Hiroshima Journal of Medical Sciences
Vol.36, No.2, 169~174, June, 1987
HIJM 36-21

A Simple Method for the Observation of Cultured Rat Hepatocytes by Scanning Electron Microscopy

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(Received February 6, 1987)

Key words: Cultured hepatocytes, Scanning electron microscopy, Three-dimensional structure, Aldehyde-prefix osmium-DMSO-osmium method

ABSTRACT

A simple method for the three-dimensional observation of inner and outer ultrastructures of the cultured hepatocyte was presented. The freeze cracked plane of the cultured cell was obtained by means of cracking the underlying substrate. This method made it possible to observe the ultrastructural features of both cell surface and cytoplasm in the same cell. Among several substrates examined in this investigation, the collagen gel sheet was the most useful for this purpose.

Hepatocyte culture system is a good model for studying liver function[18,23]. As the cells under suitable culture *milieu* maintained their various functions for a long time, they have been frequently used as a useful means to examine the biological effectiveness of drugs. Naturally, ultrastructural observation of the cultured cells give us one of the important informations for evaluation of the drug.

For the ultrastructural investigation of the cultured cells, the transmission electron microscopy (TEM) has been mainly used[3,5,7,14,17,20], and the scanning electron microscopy (SEM) has been used for three-dimensional observation[2,8,10,13,16,19].

Recently, Tanaka et al. have introduced an improved method for scanning electron microscopic observation of intracellular structure (osmium-DMSO-osmium method[22], aldehyde-prefix osmium-DMSO-osmium method[21]). They have applied this method to investigation of free cells and cultured cells and obtained good results[4]. Thus, SEM has become a very useful means to study the ultrastructural features not only of the cultured cell surface but also of its cytoarchitecture.

We will introduce a simple method for observation of inner and outer ultrastructures of the hepatocytes cultured on several substrates without complicate procedures.

MATERIALS AND METHODS

Hepatic parenchymal cells were isolated from male Wistar rats weighing 250—300g. The technique of cell isolation which was a modification of that proposed by Seglen[18], was previously described[15]. Under general anesthesia, the portal vein of the rat was cannulated and then liver was preperfused with 0.02% EDTA in calcium-free Hanks' solution. Following preperfusion for 10 min, an enzymatic perfusion in the presence of 0.05% collagenase in Hanks' solution together with 20mM $CaCl_2$ was done. These solutions were saturated with 95% O_2 and 5% CO_2 beforehand and were treated with the same gas during the procedure of separation. Then the liver was removed and thoroughly dissociated with pipetting. The cell suspension was filtrated through 200 nylon mesh filter in order to remove large cell aggregation. Filtrated cells were

gently washed 3 times with cold Hanks' solution in order to eliminate cell debris and non-parenchymal cells. Hepatocytes were finally suspended in medium 199 (M199) supplemented with 10% fetal bovine serum (FBS), 10^{-9}M insulin, 10^{-6}M dexamethasone and antibiotics (penicillin 100U/ml, streptomycin 100μg/ml).

Suspensions of hepatocytes were plated at a density of 5×10^5 cells/ml on small sized glass coverslips (5 × 20 mm) coated with 0.1% poly-L-lysin[16] or rat tail collagen gel (80μg/cm^2)[25] in plastic dishes (9.6cm^2, NUNC). They were incubated for 24 hr at 37°C in a humidified environment of 5% CO_2 : 95% air. After cultivation for 24 hr, cells were rinsed with PBS and were fixed with 0.5% paraformaldehyde — 0.5% glutaraldehyde mixture buffered with 0.1M phosphate buffer (pH 7.4) (PB) for 30 min at room temperature[4]. After rinsing with PB, the specimens were postfixed with 1% OsO_4 in PB for 30 min at room temperature. After a brief rinsing with PB, they were transferred into 25% and 50% dimethyl sulfoxide (DMSO) solution for 30 min respectively. They were then quickly frozen in liquid nitrogen. A poly-L-lysin-coated coverslip with hepatocytes was clipped its both ends with two forceps and was snapped in liquid nitrogen. As collagen gel sheet readily cracked leaving on the glass slips immediately it was frozen, a snapping procedure was not done.

