Altered Cell Motility Behaviour and Reorganization of Contractile Proteins after Primary Monolayer Cultures of Isolated Hepatocytes

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***Objective*: The aim of this study is to elucidate the temporal changes of the cell motility and the localization of contractile proteins in isolated hepatocytes after primary monolayer cultures.**
***Methods*: Cultured hepatocyte couplets and triplets were observed at 4 hr and 24 hr with time-lapse video recording using video-enhanced contrast, differential interference contact (VEC-DIC) microscopy. The distribution of actin and myosin was examined by immunofluorescence, transmission electron microscopy, and electron microscopy using a whole cell mount.**
***Results*: In 4 hr-cultured hepatocyte couplets, bile canalicular contractions, mediated by the surrounding actin and myosin, were spontaneous and forceful. At 24 hr, the cells spread and flattened out, and VEC-DIC microscopy revealed rope-like fibers, possibly stress fibers in the flattened cytoplasm. Stress fiber motilities were active and independent of other cell movements. The saltatory movement of the vesicles in the cytoplasm was clearly visualized with time-lapse recording. However, the bile canaliculi between two hepatocytes were closed at 24 hr, and the canalicular contractions were no longer seen. Actin and myosin were found in the same area as stress fibers were observed by VEC-DIC microscopy.**
***Conclusions*: It was possible to visualize high-resolution images of the dynamic cell function in the living hepatocytes, using VEC-DIC microscopy with time-lapse recording. The changes in the cell motility pattern may be ascribed to the reorganization of the contractile proteins in isolated hepatocytes after monolayer cultures. Four hr-cultured hepatocyte couplets are considered to be similar to the liver in a living state in terms of the cell morphology and the physiological motility function of the canaliculi.**

Key words: contractile proteins, isolated rat hepatocyte couplets, canalicular contraction, stress fibers, VEC-DIC microscopy

INTRODUCTION

Actin and myosin have been found in all eukaryotic cells including hepatocytes [1, 2]. Actin filaments are present throughout the hepatocyte cytoplasm but they are particularly abundant near the bile canaliculi, as many techniques have revealed [3-5]. The question remains regarding what is the action of actin in hepatocytes? Our previous observation of active bile canalicular contractions in freshly prepared isolated hepatocytes using time-lapse cinematography provided the first conclusive evidence that the canaliculi are contractile [6]. As cytochalasin and phalloidin inhibit bile canalicular contractions, actin filaments are thought to mediate this motility [7, 8].

Stress fibers, which are composed of microfilament bundles, are prominent in well-spread non-muscle cells [9], and they are also present in some cells in situ [10]. Microfilaments, which measured 5-7 nm in diameter, are considered to be formed by polymerized actin in non-muscle cells. Immunofluorescent studies have revealed that stress fibers contain actin [11], myosin [12], α-actinin [13], tropomyosin [14] and filamin (15); it was thus theorized that these fibers function as contractile organelles.

Video-enhanced contrast, differential interference contact (VEC-DIC) microscopy, a powerful microscopic technique, was introduced by Allen *et al*. [16, 17]. This microscopic technique permits the direct visualization of organelles such as microtubules in living cells [18]. In cultured hepatocytes, microfilament bundles are found in the cytoplasm [18-20], but their functional significance has not yet been elucidated. We herein report the temporal changes of cell morphology and motility of isolated hepatocytes after a primary monolayer culture, using VEC-DIC as a new microscopic technique.

MATERIALS AND METHODS

Primary Cultured Hepatocyte Preparation

Isolated hepatocytes were prepared from adult female Wistar rats weighing 170-230 g. These rats were fed with laboratory pellet rat diet and tap water ad libitum. They were given humane care in compliance with institutional guidelines. Primary cultured rat hepatocytes were obtained with a previously reported procedure [6, 21]. Liver perfusion and dissociation were based on Seglen's method [22], as modified by Laishes and Williams [23]. Initial washout perfusion through the portal vein was performed with Ca^{2+} and Mg^{2+}-free Hanks' balanced salt solution containing 0.5 mmol/L EGTA for 4 min at 40 ml/min. Next, L-15

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medium containing 0.05% type 1 collagenase (Sigma, St. Louis, MO) was perfused for 8 min at 25 ml/min.

