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Three-dimensional identification of actin filaments in phalloidin-treated rat livers by quick-freezing and deep-etching method

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Summary. An increase in microfilaments in phalloidin-treated hepatocytes of Wistar rats was identified three-dimensionally with myosin subfragment 1 (S1) on replica membranes, using the quick-freezing and deep-etching method. Almost all of the reticular microfilaments around the bile canaliculi and beneath the lateral cell membranes were decorated on their surfaces by S1 attachment. Some showed periodic structures. However, thinner filaments with diameters of 4–7 nm were not decorated by S1. Bundled intermediate filaments around the bile canalicular microfilaments and intermediate filaments localized among cell organelles had smooth surfaces without S1 decoration. The microfilaments decorated by S1 were attached directly to bundled intermediate filaments. The quick-freezing and deep-etching method is useful in analysing cytoskeletal pathology and can be applied to histochemical fields.

Key words: Quick-freezing – Deep-etching – Myosin subfragment 1 – Microfilament – Intermediate filament

Introduction

The cytoskeleton of hepatocytes plays an important role in the pathogenesis of some liver diseases, especially cholestasis and alcoholic liver diseases. Denk et al. (1981) postulated a "cytoskeleton disease" following study of Mallory bodies in liver tissues.

Several methods have been developed electron microscopically (French et al. 1982), histochemically (Ishikawa et al. 1969) and immunohistochemically (Osborn et al. 1982) in order to examine the cytoskeleton in hepatocytes. As it consists of three-dimensional structures of filaments, we needed to observe the ultrastructure three-dimensionally. The quick-freezing and deep-etching method is one of the newer techniques in electron microscopy (Heuser and Kirschner 1980), by which we can examine stereographic cytoskeletons of unmodified fresh cells and tissues at high resolution. We have already reported the pathological changes of hepatocyte cytoskeletons in phalloidin-treated intrahepatic cholestasis (Naramoto 1988a, b) and extrahepatic obstruction (Naramoto et al. 1988).

It is known that bile canalicular microfilaments increase significantly in phalloidin-treated rat livers and induce cholestasis (Gabbiani et al. 1975). In the present study, we tried to identify these filaments three-dimensionally using myosin subfragment 1 (S1) on the replica membranes and to clarify the interactions of actin filaments with other filaments. S1 corresponds to the two head structures of the myosin molecule, and has ATPase activity and specific binding ability to actin filaments.

Materials and methods

Five male Wistar rats weighing 100–200 g were given 500 μg/kg body weight of phalloidin intraperitoneally (Sigma, St. Louis, USA) once a day for a week (Gabbiani 1975). The control livers consisted of five untreated rats. They were perfused with 2% (w/v) paraformaldehyde in 0.1 M phosphate buffer (PB), pH 7.4, for 5 min from the portal veins under Nembutal anaesthesia. Liver tissue were cut into small pieces ($2 \times 2 \times 4$ mm) with razor blades and washed in PB for 20–30 min in order to remove the soluble proteins in hepatocytes. These specimens were incubated in 0.1 M PB containing S1 (1 mg/ml) for 30 min, washed in PB and postfixed with 0.25% glutaraldehyde in PB for 30 min. Then they were immersed in 10% methanol and quickly frozen in isopentane-propane mixture (about −190° C) cooled in liquid nitrogen. The frozen surfaces were fractured in liquid nitrogen by a scalpel, deeply etched in a freeze-fracture machine, EIKO FD-3S, with a turbo molecular pump (−95° C, $2\text{–}6 \times 10^{-7}$ Torr) for 10–15 min and rotary shadowed with platinum and carbon. After being taken out of the machine, the replica specimens were coated with 2% collodion in amylacetate and the liver tissues were dissolved in house-

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hold bleach (sodium hypochlorite). The replica membranes were put on grids, immersed in amylacetate solution to dissolve the collodion films and observed in Hitachi HS-9 electron microscope.

For conventional ultrathin sections, the liver tissues were prefixed with 2.5% glutaraldehyde and postfixed with 1.5% osmium tetroxide. They were routinely dehydrated in a graded series of ethanol and embedded in Quetol 812. Ultrathin sections were cut with diamond knives. They were doubly stained with uranyl acetate and lead citrate and then observed in Hitachi HS-9 electron microscope.