Cracked specimens were replaced in PB and thoroughly rinsed. They were then postfixed in 1% OsO_4 for 1 hr and macerated with 0.1% OsO_4 for 2 to 4 days at 20°C to remove excess cytoplasmic matrix (the osmic maceration procedure[22]).

After conductive staining with 2% tannic acid and 1% OsO_4, they were dehydrated in a graded ethanol series and critical point dried without treatment of isoamyl acetate. The coverslip with dried cells was obliquely mounted on a brass sample stage with Aron Alpha (Toa Synthetic Chemical Co.). Subsequently, they were coated with platinum at 30 Å in thickness in an ion sputter coater (POLARON E5150) and observed under a field emission SEM (Hitachi S-800) operated at 25kV.

RESULTS

After cultivation for 24 hr, parenchymal cells were spread and flattened on the substrates (Fig. 1). The polyhedral cells which enlarged and became confluent in some places constituted trabecula. Each cell was covered with numerous microvilli on its surface. In the case of polycation coated glass, hepatocytes were cracked perpendicularly through the monolayer in the cracked plane of glass or at a short distance from the cracked edge. Using this method, we could observe structures of both cell surface and cytoplasm (Fig. 2). In the cracked surface, numerous round shaped mitochondria, parallel arrays of rough endoplasmic reticulum (RER), smooth endoplasmic reticulum (SER) and a few lipid droplets were observed in the cells three-dimensionally (Fig. 3). Mitochondria were mainly spherical or ellipsoidal in shape. Their cristae appeared lamellar or tubular. Some tubular cristae were observed to be continuous to the periphery of lamellar cristae (Fig. 7). Continuity between RER and SER was occasionally recognized, as shown in Fig. 4. Cisternae of SER were tubular in shape (80nm in diameter) and branched off to form a closely meshed plexus beneath the plasma membrane. SER was more predominant at the free side than the opposite side, substrate side. This suggests that the cells attached to the substrate have a polarity in function.

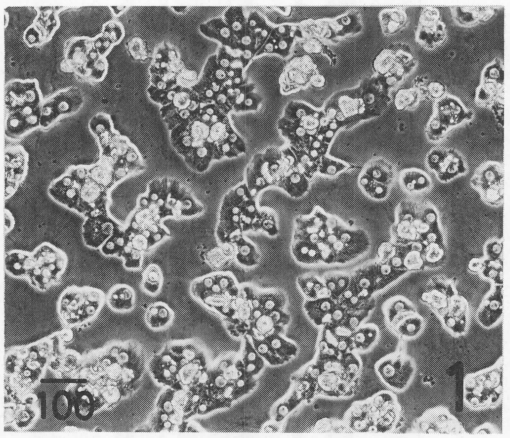


Fig. 1. A photomicrograph of 24-h-cultured hepatocytes on the collagen gel sheet, taken by phase contrast microscopy. Cells were spread and flattened on the gel × 63

In the case of poly-L-lysin, however, most of cells were detached from the cracked edge of

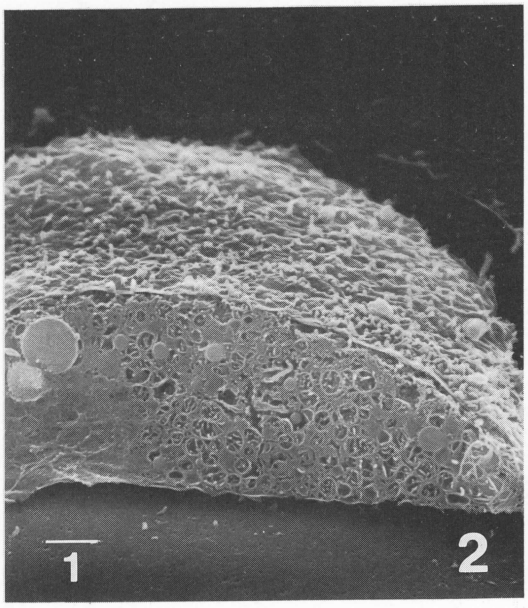


Fig. 2. A freeze cracked hepatocyte cultured on poly-L-lysin-coated glass coverslip. By cracking the glass slip, the ultrastructural features of both cell surface and cytoplasm can be obtained from one cell. Cell surface is covered with numerous microvilli and various kinds of cell organelles are apparently observed on cracked plane. × 7,000

