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PRIMARY CULTURE OF HUMAN HEPATOCYTES FROM SMALL SIZE SAMPLE

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ABSTRACT: *Human and rat hepatocytes were isolated by nonperfusion method and cultured for longer than 15 days. Human liver biopsy sample and rat liver were used as hepatocyte source. Several physical and chemical factors which were influencing on hepatocyte isolation procedure were examined and a batch isolation procedure was established for small size sample of rat liver. Isolated hepatocytes showed normal morphlological characteristics in microscopy and electron microscopical examinations and a morphological response to phalloidin. Isolated cells were cultured as a monolayer and proven to have intact morphological characteristics for longer than 15 days. Because human liver sample is harder and tighter compared with rat liver, a standard procedure for rat hepatocytes was slightly modified to reduce mechanical damage. Similarly with rat hepatocytes, isolated human hepatocytes showed a normal morphological characteristics and could be cultured for longer than 15 days. Human and rat hepatocytes were examined on their functional integrities including cytochrome-P450 related enzyme activity and it's inducibility, hormonal inducibility of AIB uptake and TAT activity, albumin synthesis, DNA synthesis, cellular protein maintenance. In all parameters used in the present study, human and rat hepatocytes showed normal functional characteristics.*

Key words: *Hepatocytes, Human, Rat, Primary culture, Morphology, Liver-specific function*

INTRODUCTION

Liver is an important organ for the homeostasis of living organism and has many well organized functions. Several *in vitro* models were suggested for the studies

on liver including isolated perfused liver, liver slice, transformed cell line, isolated hepatocytes, primary hepatocytes culture and so on. By using these models in experiments, many invaluable informations were acquired. But these methods itself possessed technical limitations. Because unfractionated tissue is used in isolated perfused liver and liver slice method, it is impossible to discriminate the functions between parenchymal cell and nonparenchymal cells. These systems are not suitable for pharmacological and toxicological studies which requirs an sustained drug metabolism and a long time. Even though transformed cell lines have homogeneity and stability, it lost well differentiated liver functions. To solve these limitations, primary hepatocytes culture was developed and applied to many studies on liver (Grisham, 1979).

Until now, the most prevail isolation method of hepatocytes was collagenase perfusion technique developed by Berry and Friend (1969). By the perfusion technique, large number of hepatocytes could be acquired in short time and viability of isolated cell was relatively high and contamination by blood-originated cells could be minimized in preparation (Seglen, 1972; Bissell *et al.*, 1973; Pariza *et al.*, 1975). But relatively intact blood vessel and large mass of organ or tissue were required. In the case of human, material for hepatocytes preparation was usually whole organ or lobe (Ismail *et al.*, 1991). The difficulty of acquiring adequate sample is a major obstacle in a popular application of human hepatocytes to many biological and medical researches. Recently, there was a large progress in biotechnology and many human-specific drugs and their candidate including cytokines were developed (Higuchi and Nakamura, 1991; Nakamura *et al.*, 1989). Accordingly, the need for primary human hepatocytes was grown in research area of human specific disease and function such as hepatitis and cirrhosis (Prince *et al.*, 1984; Shimizu *et al.*, 1986; Gripon *et al.*, 1988; Rijntjes *et al.*, 1988; Ochiya *et al.*, 1989; Thorton *et al.*, 1991).

In the present study, human hepatocytes were successfully isolated from small size sample acquired from biopsy and pathological examination. Culture method was also established and long-term culture was successfully tried. Cultured hepatocytes ware examined morphologically and functionally and seemed to keep many liver specific characteristics. In all experiments, rat hepatocytes isolated and cultured by same method with human was compared and used as an reference standard.

MATERIALS AND METHOD

Liver Samples

Rat livers were obtained from male Sprague-Dawley rats weighing 200 to 250 gm which were maintained in GERI, KIST. Animals were sacrificed by cervical dislocation and livers were dissected asseptically as soon as possible and blood was washed three times with Ca, Mg-free HBSS (Gibco Lab. Grand Islang, New York). Human liver samples were obtained by surgical biopsy and kept in ice-cold HBSS. They were transferred to laboratory within 1 hour and used as samples for hepatocyte isolation.

Isolation of Hepatocytes

Rat samples were made to small size by following methods; slicing with Stadie-Riggs Tissue slicer (Thomas Scientfic Co., N.J.), chopping with sharp blade (Dorco Co., Seoul) and injection of collagenase (type I, Sigma Chem. Co., St. Louis, M.O.) solution through 28 guage needle followed by chopping. Small sized samples was incubated with collagenase (300 unit/ml) in the presence or absence of trypsin (Sigma), DNase (Boehringer Mannheim Gmbh, Germany), calcium in 37℃ shaking water bath. Supernatant of liver digest was collected at intervals of 10 min and filtered through Nylon mesh (210 μm, Spectrum, T.X.). The filtrate was mixed with same volume of 10% FCS in HBSS and centrifuged at 50 g for 4 min. The hepatocyte pellet was resuspended in 10% FCS in HBSS and stored on ice. Digestion and harvesting step was repeated five to six times. Hepatocytes were washed three times with same buffer. Human sample were sliced to 1 to 2 mm depth with sharp blade and washed with HBSS to remove remaining blood. Collagenase digestion and hepatocyte isolation was performed similarly with rat liver but according to the hardness of sample, collagenase was used in concentration of 300 to 600 unit/ml. To determine viability and cell number, final cell suspension was mixed with 9 volume of 0.4% Trypan blue and cells were counted with Haemocytometer under microscope.