Results

In the control rats, bile canaliculi had multiple microvilli on the conventional ultrathin sections (Fig. 1, inset). However, the microfilamentous layers were difficult to identify. The quick-freezing and deep-etching method showed small amounts of the microfilamentous network around the bile canaliculi (Fig. 1). Microfilaments were also seen in cross-fractured microvilli.

In the conventional ultrathin sections of phalloidin-treated livers, abundant filamentous structures were observed around the dilated bile canaliculi (Fig. 2a) and beneath the lateral cell borders (Fig. 2b). By the quick-freezing and deep-etching method, the three-dimensional architecture of the increased microfilamentous network could be visualized clearly around the bile canaliculi (Fig. 2c) and at the cell border (Fig. 2d). The microfilaments branched out one after another, and were directly attached to the cytoplasmic sides of cell membranes (Fig. 2c) or vesicular membranous structures (Fig. 2d).

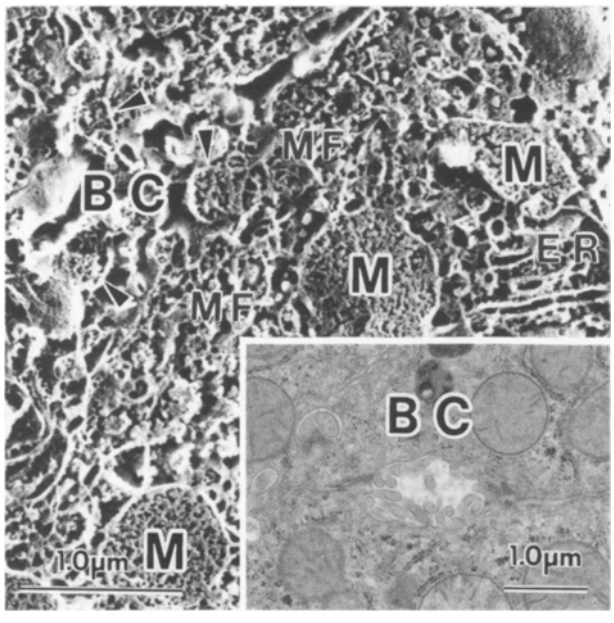


Fig. 1. Replica electron micrograph of control hepatocytes around a bile canaliculus (*BC*). Small amounts of microfilaments (*MF*) are localized around the canaliculus. Microfilaments are also seen in cross-fractured microvilli (*arrowheads*). *M*, Mitochondria; *ER*, rough surfaced endoplasmic reticulum. ×22000. *Inset:* Conventional ultrathin section. Microfilaments are rarely identified. ×11000

The microfilaments were 7–10 nm in diameter (Fig. 2c, d) and attached directly to other filaments.

In S1-decorated specimens, almost all of the bile canalicular microfilaments were ruggedly decorated on their surfaces with S1 and the thicknesses of the filaments ranged from 15 to 20 nm (Figs. 3, 4a, b). Periodic structures were observed in some areas (Fig. 3). Some thinner filaments with diameters of 4–7 nm were not decorated by S1 (Figs. 3, 4a, inset). These thinner filaments were connected directly to actin filaments on both ends as cross-linking filaments. Microfilaments beneath the lateral cell membranes and in interdigitating portions were also decorated by S1 (Fig. 4b). However, straight bundles of intermediate filaments with diameters of 10–15 nm around the increased microfilaments had smooth surfaces without S1 decoration. The S1-decorated microfilaments showed end-to-side attachments to the non-decorated intermediate filaments (Fig. 4b, arrowheads). However, intermediate filaments localized among cell organelles were not decorated by S1 (Fig. 4c), and microfilaments were rarely observed in these areas.

Discussion

It is well known that actin filaments of normal hepatocytes are mainly localized around bile canaliculi and under the cell membranes. Polygonal patterns of actin filament distribution in hepatocytes have been shown by the anti-actin antibody method (Farrow et al. 1971) or the *N*-(7-dimethyl amino-4-methyl coumrinyl) maleimide (DACM) – heavy meromyosin (HMM) method (Namihisa et al. 1980) combined with fluorescent microscopy. These actin filaments are decorated specifically with HMM, which is a subunit of myosin and forms arrowhead-like structures (Ishikawa et al. 1969).