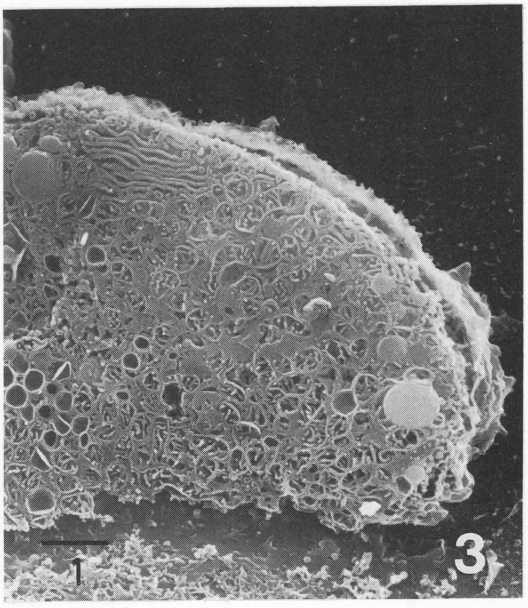


Fig. 3. Higher magnification view of cracked plane of hepatocytes cultured on poly-L-lysin-coated glass slip. Numerous spherical or ellipsoidal shaped mitochondria and their inner features, parallel arrays of RER, tubular SER, vacuoles and lipid droplets can be three-dimensionally observed. × 9,000

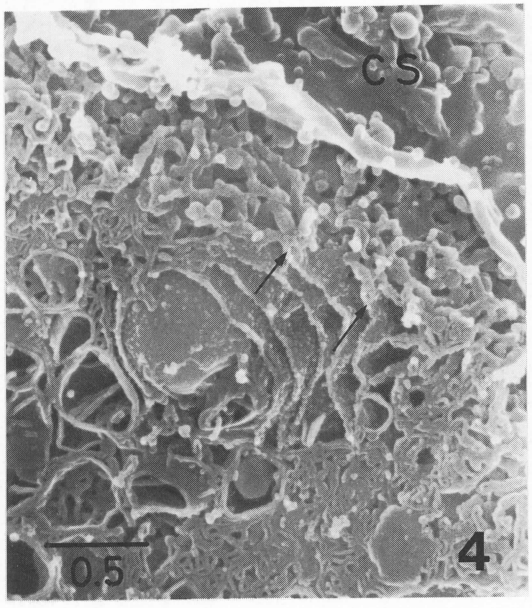


Fig. 4. Beneath the plasma membrane, tubular SER branches off to form a closely meshed plexus. Moreover, they occasionally continue to parallel arrayed RER (indicated by arrows).
CS: cell surface. × 27,500

