Efficient Induction of Hepatocyte Spheroids in a Suspension Culture Using a Water-Soluble Synthetic Polymer as an Artificial Matrix[1]

Keisuke Yamada, Masamichi Kamihira,[2] Ryuji Hamamoto, and Shinji Iijima

Department of Biotechnology, Graduate School of Engineering, Nagoya University, Chikusa-ku, Nagoya 464-8603

Received for publication, November 6, 1997

The preparation of hepatocyte spheroids by adding a water-soluble synthetic polymer as an artificial matrix was performed in a cell suspension system. Cell-aggregation was promoted without cytotoxicity by adding Eudragit (a copolymer of methacrylic acid and methylmethacrylate) to the culture medium. Spheroid-like cell aggregates, whose liver functions were enhanced, were effectively formed in the presence of 0.1% Eudragit, independent of the cultural substratum. Moreover, the mass preparation of spheroids could be achieved with a high production yield by means of a suspension culture in a spinner flask. In this case, the polymer protected the cells from damage due to agitation. The spheroids induced with Eudragit expressed high liver functions, such as albumin secretion, ammonia removal, and urea synthesis. On histological observation, the spheroids showed a well-developed cell adhesion apparatus and bile canaliculi. In addition, a higher calcium ion concentration in the cells of spheroids was observed compared with in monolayer cells.

Key words: artificial liver, artificial matrix, Eudragit, hepatocyte, spheroid.

Downloaded from http://jb.oxfordjournals.org/ at Islamic Azad University on October 1, 2012

Cell-matrix and cell-cell interactions are important in the maintenance and growth of differentiated tissues and also in embryonic organogenesis (*1*). In hepatocyte cultures, the interactions have been extensively investigated in a two-dimensional monolayer culture system on various cultural substrata coated with extracellular matrix components such as collagen or fibronectin (*2, 3*). These studies revealed that not only growth factors and hormones but also these interactions were important for maintaining the expression of liver specific functions.

On the other hand, hepatocytes exhibit a different morphology depending on the surface of a cultural substratum. For example, the cells spread over and form a flat monolayer on a collagen-coated culture dish. Hepatocytes were in contact with each other, with a round shape, on a dish coated with a laminin-rich, tumor-derived EHS gel. It was found that hepatocytes cultured on the EHS gel expressed enhanced liver functions (*4-6*). Thus, it is reasonably assumed that the cell adherent matrix influenced cell-cell contact and cell morphology, and the high liver functions were induced by the morphological change (*2, 3, 5*).

In recent years, another characteristic morphological form of hepatocytes, spheroids, has attracted great attention. When hepatocytes were cultured on proteoglycan-coated or positively-charged dishes, the cells formed three-dimensional, high cell density packed, multicellular aggregates called spheroids (*7-9*). Some researchers have reported spheroid formation using various cultural substrata (*10-13*). Hepatocyte spheroids have also been observed to exhibit long-term enhanced liver functions compared with a monolayer culture (*10, 11*). Since hepatocyte spheroids exhibit and maintain high liver functions, they have been expected to be applicable to a bioartificial liver support system. In most cases, however, the preparation of hepatocyte spheroids depends on a cultural substratum and requires a large surface area for initial cell attachment. Therefore, the supply of a surface area has been a limiting factor for the preparation of large amounts of spheroids sufficient for constructing a bioartificial liver. As an approach to solve this problem, Ueno *et al.* used a thermo-sensitive gel matrix as a culture substratum for harvesting spheroids (*13*). Alternatively, Sakai *et al.* and Wu *et al.* attempted to induce hepatocyte spheroids in a suspension culture (*14, 15*). However, they only incubated the cell suspension in a spinner flask with stirring, and spontaneous cell aggregation was induced.

In the present study, we developed a method for inducing hepatocyte spheroids with high liver functions independent of a cultural substratum by means of a static culture in a petridish or a stirred culture in a spinner flask, in which a water-soluble synthetic polymer was used as an artificial matrix.

[1] This work was supported in part by a Grant-in-Aid for Scientific Research from the Ministry of Education, Science, Sports and Culture of Japan, and in part by the Ciba-Geigy Foundation (Japan) for the Promotion of Science.

[2] To whom correspondence should be addressed. Phone: +81-52-789-4277, FAX: +81-52-789-3221, E-mail: kamihira@proc.nubio.nagoya-u.ac.jp

Abbreviations: C/EBP, CCAAT/enhancer binding protein; DAPI, 4′,6-diamidino-2-phenylindole dihydrochloride; EHS, Engelbreth-Holm-Swarm; HNF-1,3, and 4, hepatocyte nuclear factor-1,3, and 4; TEM, transmission electron microscopy.

© 1998 by The Japanese Biochemical Society.