Phalloidin is one of the toxic substances produced by the mushroom *Amanita phalloides* (Gabbiani et al. 1975). It is generally accepted that phalloidin treatment induces an increase of microfilaments in hepatocytes, by facilitating the polymerization of globular actin to filamentous actin. In the phalloidin-treated rat livers, actin proteins were shown around the dilated bile canaliculi by immunofluorescence studies (Ohta et al. 1988). We have reported the three-dimensional architecture of the increased microfilaments in phalloidin-treated hepatocytes by the quick-freezing and deep-etching method (Naramoto 1988a, b). In the present study, most of bile canalicular microfilaments were identified as actin filaments on replica membranes by S1 decoration. The S1 is a part of HMM and has an ability to bind with actin filaments. The S1 can penetrate the cytoplasm more easily than HMM, because of its lower molecular weight.

Some thin filaments without S1 decoration were localized as cross-linking filaments among the increased bile canalicular microfilaments. The undecorated filaments were 4–7 nm in diameter and thinner than actin filaments. It is probable that they are new types of cytoskeletal structures outside the three major classes of cytoskeletons (microfilaments, intermediate filaments and microtubules; Roberts 1987). Recently six proteins of

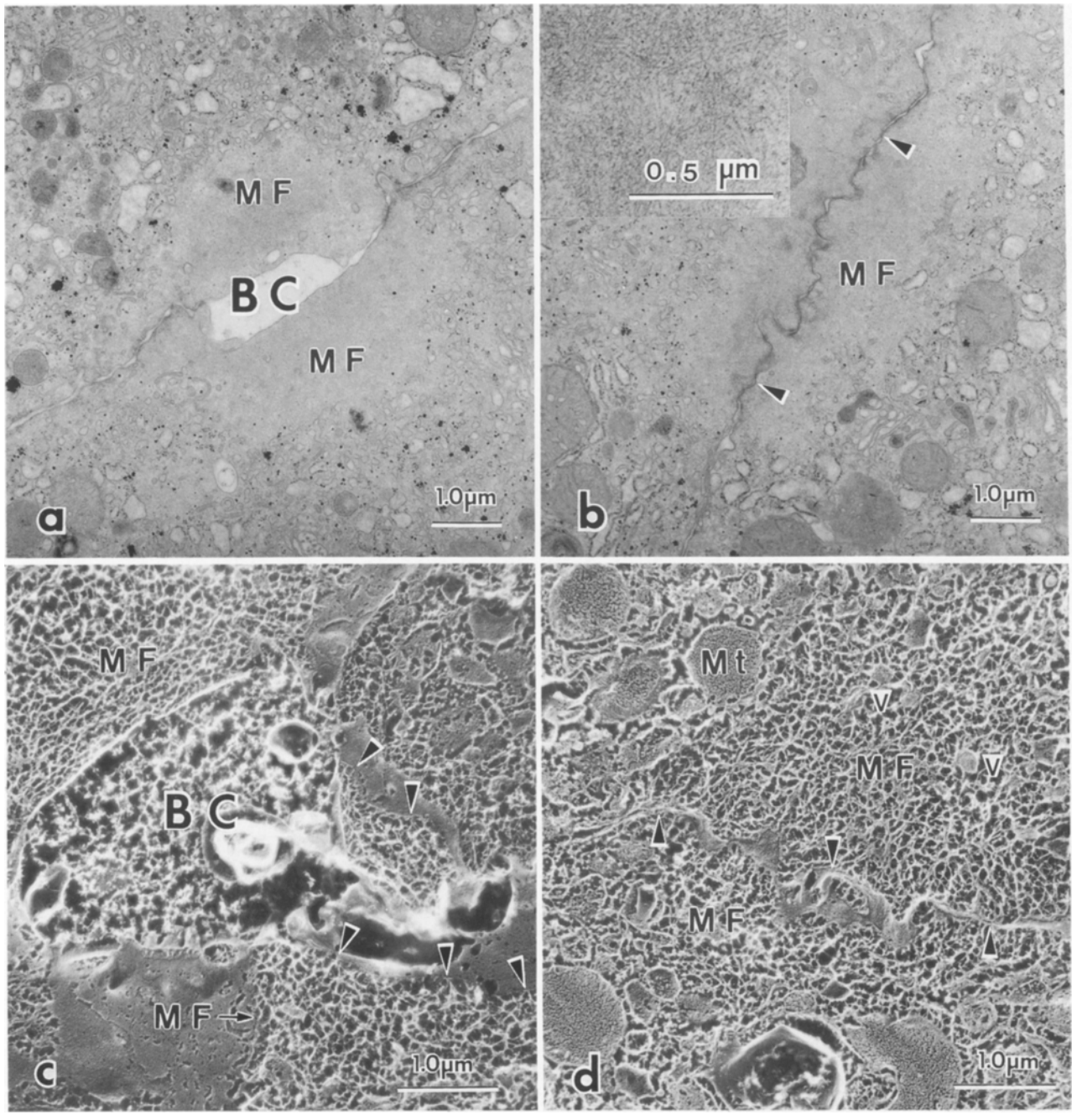


Fig. 2. a, b. Conventional ultrathin sections of hepatocytes of phalloidin-treated rats. Microfilaments (*MF*) increase around bile canaliculi (*BC*) as shown in **a** and under lateral cell membranes (**b**). *Arrowheads* indicate the cell to cell border. ×11000 *Inset:* High magnification of microfilaments. ×46000. **c, d** Replica electron micrographs of hepatocytes without S1 treatment. Three-dimensional architecture of the increased microfilamentous network (*MF*) can be visualized clearly around bile canaliculus (**c**) and at cell borders (**d**). *Arrowheads* in **c** indicate the direct attachment of microfilaments to cytoplasmic sides of the bile canalicular membrane. *Mt*, Mitochondria; *V*, Vesicular membranous structure. ×16000

fine filaments have been identified – spasmin, echinonematin, nematode MSP, tektin, giardin and titin (Roberts 1987). It has been proposed that their functions contribute to cell morbility and cytoarchitecture. However, there are no reports of localization of these fine filaments in hepatocytes. It has also been reported that myosin proteins are localized in enlarged areas around the dilated bile canaliculi in the same way as the actin filaments are found in phalloidin-treated rat livers (Ueno et al. 1988). However, it is not known whether the increased myosin proteins are filamentous structures or not. One possibility is that the fine filaments identified as cross-linkers in this experiment are composed of myosin or other actin-binding proteins. Functionally, actomyosin interaction with the calcium-calmodulin system is needed for peristaltic contraction of bile canaliculi. Therefore, it will be interesting to clarify the ultastructural distribution of myosin and how the myosin pro-