Culture of Isolated Hepatocytes

Rat hepatocytes was resuspended in hormonal AB media, a modified Waymouth medium and cell concentration was adjusted to 0.4×10^6 cells/ml. In some case, DMEM was compared with AB media in morphological observation. Human hepatocytes were diluted with same medium but cell concentration was adjusted to 0.8×10^6 cells/ml to compensate smaller cell size than those of rat. Two ml of cell suspension was added to each well of 6 well plate (Falcon, N.J.) precoated with Vitrogen 100 (Celltrix, C.A.) and incubated at 37℃ in 5% CO_2 incubator (Flow, V.A.). Medium was changed at 4 hour after plating and every 24 hour thereafter.

Microscopical Observation

Isolated hepatocytes and cultured hepatocytes were examined on their morphological characteristics using inverted microscope and electron microscope. To test the responsiveness of cell membrane on xenobiotics, phalloidin (10 μg/ml) was exposed to isolated hepatocytes for 20 min and membrane protrusion was examined under microscope. For electron microscopic observation, isolated hepatocytes were prefixed for 2 hrs with PBS containing 2.5% glutaraldehyde and 2% paraformaldehyde and fixed with 1% osmium tetroxide. Dehydrated samples was examined with Jeol JSM-35C scanning microscope (15 kV) and Kep; 1200EX transmission electron microscope (80 kV) (Sattler *et al.*, 1982; Glauert, 1967; Kawamoto *et al.*, 1980).

Protein Determination

Sample was digested with 0.2 N NaOH or dispersed with ultrasonicator and protein was determined by Bradford methods (Bradford, 1976).

Induction of EROD Activitry by TCDD

TCDD was added in culture medium from 24 hrs and EROD activity was measured essentially as described previously (Lubert *et al.*, 1985, Blank *et al.*, 1987). The assay buffer used to determine EROD activity consisted of 0.1 M potassium phosphate (pH 7.5), 2 mg/ml bovine serum albumin, 10 μ M dicoumarol, 5 mM glucose 6-phosphate, 20 units/ml of glucose-6-phosphate dehydrogenase, and 0.5 μM NADPH. Formation of resorufin by the homogenized cell preparation after the addition of 2.5 μM NADPH. Formation of resorufin by the homogenized cell preparation after the addition of 2.5 μM ethoxyresorufin was monitored fluorimetrically (Hitachi model 650-10S) at an excitation wavelength of 550 nm and an emission wavelength of 585 nm, respectively.

AIB Uptake Determination

Hormonal induction of α-aminoisobutylic acid (AIB) transport were measured by the method described earlier (Kletzien *et al.*, 1976; Pariza *et al.*, 1976). AIB transport was preinduced in the cultured hepatocytes by the addition of 1 μM dexamethasone at 24 hrs after initial plating and 0.2 μM glucagon at 42 hrs (Pariza *et al.*, 1976). At 48 hrs, the culture medium was aspirate off and the plate was rinsed with warm Hank's-Hepes salt solution (37℃). AIB uptake was measured by incubating the cells in 2 ml of Hank's complete-Hepes medium (8 mM glucose added to Hank's-Hepes salt solution) containing 1 mM α-aminoisobutylic acid and α-amino (^{14}C) isobutylic acid (0.2 μ Ci/ml medium) for 4 min at 37℃. Indubation was terminated by aspiration off the medium and rinsing the cells several times with a total of about 20 ml of cold Hank's-Hepes salt solution. Cells were digested in 0.2 N NaOH and radioactivity was estimated with liquid scintillation counter.

TAT Activity Determination

Tyrosine aminotransferase (TAT) was preinduced by the addition of 10 μM dexamethasone at 24 hr after initial plating according to Bonney *et al.* (1974), and activity was determined by the methed of Diamondstone (1966). At 48 hr, the medium was drawn off and the plate was rinsed once with 3 ml of saline . Then 1 ml of cold homogenizing buffer (0.2 M potassium phosphate, 10 mM α-ketoglutarate, 0.04 mM pyridoxal phosphate, 1 mM EDTA, pH 7.3) was added, and the plates were immediately frozen in deepfreezer at -70℃ . They were subsequently thawed and the cells were scrapped into a test tube and frozen in the freezer and thawed twice. The resulting suspension was centrifuged at 15,000 g for 30 min, and the supernatant fluid served as the source for enzyme assay. The enzyme activity was expressed as 1 unit being equal to 1 μ mole of p-hydroxy phenylpyruvate formed in 1 min.

ELISA for Albumin Determination

Liver-specific albumin synthesis was monitored by ELISA technique and human serum albumin (Sigma Chemical Co., St. Louis, MO) was used as standard antigen (Ochiya *et al.*, 1989). Waste culture medium was harvested at intervals of 24 hr during cultivation and used as sample for ELISA determination. Sample was diluted with carbonate buffer and incubated in 96 well microplate for 24 hrs at 37℃ .

After blocking with 3% casein in TBS, mouse monoclonal antibody against human serum albumin and horseradish peroxidase conjugated antimouse IgG was used for reaction and visualization.