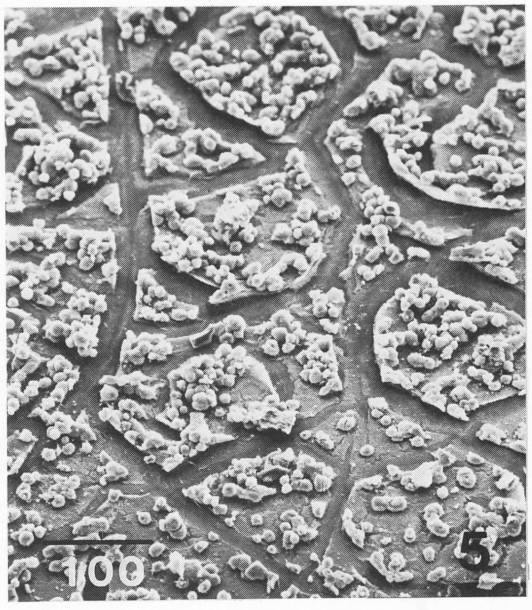
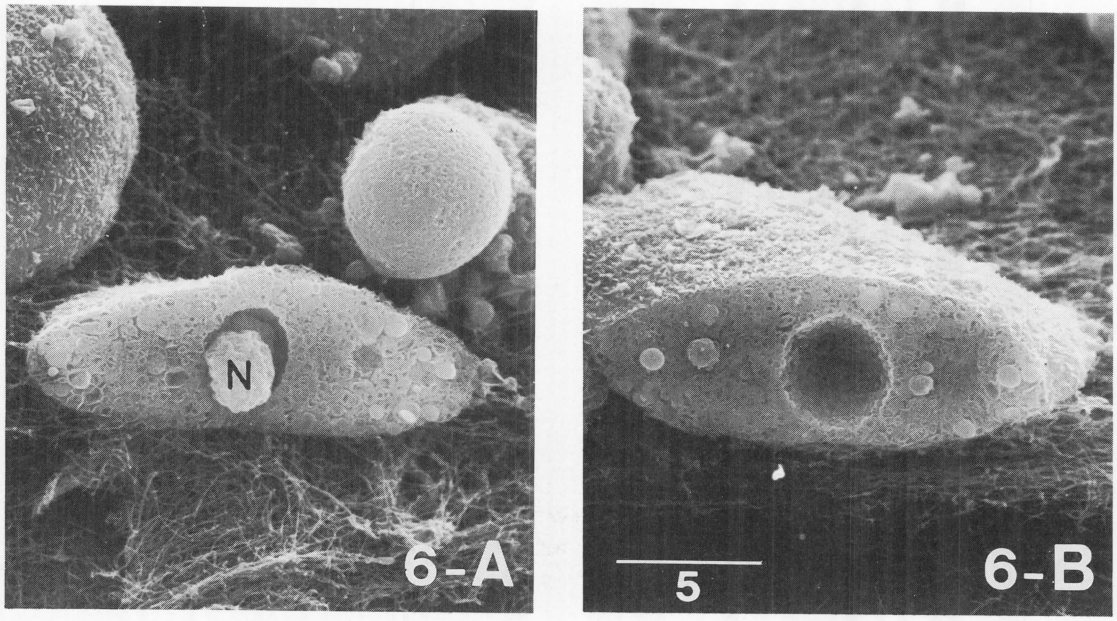


Fig. 5. Collagen gel sheet cracked immediately it was frozen in liquid nitrogen without snapping the underlying glass coverslip. As a result, hepatocytes cultured on it are passively cracked and give us many available cracked planes. × 153



Figs. 6—A and 6—B. Using the collagen gel sheet as substrate, the both cracked faces of two separated fragments of one hepatocyte can be obtained. In this case, however, nuclei (N) is not cracked and it remains in either fragment. × 3,750

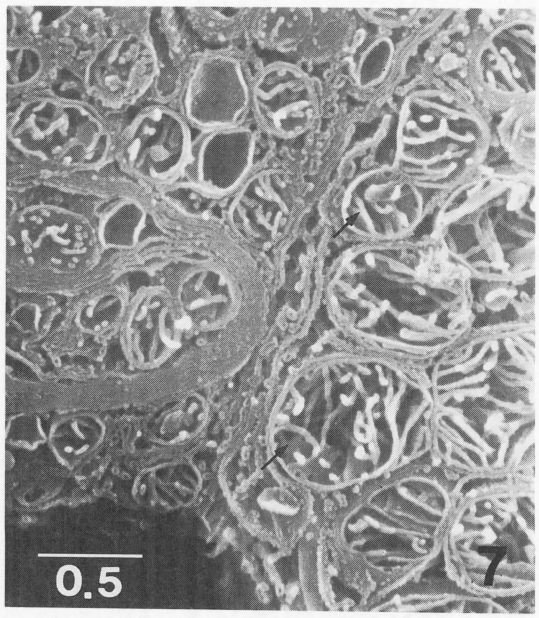


Fig. 7. The two apposing plasma membrane are nearly parallel and are separated by very narrow intercellular space. Mitochondrial cristae are lamellar or tubular, and tubular cristae extending from the lamellar ones are also observed (indicated by arrows). × 28,300

glass by the shock of clipping in liquid nitrogen. Therefore, few of cells showed appropriate cracked plane to examination. On the other hand, collagen gel sheet was easily cracked by freezing alone and provided many appropriate cracked planes without detachment of cells from the substrate (Fig. 5). In addition, the both cracked faces of two separated fragments can be observed in this case (Figs. 6—A and 6—B).

