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Three-dimensional studies of the cytoskeleton of cultured hepatocytes: a quick-freezing and deep-etching study

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Summary. The ultrastructure of the cytoskeleton of cultured mouse hepatocytes was studied by a quick-freezing and deep-etching method. Isolated mouse hepatocytes were cultured on collagen gels for 48 h, fixed in paraformaldehyde and centrifuged to prepare cell pellets. The hepatocytes were split open to remove cytoplasmic soluble proteins for replica preparations. Some specimens were decorated with anti-actin antibody or S_1 myosin fragments to identify actin filaments. They were quickly frozen in isopentane-propane mixture, fractured in liquid nitrogen, deeply etched in a freeze-fracture machine and rotary shadowed by platinum and carbon. The basal cell membranes of hepatocytes were in contact with the collagen gels and the apical surface faced the culture medium. Networks of actin filaments were attached to the apical cell membranes, but intermediate filaments were localized along the basal layer. Some intermediate filaments were associated with cell organelles, such as the endoplasmic reticulum. The Golgi apparatus was less associated with the cytoskeleton and showed synthesized materials in the cisternae. Cytoskeletal organization in cultured hepatocytes was revealed three-dimensionally, indicating that the interaction of cell membranes with collagen gels is important for the organization of the cytoskeleton.

Key words: Cytoskeleton – Hepatocytes – Quick freezing

Introduction

A number of studies have focused on cytoskeletal organization in hepatocytes (Franke et al. 1981a; French and Davies 1975; French et al. 1982, 1989; Hoshino et al. 1989; Katsuma et al. 1988; Okanoue et al. 1985, 1988; Van Eyken et al. 1987). Moreover, the cytoskeletal changes in the hepatocytes have been described in a variety of pathological conditions (Borenfreund et al. 1980; Denk and Lackinger 1986; Katsuma et al. 1987; Naramoto et al. 1990; Ohta et al. 1988). In contrast to many studies about in vivo hepatocytes, there is little information about the ultrastructures of cultures hepatocytes (Franke et al. 1981b; Mak et al. 1980; Marceau et al. 1980; Mesland et al. 1981; Prentki et al. 1979; Sattler et al. 1978).

The usefulness of culture systems depends on the ability of hepatocytes to respond in a way which resembles in vivo responses (Prentki et al. 1979). There have been some studies on the properties of hepatocytes in monolayer cultures (Franke et al. 1981b; Marceau et al. 1980; Michalopoulos and Pitot 1975; Nickola and Frimmer 1986; Ohno and Fujii 1990; Sattler et al. 1978; Sudhakaran et al. 1986) and from these it is known that cultured hepatocytes usually lose their in vivo morphology and some biological functions after culturing (Michalopoulos and Pitot 1975; Sudhakaran et al. 1986). Although the maintenance of in vivo morphology is very important for efficient biological functions, the mechanism needed to establish a stable morphology is unclear at present.

Cell contact with extracellular matrices might play a role in stabilizing the morphological orientation (Gjessing and Seglen 1980; Marceau et al. 1980; Ohno and Takasu 1989; Rapraeger et al. 1986; Sugrue and Hay 1981; Woods et al. 1984). It is generally known that collagen matrices induce the ultrastructural organization of cultured hepatocytes (Mak et al. 1980; Sattler et al. 1978). Both type I and type III collagens have been demonstrated immunohistochemically in normal liver tissues (Martinez-Hernandez 1984). The collagen fibrils from the tendon used for tissue cultures are copolymers of type I and type III collagens and the collagen gel matrix is thus suitable for studying the morphological differentiation of cultured hepatocytes (Michalopoulos and Pitot 1975; Mak et al. 1980; Nickola and Frimmer 1986; Ohno and Fujii 1990; Rubin et al. 1981; Sattler et al. 1978). We have reported that the attachment of cell membranes to collagen gels induced the polarization of cultured thyroid cells (Ohno and Takasu 1989; Takasu

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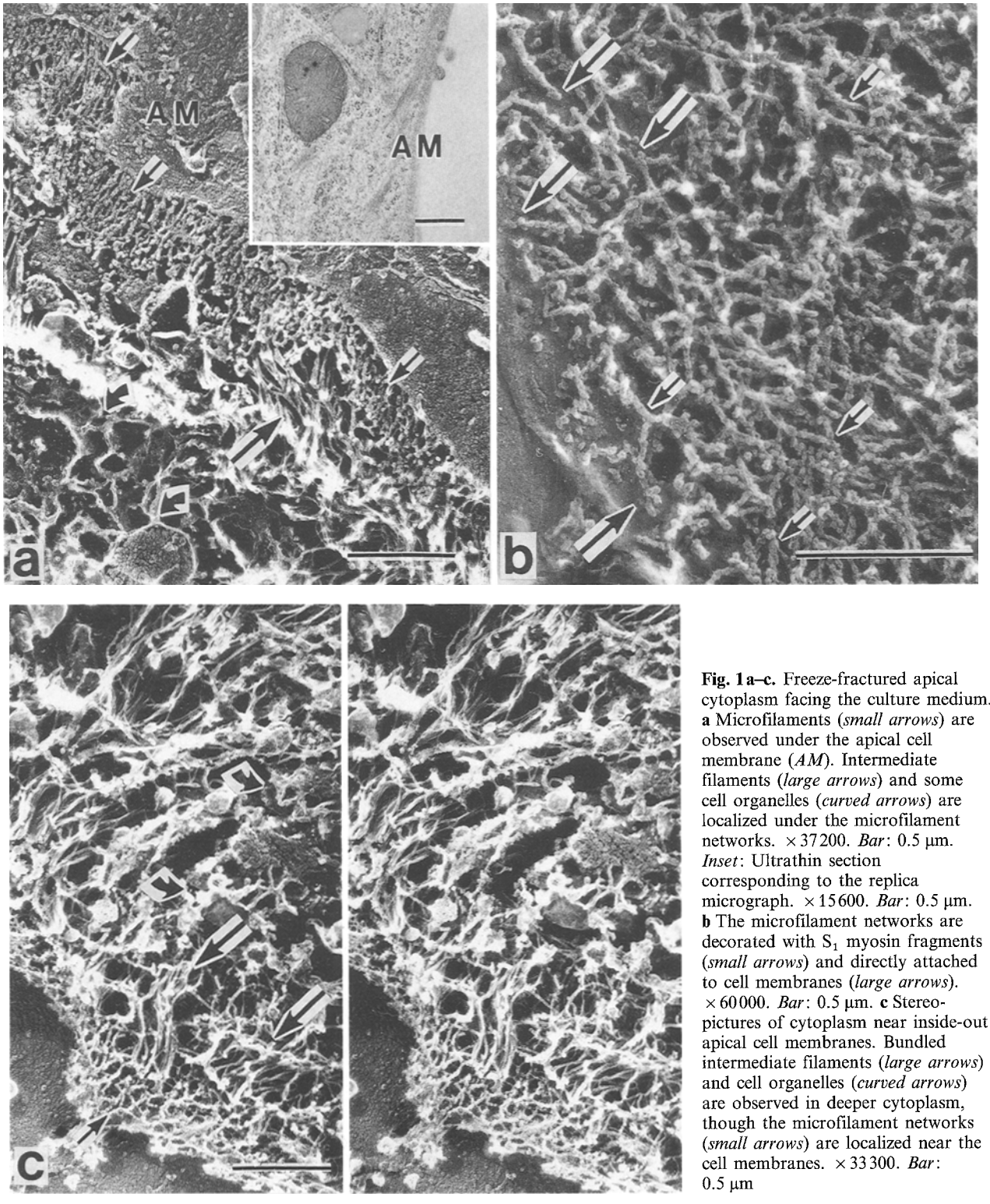