Measurement of ^{3}H Thymidine Uptake

DNA synthetic capacity was expressed as ^{3}H thymidine uptake to DNA (Pariza *et al.*, 1975; Bonney and Maley, 1975; Nakamura *et al.*, 1984; Miyazaki *et al.*, 1992). Medium was aspirated off every 24 hr after plating, and cells were exposed to ^{3}H thymidine (1 μ Ci/ml) containing medium for 2 hrs. After labelling, cellular macromolecule was precipitated and washed three times with TCA. Final pellet was dissolved in 0.2 N NaOH and radioactivity and protein contents were measured as described earlier.

RESULTS

Isolation of Hepatocytes by Non Perfusion Method

For the non-perfusion and batch isolation, rat liver was made to small piece to support enzymatic digestion. Slicing with Staddie Rigg's tissue slicer, chopping with sharp blade and injection followed chopping were used and compared. As shown in figure 1A, the injection method was the best tool in terms of cell yield and viability of first 10 min isolated sample. Collagenase concentration in digestion media was determined. Cell yield was improved by increase of collagenase concentration but at above 300 units/ml similar results were obtained (Figure 1B). Along with collagenase, trypsin and DNase were also included in the cell isolation. By addition of trypsin (0.5 mg/ml) and DNase (200 units/ml), no significant difference was obtained (Figure 1C). Usually in the case of collagenase perfusion, Ca^{++} was included in perfusion media to improve the action of collagenase as a cofactor. Figure 1D showed the effect of calcium on cell yield and viability. Cell yield was decreased to about 36% of control at 0.5 mM of calcium and viability was also affected by increase of calcium (0.5 mM). To dissociate digested hepatocytes to media, three different method was applied. Magnetic stirring and spinning with low speed spinner vessel were inadquate in viability and cell yield. Most reasonable method was shaking followed by pipetting with wide mouth pipettes (Figure 1E). This results was confirmed by following time course study. Magnetic stirring had a defect in harder than that of rat cell viability because of its grinding effects and spinning was too weak to force the digested cells to disperse in isolation media (Figure 2).

In the case of human liver sample, tissue was harder than that of rat and contain many collageneous materials (Figure 4). Chopping with sharp blade caused cell death (data were not shown) and injection of perfusion media also impossible because too high pressure was required and personal safety problem might occur. After slicing with a depth of about 2 mm, collagenase fibrile was digested by collagenase treatment and parenchymal cells were delivered to digestion media. After 7~8 times harvesting, the cell yield and viability were about 1.5×10^7/g tissue and above 85%, respectively.

Primary Culture of Hepatocytes

Isolated rat hepatocytes were applied to primary monolayer culture. As shown in Figure 3, isolated rat hepatocyte was a nearly single cell and had a round cell morphology. At 4 hr after plating, hepatocytes attached to collagen-coated dish and partial population had a spreaded morphology. In AB media, hepatocytes kept a normal spreaded morphology for at least 15 days (Figure 3). When DMEM was used as a culture medium, a change in cell population was observed and fibloblast-like cell type overgrew and culture pattern was quite different compared with AB media at 12 day after plating (data not shown).

Because isolated human hepatocytes had a rather small cell size compared with rat hepatocytes, they were plated in a concentration of 0.8×10^6 ml in AB media.