The two apposing plasma membranes were nearly parallel and were separated by very narrow intercellular space (Fig. 7). The bile canaliculus was rarely observed between adjoining cells.

DISCUSSION

Since the isolated hepatocytes were first cultured by Bissell[1], many investigators have used the culture system for studies in biology, biochemistry and others. A number of ultrastructural studies of cultured hepatocytes using TEM and SEM have been reported[2,3,5,7,8,10,13,14,16,17,19,20]. In the primary culture system, the most important problem is how one maintain well functions of the hepatocytes during culture period. It is known that the polycation and collagen gel have allowed to sustain growth of hepatocytes and other epithelial cells in culture[11,12,17,24,25].

Another problem is how one does protect the sample from stripping off from the substrate which is easily occured by the shock of crack-

ing. In order to fix firmly the cells to glass slips, several treatments to coat glass slips were tested; i.e. glass slips were coated with any of the following materials, poly-L-lysin[16], collagen gel[1,25] and gelatin[9]. Among these materials, collagen gel is the most available one but others were not. Plastic dish (NUNC), usually used for culture, was also unsuitable. Moreover, collagen gel sheet could not been cracked on plastic dish in liquid nitrogen.

From these results, it is concluded that a collagen gel-coated glass slip is useful for both cell culture and freeze cracking method.

Our simplified freeze cracking method for the scanning electron microscopic observation made it possible to show three-dimensionally the morphological features of the internal structures of hepatocytes in culture. It was clarified that SER consists of a meshwork of anastomosing tubular cisternae beneath the plasma membrane, and continues to RER (Fig. 5). The size of mitochondria is various from cell to cell.Mitochondrial cristae are flattened plate-like or tubular in shape, and tubular cristae extending from the lamellar ones were also found. We rarely observed the bile canaliculi though other investigators recognized them in cultured hepatocytes[14,16,17]. The reason for this disparity was thought to be caused by the difference of preparation of the cells. These findings show that the cultured hepatocytes maintain the same ultrastructural characteristics as those *in situ*.