MATERIALS AND METHODS

Hepatocyte Culture—Hepatocytes were isolated from 6-8-week-old Sprague-Dawley rats by means of the conventional *in situ* collagenase perfusion method and low-speed centrifugation procedure (*16*). More than 99% of the isolated cells were hepatocytes, as judged on phase-contrast microscopic observation, and more than 90% were viable, as judged with the trypan blue dye exclusion method. The isolated hepatocytes were cultured in a serum-free hormonally defined medium consisting of Williams' medium E (Gibco, New York, NY, USA) supplemented with 0.1 μM $CuSO_4 \cdot 5H_2O$, 25 nM Na_2SeO_3, 1.0 μM $ZnSO_4 \cdot 7H_2O$, 0.1 μM insulin (Sigma Chemical, St. Louis, MO, USA), 1.0 μM dexamethasone (Wako Pure Chemical, Osaka), 20 μg/liter epidermal growth factor (EGF) (Sigma Chemical), 20 mg/liter egg yolk lipoprotein (Wako Pure Chemical), 48 mg/liter gentamicin sulfate (Sigma Chemical), and 100 mg/liter chloramphenicol (Wako Pure Chemical). Eudragit S100 (a copolymer of methacrylic acid and methylmethacrylate; Röhm Pharma GmbH, Darmstadt, Germany) was added to the medium at various concentrations as an artificial matrix to induce cell aggregation. Prior to the addition, the polymer was dissolved in water and the pH was adjusted to 7.4 by adding 2 M NaOH. The polymer was originally developed as an enteric coating polymer.

For static petridish cultures, the cells were seeded at 5×10^5 cells per 35-mm diameter polystyrene tissue culture dish (catalog no. 3000-035; Iwaki Glass Works, Chiba) in 2 ml of medium. In some experiments, a positively-charged Primaria® dish (catalog no. 3801; Becton Dickinson, Bedford, MA, USA) was used for a control spheroid culture. A half volume of the medium was replaced with fresh medium without the polymer everyday.

Spinner Flask Culture—For spinner flask cultures, freshly isolated hepatocytes were inoculated into a 250 ml spinner flask (Shibata Hario, Tokyo) at 2.5×10^5 cells/ml in 100 ml of culture medium. The agitation rate was 50 rpm and the culture temperature was maintained at 37°C by circulating thermo-regulated water through a jacket. A gas mixture of 95% air and 5% CO_2 was provided at the rate of 500 ml/min through the surface of the medium. A small amount of broth was taken from the spinner flask, and used for observation of the cell morphology and analyses.

Electron Microscopic Observation—The spheroids formed were collected by centrifugation at 600 rpm, and washed extensively with a PBS solution (137 mM NaCl, 2.7 mM KCl, 8.1 mM $Na_2HPO_4 \cdot 12H_2O$, and 1.5 mM KH_2PO_4, pH 7.4). The cell pellet was dipped in a solution comprising 2% glutaraldehyde in PBS for 1 h for fixing. After washing again with PBS, the cell pellet was treated with 1% osmium tetraoxide in PBS for 1 h. The pellet was then dehydrated by soaking in a series of solutions of increasing concentrations of ethanol (30, 50, 70, 90, and 100% ethanol), and finally transferred to 100% propylene oxide. The spheroids were embedded in Spurr's resin. The spheroid-containing resin was sliced, and the resulting ultra-thin sections were stained with a solution containing uranyl acetate and lead citrate. The ultra-thin sections were observed under a transmission electron microscope (H600; Hitachi, Tokyo).

Visualization and Measurement of Calcium Ions in Hepatocytes—The medium in a petridish was carefully removed and the cells were washed twice with a modified Hanks solution (137 mM NaCl, 3.5 mM KCl, 0.44 mM KH_2PO_4, 25 mM $NaHCO_3$, 0.33 mM Na_2HPO_4, and 0.5 mM $CaCl_2$). The cells were then stained with fura2/AM (2 μM fura2/AM [Wako Pure Chemical] dissolved in the modified Hanks solution) for 30 min at room temperature, and then washed three times with the modified Hanks solution. The calcium ion density of the cells was observed under a fluorescence microscope (IX 70; Olympus, Tokyo).

When the calcium ion concentration in the cells was determined, fura 2/AM-stained cells were disrupted by sonication. After centrifugation, the fluorescence intensity of the supernatant was measured with a spectrofluorometer (FP-770; JASCO, Tokyo), with excitation at 362 nm and emission at 512 nm.

Analyses—The amount of DNA in the cultured cells was determined by the DAPI (4′,6-diamidino-2-phenylindole dihydrochloride; Wako Pure Chemical)-DNA fluorometry method after disruption of the cells by sonication (*17*). The supernatant was subjected to measurement of the fluorescence intensity using a spectrofluorometer, with excitation at 360 nm and emission at 450 nm.

The albumin concentration in the medium was determined by means of a sandwich solid-phase enzyme-linked immunosorbent assay (ELISA), using anti-rat albumin (catalog no. 55729; Organon Teknika, Durham, NC, USA) and peroxidase-conjugated anti-rat albumin antibodies (catalog no. 55776; Organon Teknika) for detection, and purified rat albumin (Sigma Chemical) as the standard.

Ammonia removal and urea synthesis were measured as follows. First, the medium was carefully removed from a cell-cultured dish, and then fresh medium containing 1.0 mM NH_4Cl was added. After the dish had been cultured for a further 4 h, the ammonia and urea concentrations in the medium were measured using diagnostic kits obtained from Wako Pure Chemical (catalog nos. 277-14401 and 279-36201, respectively). The rates of ammonia removal and urea synthesis were calculated from the decrease in the ammonia concentration and the increase in the urea concentration, respectively.

RESULTS AND DISCUSSION

Morphological Change of Hepatocytes on Addition of Eudragit in Static Cultures—In our previous study, we applied Eudragit for specific animal cell separation using an aqueous two-phase system, in which the polymer was used as a ligand-carrier (*18*). It was found that the polymer showed low cytotoxicity toward hepatocytes and promoted the aggregation of hepatocytes. Therefore, we assumed that the polymer could be used as an artificial matrix for inducing hepatocyte spheroid formation.