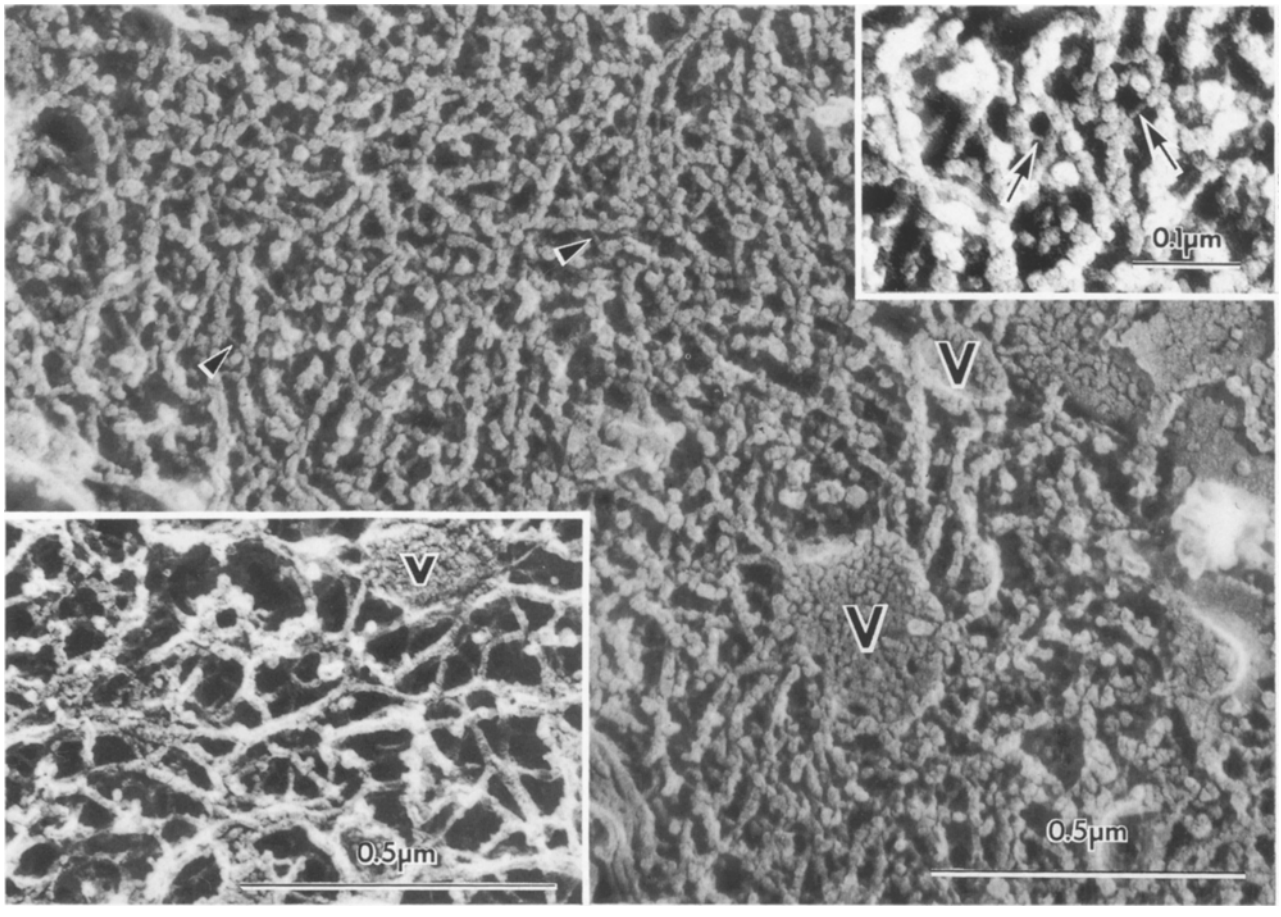