One million isolated hepatocytes were inoculated in a glass-bottom dish. In the specially prepared culture dishes used in this study, the plastic at the bottom was replaced with a glass coverslip (No. 0) to achieve high resolution for differential interference contrast (DIC) microscopy. The cells were maintained with L-15 medium which contained 10% fetal bovine serum, 10 mmol/L HEPES, penicillin (100 U/ml) and streptomycin (100 μg/ml). The viability of the isolated hepatocytes by trypan blue exclusion test was approximately 95%. These cells were pre-incubated for 4 hr at 38°C to facilitate cell attachment to dish bottoms and to allow time for recovery from the cell isolation procedure. Two hours after inoculation, the culture medium was changed to withdraw any unattached cells. With this method, approximately 10% of the isolated hepatocytes remained inseparable. The partially separated cell groups, especially couplets and triplets were then selected for this experiment.

DIC Microscopy and Image Recording

After inoculation, isolated hepatocytes were cultured for 4 or 24 hr. During the experiments, the cells were stored at 38°C using temperature-controlled plastic housing (Nikon NP-2, Japan). Four or 24 hr-cultured hepatocytes were examined by VEC-DIC microscopy [16, 17] using DIC optics (Nikon, Diaphot TMD-EY) consisting of 40 and 100 times objectives.

Images were projected onto a binary computer-compatible chalnicon video camera (Hamamatsu, C 1000-01, Waltham, MA), whose averaging function improved the signal-to-noise ratio by gathering incoming video data over several frames. The video signal was transferred to a time-lapse video cassette recorder (Panasonic NB 6050 or NV 8050) and to a 9-inch TV monitor (Panasonic WV 5360). Photographs were taken of the video image on the TV monitor screen using a Nikon F3 camera.

Immunofluorescence

Indirect immunofluorescent staining procedure followed that of Lazarides [11]. Four and 24 hr-cultured hepatocytes grown on coverslips were fixed either in paraformaldehyde in phosphate buffered saline (PBS; pH 7.2) for 30 min, or directly in cold acetone (−20°C) for 5 min. After rinsing with PBS, cells were incubated for 60 min with rabbit anti-actin or rabbit anti-myosin antibodies (Biomedical Technologies Inc., Cambridge, MA). Next, they were incubated for 30 min with FITC-conjugated goat anti-rabbit antibodies. A Nikon fluorescence microscope (Diaphot TMD-EF) equipped with a B1-filter cassette (exciting filter 420-485 nm, dichroic mirror DM 510, and absorption filter 520-560 nm) was used for examination.

Scanning Electron Microscopy

Four and 24 hr-cultured hepatocytes were fixed with 1% glutaraldehyde and post-fixed with 1% osmium tetroxide in 0.1 mol/L cacodylate buffer for 1 hr at 4°C. The cells were then dehydrated in a graded series of ethanol solutions and dried with a critical-point drying apparatus (E3100 Jumbo Series II, Polaron Equipment Ltd., Watford, England). They were coated with gold using a cold spatter etch unit (DESK-1, Denton Vacuum Inc., Cherry Hill, NJ) and then were examined under a JEOL scanning electron microscope (JSM-35, Tokyo, Japan) with a 20 KV acceleration voltage.

Transmission Electron Microscopy

Four and 24 hr-cultured hepatocytes were fixed with a fixative consisting of 1% glutaraldehyde and 0.1% tannic acid (Sigma) in the buffer. The buffer itself (pH 7.0) contained 25 mmol/L PIPES, 50 mmol/L KC1 and 5 mmol/L $MgCl_2$ [18, 24]. After the hepatocytes were fixed for 30 min at room temperature, they were rinsed with the buffer and then were post-fixed with 0.1% osmium tetroxide for 20 min at 4°C [25]. The cells were then soaked in 1% aqueous uranyl acetate for 30 min at room temperature [3], dehydrated in a graded series of ethanol, and embedded in Epon. Ultrathin sections were cut with a diamond knife on an ultramicrotome (LKB 2088 Ultratome V, Stockholm, Sweden), and they were stained in saturated aqueous uranyl acetate solution and Sato's lead solution [26]. The specimens were examined under a Philips EM 400T transmission electron microscope (Philips Electronic Instruments Inc., Mohawk, N.J.) with a 60 KV acceleration voltage.