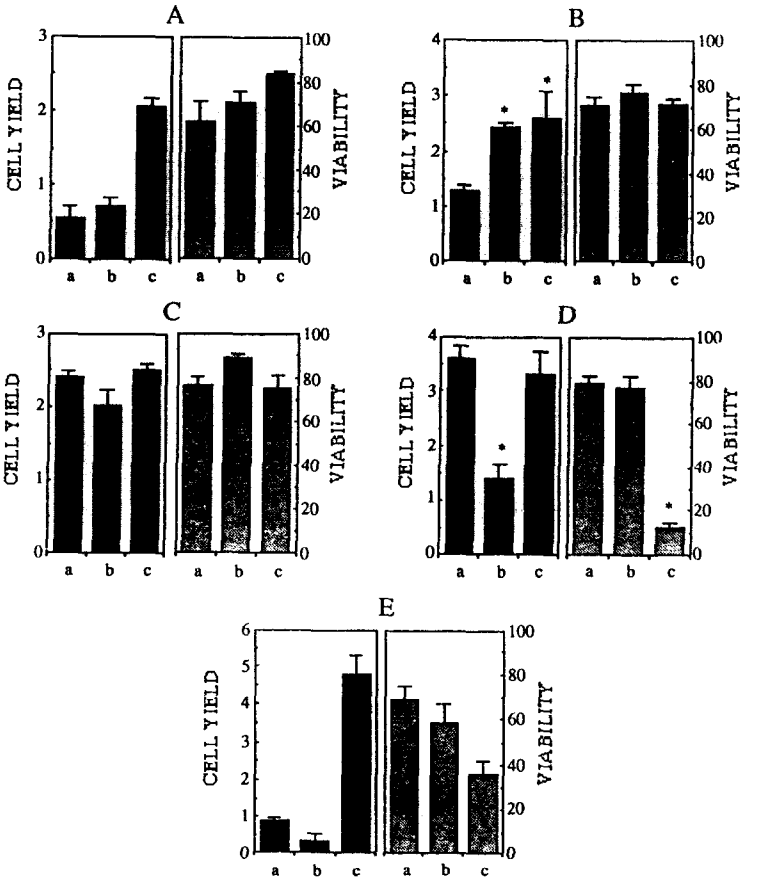

Figure 1. Effects of hepatocytes isolation conditions on cell yield and viability in collagenase liver digestion. Samples were exposed for 10 minutes to defined conditions and cells were harvested to determine cell concentration and viability. Cell yield was expressed as cell number$\times 10^6$/g tissue and viability as percent of viable cell. A. Sample preparation methods, a; slicing, b; chopping, c; injection followed by chopping. B. Collagenase concentration, a; 150 units/ml, b; 300 units/ml, c; 600 units/ml, C. Other enzymes, a; collagenase alone, b; collagenase plus trypsin, c; collagenase plus DNase, D. Calcium, a; none, b; 0.5 mM, c; 5.0 mM, E. Mechanical shearing methods, a; shaking followed pipetting, b; spinning, c; magnetic stirring. The asterisk indicates $P \leq 0.05$ when compared with the control value.

As shown in Figure 3, isolated hepatocytes had a round morphology and at 24 hr after plating cell spreading could be observed. Thereafter human hepatocytes showed very similar pattern to rat hepatocytes and kept an normal spreaded morphology for longer than 12 days.

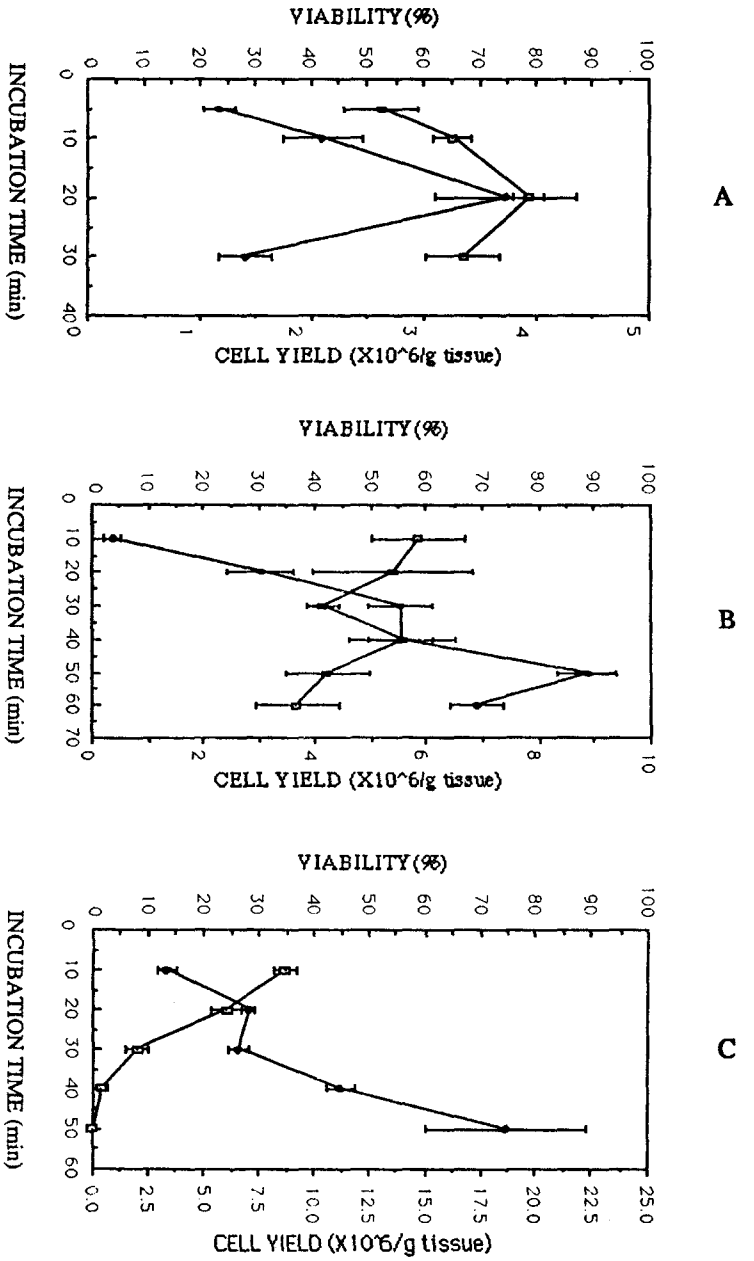

Figure 2. Time interval exmination of cell yield and viability of collagenase digestion under shaking and pipetting (A), low-speed spinning by spinner vessel (B), and low speed magnetic stirring (C). Detailed method was same as figure 1.

Morphological Obervation of Isolated Rat Hepatocytes

Cellular integrity of isolated rat hepatocytes was examined by SEM and TEM. Figure 4 showed that hepatocyes were isolated as an intact state and had many microvilli and intracellular microorganelle. This result indicated that hepatocytes were not damaged by isolation procedure and exposure of collagenase. Phalloidin, a microbial toxin produced by *Amanita phalloides*, is a selective hepatotoxic agent and requires intact membrane to induce acute membrane deformity. Hepatocytes isolated by batch digestion was vulnerable to phalloidin and formed an acute flower-shaped membrane protrusion as an evidence of integrity related with membrane and intracellular cytoskeleton such as actin fibrile (Figure 4).