REFERENCES

1. **Bissell, D.M., Hammaker, L.H. and Meyer, U.A.** 1973. Parenchymal cells from adult rat liver in nonproliferating monolayer culture. J. Cell Biol. **59**: 722—734.
2. **Chenery, R., George, M. and Krishna, G.** 1981. The effect of ionophore A23187 and calcium on carbon tetrachloride-induced toxicity in cultured rat hepatocytes. Toxicol. Appl. Pharmacol. **60**: 241—252.
3. **Feltkamp, C.A. and van der Waerden, A.W.M.** 1983. Junction formation between cultured normal rat hepatocytes. J. Cell Sci. **63**: 271—286.
4. **Fukudome, H. and Tanaka, K.** 1986. A method for observing intracellular structures of free cells by scanning electron microscopy. J. Microsc. **141**: 171—178.
5. **Furukawa, K., Mochizuki, Y. and Sawada, N.** 1984. Properties of peroxisomes and their induction by clofibrate in normal adult rat hepatocytes in primary culture. In Vitro **20**: 573—584.
6. **Hauschka, S.D. and Konigsberg, I.R.** 1966. The influence of collagen on muscle clones. Proc. Natl. Acad. Sci. USA **55**: 119—126.
7. **Heine, U.I., Wilson, M.J. and Munoz, F.** 1984. Characterization of rat liver cells transformed in culture by DL-ethionine. In Vitro **20**: 291—301.
8. **Homma, S., Nagamori, S., Hasumura, S., Fujise, K., Sujino, H., Kameda, H., Kirino, Y., Hataba, Y. and Suzuki, T.** 1982. The morphological studies on characteristics of isolated parenchymal and non-parenchymal cells of the rat liver. J. Electron Microsc. **31**: 389—396.
9. **Howrot, E.** 1980. Cultured sympathetic neurons: effects of cell-derived and synthetic substrata on survival and development. Dev. Biol. **74**: 136—151.
10. **Knipe, S.M., Klauning, J.E., Gunning, W.T. and Goldblatt, P.J.** 1981. Ultrastructural observation of mouse hepatocytes in primary culture. Micron **12**: 209—210.
11. **Mckeehan, W.L. and Ham, R.G.** 1976. Stimulation of clonal growth of normal fibroblasts with substrata coated with basis polymers. J. Cell Biol. **71**: 727—734.
12. **Michalopoulos, G. and Pitot, H.C.** 1975. Primary culture of parenchymal liver cells on collagen membranes. Exp. Cell Res. **94**: 70—78.
13. **Miettinen, A., Virtanen, I, and Linder, E.** 1978. Cellular actin and junction formation during reaggregation of adult rat hepatocytes into epithelial cell sheet. J. Cell Sci. **31**: 341—353.
14. **Moses, R.L., Delcarpio, J.B., McNeal, M.M. and Davis, R.A.** 1982. Ultrastructure of cultured hepatocytes from fat- and cholesterol-fed rats. Virchows Archi. (Cell Pathol.) **41**: 95—106.
15. **Nakagawa, S., Yoshida, S., Hirao, Y., Kasuga, S. and Fuwa, T.** 1985. Cytoprotective activity of components of garlic, ginseng and ciuwjia on hepatocyte injury induced by carbon tetrachloride *in vitro*. Hiroshima J. Med. Sci. **34**: 303—309.
16. **Penasse, W., Bernaert, D., Mosselmans, R., Wanson, J.C. and Drochmans, P.** 1979. Scanning electron microscopy of adult rat hepatocytes *in situ*, after isolation of pure fractions by elutriation and after culture. Biol. Cellulaire **34**: 175—186.
17. **Sattler, C.A., Michalopoulos, G., Sattler, G.L. and Pitot, H.C.** 1978. Ultrastructure of adult rat hepatocytes cultured on floating collagen membranes. Cancer Res. **38**: 1539—1549.
18. **Seglen, P.O.** 1976. Preparation of isolated rat liver cells. Methods in Cell Biology **13**: 29—83.
19. **Sorensen, E.M.B., Smith, N.K.R., Boecker, C.S. and Acosta, D.** 1984. Calcium amelioration of cadmium-induced cytotoxicity in cultured rat hepatocytes. In Vitro **20**: 771—779.
20. **Stacey, N.H. and Fanning, J.C.** 1981. Ultrastructural changes in isolated rat hepatocytes after incubation with carbon tetrachloride. Toxicology **22**: 69—77.
21. **Tanaka, K. and Mitsushima, A.** 1984. A prepa-

ration method for observing intracellular structures by scanning electron microscopy. J. Microsc. **133**: 213—222.

22. **Tanaka, K. and Naguro, T.** 1981. High resolution scanning electron microscopy of cell organelles by a new specimen preparation method. Biomed. Res. **2 Suppl.**: 63—70.
23. **Tanaka, K., Sato, M., Tomita, Y. and Ichihara, A.** 1978. Biochemical studies on liver functions in primary cultured hepatocytes of adult rats. I. Hormonal effects on cell viability and protein synthesis. J. Biochem. **84**: 937—946.
24. **Yang, J., Flynn, D., Larson, L. and Hamamoto, S.** 1982. Growth in primary culture of mouse submandibular epithelial cells embedded in collagen gels. In Vitro **18**: 435—442.
25. **Yang, J., Richards, J., Bowman, P., Guzman, R., Enami, J., McCormick, K., Hamamoto, S., Pitelka, D. and Nandi, S.** 1979. Sustained growth and three-dimensional organization of primary mammary tumor epithelial cells embedded in collagen gels. Proc. Natl. Acad. Sci. USA **76**: 3401—3405.