Whole Cell Mount Procedure

EM gold grids (J.B. EM Service Inc., Montreal, Canada) were forvar- and carbon-coated and sterilized by exposure to ultraviolet light. The grids were put in culture dishes before isolated hepatocyte inoculation. Twenty four hr-cultured hepatocytes grown on grids were either lysed and then fixed or lysed and fixed simultaneously. The buffer used for lysis and/or fixation consisted of 25 mmol/L PIPES (pH 6.9), 1 mmol/L EGTA, 1% polyethylene glycol 6000 (Sigma), and 0.5 mmol/L $MgCl_2$ [18]. This buffer contained 0.1% Triton X-100 (Sigma) and 1% glutaraldehyde for lysis and fixation, respectively. After 30 min of fixation, the cells were washed with the buffer and post-fixed with 1% osmium tetroxide for 1 min. They were then dehydrated in a graded series of ethanol, stained with 0.5% uranyl acetate in 70% ethanol for 1 min, and critical-point dried. The specimens that were mounted following the whole cell mount procedure were examined at 100 KV accelerating voltage under the same transmission electron microscope as described previously.

RESULTS

In time-lapse photography of 4 and 24 hr-cultured hepatocytes, marked differences in cell motility were observed under VEC-DIC microscopy. In 4 hr-cultured couplets or triplets, several types of motility were present throughout the cytoplasm. Some of this movement was random and Brownian-like. Other prominent vesicular or vacuolar movement was found, especially in the pericanalicular regions. A striking finding was that the bile canaliculi between two hepatocytes contracted spontaneously and forcefully, independent of other cellular motility. Figure 1 represents typical bile canalicular contractions of hepatocyte triplets observed by VEC-DIC microscopy. The contractions were regular

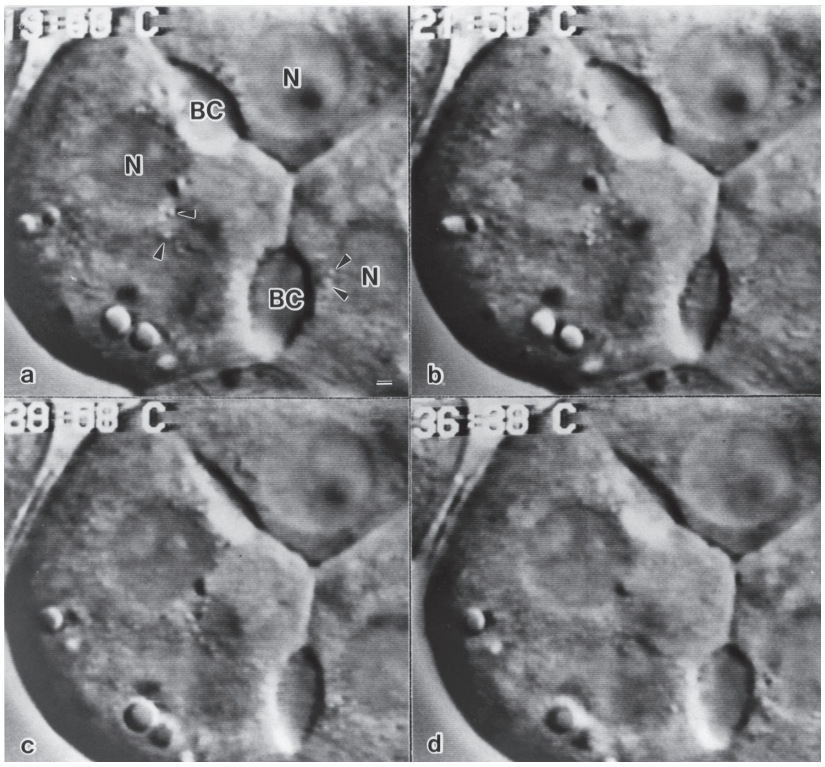

Fig. 1 Representative VEC-DIC micrographs of 4-hr cultured hepatocytes.
Figures a, b, c, and d show a sequence of the bile canalicular contractions in hepatocyte triplets. A time lapse series is shown with a real time value in the upper left corner of each micrograph. Bile canaliculi (BC) show spontaneous and dynamic contractions. Active vesicular movements (arrowheads) are clearly recognized in the pericanalicular and perinuclear regions. A bar indicates 1 μm.