Change of Cellular Protein Amount During Cultivation

After initial plating, cells were harvested every 24 hr and protein concentration was determined. At 24 hr, protein amount was decreased to 78% of initial but thereafter protein concentration was unchanged. Cellular protein of 96 hr cultured sample was 70.5% of initial. These results showed that hepatocytes were successfully maintained under culture condition used in present study (Figure 5).

Functional Characterization of Cultured Hepatocytes

Human and rat hepatocytes were examined on hormonal response. Table 1

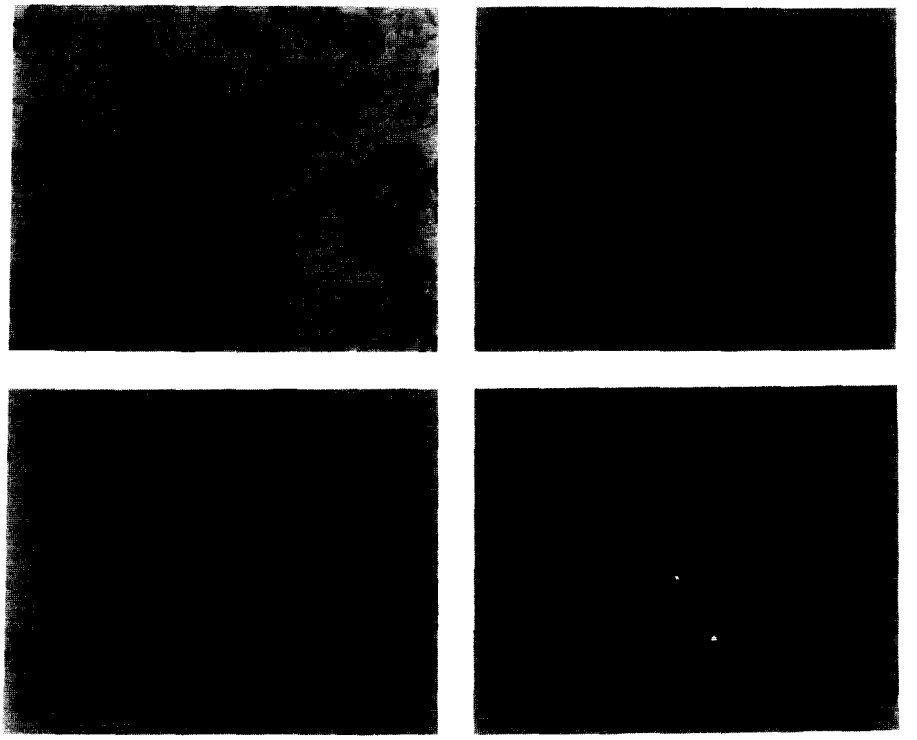

Figure 3. Examination on morphological characteristics of isolated and cultured hepatocytes. A, rat hepatocytes at 0 time; B, rat hepatocytes at 12 days; C, human hepatocytes at 0 time; D, human hepatocytes at 12 dyas.

showed an induction of α-aminoisobutyric acid (AIB) uptake by dexamethasone and glucagon. By the treatment of glucagon alone, AIB uptake was increased to 1.5 fold of uninduced control in rat hepatocytes. Dexamethasone showed a strong synergism to an effect of glucagon and the level of AIB uptake was 5.5 fold of uninduced control. Human hepatocytes responded similarly to rat hepatocytes. Dexamethasone stimulated AIB uptake in human hepatocytes and the level was nearly same with that of rat hepatocytes. Second parameter was tyrosine aminotransferase (TAT) induction by dexamethasone (Table 2). Rat hepatocytes responded well to dexamethasone. By addition of dexamethasone (10 μM) to culture media for 24 hrs, TAT was induced to 7.3 fold of uninduced control. But in the case of human, uninduced background level was relatively low compared with rat and the effect of dexamethasone could not be observed. The difference between AIB uptake and TAT showed that TAT in human hepatocytes might be controlled by a different mechanism compared with rat hepatocytes.

To measure drug metabolizing activity and gene turn-on process in nucleus, an induction process of EROD activity in rat and human hepatocytes by TCDD

