular capillary wall. The border between the matrix and lamina densa was unclear. The fibrils organizing the networks of lamina densa of the glomerular loop were thickened, with some decoration. Connecting fibrils were disrupted in the areas of endothelial detachment. It is suggested that prolonged tissue injury with endothelial detachment might induce mesangial sclerosis composed of fine fibrillar networks. The increase in density of the networks seemingly interfere with the contractile function of mesangial cells, which is followed by alteration of mesangial flow.

**Key words:** Masugi nephritis – Quick freezing – Deep etching – Mesangial sclerosis

## Introduction

Mesangial sclerosis or a significant increase of mesangial matrix is the characteristic feature of chronic glomerulonephritis. The analysis of the development and fate of matriceal change in chronic glomerulonephritis is important because in some cases progressive mesangial sclerosis results in global glomerular sclerosis (Striker et al. 1984). Further, an increase in obsolescent glomeruli correlates with a deterioration of renal function that is often accompanied by persistent proteinuria (Shigematsu et al. 1985). Moreover, progressive mesangial sclerosis is commonly accompanied by atrophy of the nephron and interstitial fibrosis, which end with chronic renal failure. Mesangial sclerosis is divided into axial proliferation and circumferential mesangial interposition, which are histologically termed as mesangial proliferative glomerulonephritis and membranoproliferative glomerulonephritis respectively. The chronic phase of accelerated Masugi nephritis is a suitable model for analysing the organization and development of the mesangial sclerosis (Shigematsu and Kobayashi 1973; Kondo and Shigematsu 1980). Three-dimensional observation on the changes in extracellular matrix of the mesangium and basement membrane has not been fully performed in this model.

The quick-freezing and deep-etching method, which is one of the new techniques in electron microscopy, has the advantage of allowing the examination of the three-dimensional ultrastructure of tissues and cells in vivo at high resolution (Heuser and Kirschner 1980; Ohno 1985; Ohno and Takasu 1989). We have already applied this method to various disease models of hepatocytes (Naramoto 1988; Naramoto et al. 1988, 1990; Ohno and Fujii 1990). The quick-freezing and deep-etching method is also suitable for examining the skeletal organization of the extracellular matrix. We have previously reported the three-dimensional ultrastructures of damaged glomeruli in serum sickness nephritis (Naramoto et al. 1991). In the present study, we examined three-dimensional changes of capillary wall as well as mesangial matrix with special attention to the basement membrane, where rabbit nephrotoxin and anti-rabbit Ig G were located (Masugi 1980).

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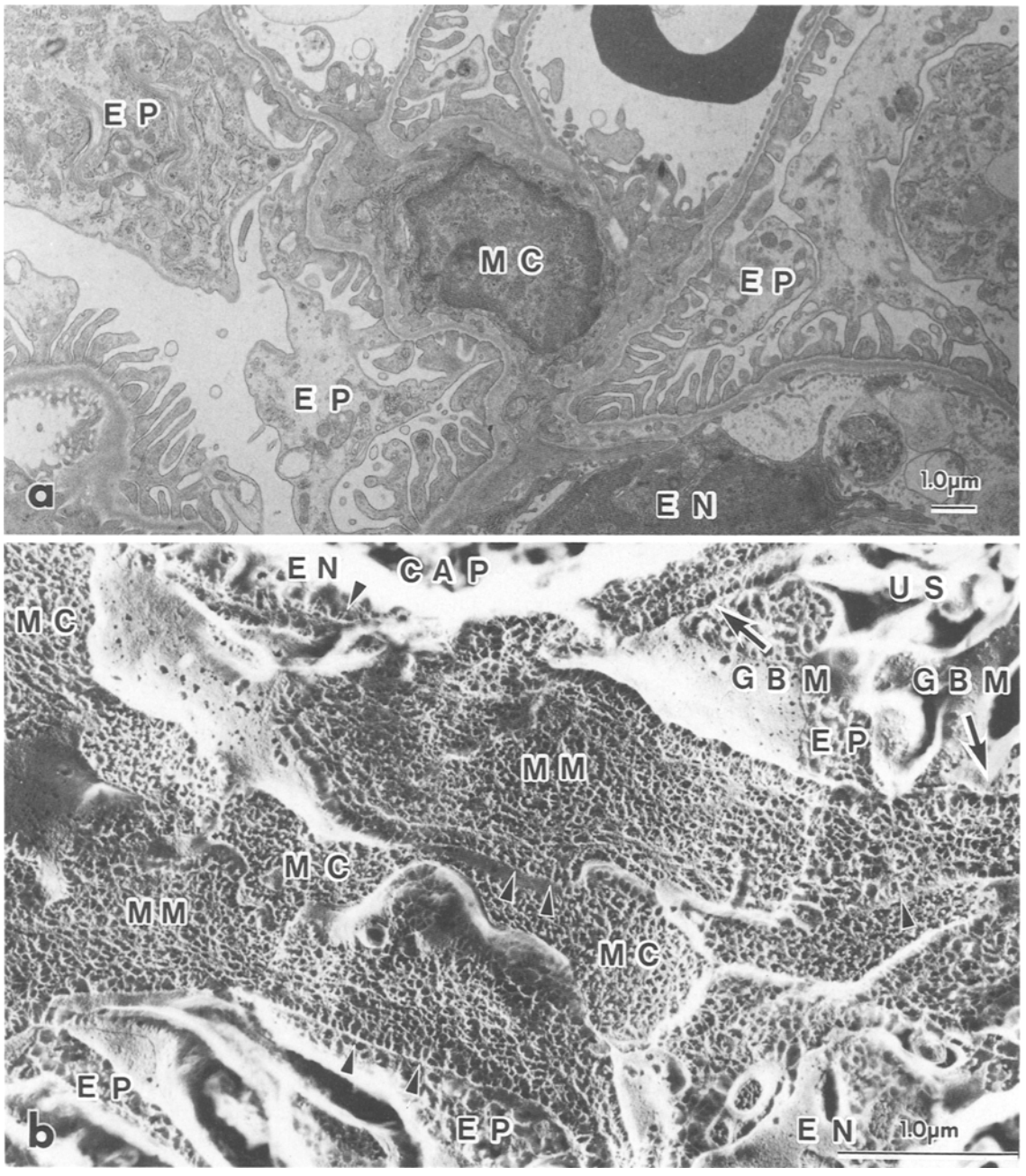