Fig. 1 a–c. Freeze-fractured apical cytoplasm facing the culture medium. **a** Microfilaments (*small arrows*) are observed under the apical cell membrane (*AM*). Intermediate filaments (*large arrows*) and some cell organelles (*curved arrows*) are localized under the microfilament networks. ×37200. *Bar*: 0.5 μm. *Inset*: Ultrathin section corresponding to the replica micrograph. ×15600. *Bar*: 0.5 μm. **b** The microfilament networks are decorated with S_1 myosin fragments (*small arrows*) and directly attached to cell membranes (*large arrows*). ×60000. *Bar*: 0.5 μm. **c** Stereo-pictures of cytoplasm near inside-out apical cell membranes. Bundled intermediate filaments (*large arrows*) and cell organelles (*curved arrows*) are observed in deeper cytoplasm, though the microfilament networks (*small arrows*) are localized near the cell membranes. ×33300. *Bar*: 0.5 μm

et al. 1988), but the mechanism involved in the localization of cell organelles in cultured hepatocytes is unknown. In recent years, more attention has been directed towards the role of cytoskeleton in the determination of morphological orientation (Feldmann 1989; French et al. 1982, 1989; Marceau et al. 1980; Nickola and Frimmer 1986; Ohta et al. 1988; Rapraeger et al. 1986; Woods et al. 1984).

Transmission and scanning electron microscopy have been used to study the ultrastructure of hepatocytes in liver (Ohta et al. 1988; Okanoue et al. 1988). However, current knowledge regarding three-dimensional ultrastructure of cultured hepatocytes is limited. Recently, the quick-freezing and deep-etching method has been introduced in the field of cell biology, which provides three-dimensional images of the ultrastructural organi-

zation (Gotow and Hashimoto 1989; Heuser and Kirschner 1980; Hirokawa et al. 1984; Isobe and Shimada 1986; Meller 1985; Ohno 1985). Although the method has proved useful for the three-dimensional analysis, there has also been a report of cultured hepatocytes with this method (Ohno and Fujii 1990). The purpose of this study is to analyse the ultrastructures of normal hepatocytes cultured on collagen gels by the quick-freezing and deep-etching method. The cytoskeletons in hepatocytes appear to be important in various physiological phenomena such as maintenance of cell shapes and secretory processes (Feldmann 1989; French et al. 1982; Prentki et al. 1979). Special attention has been paid to the organization of cytoskeleton, microfilaments and intermediate filaments, throughout the cytoplasm of cultured hepatocytes.

Materials and methods

Normal male DDY mice weighing 20–25 g were anaesthetized intraperitoneally with pentobarbital sodium solution (5 mg/100 g body weight) and perfused via the left ventricle with oxygenated Ca^{2+}-free Hanks' balanced salt solution (CF-HBSS) supplemented with 10 mM hydroxyethylpiperazine ethanesulphonic acid (HEPES), pH 7.4, at 37° C. The excised livers were cut into small pieces, suspended in the 0.05% collagenase solution in CF-HBSS and shaken at 37° C for 15 min (Ohno and Fujii 1990). The cell suspension was centrifuged at 50 *g* for 2 min and the first crop of cells was discarded. Two successive crops of cells were obtained from two similar incubations in the collagenase solution containing 5 mM $CaCl_2$. The viability of the cells was amounted to 80–95% by trypan blue dye exclusion test.

Petri dishes (60 mm) were treated with the solution of rat tail collagens (Bornstein 1958). The separated cells were plated at 2.0×10^6 viable cells/petri dish and maintained in the mixed medium of Dulbecco's modified Eagle's medium (45%) (Flow Laboratories, McLean, Virginia, USA) and RPMI 1640 medium (45%), containing 10% newborn calf serum at 37° C in 5% CO_2-95% air. The mixed medium was also supplemented with 1×10^{-6} M insulin, 2×10^{-6} M hydrocortisone-21-hemisaccinate (sodium salt), 2×10^{-6} M dexamethasone and 50 mg/l kanamycin. After overnight incubation, the used medium was discarded and the cells were cultured in the new medium at 37° C in 5% CO_2-95% air for 48 h. The cultured cells were identified as hepatocytes by peroxisomal reactivities induced with clofibric acid, as published elsewhere (Ohno and Fujii 1990).