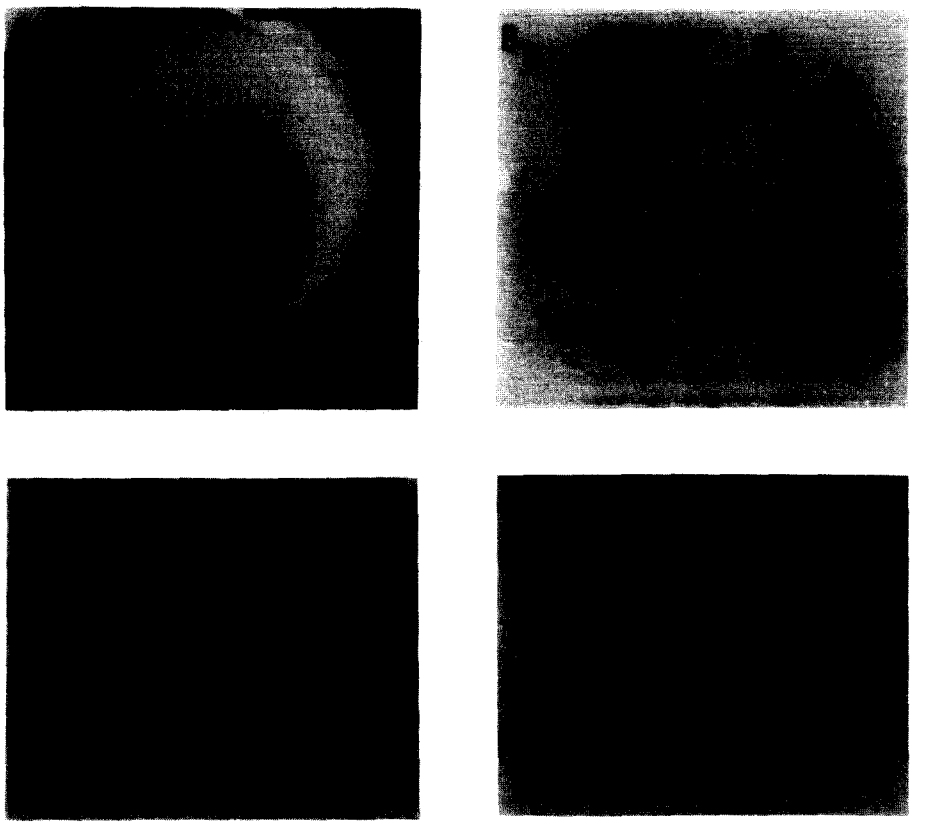

Figure 4. Microcsopic examination of hepatocytes. A, scanning electron micrograph of isolated rat hepatocytes (×2,000); B, transmission electron micrograph of isolated rat hepatocytes (×3,000); C, photomicrograph of phalloidin (10 μg/ml) treated hepatocytes; D, collagen fibrile observed during collagenase digestion of human liver.

was examined (Figure 6, 7). TCDD caused an significant induction from 24 hr after TCDD treatment in rat hepatocytes. At 96 hr after plating, EROD specific activity was increased to more than 6 fold of uninduced control. Human hepatocytes also responded well to TCDD and showed similar induction profile to rat hepatocytes. At 96 hr, TCDD augmented EROD activity to 9 fold of control. One interesting observation was that uninduced EROD activity was decline at early culture time as shown in other previous report but rebound to level of 0 time at 96 hr. Different loading cell density might be a reason for this phenomenon (Figure 8). Uninduced EROD activity showed an positive relationship with cell concentration and 0.64×10^6 cells/well was a critical point. When expressed as an specific activity, EROD activity of high density (0.8×10^6 cells/well) was about 2 fold of low concentration (0.32×10^6 cells/well).

Albumin synthesis is a characteristic function of hepatocytes. Because AB media contained high concentration of albumin, DMEM was used as a culture medium. As shown in Table 3, human hepatocytes produced 1.88 ng albumin/mg protein for the first 24 hr and 0.98 ng albumin/mg protein for the last 24 hr. Because DMEM was not suitable for hepatocytes as shown above, albumin synthesis was decreased slightly. But until 96 hr after plating, albumin synthesis could be maintained at an reasonable level.

Because most parenchymal hepatocytes stay in G_0 phase in *in vivo* state, DNA synthesis is quite low. But recently, many growth factor was discovered which stimulated the growth of hepatocytes *in vitro*. And it was reported that cultured hepatocytes contained the capacity to synthesize cellular DNA and were good model of hepatic regeneration. Figure 9 showed the time course of ^{3}H-thymidine uptake of cultured rat hepatocytes. Hepatocytes prepared by batch isolation con-

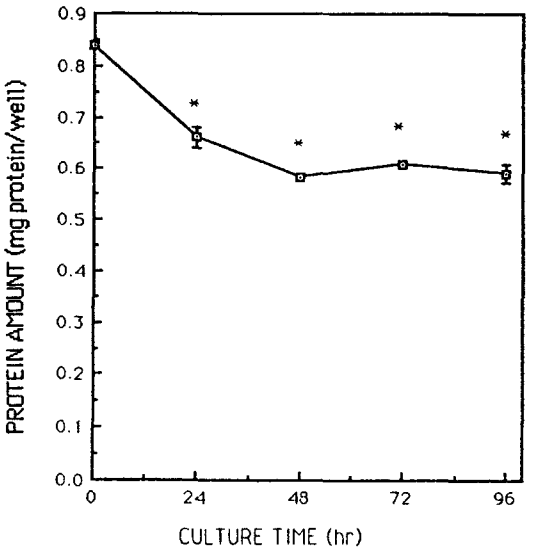

Figure 5. Change of cellular protein amount during cultivation of rat hepatocytes in AB medium. Cells were cultured for 96 hr and harvested every 24 hr after initial plating for protein determination. The asterisk indicates $P\leq0.05$ when compared with the value at 0 time.