**Fig. 1 a, b.** Electron micrographs of mesangial area in control glomeruli. **a** Conventional ultrathin section. Small amounts of amorphous mesangial matrix are seen around a mesangial cell (*MC*). *EP*, podocyte; *EN*, endothelial cell. ×7,000. **b** Replica electron micrograph of mesangium. Mesangial matrix (*MM*) forms loose networks of fibrils with diameters of 8–15 nm. The diameters of such network pores range from 20 to 45 nm. The fibrillar networks are connected with the cell membranes of MC, EN and EP by connecting fibrils (*arrowheads*). *GBM*, glomerular basement membrane; *CAP*, capillary space; *US*, urinary space. ×29,000

## Materials and methods

Accelerated Masugi nephritis was induced in six male Wistar rats as reported in detail previously (Shibata et al. 1985). Briefly, 0.5 ml of γ-globulin-rich rabbit serum mixed with an equal volume of Freund's complete adjuvant was injected into footpads and subcutis of the backs of rats. Eight days after the first immunization, 0.5 ml rabbit nephrotoxic serum was injected into the tail veins to induce nephritis. The rats were sacrificed at 1 month under Nembutal anaesthesia. A control group was consisted of six untreated rats.

For morphological examination the kidneys were perfused with 2% (w/v) paraformaldehyde in 0.1 M phosphate buffer (PB), pH 7.4, via the abdominal aorta, for 5 min. The renal cortices were immediately cut into small pieces (2 × 2 × 4 mm) with razor blades and washed in PB for 30 min to remove the soluble proteins from the tissue surfaces (Naramoto et al. 1990). They were postfixed with 0.25% glutaraldehyde in PB for 30 min. They were rinsed with 10% methanol and quickly frozen in isopentane-propane mixture (around −190° C) cooled in liquid nitrogen. The tissue surfaces of the frozen specimens were fractured with a scalpel in the liquid nitrogen. They were deeply etched in an Eiko FD-3S machine at −95° C and $2.7–8.0 \times 10^{-5}$ Pa ($2–6 \times 10^{-7}$ Torr) for 15–20 min and rotary shadowed with platinum and carbon. The replica membranes with the specimens were taken out and immediately