Split hepatocytes were prepared according to the cell splitting method of Ohno (1985), as follows. The cultured hepatocytes were fixed with 2% paraformaldehyde in 0.1 M phosphate buffer (PB), pH 7.4, for 20 s and centrifuged to prepare cell pellets in PB. Clean glass coverslips with the circles of 4 mm diameter marked with a diamond pen were pretreated with 3-aminopropyl triethoxy silane and glutaraldehyde to make the cells attach to the coverslips (Büechi and Bächi 1979). Small drops of suspension cells were put in the circles on one coverslip and immediately covered with another coverslip to sandwich the hepatocytes. The following manipulation was done under a phase-contrast light microscope. The top coverslip was gently pressed with pincettes to make the flat cells attach to each coverslip. Some proteins, exposed at the extracellular surface of cell membranes, were bound to each coverslip by the action of glutaraldehyde. A lot of PB was added between the two coverslips and then the coverslips were plunged into PB. The two coverslips were gradually separated during 10–20 min. The hepatocytes could be consequently split open and randomly attached to each coverslip. The split cells were washed in PB to remove cytoplasmic soluble proteins and postfixed with 2.5% glutaraldehyde in PB for 30 min for replica preparations.

In order to demonstrate actin filaments the split hepatocytes were treated with 0.1 M lysine in PB for 30 min to block free aldehyde residues of glutaraldehyde. They were immunostained with primary rabbit anti-actin antibody (Miles Scientific, Naperville, Ill.) for 30 min at a dilution of 1:10 and with secondary goat anti-rabbit immunoglobulin G coupled to peroxidase for

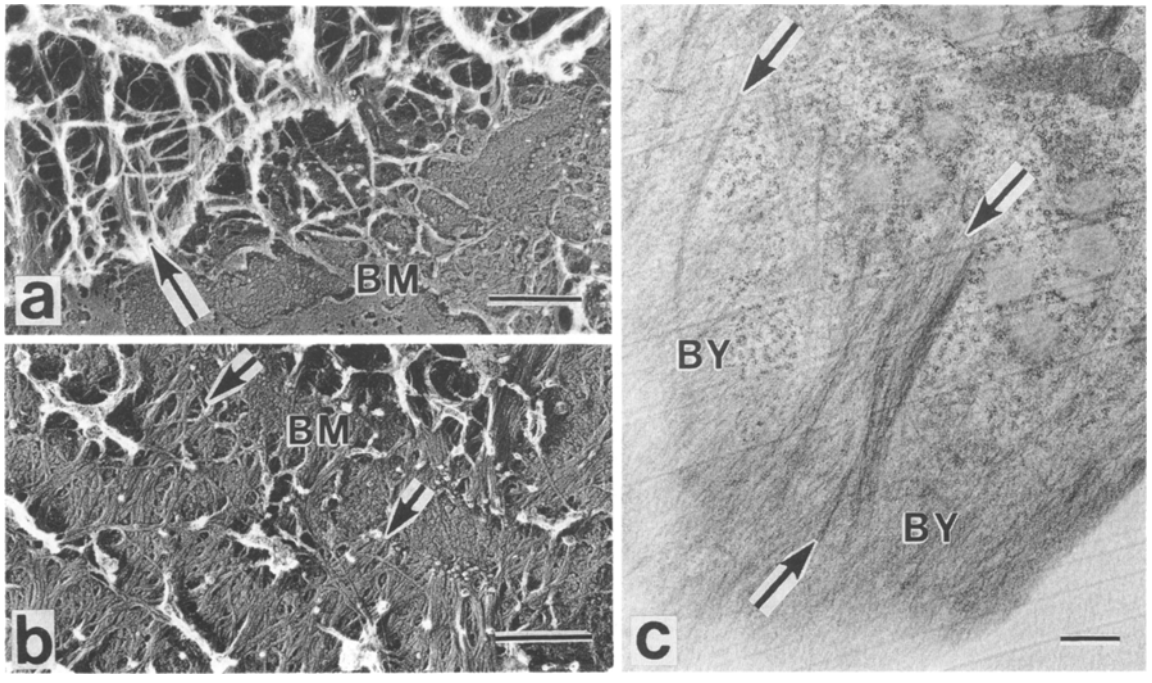


Fig. 2. **a** Obliquely freeze-fractured basal cytoplasm. Intermediate filaments (*arrows*) are interconnected each other and localized along the basal cell membranes (*BM*). ×26000. *Bar*: 0.5 μm. **b** Horizontally freeze-fractured basal cytoplasm. Bundled intermediate filaments (*arrows*) are densely distributed on the cell membranes. ×26000. *Bar*: 0.5 μm. **c** Ultrathin section showing bundled cytoskeletons (*arrows*) in the basal cytoplasm (*BY*). ×14800. *Bar*: 0.5 μm