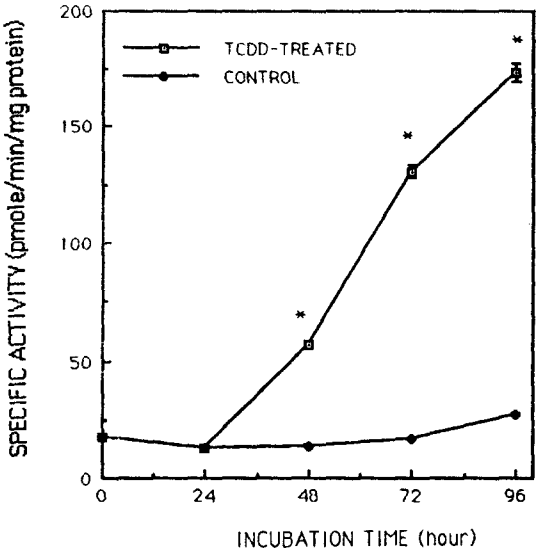

Figure 6. *In vitro* induction profile of EROD activity in primary rat hepatocytes culture. TCDD was treated at 10 nM from 24 hr after plating. The asterisk indicates $P \leq 0.05$ when compared with the control values.

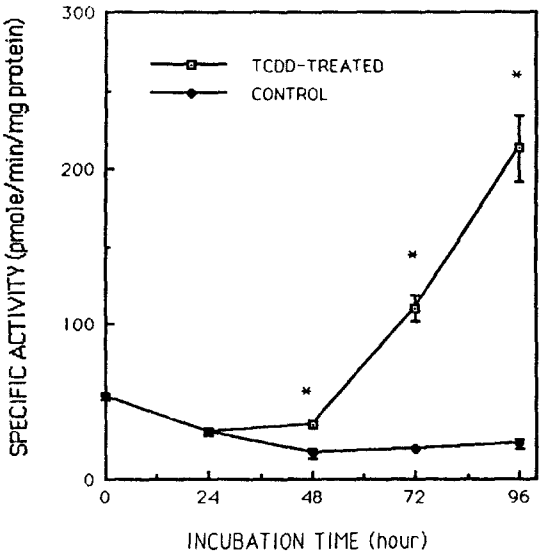

Figure 7. *In vitro* induction profile of EROD activity in primary human hepatocytes culture. TCDD was treated at 10 nM from 24 hr after plating. The asterisk indicates $P \leq 0.05$ when compared with the control values.

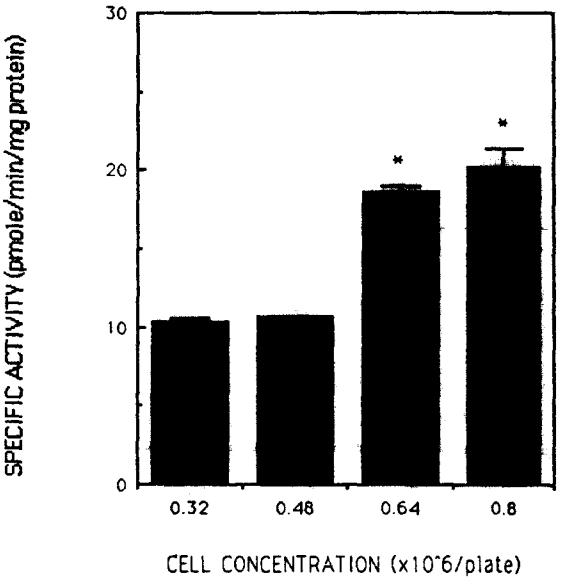

Figure 8. Change of uninduced EROD activity in different cell densities. Cells were cultivated for 96 hr and harvested to determine EROD activity. The asterisk indicates P≤0.05 when compared with the lowest concentration value.

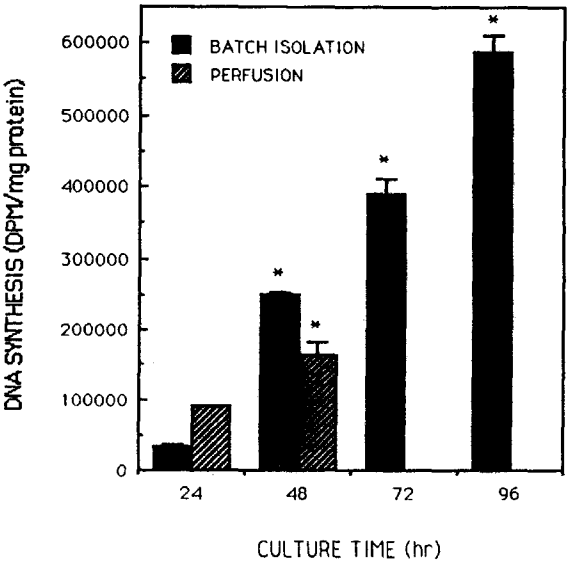

Figure 9. Induction profile of ^{3}H-thymidine incorporation in primary rat hepatocytes culture. Hepatocytes isolated by batch isolation and perfusion were compared. Cells were cultured for 96 hr and harvested every 24 hr after initial plating for assay. The asterisk indicates P≤0.05 when compared with the 24 hr value.

tained a large capacity of DNA synthesis. From 48 hr, DNA synthesis was strongly stimulated and level at 96 hr was 17 fold of the first 24 hr cultured cells. Hepatocytes isolated by perfusion also showed a stimulation in 48 hrs' culture. Human hepatocytes isolated by batch digestion showed a similar pattern with rat hepatocytes and ^{3}H-thymidine uptake was 2.3 fold stimulated at 72 hr and 96 hr cultivation compared with the first 24 hr (Figure 10). These results showed that the isolation or culture condition gave stimulation signals to hepatocytes and presented an *in vitro* regeneration condition to cultured hepatocytes.