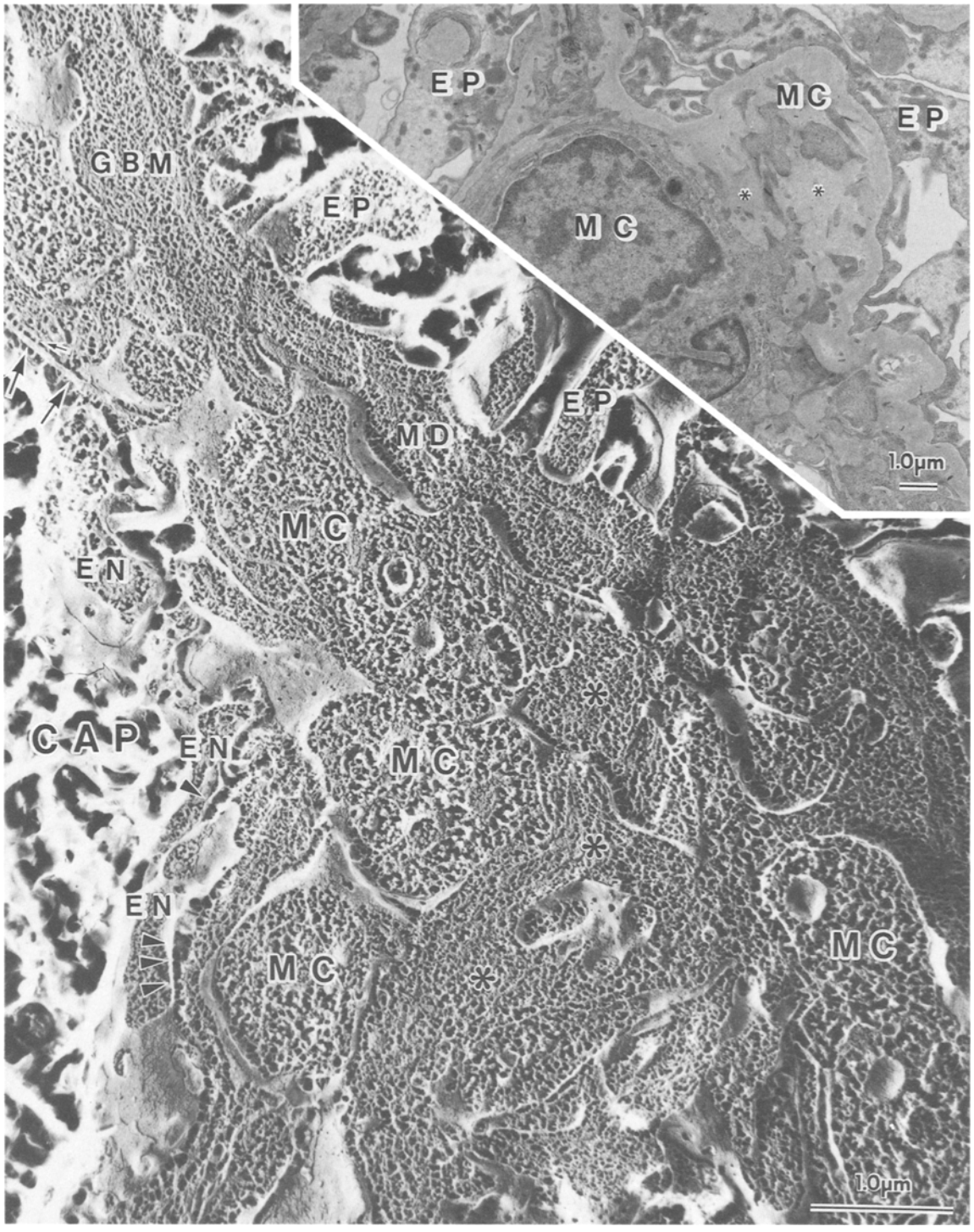


**Fig. 2.** Replica electron micrograph of mesangial area in Masugi nephritis 1 month after nephrotoxin injection. The *stars* show increased mesangial matrix composed of fine fibrillar networks. The fibrils spread around mesangial cells and directly reach the mesangial lamina densa (*MD*) and peripheral basement membrane (*GBM*). The fibrils are directly attached to the membranes of mesangial cells (*MC*). *Large arrows* indicate a thicker fibril, which is connected with cross-bridges (*small arrows*). The connecting fibrils are disrupted in the area of endothelial detachment (*arrowheads*). For other abbreviations, see Fig. 1. ×24,000. *Inset:* Corresponding electron micrograph in conventional ultrathin section. The *stars* show amorphous mesangial matrix. ×6,500

coated with 2% collodion. The kidney tissues were dissolved in sodium hypochlorite. The replica membranes were put on grids and immersed in amylacetate solution to dissolve the collodion. They were then observed in a Hitachi HS-9 electron microscope.

Small pieces of renal cortices were doubly fixed with 2.5% glutaraldehyde in PB for 1 h and 1.5% osmium tetroxide in PB for 1 h. They were dehydrated in a graded series of ethanol and embedded in Quetol 812 (epoxy resin). Ultrathin sections were cut with diamond knives and were stained with uranyl acetate and lead citrate (Naramoto et al. 1990).