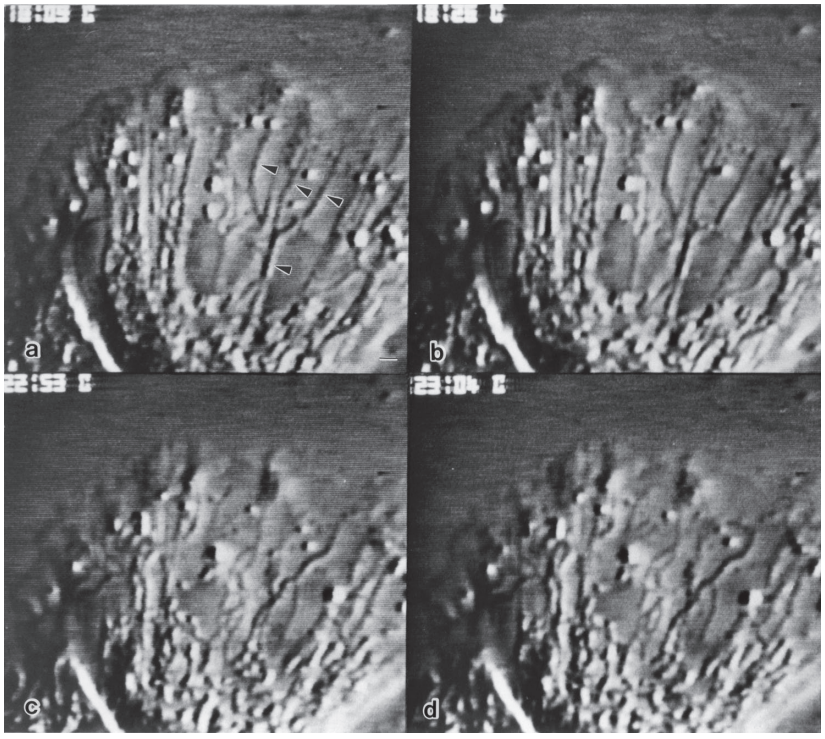

Fig. 2 Representative VEC-DIC micrographs of 24-hr cultured hepatocytes.
Figures a, b, c, and d show a time-lapse sequence of the flattened cytoplasm. The real time is shown in the upper left corner of each micrograph. Rope-like fibers indicated by arrowheads are found in the flattened cytoplasm, and they were straight or curved, intersecting each other at various points and angles. The rope-like fibers show dynamic motilities such as stretching, retracting, and squirming. It is also noted that three sizes of vesicles show active saltatory movements in the flattened cytoplasm. Bile canalicular contractions are no longer seen at 24-hr cultured hepatocytes. A bar indicates 1 μm.

and repetitive.

The isolated hepatocyte couplets and triplets spread and flattened out at 24 hr after a monolayer culture. Several rope-like fibers (RFs), approximately 0.3-1.2 μm in apparent diameter were seen in the flattened cytoplasm, especially on the cell periphery, and they ran almost parallel to the long axis of the cell body. The RFs were either straight or curved, thus intersecting each other at various points and angles. In time-lapse photography taken 36 times real time, the RF showed active movements such as stretching, retracting, and squirming (Fig. 2), which were independent of cell ruffling or other cell movement. They moved intermittently toward or away from the cell margin. The bile canaliculi between two hepatocytes were closed, and the canalicular contractions could not be seen at 24 hr. Three sizes of vesicles or vacuoles showed saltatory movement over distances from 5-15 μm in the flattened cytoplasm. The medium vesicles (0.3-0.8 μm moved faster and more actively than the small (0.1-0.3 μm in diameter) and large (1.0-1.6 μm in diameter) vesicles. Some of these vesicles appeared to move by