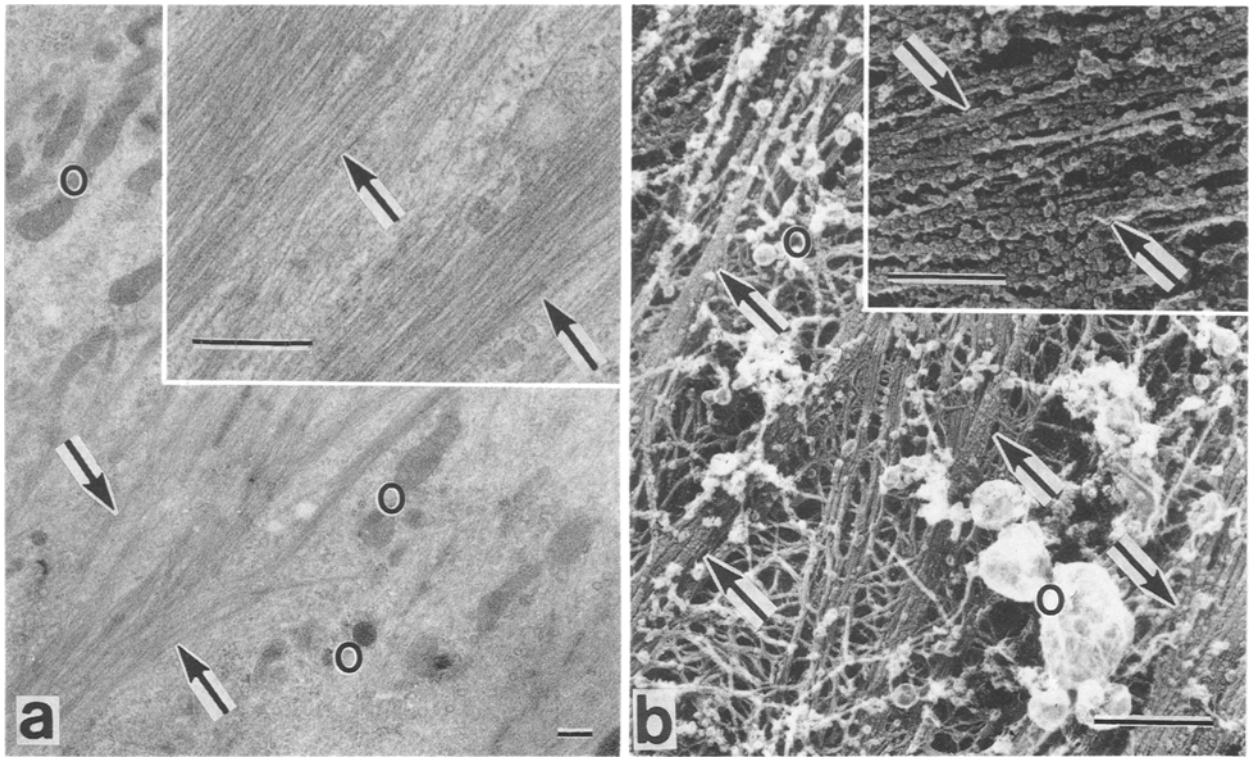


Fig. 3. **a** Some bundled cytoskeletons (*arrows*) are observed in deep cytoplasm. *O*, Cell organelles. ×9500. *Bar*: 0.5 μm. *Inset*: Higher magnification of bundled cytoskeletons. ×31000. *Bar*: 0.5 μm. **b** On the replica membranes, some intermediate filaments are decorated with the granular structures (*arrows*). *O*, Cell organelles. ×31000. *Bar*: 0.5 μm. *Inset*: The granular structures are immunostained with anti-actin antibody (*arrows*). ×31000. *Bar*: 0.5 μm

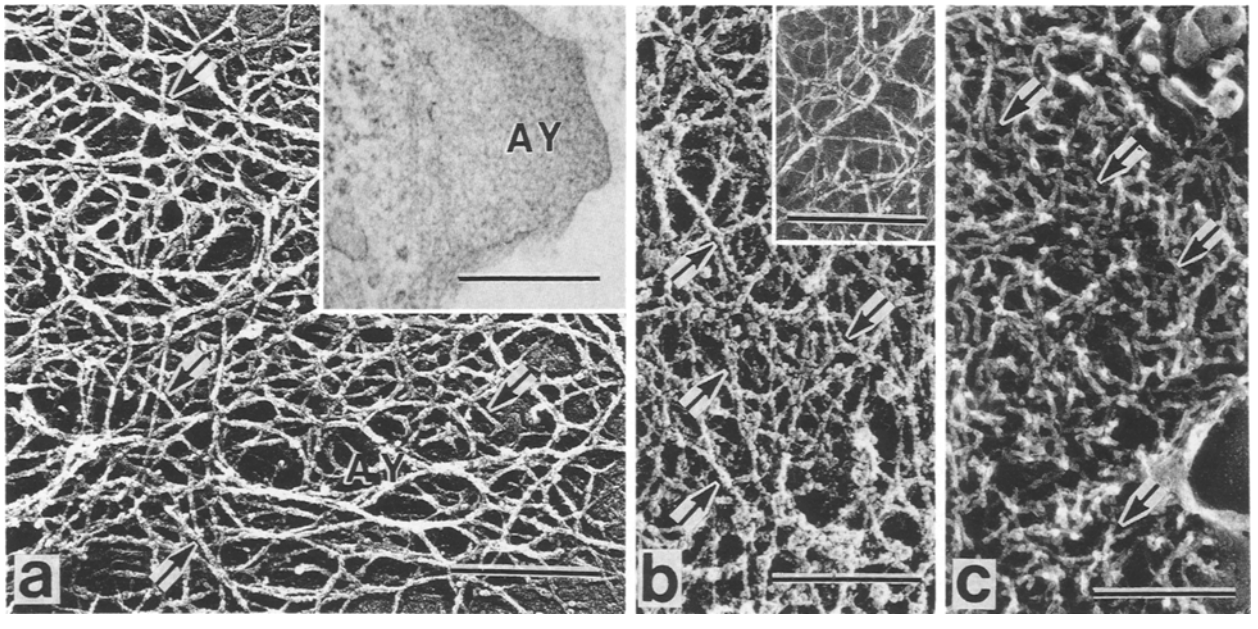


Fig. 4a–c. Replica electron micrographs of the peripheral cytoplasm. **a** Many microfilaments (*arrows*) are localized in the peripheral cytoplasm (*AY*). ×39600. *Bar*: 0.5 μm. *Inset*: Ultrathin section. Peripheral regions lack large cell organelles. ×38000. *Bar*: 0.5 μm. **b** The microfilaments are immunostained with anti-actin antibody to form aggregated DAB reaction products (*arrows*). ×39600. *Bar*: 0.5 μm. *Inset*: Immunocytochemical control. ×36000. *Bar*: 0.5 μm. **c** The microfilaments are decorated with S_1 myosin fragments (*arrows*). ×37000. *Bar*: 0.5 μm