Figure 1 shows the morphological observation of hepatocytes cultured in medium containing various concentrations of Eudragit. When Eudragit was added to the medium, the cells aggregated from the beginning of the culture and several cells loosely agglomerated into multicellular aggregates on day 1, whereas the cells became weakly attached to the dish surface without Eudragit (data not shown). At this point, no big differences in cell morphology depending on the Eudragit concentration were observed, but the agglomerated cells tended to become attached to the dish surface as the polymer concentration

Downloaded from http://jb.oxfordjournals.org/ at Islamic Azad University on October 1, 2012

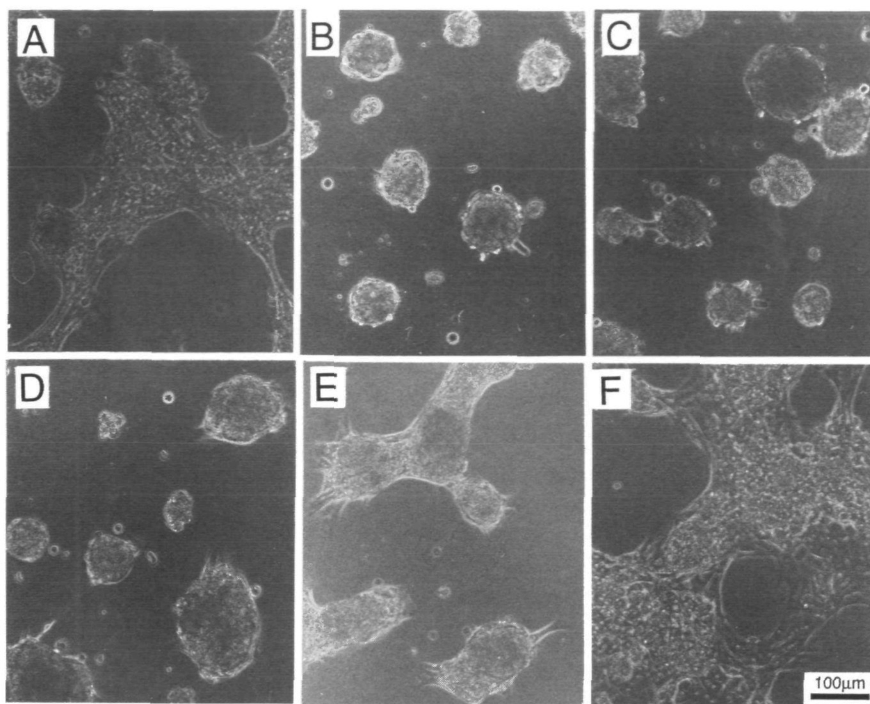

Fig. 1. **Photographs of hepatocytes with Eudragit in static cultures in petridishes on day 5.** The cells were seeded at 5×10^5 cells per 35-mm-diameter polystyrene tissue culture dish in 2 ml of culture medium. Eudragit was added to the medium at various concentrations: no addition (A), 0.05% (B), 0.1% (C), 0.18% (D), 0.26% (E), and 0.5% (F).

increased (data not shown). The cell-cell attachment became tight and spheroids were formed on day 5 (Fig. 1). When 0.05-0.18% Eudragit was added, the shape of the aggregates was smooth and spherical, and more than 80% of the cells formed spheroids. The average diameter of spheroids became large as the polymer concentration increased. On the other hand, the cell aggregates became attached to the dish surface and did not form floating spheroids at Eudragit concentrations above 0.26%. At high Eudragit concentrations, the polymer became attached to not only the cell surface but also the dish surface. Therefore, the cell aggregates became attached to the dish surface and then spread on the surface.

With the conventional surface-dependent spheroid formation method (for example, culturing on a Primaria® dish or proteoglycan-coated dish), cells first became weakly attached to the substratum, and then gradually became detached and formed spheroids simultaneously. Thus, although the process of spheroid formation was different between the Eudragit and conventional procedures, the morphology of the cell aggregates was very similar. In the present study, normal tissue culture dishes without special surface modification were used for the static cultures. Without the polymer, the cells became weakly attached and spread on the dish surface and had not formed spheroids on day 5 (Fig. 1), indicating that spheroid formation was induced by the polymer.

Correlation between Morphological Changes and Liver Functions—Figure 2 shows the changes in DNA content and albumin secretion rate during the culture period. The total amount of DNA in a dish did not drastically change throughout a culture (Fig. 2A). This means that the cell density did not change. As can be seen in Fig. 2B, the dependency of albumin secretion on the Eudragit concentration was observed. This was also related to the cell morphology; the hepatocytes clearly formed floating spheroids with 0.1% Eudragit, but they tended to become attached to the dish surface with above 0.26% Eudragit, and the cells formed multicellular aggregates on the dish surface rather than spheroids. The ability of albumin secretion was similar in all cases but higher than the control level at the beginning of the culture. As the culture proceeded, the albumin secretion increased with 0.1% Eudragit, with which the cells formed spheroids, but it was not enhanced in the culture without Eudragit, in which the cells became attached and spread on the dish surface. The albumin secretion of spheroids induced by 0.1% Eudragit addition was about 3-fold that of a monolayer on day 7. The enhanced liver functions of spheroids were coincident with other reports (*9, 10*).

The other liver functions of the spheroids induced by Eudragit, ammonia removal, and urea synthesis, exhibited a similar tendency to in the case of albumin secretion. With around 0.1% Eudragit, ammonia removal and urea synthesis were the highest (data not shown). Therefore, 0.1% Eudragit was selected for spinner flask cultures.