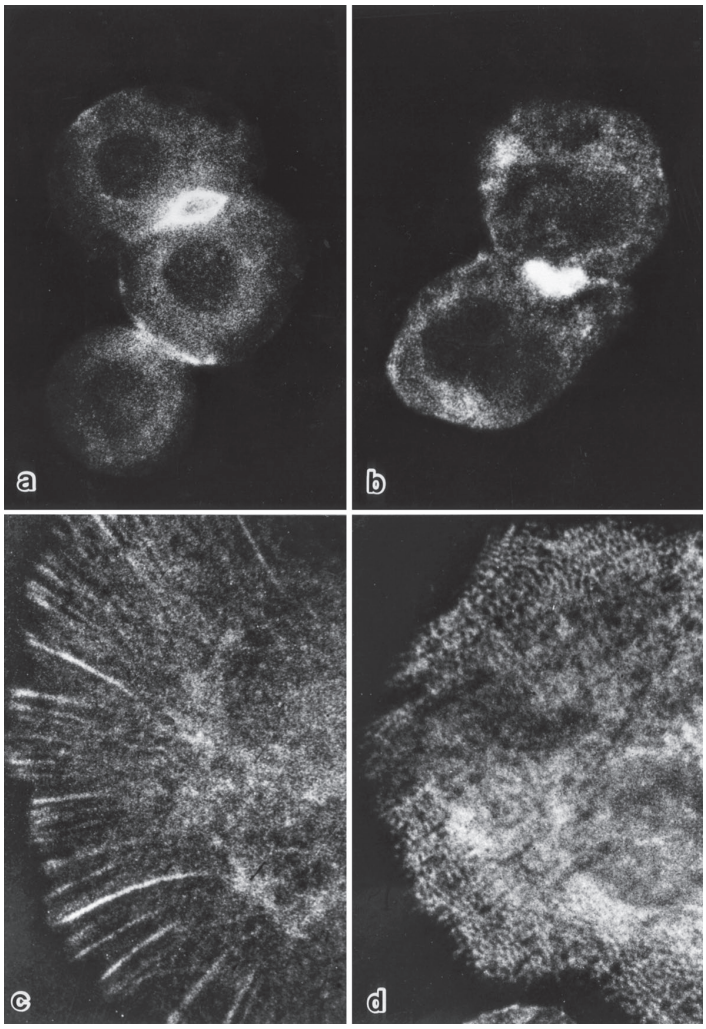

Fig. 3 Immunofluorescence micrographs of 4-hr and 24-hr cultured hepatocytes.
a, b. 4-hr cultured hepatocytes. **c, d.** 24-hr cultured hepatocytes.
a. Stained with actin antibody. Actin is localized around the bile canaliculi.
b. Stained with myosin antibody. Myosin fluorescence is located around the canaliculi.
c. Stained with actin antibody. The actin fluorescence is recognized in a linear form in the flattened cytoplasm.
d. Stained with myosin antibody. Myosin is present in a dot form in the flattened cytoplasm.

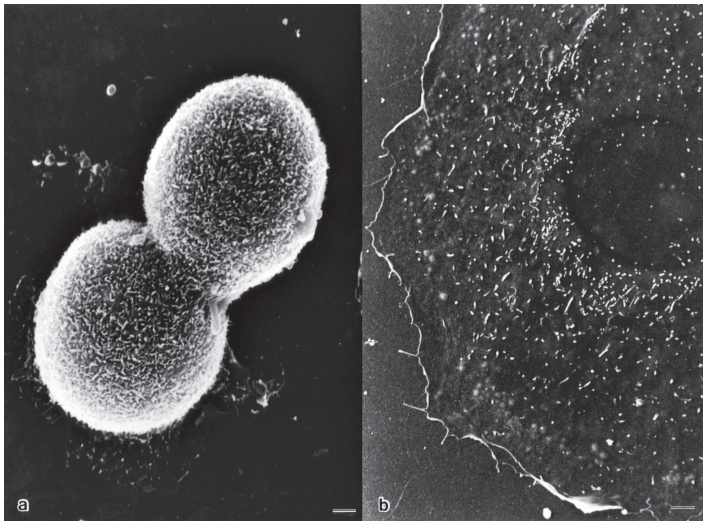

Fig. 4 Scanning electron micrographs of 4-hr and 24-hr cultured hepatocytes.
a. 4-hr cultured hepatocytes. Paired hepatocytes are spherical and covered with uniform microvilli. A bar indicates 1 μm.
b. 24-hr cultured hepatocytes. It is noted that cultured hepatocytes spread and flattened out at 24 hr after a monolayer culture. A bar indicates 1 μm.

rolling along the RFs.

By means of immunohistochemistry, actin- and myosin-specific fluorescence was observed, particularly around the bile canaliculi in 4 hr-cultured hepatocytes (Fig. 3). At 24 hr, actin fluorescence was linear in the areas in which the RFs were revealed by VEC-DIC microscopy. As a result, the RFs were found to correspond to stress fibers (actin bundles). Faint myosin fluorescence appeared as dots in the flattened cytoplasm.

Scanning electron microscopy revealed 4 hr-cultured hepatocytes to have spherical contours and uniform microvilli-covered surfaces. In contrast, at 24 h after a monolayer culture, the cells spread and flattened out (Fig. 4).