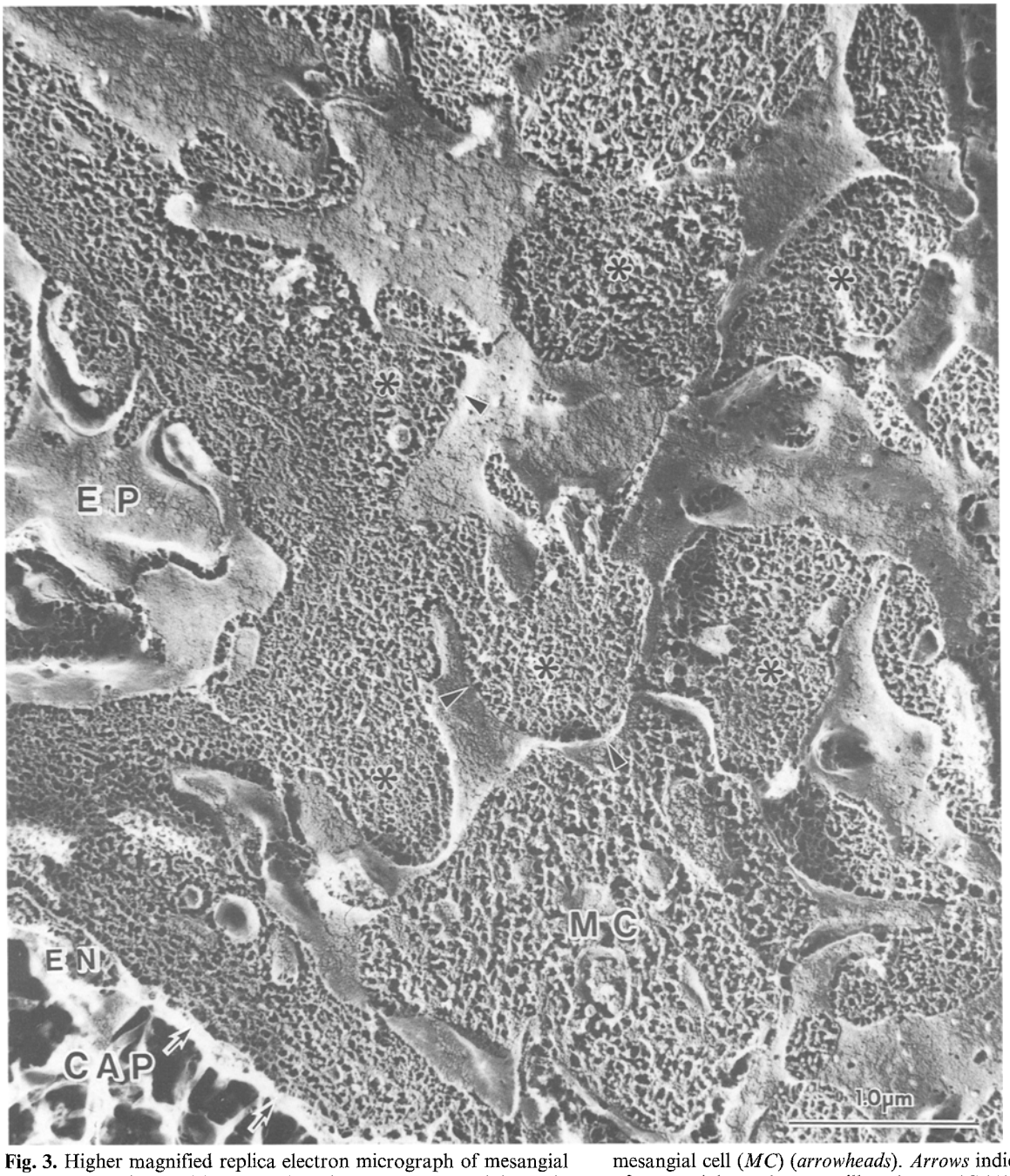


**Fig. 3.** Higher magnified replica electron micrograph of mesangial area in Masugi nephritis. *Stars* show increasing mesangial matrix composed of fine fibrillar networks, which resemble the networks of GBM. These fibrils are directly attached to the membrane of mesangial cell (*MC*) (*arrowheads*). *Arrows* indicate the denudation of mesangial matrix to capillary lumen (*CAP*) due to the detachment of endothelial cell (*EN*). × 32,000

## Results

In the control rats, small amounts of amorphous mesangial matrix were seen around mesangial cells in conventional ultrathin sections (Fig. 1 a). However, the quick-freezing and deep-etching mehod revealed that the mesangial matrix formed loose networks of fibrils with diameters of 8–15 nm (Fig. 1 b). The diameters of such network pores ranged from 20 to 45 nm. The fibrillar networks were connected with the cell membranes of mesangial cells, endothelial cells and podocytes via connecting fibrils. However, the fibrils of the mesangial matrix ran continuously into the lamina densa of paramesangium or peripheral basement membrane. The lamina densa also formed fine fibrillar networks.