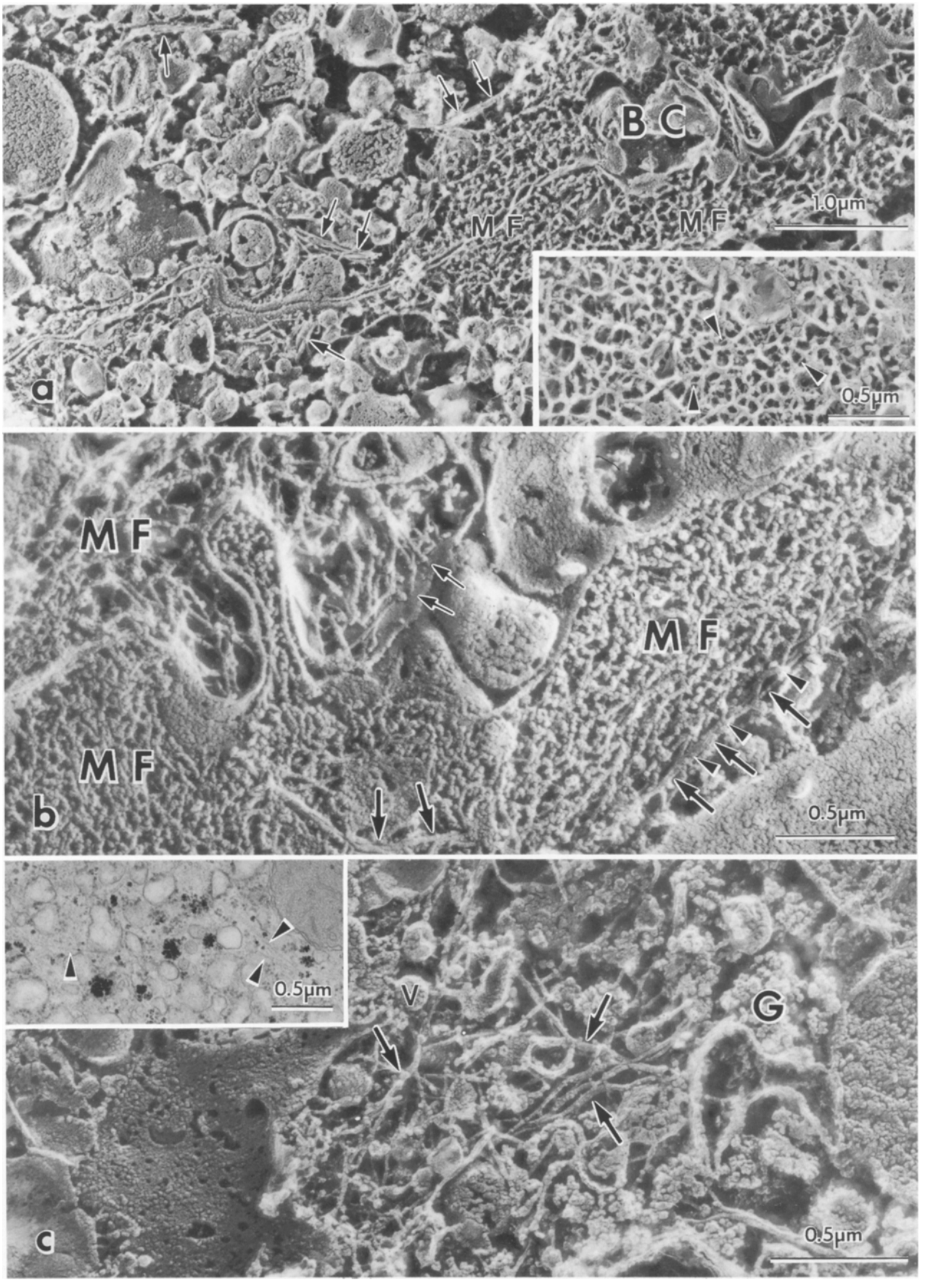