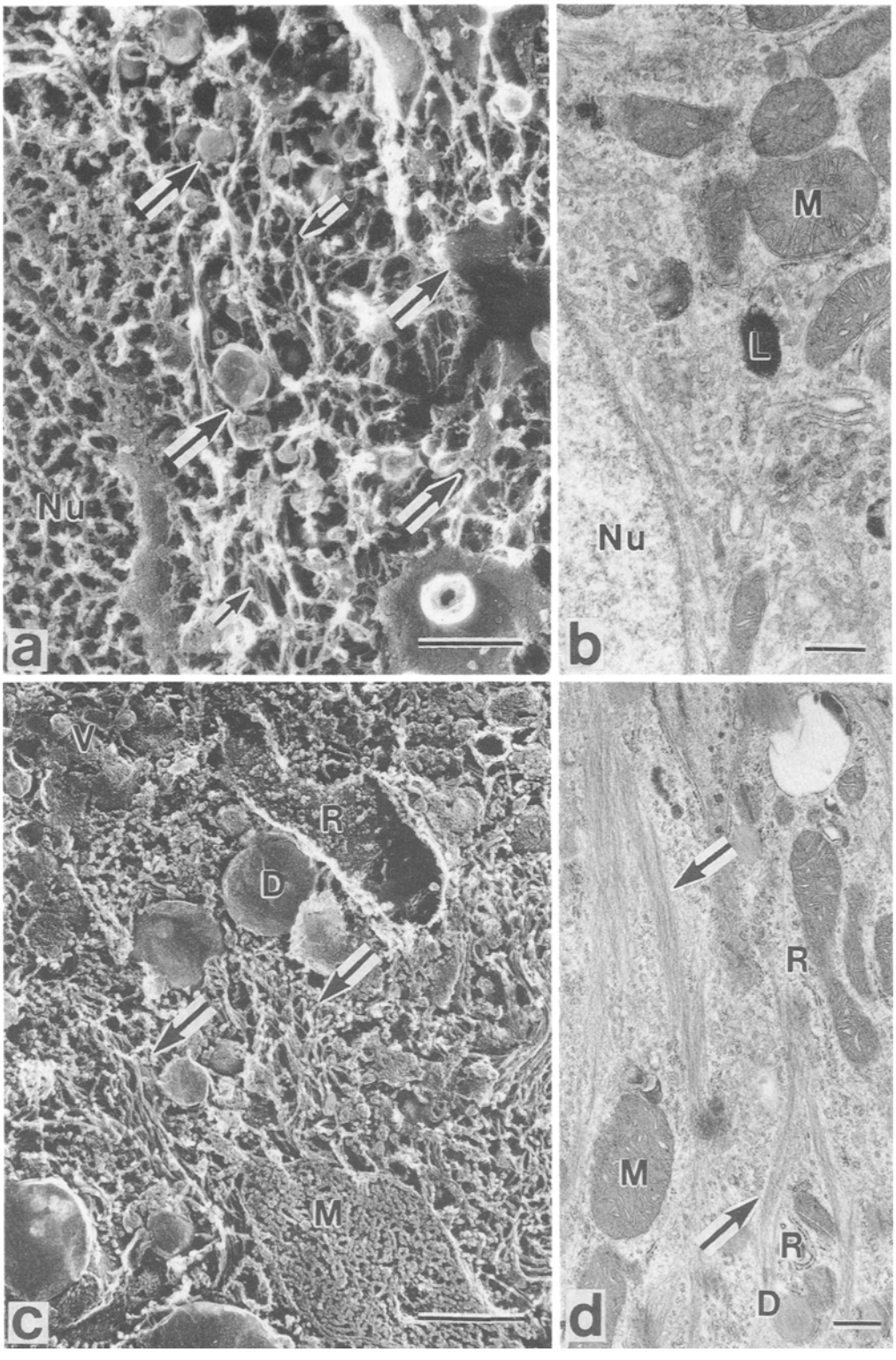


Fig. 5. **a** Replica electron micrograph of deep cytoplasm near the nucleus (*Nu*). Many cell organelles (*large arrows*) and intermediate filaments (*small arrows*) can be identified. ×31000. *Bar*: 0.5 μm. **b** Ultrathin section corresponding to the replica micrograph. *M*, Mitochondria; *L*, lysosomes. ×18000. *Bar*: 0.5 μm. **c** Freeze-fractured intermediate filaments (*arrows*) are localized among cell organelles. *D*, Lipid droplets; *M*, mitochondria; *V*, vesicles; *R*, endoplasmic reticulum. ×31000. *Bar*: 0.5 μm. **d** Ultrathin section. Bundled cytoskeletons (*arrows*) are distributed throughout the cytoplasm. *M*, Mitochondria; *R*, endoplasmic reticulum; *D*, lipid droplets. ×13600. *Bar*: 0.5 μm

30 min at a dilution of 1:20 (Ohno and Takasu 1989; Takasu et al. 1988). They were fixed again with 0.25% glutaraldehyde for 15 min to stabilize antigen-antibody complexes and were incubated in 3,3′-diaminobenzidine tetrahydrochloride and hydrogen peroxide solution for 4 min. As the immunostaining control, some hepatocytes were treated with normal rabbit serum as the primary antibody and then processes as described above.

To demonstrate the S_1 myosin fragment (Sigma, St Louis, MO, USA) the fragments were obtained from rabbit muscle. The split hepatocytes were incubated in 1 mg/ml S_1 myosin fragments for 30 min at room temperature (Naramoto et al. 1990; Ohno and Takasu 1989). They were washed in PB, fixed with 0.25% glutaraldehyde for 10 min and prepared for replica membranes as described below.