In the glomeruli of accelerated Masugi nephritis, the proliferation of mesangial cells and the increase of mesangial matrix predominated (Figs. 2, 3). Increased mesangial matrix was composed of amorphous materials in conventional ultrathin sections (Fig. 2, inset), but the

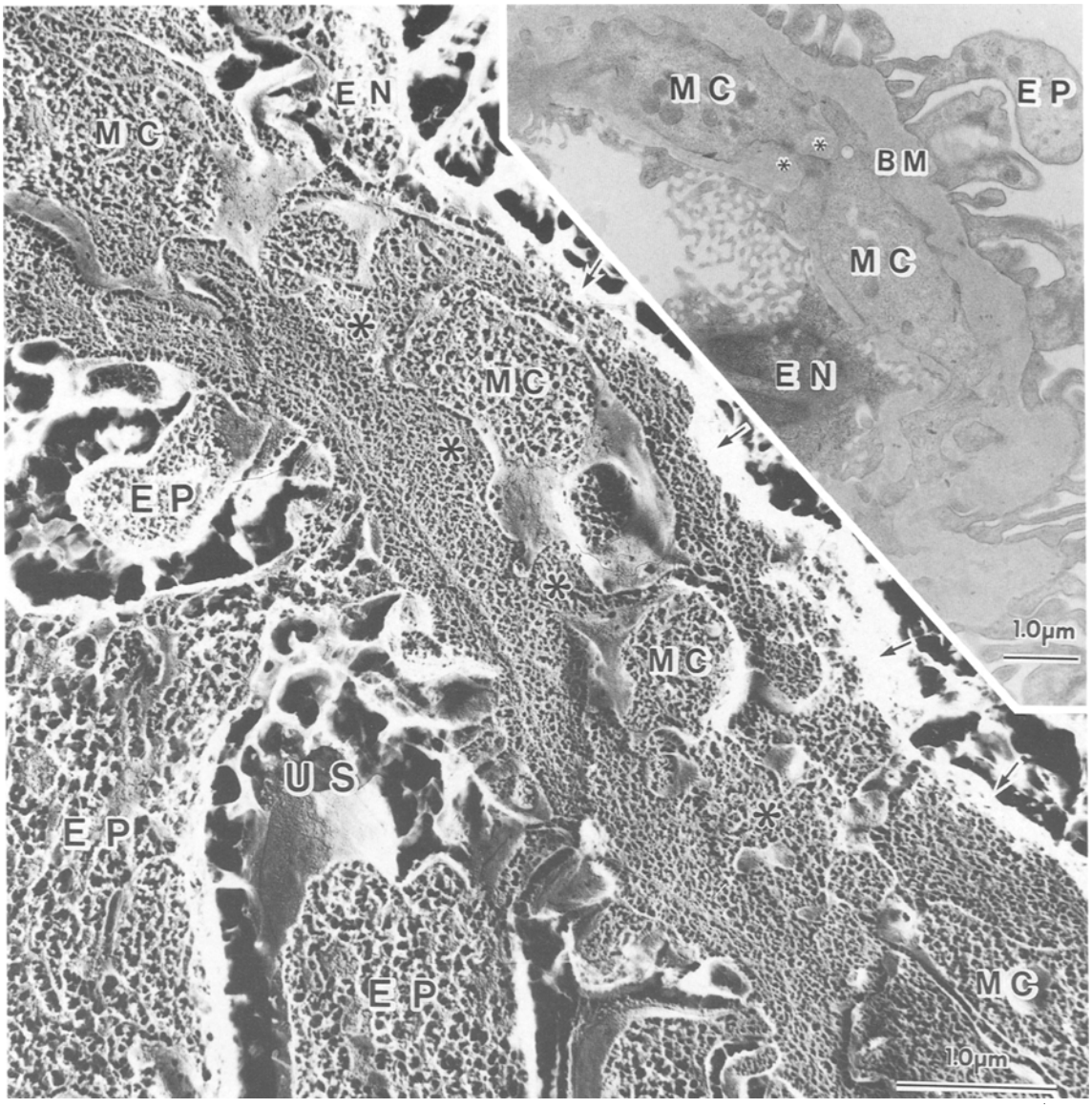


**Fig. 4.** Replica electron micrograph of mesangial interposition in Masugi nephritis. *Stars* show the produced matrix composed of fine fibrillar networks around the mesangial cell processes (*MC*). The border to the lamina densa is unclear. *Arrows* indicate the detachment of endothelial cells (*EN*). ×24,000. *Inset:* Corresponding figure of conventional ultrathin section. *Stars* show newly formed matrix. *BM*, basement membrane. ×11,000

quick-freezing and deep-etching method revealed increased fine fibrillar networks in the mesangial matrix (Figs. 2, 3). The diameters of mesh pores were about 15 nm. These constitutional fibrils with diameters of 10–20 nm extended continuously around mesangial cells and were directly associated with the lamina densa of paramesangium and peripheral basement membrane at the mesangial angle. They were also attached to the cell membranes of mesangial cells, endothelial cells and podocytes by connecting fibrils. Moreover, thicker fibrils with diameters of 20–30 nm were localized in the fibrillar networks and connected with cross-bridges with diameters of 7–10 nm.