Spheroid Formation in Spinner Flask Cultures—As the next step, spheroid formation induced by Eudragit was examined in a suspension culture in a spinner flask. Figure 3 shows the cell morphology at 24 h (Fig. 3, A and C) and 48 h (Fig. 3, B and D) after inoculation. Without Eudragit, the cells did not aggregate in a spinner flask culture (Fig. 3, A and B). Approximately 80% of the hepatocytes agglomerated rapidly (Fig. 3C) and formed spheroids effectively within 48 h (Fig. 3D), when the polymer was added to the medium. The diameter of spheroids formed was around 100 μm. In static cultures in petridishes, it took 3-5 days for the formation of spheroids, whereas spheroids formed within 2 days in spinner flask cultures (Fig. 3D). This suggested that the spheroid formation was promoted by agitation. Without the polymer, the cells were damaged by agitation and most of the cells died within a day, as judged

Downloaded from http://jb.oxfordjournals.org/ at Islamic Azad University on October 1, 2012

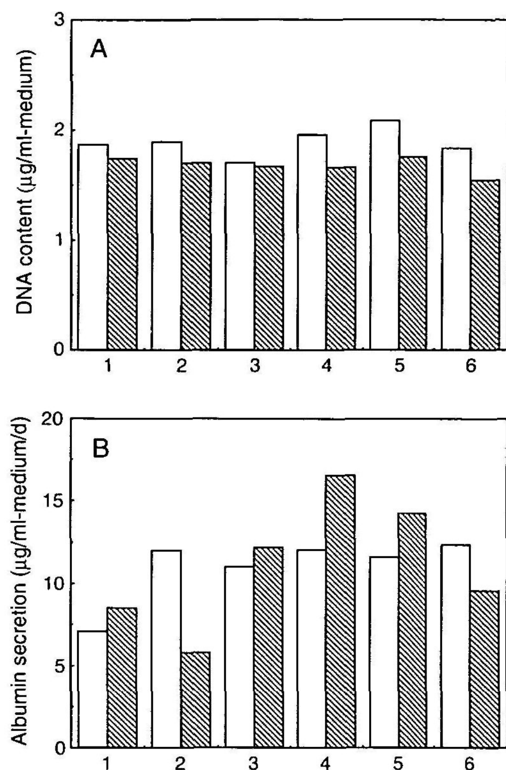

Fig. 2. **Effects of the Eudragit concentration on the DNA content and albumin secretion.** The cells were seeded at 5×10^5 cells per 35-mm-diameter polystyrene tissue culture dish in 2 ml of culture medium. In some experiments, a collagen-coated dish was used for a monolayer culture. Columns: 1, 0% Eudragit control; 2, 0% Eudragit monolayer; 3, 0.05% Eudragit; 4, 0.1% Eudragit; 5, 0.18% Eudragit; 6, 0.26% Eudragit. In each case, the left open bars represent the values on day 2 in the cultures and the right hatched bars those on day 7 in the cultures. (A) The amount of DNA in the cultured cells was determined by the DAPI-DNA fluorometry method after disruption of the cells by sonication. (B) The albumin concentration in the medium was measured by means of a sandwich solid-phase enzyme-linked immunosorbent assay (ELISA).

on trypan blue dye staining of the cells (data not shown). Therefore, protection of the cells from the damage due to agitation by the polymer in spinner flask cultures could be expected.

After 2 days culture in a spinner flask, the spheroids formed were transferred to a normal tissue culture dish in order to measure liver functions. The spheroids also exhibited high albumin secretion ability compared with monolayer cultures in petridishes, and the higher level was maintained throughout the 7 day culture, while most of the cells died and little albumin was produced in the culture without the polymer (Fig. 4). Furthermore, the other liver functions, ammonia removal and urea synthesis, on day 4 were higher compared with in a monolayer culture or a spinner culture without the polymer (Table I). These results indicate that spheroids prepared using the polymer in a spinner flask also express high liver functions, and that this method is promising for the preparation of large amounts of spheroids for a hybrid-type liver support system.

Histological Features of Spheroids Induced by Eudragit Addition—Some researchers also reported that high liver functions were expressed and maintained for a longer time than in the case of a monolayer, when hepatocytes formed spheroids (*10, 11*). One of the reasons for this high expression is the well-developed cell-cell interactions through cell-cell junctional complexes such as tight junctions and desmosomes, and a kind of cell communication may activate differentiated functions. Since such cell-cell junctional complexes are observed in liver tissue, the environment of hepatocytes in spheroids may be closer to *in vivo* conditions compared with in a monolayer.

To determine whether or not spheroids induced by Eudragit possess such a characteristic structure, cells were histologically observed by TEM (Fig. 5). The cells in spheroids induced by Eudragit were histologically indistinguishable from conventional spheroids (*9, 19*). As shown in Fig. 5A, the hepatocytes possessed a large round nucleus and abundant cytoplasm organelles. The cells were in close contact with junctional complexes (Fig. 5B), and bile canaliculi were also observed (Fig. 5C). The results suggest

Downloaded from http://jb.oxfordjournals.org/ at Islamic Azad University on October 1, 2012