For quick freezing and deep etching all specimens on the marked circles of coverslips were rinsed in 10% methanol in distilled water to avoid the formation of ice crystal (Heuser and Kirschner 1980). The hepatocytes on the coverslips were blotted with filter paper to remove excess fluid and quickly frozen in the rapidly stirred mixture of isopentane-propane (1:2) cooled by liquid nitrogen (Jehl et al. 1981). The careful removal of excess fluid from the coverslip immediately before freezing was critical to obtaining good freezing. The quick-freezing technique by liquid cryogen was suitable for cultured monolayer cells, because it avoided a compression of the specimens. They were transferred into liquid nitrogen, shaken sharply to remove the adherent isopentane-propane mixture and fractured with a scalpel in the liquid nitrogen. The frozen specimens were introduced into an Eiko FD-3S freeze-fracture machine and deeply etched at −95° C under the vacuum conditions of 10^{-7}–10^{-8} Torr ($2.7–8.0 \times 10^{-5}$ Pa) for 30–60 min. After deep etching, the specimens on the rotary stage were first shadowed with platinum at the angle of 37° for several seconds and then rotary shadowed up to the total thickness of about 2 nm. They were additionally coated with carbon at the angle of 90°. A dop of 2% collodion in amylacetate solution was put on the replicas as soon as the specimens were taken out from the machine, to prevent the replicas from breaking into pieces during the following digestion procedure. The replicas coated with dried collodion were treated in household bleach for 15–30 min to dissolve the cellular components. The coated replicas became detached from the coverslips and were transferred to distilled water. The large replicas (4 mm in diameter) in the distilled water were cut into small pieces with scissors. They were picked up on Formvar-filmed copper grids and then immersed in amylacetate solution to dissolve the dried collodion. All replicas were observed in Hitachi HS-9 and H-700 electron microscopes at accelerating voltages of 75–100 kV. Some stereo-pictures were taken at tilting angles of ±5°. Electron micrographs were printed from the inverted negative films to make platinum deposits appear white and the background appear dark.

To prepare ultrathin sections the monolayer hepatocytes were fixed with 2.5% glutaraldehyde in PB for 30 min. They were washed in PB and postfixed with 1% osmium tetroxide for 30 min. They were routinely dehydrated in a graded series of ethanol and embedded by upside-down gelatin capsules containing Epon 812 (Ohno et al. 1982). The Epon blocks were detached from plastic dishes in liquid nitrogen. Ultrathin sections were cut with a diamond knife and mounted on copper grids. They were doubly stained with uranyl acetate and lead citrate and then observed in a Hitachi HS-9 electron microscope.

Results

Networks of microfilaments under apical cell membranes were observed three-dimensionally on the replica membranes (Fig. 1a). Intermediate filaments and cell organelles were localized under the microfilament networks. However, the cytoskeleton was difficult

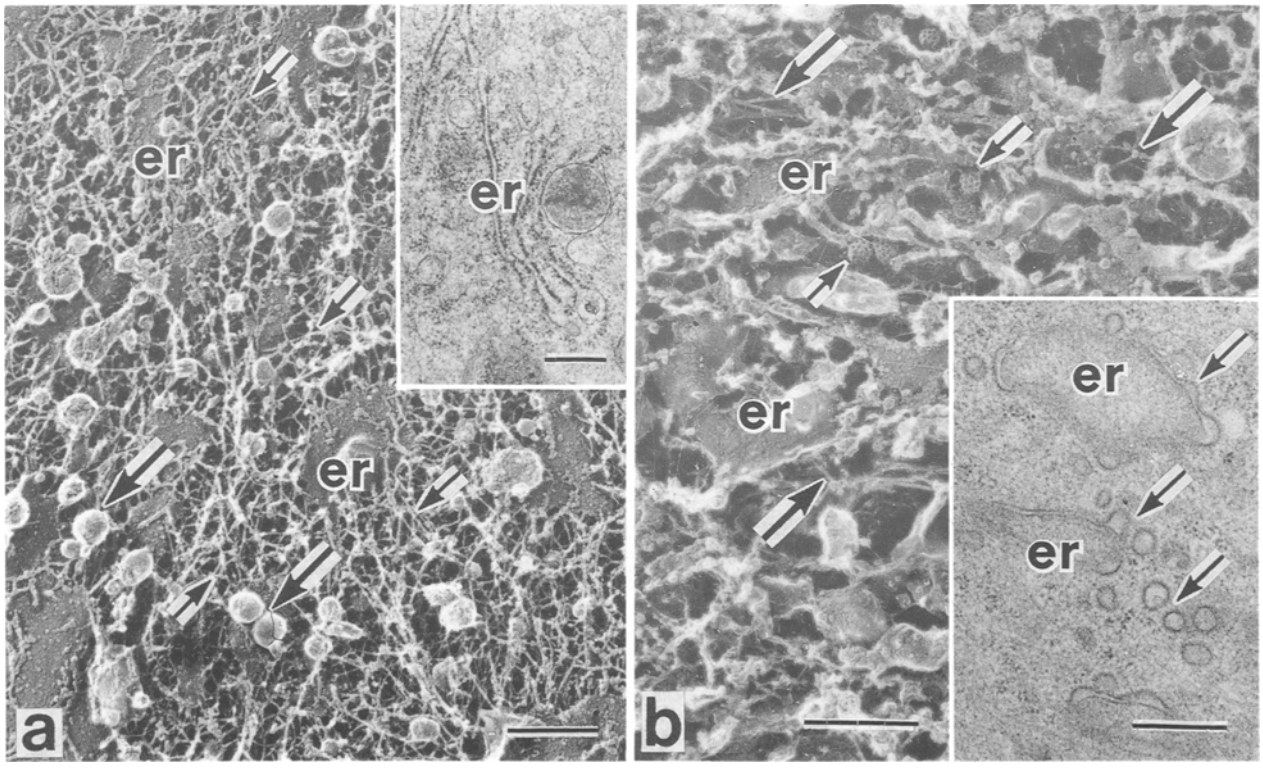
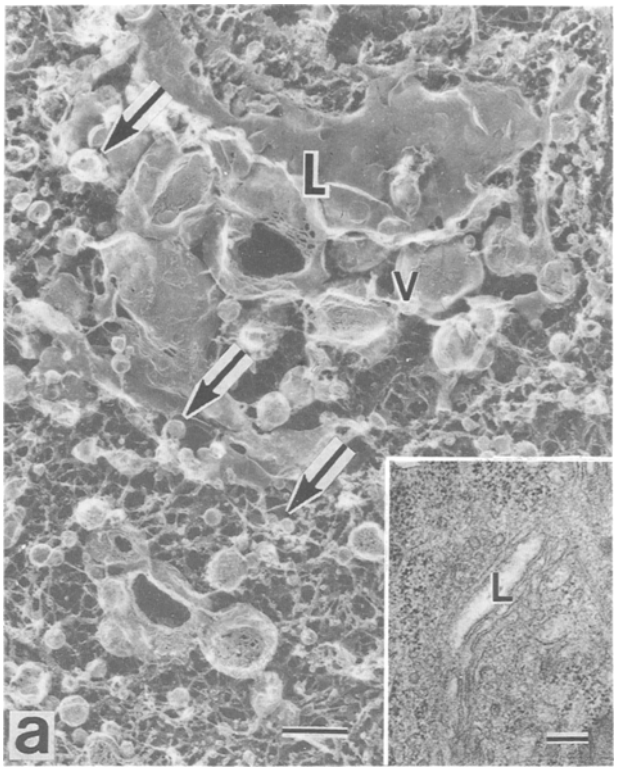