Newly formed matrix of fine fibrillar networks, which showed almost the same organization as in the axial portion, was also seen in the areas of mesangial interposition in the glomerular capillary wall (Fig. 4). Network structures around mesangial processes were transferred into the constituent networks of lamina densa, but the border between them was unclear. The fibrils were also attached to the mesangial processes by connecting fibrils. The fibrils organizing lamina densa were ruggedly decorated on their surfaces and thickened up to about 20 nm in diameter, in contrast to the control lamina densa, which was composed of comparatively smooth surfaced fibrils with diameters of 8–15 nm (Fig. 5). The organization of fibrillar networks was not changed. The destruction of the networks or enlargement of mesh pores were not observed in the basement membrane. Endothelial detachment from the basement membrane was seen in conventional ultrathin sections (Fig. 6a), sometimes in the areas of migratory cell infil-

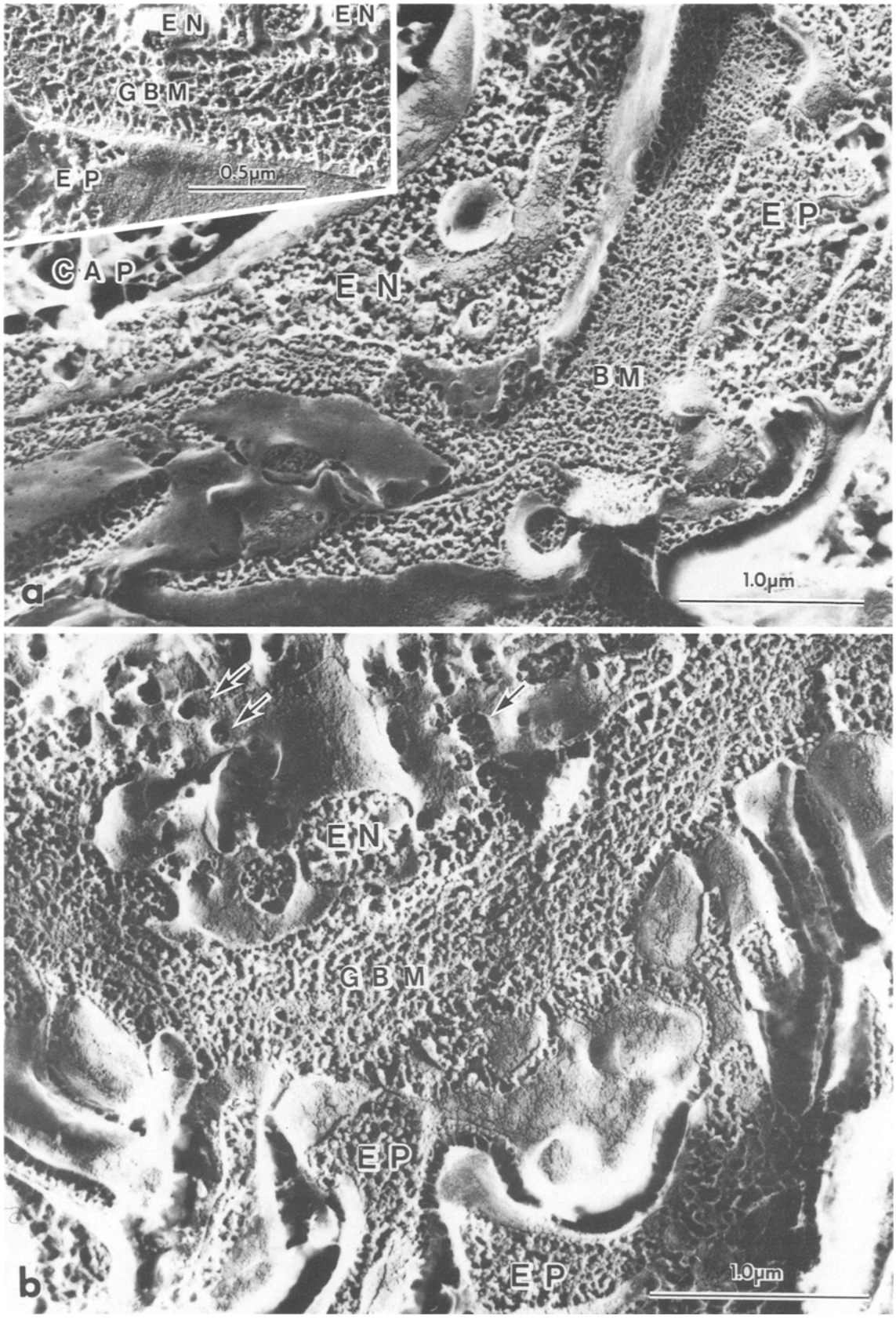


**Fig. 5a, b.** Replica electron micrographs of peripheral basement membrane (*GBM*). **a** The fibrils organizing the lamina densa (*BM*) are ruggedly decorated on their surface. The organization of fibrillar network has not been changed as compared with control glomerular basement membrane (*inset: ×43,000*). *×34,000.* **b** Higher magnification of GBM. Thickening of the fibrils is clearly demonstrated. *Arrows* indicate the fenestration of an endothelial cell. ×41,000