Through this method for transmission electron microscopy, the ultrastructures of microfilaments, microtubules, and intermediate filaments were well

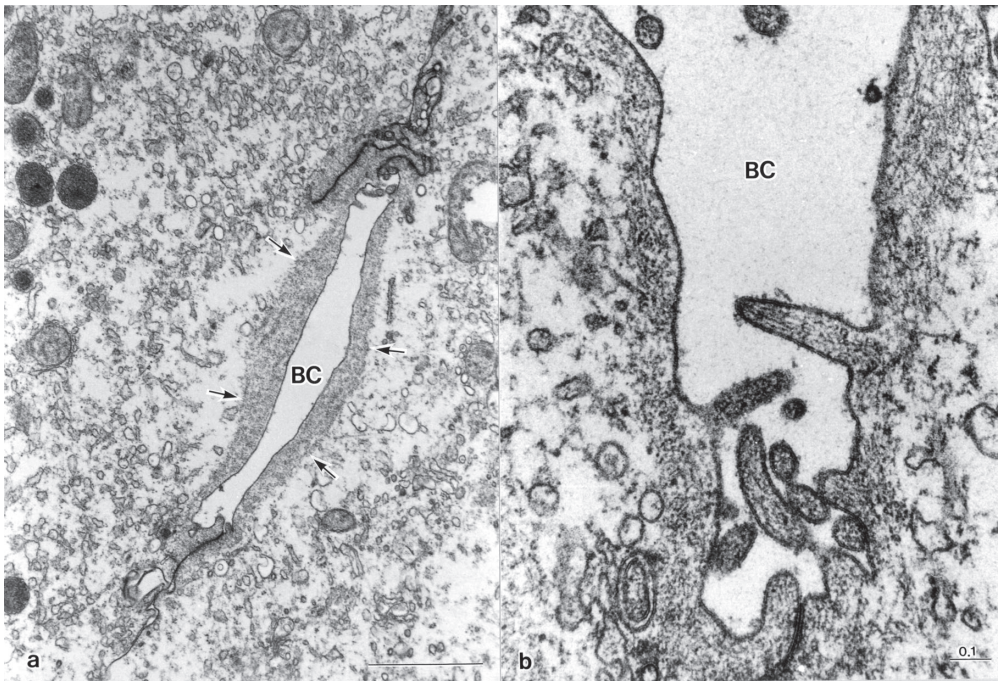

Fig. 5 Transmission electron micrographs of 4-hr cultured hepatocyte couplets.
a. Microfilament bundles (arrows) are present around a bile canaliculus (BC) between two hepatocytes. A bar indicates 1 μm.
b. At high magnification, microfilaments extend into the core of microvilli, associated with the bile canalicular membranes. A bar indicates 0.1 μm.