Fig. 6. a Rough-surfaced endoplasmic reticulum (*er*) and vesicles (*large arrows*) are localized with intermediate filaments (*small arrows*). ×24000. *Bar*: 0.5 μm. *Inset*: Ultrathin section. ×16000. *Bar*: 0.5 μm. **b** Coated vesicles (*small arrows*) and pits are localized near the membranes of endoplasmic reticulum (*er*). Much less intermediate filaments (*large arrows*) are associated with the cell organelles. ×29600. *Bar*: 0.5 μm. *Inset*: Ultrathin section. *Arrows* indicate coated vesicles or pits. *er*, Endoplasmic reticulum. ×24800. *Bar*: 0.5 μm



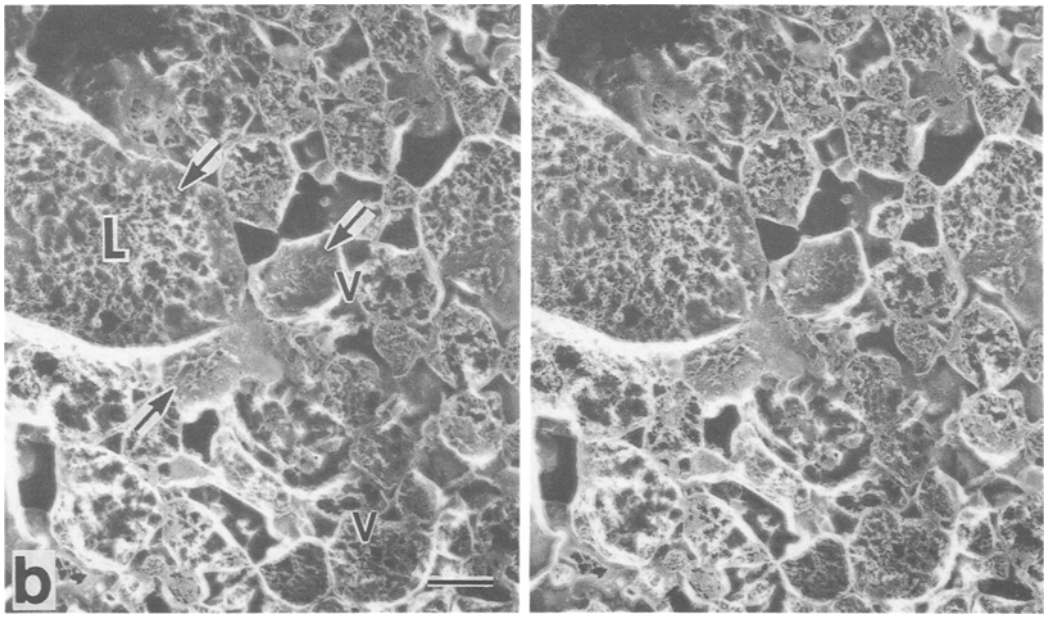


Fig. 7. a Replica electron micrograph of Golgi apparatus in split hepatocytes. Golgi lamellae (*L*), vacuoles (*V*) and vesicles (*arrows*) are identified from the view of cytoplasmic sides. ×17200. *Bar*: 0.5 μm. *Inset*: Ultrathin section. ×10100. *Bar*: 0.5 μm. **b** Stereo-pictures of freeze-fractured Golgi apparatus. Some materials (*arrows*) can be observed within the lumens, but less cytoskeletons are associated with membranes of the Golgi apparatus. *L*, Golgi lamellae; *V*, Golgi vacuoles. ×17200. *Bar*: 0.5 μm

to see under apical cell membranes on ultrathin sections (Fig. 1a, inset). The microfilaments were decorated with S_1 myosin fragments (to demonstrate actin filaments) and directly attached to cytoplasmic sides of cell membranes (Fig. 1b). Stereo-pictures of apical cytoplasm were observed from the fractured cytoplasmic side (Fig. 1c). The microfilament networks were mainly localized under the apical cell membranes. Bundled intermediate filaments were also observed in deeper cytoplasm and some cell organelles were associated with the cytoskeleton.

The basal cytoplasm attached to coverslips was freeze-fractured in liquid nitrogen (Fig. 2a, b). Many intermediate filaments were randomly distributed along basal cell membranes (Fig. 2a). The horizontally freeze-fractured basal cytoplasm showed that bundled intermediate filaments were localized on the cell membranes (Fig. 2b), which correspond to those on ultrathin sections (Fig. 2c). Tightly bundled cytoskeleton was also observed in the deep cytoplasm on ultrathin sections (Fig. 3a). Lateral connections of bundled intermediate filaments were observed three-dimensionally on replica membranes (Fig. 3b). Granular structures were observed between the parallel intermediate filaments, but they were not identified among randomly running intermediate filaments. These structures were immunostained with anti-actin antibody to form rugged contours along intermediate filaments (Fig. 3b, inset).

The peripheral cytoplasm was freeze-fractured in the liquid nitrogen (Fig. 4). Although few cell organelles were observed in this area, many microfilaments were localized near the cell membranes (Fig. 4a). The microfilaments were immunostained with anti-actin antibody to identify actin proteins (Fig. 4b). Moreover,

they were decorated with S_1 myosin fragments to form rugged structures (Fig. 4c).