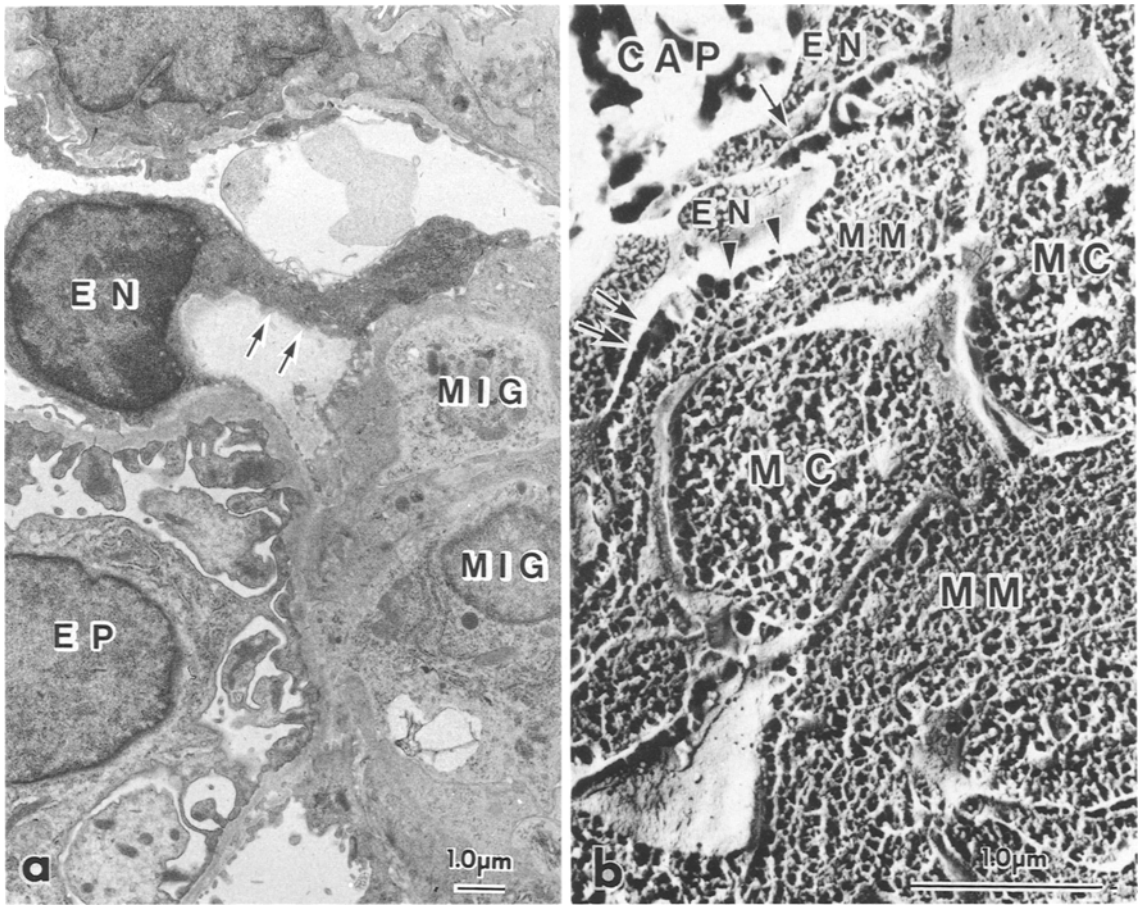


**Fig. 6a, b.** Electron micrograph of endothelial detachment in Masugi nephritis. **a** Conventional ultrathin section. *Arrows* indicate the detachment of endothelial cell (*EN*) in the area of migratory cell (*MIG*) infiltration. ×6,600. **b** Replica electron micrograph of corresponding area. Connecting fibrils between mesangial matrix (*MM*) and endothelial cell are disrupted, as shown by *arrows*. In contrast, *arrowheads* indicate the preserved connecting fibrils. ×30,000

tration. The disruption of the connecting fibrils from lamina densa was observed in these areas by the quick-freezing and deep-etching method (Fig. 6b).