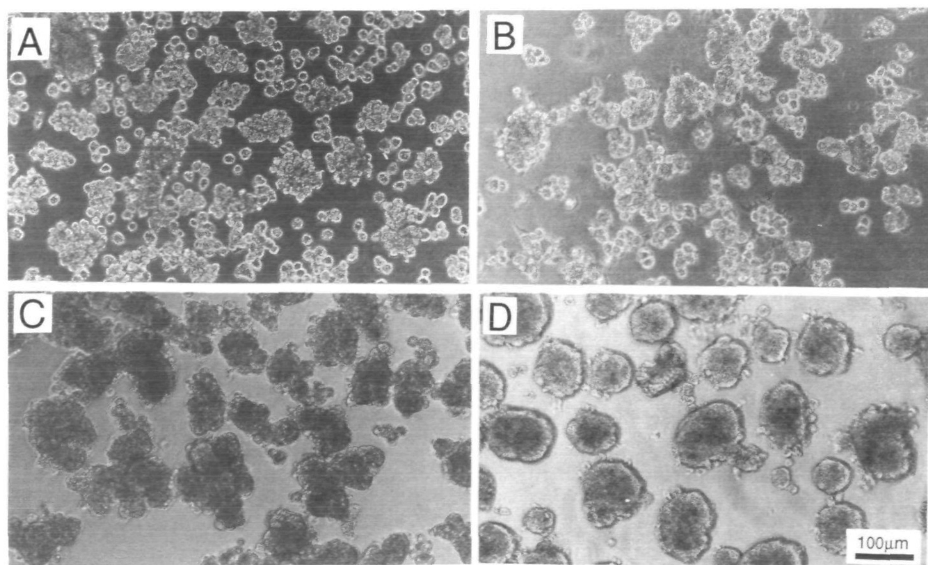

Fig. 3. **Photographs of hepatocytes in spinner flask cultures.** Hepatocytes isolated from a Sprague-Dawley rat were inoculated into a 250 ml spinner flask at 2.5×10^5 cells/ml in 100 ml of culture medium. The cells were cultured in the absence (A, B) or presence (C, D) of 0.1% Eudragit. The cells were observed at 24 h (A, C) and 48 h (B, D).

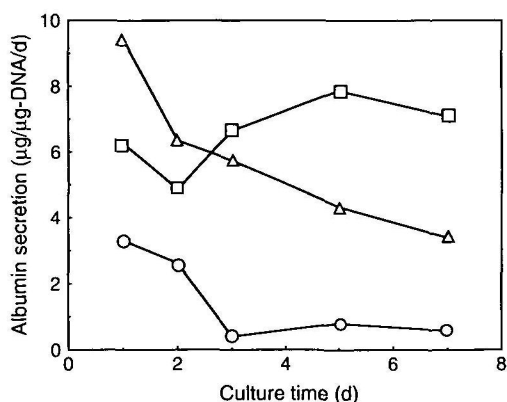

Fig. 4. **Ability of albumin secretion of spheroids in spinner flask cultures.** Hepatocytes were cultured in a spinner flask for 48 h in the absence (circles) or presence of 0.1% Eudragit (squares). The cells were then transferred to a normal plastic petridish and cultured for 5 days. As a control, the cells were seeded into a collagen-coated dish and cultured for 7 days (triangles). The methods for measurement of the DNA content and albumin concentration were the same as in Fig. 2.

TABLE I. **Ammonia removal and urea synthesis of cultured hepatocytes.**

	Ammonia removal (μmol/h/1$\times 10^6$ cells)	Urea synthesis (μmol/h/1$\times 10^6$ cells)
Monolayer (static culture)	0.252	0.130
Eudragit 0.1% (static culture)	0.798	0.325
No addition (spinner culture)	0.168	N.D.
Eudragit 0.1% (spinner culture)	0.606	0.257

N.D.: Not detectable. The culture conditions were the same as in Fig. 4.

that the spheroids show an organized tissue-structure and that this structure may contribute the high expression of liver functions.

Localization of the Calcium Ion Concentration in Spheroids—Hepatocytes in a spheroid are known to be bound through well-developed gap junctions (*9, 19, 20*). Gap junctions are intercellular membrane channels which link neighboring cells, which are responsible for the reciprocal exchange of small molecules and ions of less than 1 kDa, including second messengers such as cAMP, inositol triphosphate and calcium ions (*21-23*). Gap junctional intercellular communication is considered to play a crucial role in the maintenance of homeostasis, morphogenesis, cell differentiation and growth control in multicellular organisms (*24*). We observed enhanced albumin secretion in a monolayer culture, when a calcium ionophore, A23187, was added to the medium (Hamamoto *et al.*, unpublished results). This suggested that calcium ions might play an important role in the expression of the liver functions in hepatocytes. Therefore, we measured the cytosolic calcium ion concentration of the spheroids and compared it with that of a monolayer.

Figure 6 shows the calcium ion concentration in the cells determined using a calcium-binding fluorescence reagent, fura 2/AM. The fluorescence intensity of the cells was high