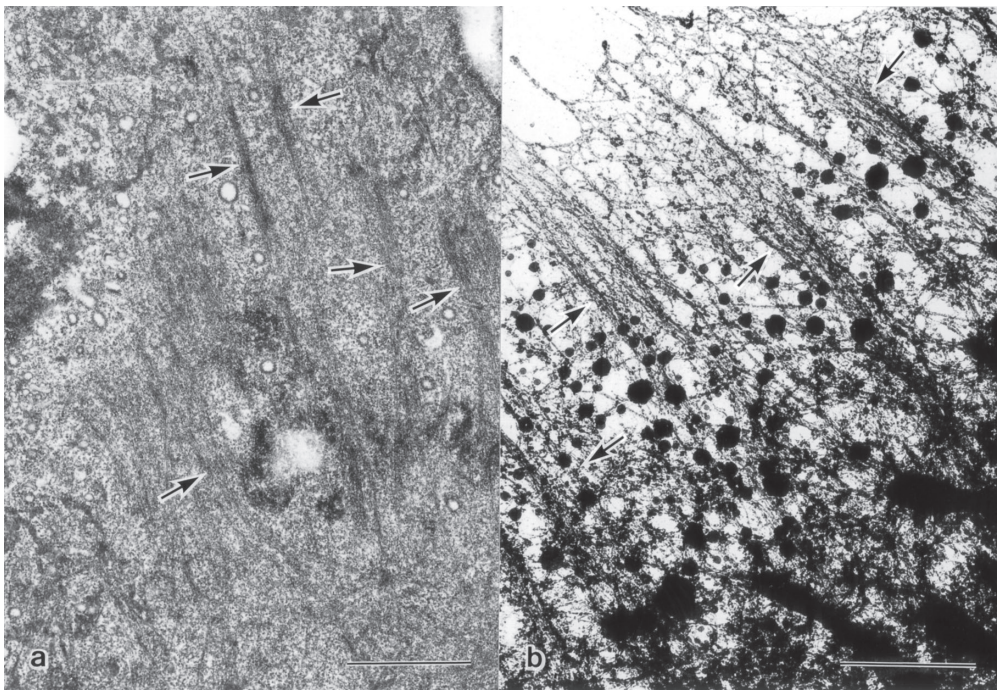

Fig. 6 Electron micrographs of 24-hr cultured hepatocyte couplets.
a. Transmission electron micrograph. Microfilament bundles (arrows) are seen running towards the cell margin. A bar indicates 1 μm.
b. Whole cell mount electron micrograph. Microfilament bundles (arrows) are clearly recognized in the flattened cytoplasm. Some vesicles appear to be associated with microfilament bundles. A bar indicates 1 μm.

preserved and recognized in cultured hepatocytes. A meshwork of microfilaments, measuring 5-7 nm in diameter, was seen in the pericanalicular regions of 4 hr-cultured hepatocytes (Fig. 5). These microfilaments were particularly abundant around the bile canaliculi and they extended into the cores of the microvilli.

In 24 hr-cultured hepatocytes, microfilament bundles, which were not seen in the 4 hr-cultured cells, were observed in the flattened cytoplasm (Fig. 6). The microfilament bundles were seen radiating toward the cell margin and they were associated with the cortical microfilaments. Some small vesicles were

present between the microfilament bundles and they were closely associated with them. Microtubules and intermediate filaments were randomly visualized in the flattened cytoplasm of 24 hr-cultured hepatocytes. By electron microscopy using whole cell mounts, the structure of cytoskeletons was clearly recognized in 24 hr-cultured hepatocytes. The microfilament bundles radiating toward the cell margin could be observed in the flattened cytoplasm (Fig. 6), although no cortical microfilaments were preserved. The microfilament bundle distribution coincided with distribution of the RFs observed by VEC-DIC microscopy. Some vesicles appeared to be associated with the microfilament bundles. The microtubules were distributed more randomly than the microfilament bundles in the flattened cytoplasm of the 24 hr-cultured hepatocytes.

DISCUSSION

The present study synthesized several methods used to study cell motility: time-lapse recording using VEC-DIC microscopy, immunohistochemistry for actin and myosin, transmission electron microscopy and whole cell mount electron microscopy. We documented differences in the cell motility of 4 and 24 hr-cultured hepatocytes by VEC-DIC microscopy and then correlated those results with the localization and distribution of contractile proteins using immunofluorescence and electron microscopy. With VEC-DIC microscopy, it is possible to visualize high-resolution images of the dynamic cell function in living hepatocytes. We observed the movements of intracellular organelles such as vesicles, which could not be identified with phase-contrast microscopy.

Bile canaliculi of 4 hr-cultured hepatocytes contracted forcefully. Bile canalicular motility in vitro is highly ordered [6, 21], and it is mediated by actin filaments since cytochalasin and phalloidin inhibit this motility [7, 8]. Our previous studies showed that the contractions are dependent of Ca^{2+}, calmodulins and ATP [27, 28]. In this study, actin- and myosin-specific fluorescence was particularly strong around the bile canaliculi in the 4 hr-cultured couplets and triplets. By electron microscopy, a microfilament meshwork was found to be present near the canaliculi, and it was also associated with the bile canalicular membranes. As a result, both actin and myosin are suggested to be involved in the bile canalicular motility in 4 hr-cultured hepatocytes. We also demonstrated active bile canalicular contractions in living rats using a sophisticated technique [29]. The results indicate that canalicular motility plays a dynamic role in bile flow, thus propelling bile from one portion to another in a living state. Therefore, not only contractile protein distribution but the physiological motility function of bile canaliculi in 4-hr-cultured hepatocyte couplets is similar to those of the liver in a living state. Since the stress fibers in the flattened hepatocytes recognized at 24 hr are not present in the liver of rats and human, 4 hr-cultured hepatocytes should be used for the study of cell morphology and function in the liver.