## Discussion

We have found that the backbone structure of mesangial sclerosis is composed of fine fibrillar networks resembling the lamina densa. We have already demonstrated that laminin was one of the components organizing the fine fibrils using this methodology combined with immunohistochemistry (Naramoto et al., unpublished data). Other components include type IV collagen, fibronectin, entactin and glycosaminoglycan, mainly produced by the increased numbers of mesangial cells (Ishimura et al. 1989). The fibrillar networks are formed by a self-assembly mechanism (Yurchenco and Furthmayr 1984). Mesangial sclerosis results from an increased synthesis of the components and disturbance of their degradation in the mesangial compartment (Romen and Morath 1979). Functionally an increase of newly formed mesangial network structures seems to interfere with the contractile ability of the mesangial cells. It is kown that the contraction and relaxation of normal mesangial cells play an important role in the regulation of glomerular filtration through the interconnecting fibrillar networks in the mesangial matrix (Sakai and Kriz 1987; Mundel et al. 1988) as shown in this study. Therefore mesangial sclerosis probably impairs the regulation of filtration.

Secondly, the increased mesangial networks may alter the mesangial flow. The mesangial pathway is supposed to correspond to the joints of pores of fibrillar networks in the mesangial matrix. Material may easily be trapped and accumulate in the increased, disorganized and more finely fibrillar networks of the mesangial matrix in the prolonged inflammation. Further, these materials, which contain growth factors to mesangial cells, such as interleukin 1, platelet-derived growth factor, epidermal growth factor and insulin-like growth factor, may activate and accelerate mesangial cells to produce additional matrix (Kashgarian 1985). Thus, these processes indicate a vicious circle between mesangial sclerosis and the impairment of mesangial flow and may contribute to the progression of glomerulosclerosis.

In addition to the fine fibrils mainly composed of the matrix, we demonstrated thicker fibrils with diameters of 20–30 nm in the increased mesangium. The thicker fibrils which resemble type I collagen are connected with the fine fibrillar networks via cross-bridges.

It is known that collagen fibres of types I and III (interstitial collagens) are scarce in normal glomeruli (Roll et al. 1980). However, it has been reported that cultured mesangial cells produced type I collagen (Ishikawa et al. 1980). Therefore, mesangial cells could produce type I collagen under pathological conditions including mesangial sclerosis. Moreover, focal adhesion to Bowman's capsule may induce an increase of interstitial collagens in the mesangial matrix (Striker et al. 1984).

Newly formed fine fibrillar networks were also seen in the areas of mesangial interposition, which is one of the characteristic changes in the progressive glomerulonephritis. The mesangial cells do not extend themselves into the peripheral capillary wall under normal physiological conditions, possibly because endothelial cells or podocytes secrete the heparin-like materials which inhibit the mesangial cell proliferation (Castellot and Hoover 1986). In Masugi nephritis, however, severe damage to endothelial cells may allow the proliferation or elongation of mesangial cells into the oedematous or lytic peripheral subendothelial space. These interposed mesangial cells also produce the matrix of fine fibrillar networks around themselves in the peripheral capillary loop. Thus, newly formed fine fibrillar networks in the axial portions and mesangial interposition seem to result from the impaired recovery from the lysis of extracellular matrix like mesangiolysis.

In our experiment, detachment of endothelial cells from the basement membrane was seen only partially, where their connecting fibrils in lamina rara interna were disrupted. This finding presumably explains how the detachment of endothelial cells occurs. The connecting fibrils on the endothelial cells are easily disrupted under some pathological conditions. We have already reported that the connecting fibrils are disrupted in the lamina rara externa in serum sickness nephritis and that detachment of podocytes ensued (Naramoto et al. 1991). Prolonged injury with detachment of endothelial cells in chronic Masugi nephritis seemingly resulted in failure in the repair of glomerular structure, followed by progressive sclerosis.

In accelerated Masugi nephritis, nephrotoxic rabbit globulin and anti-rabbit Ig G are known to be linearly observed along the peripheral basement membrane using an immunofluorescent method (Masugi 1980). Ultrastructural changes of fine skeletal organization were not observed on conventional ultrathin sections. In the present study, the quick-freezing and deep-etching method demonstrated that the fibrils organizing the networks of lamina densa were diffusely thickened with some decorations. It has been reported that autologous antibody against nephrotoxin was located throughout the glomerular basement membrane by immunoelectron microscopy (Hoedemaeker et al. 1972). Therefore, nephrotoxin and anti-rabbit Ig G may be associated with the fibrils organizing the networks of basement membrane. The matriceal fibrils of glomerular basement membrane are known to consist of type IV collagen, laminin, entactin, nidogen, etc. (Laurie et al. 1984), although the injection of monoclonal antibodies against these proteins could not induce the proliferative glomerulonephritis like Masugi nephritis (Abrahamson and Caulfield 1982; Yaar et al. 1982). The principal antigens and their distribution in the Masugi nephritis have not been elucidated. The quick-freezing and deep-etching method combined with immunohistochemistry will clarify the antigenic sites and their distribution three-dimensionally in the near future.

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