Fig. 3. Replica electron micrographs of hepatocytes decorated with S1. Almost all of the bile canalicular microfilaments show periodic structures with the decoration of S1 and are attached directly to vesicular structures (*V*). *Arrowheads* indicate thin filaments without S1 decoration. ×83000. *Right upper inset:* High magnification view of undecorated thin filaments (*arrows*). These filaments with the diameters of 4–7 nm are connected directly with actin filaments as cross-linking filaments. ×130000. *Left lower inset:* Smooth surfaced microfilaments without S1 treatment. *V*, Vesicular membranous structure. ×83000

teins bind with the actin filaments, by using the quick-freezing and deep-etching method combined with immunohistochemistry.

We also reported an increase of intermediate filaments in phalloidin-treated rat livers (Naramoto 1988a, b). Bundled intermediate filaments were seen around the increased microfilaments and seemed to be formed as a part of the reactive changes to the cholestasis. In this study, we have demonstrated that the decorated microfilaments are directly attached to the bundled intermediate filaments. Although the interaction of three major cytoskeletal filaments plays an important role in the functional expression of the hepatocyte cytoskeleton (Dubin et al. 1980), their three-dimensional connections have not yet been clarified. Actin filaments probably contract at the connecting point with intermediate filaments as one fulcrum. The intermediate filament bundles around bile canalicular microfilaments seem to correspond to the pericanalicular sheath, reported by Katsuma et al. (1988).

Fig. 4a–c. Electron micrographs of hepatocytes decorated with S1. **a** Almost all of the bile canalicular microfilaments are ruggedly decorated on their surfaces (*MF*). *Arrows* indicate bundled intermediate filaments. *BC*, Bile canaliculus. ×24000. *Inset*: Some thin filaments (*arrowheads*) with the diameters of 4–7 nm are not decorated with S1 and connect directly to actin filaments on both ends as cross-linking filaments. ×29000. **b** Three-dimensional organizations of cytoskeleton under the lateral cell membranes including interdigitating portions. Almost all of microfilaments (*MF*) are decorated with S1. The decorated microfilaments enter into the interdigitating portions and are attached directly to the cell membranes (*small arrows*). Straight bundles of intermediate filaments (*large arrows*) outside the S1-decorated microfilaments have smooth surfaces without S1 decoration. *Arrowheads* indicate direct connections between microfilaments (*MF*) and intermediate filaments (*large arrows*). ×43000. **c** Electron micrographs of the cytoplasm near the nucleus. Intermediate filaments (*arrows*) among cell organelles are not decorated with S1. Microfilaments are rarely present in these areas. *G*, Glycogen granule. ×54000. *Inset*: Conventional ultrathin sections corresponding to the areas. *Arrowheads* show intermediate filaments. ×22000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