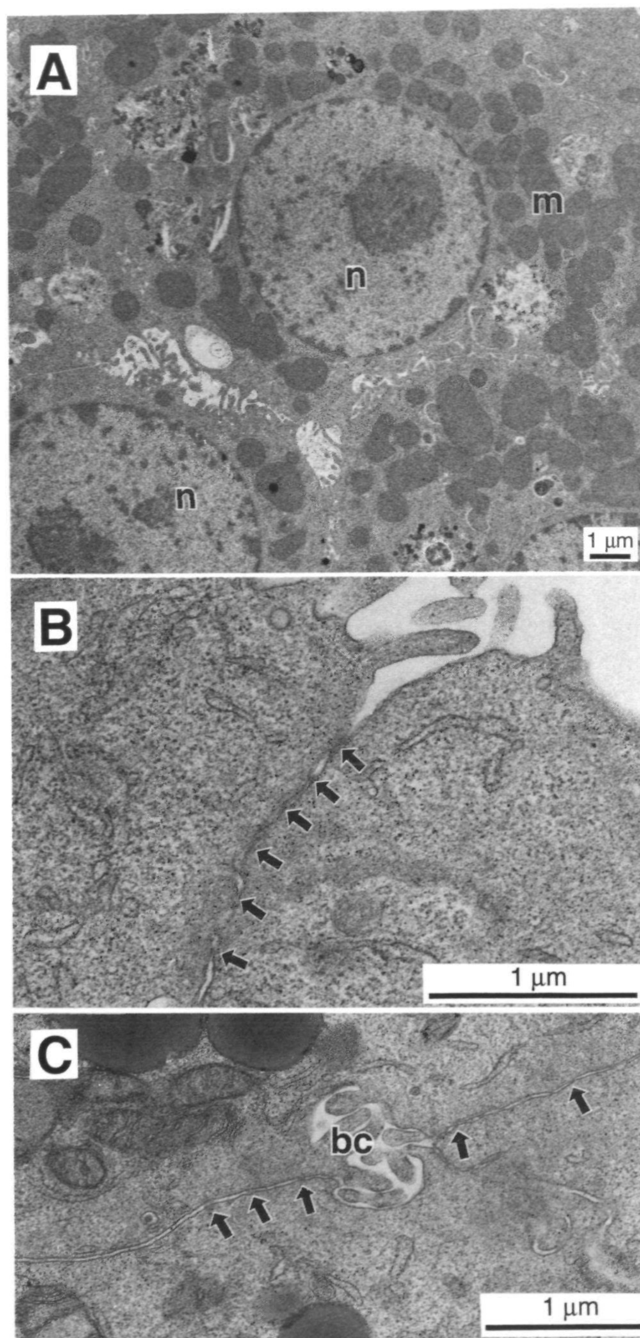

Fig. 5. **Transmission electron microscopic observation of spheroids induced by Eudragit.** The spheroids formed were collected and washed extensively with a PBS solution. The cell pellet was dipped in a solution containing 2% glutaraldehyde for 1 h for fixing, and then treated with 1% osmium tetraoxide for 1 h. The pellet was then dehydrated by soaking in a series of solutions of increasing concentrations of ethanol, and finally transferred to 100% propylene oxide. The spheroids were embedded into Spurr's resin. The spheroid-containing resin was sliced, and the resulting ultra-thin sections were stained with a solution containing uranyl acetate and lead citrate. The ultra-thin sections were observed under a TEM. (A) The cells possessed a large round nucleus (n), abundant mitochondria (m), and abundant other cytoplasmic organelles. (B) Junctional complexes (arrows) developed at the interface for cell-cell attachment. (C) A bile canaliculus (bc) was formed between the cells. The bile canaliculus was sealed by junctional complexes (arrows).

Downloaded from http://jb.oxfordjournals.org/ at Islamic Azad University on October 1, 2012