Stress fibers can be satisfactorily visualized in several living cells by phase contrast and DIC microscopy [30-32]. Moreover, actin bundles are mainly recognized as submembranous bundles on the adhesive side of the cell, and they correspond to the stress fibers observed via phase contrast microscopy in living cells [30]. In the present study, stress fibers were found in the flattened cytoplasm by means of VEC-DIC microscopy when isolated hepatocytes spread and flattened out at 24 hr after culture. Time-lapse recording of flattened hepatocytes revealed active stress fiber motility such as stretching and retracting, which was independent of cell ruffling or other cell movement. Interestingly, the bile canaliculi between two hepatocytes were closed at 24 hr, and the canalicular contractions were no longer seen. The cell motilities of cultured hepatocytes at 24 hr were completely different from those at 4 hr. Microfilament bundle motility in cultured cells has been reported and discussed by many researchers (listed in reference 33). Although the microfilament bundles were long considered as only cytoskeletal elements, the demonstration of immunofluorescence using contractile protein antibodies [11-15] and ultrastructural investigations [9, 34, 35] has led to the speculation that microfilament bundles may have a contractile function. Cytochalasin and phalloidin as actin inhibitors dramatically rearrange actin in cells, as revealed by immunofluorescence microscopy [36, 37] and whole cell mount electron microscopy [38]. This speculation is supported by experiments showing that stress fibers contract when Mg-ATP is added to extracted cell models [31, 34].

In primary cultured hepatocytes, stress fibers have been observed on the cell periphery several days after culturing [19, 20]; however, stress fiber motility and functional significance are still unknown. Four hr-cultured hepatocytes do not have any stress fibers in the cytoplasm, but they have much actin and myosin, especially around the bile canaliculi. On the other hand, stress fibers were found in the flattened cytoplasm of 24 hr-cultured hepatocytes, whereas the closed canaliculi did not show active contractions. It is speculated that stress fibers recognized at 24 hr may be formed by a long-time monolayer culture. Stress fiber formation after monolayer cultures may be related to the reorganization of contractile proteins in culture so that isolated hepatocytes can attach to the substratum, thus leading to a trabecular pattern of epithelial cell sheets [19]. This speculation is supported by the studies using interference reflection microscopy, which revealed a close relationship between stress fibers and the substratum [32, 39, 40].

Of interest is the fact that stress fibers in 24 hr-cultured hepatocytes were shown to move actively. As some vesicles moved by rolling along stress fibers, stress fiber motility may be related to vesicular movement in cultured hepatocytes. In plant cells, microfilament bundles move actively and are responsible for endoplasmic streaming [41]. Strong supporting evidence from a microinjection study with fluorescence-labeled actin molecules using time-lapse photography shows the assembly and reorganization of stress fibers in living fibroblasts [42, 43]. It has been speculated that stress fibers are the contractile element involved in cytoplasmic movement. As cytoskeletons, they are essential, allowing cultured cells to adhere to the substratum. Allen and his coworkers reported particle transport along microtubules in thin living cells by

means of VEC-DIC microscopy [18]. Judging from the results of immunofluorescence and electron microscopy, the microtubules they mention are considered to differ from our RFs in terms of the width and the distribution.

Over the past 10 years, members of the Rho family have been implicated in many different cellular events, including actin organization, cell adhesion and cell migration. The Rho small G protein family consists of the Rho, Rac, and Cdc42 subfamilies and regulates formation of stress fibers and focal adhesions in many types of cultured cells through reorganization of the actin cytoskeleton [44]. Unfortunately, the precise mechanisms in the regulation of these processes are poorly understood in cultured hepatocytes. Further study is thus needed to clarify the association between the Rho family and the reorganization of the actin cytoskeleton in primary monolayer cultures of isolated hepatocytes.

In summary, this study revealed altered motilities in primary cultured hepatocytes using VEC-DIC microscopy with time-lapse recording. In 24 hr-cultured cells, stress fibers were observed to move, whereas 4 hr-cultured cells featured active bile canalicular contraction. Moreover, altered contractile protein distribution was demonstrated by both immunofluorescence and electron microscopy. These findings indicate that actin and myosin are involved in bile canalicular contraction at 4 hr and stress fiber motility at 24 hr in cultured hepatocytes. The changes in the cell motility pattern may be ascribed to the reorganization of the contractile proteins in isolated hepatocytes after monolayer cultures.

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