Figures 5–7 illustrate electron micrographs of the perinuclear cytoplasm. Many cell organelles and intermediate filaments were localized near the nucleus (Fig. 5a, b). Some cell organelles were observed among networks of intermediate filaments (Fig. 5c, d). Lamellae of the endoplasmic reticulum were freeze-fractured in the liquid nitrogen, and their luminar sides contained granular materials (Fig. 5c). Figure 6a illustrates the areas of the rough-surfaced endoplasmic reticulum in split hepatocytes. Intermediate filaments were associated with the cytoplasmic surface of endoplasmic reticulum membranes. In other cytoplasm, coated vesicles and pits were formed on the endoplasmic reticulum and much less intermediate filaments were localized in this area (Fig. 6b). The Golgi apparatus was observed from the cytoplasmic side in split hepatocytes (Fig. 7a). Stereo-pictures illustrate that the Golgi apparatus showed synthesized materials in the fractured lumens (Fig. 7b).

Discussion

The quick-freezing and deep-etching method is a useful technique for studying three-dimensional ultrastructure of cultured hepatocytes. It has the advantage that it allows a view of whole cytoplasm, in contrast to ultrathin sections. Our method has two principle characteristics. Firstly, the cell-splitting method used in the present study enabled us to visualize the cytoskeleton in hepatocytes after removing cytoplasmic soluble proteins. It is well known that the cytoskeleton is mainly composed of microfilaments, intermediate filaments and microtubules, which are thought to be responsible for determining intracellular ultrastructure and functions (Feldmann 1989; French et al. 1989; Knapp et al. 1983; Prentki et al. 1979). In our study, the split hepatocytes appeared to be well suited for use in labelling procedures such as those used with S_1 myosin fragments and anti-actin antibody. Secondly, as a quick-freezing step, metal contact freezing by liquid helium is not always practical due to difficulties of handling and high cost. So we reported a simple method of plunging specimens in the isopentane-propane mixture cooled by liquid nitrogen.

The ultrastructure determined by cell attachment to matrices is an important factor in influencing protein metabolism in cultured hepatocytes (Sudhakaran et al. 1986). Our primary interest was to determine whether the monolayer hepatocytes formed different cytoskeletal organizations when they were cultured on collagen gels. It has been reported that the addition of collagens to culture medium resulted in the stabilization of cultured hepatocytes (Mak et al. 1980; Michalopoulos and Pitot 1975; Nickola and Frimmer 1986; Rubin et al. 1981). In the present study, the interaction of collagen gels with cell membranes induced the formation of basal cytoplasm. Therefore, the effects of collagen gels on intracellular ultrastructure might be mediated via unknown receptors for some extracellular molecules (Rapraeger et al. 1986; Rubin et al. 1981). It is one possibility that proteoglycans act as matrix receptors to stabilize the basal cytoplasm of cultured hepatocytes, as reported in cultured fibroblasts (Woods et al. 1984). The existence of adhesive surfaces determined the orientation of basal cytoplasm.

The present study also calls attention to the role of collagen gels in controlling the organization of the cytoskeleton. As the elucidation of functions of the cytoskeleton has been the focus of recent studies, the relationship between cytoskeletons and matrices has been discussed in some cultured cells (Marceau et al. 1980; Rapraeger et al. 1986; Sugrue and Hay 1981; Woods et al. 1984). In the present study, the basal cell membrane was in contact with collagen gels and the apical membrane surface was oriented towards the culture medium, resulting in the different cytoskeletal organization. This indicates that the collagen gel is an important element in maintaining the basal cytoskeleton. The basal arrangement of intermediate filaments served as a mechanical support for cell structures, as reported in other cells (Lazarides 1980; Lehto et al. 1978). It is probable that the collagen gels induce the reorganization of the cytoskeleton through their binding sites on the cell membrane. The connections between cell membranes and the cytoskeleton may play an important role in many cellular phenomena, as reported in cultured thyroid cells (Takasu et al. 1988). The richness of intermediate filaments in the basal cytoplasm agrees with the view that the cytoskeleton is involved in the maintenance of ultrastructure in vitro (Borenfreund et al. 1980; Marceau et al. 1980). However, the distribution of microtubules was not a prime concern of the present study.

The morphological structure of hepatocytes removed from their native architecture differs from that observed in intact tissues (Prentki et al. 1979). The disappearance of tissue elements is a first step in disturbing the maintenance of in vivo ultrastructure. It has been reported that the genetic expression of intermediate filaments changes with the cellular environment and that their polypeptide composition is modified in cultured hepatocytes (Franke et al. 1981a, b). The peripheral sheet of intermediate filaments seen in in vivo hepatocytes (Franke et al. 1981a, Katsuma et al. 1988; Ohta et al. 1988; Van Eyken et al. 1987) was not formed in cultured hepatocytes (Franke et al. 1981b). However, intermediate filaments in the basal cytoplasm play an important role in the maintenance of cell shape. Moreover, it is reasonable to postulate that the intermediate filaments in cultured hepatocytes function as frameworks for the organization of various cell organelles, as described in other cells (French et al. 1982; Lazarides 1980; Okanoue et al. 1985). These changes in the cytoskeleton seem to be a cellular adaptation to the culture conditions.

It has been reported that microfilaments are required for the secretion of bile acids and proteins and the endocytosis of serum proteins. Cultured hepatocytes have commonly been used as a model system to study a variety of basic cellular functions (Prentki et al. 1979; Sudhakaran et al. 1986). In liver tissues, the microfilament networks are mainly localized in the peripheral cytoplasm facing bile canaliculi and space of Disse (French and Davies 1975; Hoshino et al. 1989). In our cultured hepatocytes, microfilaments were localized in the apical cytoplasm as reported before (Mak et al. 1980), but intermediate filaments predominated in the basal cyto-

plasm. In contrast to the hepatocytes reorganized into tissue organization, the monolayer hepatocytes underwent less functional alteration (Sudhakaran et al. 1986). The collagen gels were responsible for the formation of monolayers and microvillus formation was induced on apical cell membranes. Functionally, microvilli have an important role in the transfer of materials between the cell and its environment. However, it remains to be determined whether the morphological polarity of cultured hepatocytes contributes to a different metabolic activity in vitro. The promising quick-freezing and deep-etching method will be applied to clarifying the relationship between three-dimensional ultrastructure of cultured hepatocytes and their functions.

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