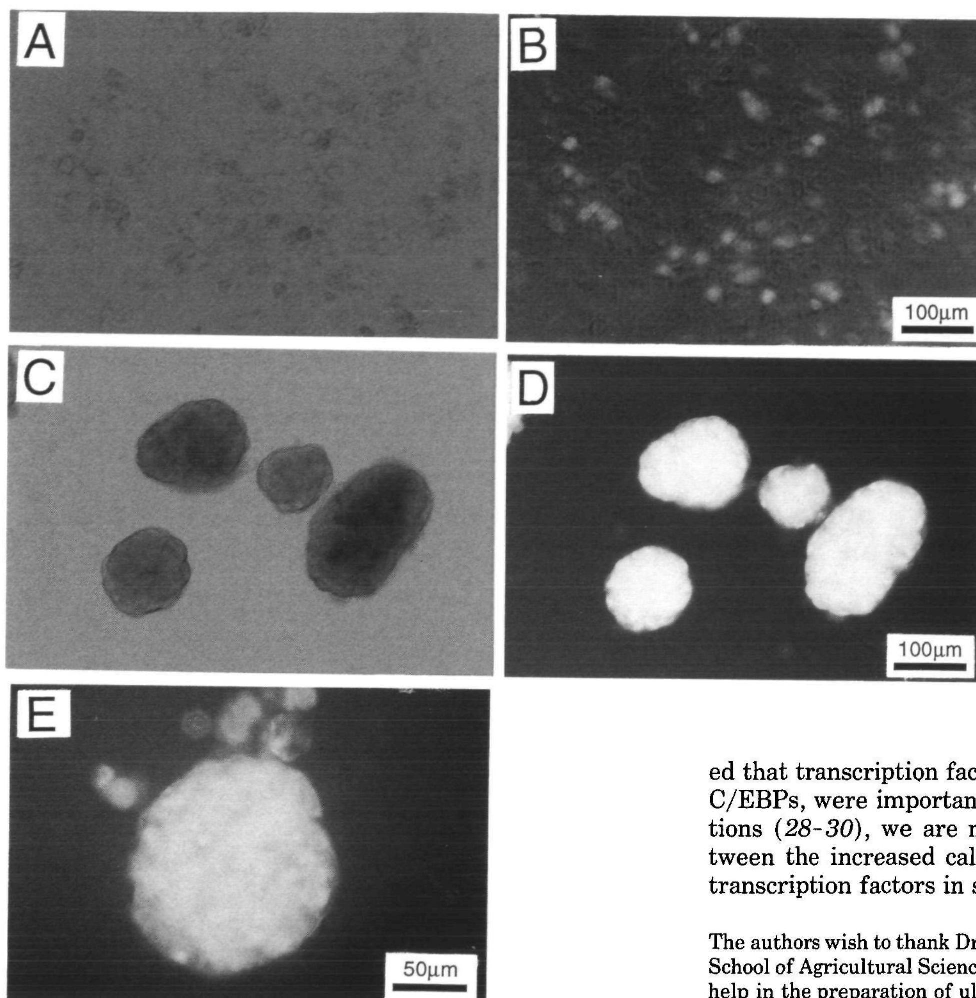

Fig. 6. **Fluorescence microscopic observation of hepatocytes stained with fura2/AM on day 5.** Cells were cultured on collagen-coated dishes (A, B) or cultured with the addition of 0.1% Eudragit (C-E). The cells were stained with fura2/AM (B, D, E). For staining, the cells were washed twice with a modified Hanks solution, stained with a 2 μM fura2/AM solution for 30 min at room temperature, and then washed three times with the modified Hanks solution. The calcium ion density of the cells was observed under a fluorescence microscope.

in the spheroids compared with in a monolayer, and the intensity in spheroids normalized as to the DNA content was about 1.5-fold that in a monolayer. A high concentration in the cells was also observed in conventional spheroids induced by using a Primaria® dish (data not shown). The high calcium ion concentration was maintained throughout the 7 day culture. In particular, strong fluorescence intensity was observed in the inner part of cells in spheroids under a confocal laser microscope (data not shown). This is coincident with the distribution of cytochrome P-450 reported by Hu *et al.* (*25*). Cytochrome P-450s play a major part in the metabolism of endogenous compounds and in the detoxication of xenobiotic molecules in hepatocytes, and it was reported that the expression of a kind of cytochrome P-450 was regulated by the calcium ion concentration in the cells (*26*). In addition, a change in the calcium ion concentration controls the cytoskeleton by changing the degree of the association and dissociation of actin (*27*). As described above, for a hepatocyte, the cell morphology and cell-cell interaction are very important for growth and differentiation (*7*). Thus, calcium ions, as a second messenger, can be transmitted from cell to cell through well-developed gap junctions inside the spheroids and the high calcium ion concentration may play an important role in the morphological change of hepatocytes and the high expression of liver-specific functions. Since many authors reported that transcription factors, such as HNF-1,3, and 4, and C/EBPs, were important for the expression of liver functions (*28-30*), we are now studying the relationship between the increased calcium ion concentration and these transcription factors in spheroids.

The authors wish to thank Drs. Hiroshi Miyake and Kanjun Hirunagi, School of Agricultural Science, Nagoya University, for the advice and help in the preparation of ultra-thin sections for TEM observation.

REFERENCES

1. Gumbiner, B.M. (1996) Cell adhesion: The molecular basis of tissue architecture and morphogenesis. *Cell* **84**, 345-357
2. Ben-Ze'ev, A., Robinson, G.S., Bucher, N.L., and Farmer, S.R. (1988) Cell-cell and cell-matrix interactions differentially regulate the expression of hepatic and cytoskeletal genes in primary cultures of rat hepatocytes. *Proc. Natl. Acad. Sci. USA* **85**, 2161-2165
3. Hansen, L.K., Mooney, D.J., Vacanti, J.P., and Ingber, D.E. (1994) Integrin binding and cell spreading on extracellular matrix act at different points in the cell cycle to promote hepatocyte growth. *Mol. Biol. Cell* **5**, 967-975
4. Bissell, D.M., Arenson, D.M., Maher, J.J., and Roll, F.J. (1987) Support of cultured hepatocytes by a laminin-rich gel. *J. Clin. Invest.* **79**, 801-812
5. Dunn, J.C., Tompkins, R.G., and Yarmush, M.L. (1991) Long-term in vitro function of adult hepatocytes in a collagen sandwich configuration. *Biotechnol. Prog.* **7**, 237-245
6. Rojkind, M., Gatmaitan, Z., Mackensen, S., Giambrone, M.A., Ponce, P., and Reid, L.M. (1980) Connective tissue biomatrix: Its isolation and utilization for long-term cultures of normal rat hepatocytes. *J. Cell Biol.* **87**, 255-263
7. Landry, J., Bernier, D., Ouellet, C., Goyette, R., and Marceau, N. (1985) Spheroidal aggregate culture of rat liver cells: histotypic reorganization, biomatrix deposition, and maintenance of functional activities. *J. Cell Biol.* **101**, 914-923
8. Koide, N., Shinji, T., Tanabe, T., Asano, K., Kawaguchi, M., Sakaguchi, K., Koide, Y., Mori, M., and Tsuji, T. (1989) Continued high albumin production by miltícellular spheroids of adult rat hepatocytes formed in the presence of liver-derived

Downloaded from http://jb.oxfordjournals.org/ at Islamic Azad University on October 1, 2012

proteoglycans. *Biochem. Biophys. Res. Commun.* **161**, 385-391

9. Koide, N., Sakaguchi, K., Koide, Y., Asano, K., Kawaguchi, M., Matsushima, H., Takenami, T., Shinji, T., Mori, M., and Tsuji, T. (1990) Formation of multicellular spheroids composed of adult rat hepatocytes in dishes with positively charged surfaces and under other nonadherent environments. *Exp. Cell Res.* **186**, 227-235
10. Matsushita, T., Ijima, H., Koide, N., and Funatsu, K. (1991) High albumin production by multicellular spheroids of adult rat hepatocytes formed in the pores of polyurethane foam. *Appl. Microbiol. Biotechnol.* **36**, 324-326
11. Sakai, Y. and Suzuki, M. (1991) Formation of spheroids of adult rat hepatocytes on polylysine-coated surfaces and their albumin production. *Biotechnol. Tech.* **5**, 299-302
12. Tobe, S., Takei, Y., Kobayashi, K., and Akaike, T. (1992) Receptor-mediated formation of multicellular aggregates of primary cultured adult rat hepatocytes on lactose-substituted polystyrene. *Biochem. Biophys. Res. Commun.* **184**, 225-230
13. Ueno, K., Miyashita, A., Endoh, E., Takezawa, T., Yamazaki, M., Mori, Y., and Satoh, T. (1992) Formation of multicellular spheroids composed of rat hepatocytes. *Res. Commun. Chem. Pathol. Pharmacol.* **77**, 107-120
14. Sakai, Y., Furukawa, K., and Suzuki, M. (1992) Immobilization and long-term albumin secretion of hepatocyte spheroids rapidly formed by rotational tissue culture methods. *Biotechnol. Tech.* **6**, 527-532
15. Wu, F.J., Friend, J.R., Hsiao, C.C., Zilliox, M.J., Ko, W.J., Cerra, F.B., and Hu, W.S. (1996) Efficient assembly of rat hepatocyte spheroids for tissue engineering applications. *Biotechnol. Bioeng.* **50**, 404-415
16. Seglen, P.O. (1976) Preparation of isolated rat liver cells. *Methods Cell Biol.* **13**, 29-83
17. Hamada, S. and Fujita, S. (1983) DAPI staining improved for quantitative cytofluorometry. *Histochemistry* **79**, 219-226
18. Hamamoto, R., Kamihira, M., and Iijima, S. (1996) Specific separation of animal cells using aqueous two-phase systems. *J. Ferment. Bioeng.* **82**, 73-76
19. Asano, K., Koide, N., and Tsuji, T. (1989) Ultrastructure of multicellular spheroids formed in the primary culture of adult rat hepatocytes. *J. Clin. Electron Microscopy* **22**, 243-252
20. Spray, D.C., Fujita, M., Saez, J.C., Choi, H., Watanabe, T., Hertzberg, E., Rosenberg, L.C., and Reid, L.M. (1987) Proteoglycans and glycosaminoglycans induce gap junction synthesis and function in primary liver cultures. *J. Cell Biol.* **105**, 541-551
21. Loewenstein, W.R. (1979) Junctional intercellular communication and the control of growth. *Biochim. Biophys. Acta* **560**, 1-65
22. Nathanson, M.H. and Burgstahler, A.D. (1992) Coordination of hormone-induced calcium signals in isolated rat hepatocyte couplets: demonstration with confocal microscopy. *Mol. Biol. Cell* **3**, 113-121
23. Rose, B., Simpson, I., and Loewenstein, W.R. (1977) Calcium ion produces graded changes in permeability of membrane channels in cell junction. *Nature* **267**, 625-627
24. Kumar, N.M. and Gilula, N.B. (1996) The gap junction communication channel. *Cell* **84**, 381-388
25. Hu, W.S., Friend, J.R., Wu, F.J., Sielaff, T., Peshwa, M.V., Lazar, A., Nyberg, S.L., Remmel, R.P., and Cerra, F.B. (1997) Development of a bioartificial liver employing xenogeneic hepatocytes. *Cytotechnology* **23**, 29-38
26. Gonzalez, F.J. and Lee, Y.H. (1996) Constitutive expression of hepatic cytochrome P450 genes. *FASEB J.* **10**, 1112-1117
27. Sobue, K., Kanda, K., Tanaka, T., and Ueki, N. (1988) Caldesmon: a common actin-linked regulatory protein in the smooth muscle and nonmuscle contractile system. *J. Cell. Biochem.* **37**, 317-325
28. Xanthopoulos, K.G., Prezioso, V.R., Chen, W.S., Sladek, F.M., Cortese, R., and Darnell, J.E., Jr. (1991) The different tissue transcription patterns of genes for HNF-1, C/EBP, HNF-3, and HNF-4, protein factors that govern liver-specific transcription. *Proc. Natl. Acad. Sci. USA* **88**, 3807-3811
29. van Ooij, C., Snyder, R.C., Paeper, B.W., and Duester, G. (1992) Temporal expression of the human alcohol dehydrogenase gene family during liver development correlates with differential promoter activation by hepatocyte nuclear factor 1, CCAAT/enhancer-binding protein α, liver activator protein, and D-element-binding protein. *Mol. Cell. Biol.* **12**, 3023-3031
30. Nishiyori, A., Tashiro, H., Kimura, A., Akagi, K., Yamamura, K., Mori, M., and Takiguchi, M. (1994) Determination of tissue specificity of the enhancer by combinatorial operation of tissue-enriched transcription factors: Both HNF-4 and C/EBPβ are required for liver-specific activity of the ornithine transcarbamylase enhancer. *J. Biol. Chem.* **269**, 1323-1331

Downloaded from http://jb.oxfordjournals.org/ at Islamic Azad University on October 1, 2012