Table 1. Induction of α-aminoisobutyric acid (AIB) uptake by dexamethasone and glucagon in rat and human hepatocyte culture.

Origin	Hormone Treatment	AIB uptake (nmole/mg protein/min)
Rat	none	0.83± 0.12
	glucagon	1.30± 0.06
	glucagon+ dexamethasone	4.58± 0.16
Human	none	–
	glucagon	1.38± 0.04
	glucagon+ dexamethasone	4.43± 0.15*

The asterisk indicated P≤0.05 when compared with the control values.

Table 2. Induction of tyrosine aminotransferase (TAT) by dexamethasone in rat and human hepatocyte culture.

Origin	Dexamethasone induction	TAT activity (mUnits/mg protein)
Rat	–	38.83± 4.90
	+	281.91± 16.60*
Human	–	23.30± 6.34
	+	22.48± 4.92

The asterisk indicates P≤0.05 when compared to the control values.

Table 3. Albumin synthesis in primary human hepatocyte culture. The amount of albumin was determined by ELISA technique.

Culture time (hr)	Albumin synthesis (ng albumin/24 hr/mg protein)
24	1.88
48	1.54
72	1.21
96	0.98

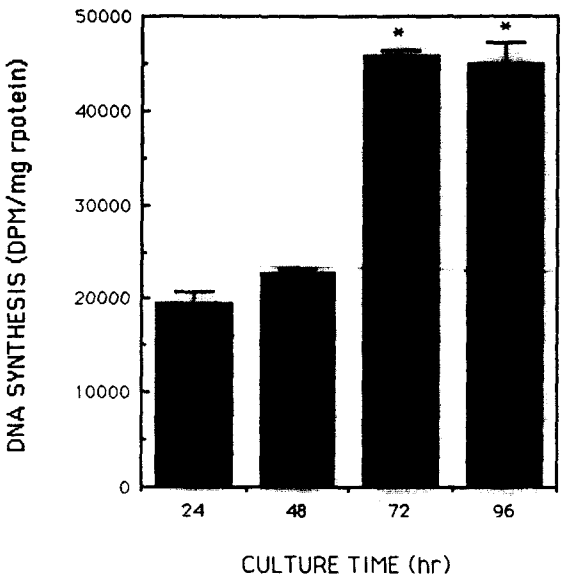

Figure 10. Induction profile of ^{3}H-thymidine incorporation in primary human hepatocytes culture. Cells were cultured for 96 hr and harvested every 24 hr after initial plating for determination. The asterisk indicates $P \leq 0.05$ when compared with the 24 hr value.

DISCUSSION

The purpose of present study was to creat an *in vitro* model system for the study on liver. Even though several *in vitro* testing systems were reported, primary hepatocyte culture seemed to be an excellent model owing to its many advantages. The prevail method for the isolation of hepatocytes from animal or organ was collagenase perfusion technique (Berry and Friend; 1969; Seglen 1972). Other methods also reported and the underlying principle for these methods was chopping or slicing followed by enzymatic digestion with collagenase, hyaluronidase and trypsin. But these methods had some problems on viability and cell yield and required a long time and large labour (Nau *et al.*, 1978; Armato *et al.*, 1978 a and b; Tanaka *et al.*, 1978). Perfusion technique was developed for rat hepatocytes was applied for other species including human. In the case of human, whole organ, lobe or relatively large tissue sample was donated from organ donor and perfusion was performed through blood vessel by using large cannular such as 14 guage. The enzymes used were collagenase, hyaluronidase, DNase and dispase (Rijntes *et al.*, 1988; Gripon *et al.*, 1988; Ochiya *et al.*, 1989; Babaev *et al.*, 1989; Thornton *et al.*, 1991; Ismail *et al.*, 1991). To perform perfusion, sample size was at least 20~30 g and sometimes 100~200 g liver tissue was used. Since organ donation was not common in this country, it was impossible to acquire an adequate sample for perfusion and thus, only small size sample for pathological and diagnostic examination could be acquired. Because the size was too small and many blood circulation vessels were cut out, these samples could not be applicable for perfusion. Accordingly, batch isolation was adopted as cell isolation method at pre-

sent study.

For rat hepatocytes isolation, most reasonable method was an injection and chopping followed by shaking and pipetting. Digestion enzyme was collagenase (300 units/ml) in HBSS and addition of calcium or other enzymes did not produce better results than collagenase alone. After repeated isolation and washing, cell viability was above than 85% and cell yield was about 1×10^7/g wet liver. This result was comparable with previous reports. In isolation of human hepatocytes, chopping method was substituted with slicing to reduce mechanical damage caused by mincing. The yield and viability of human hepatocytes were almost same with those of rat. To support a long term study such as viral infection and chronic exposure experiments, long term culture was tried. The monolayer culturing method on collagen coated culture plate was suitable for hepatocyte of both rat and human. In both case, parenchymal cells showed normal spreaded morphology at 24 hr after plating. Cellular protein concentration also maintained during culture. Culture media used in present study was AB media (a modified Waymouth'media) and suitable for both rat and human hepatocytes. For above 15 days, culture was maintained and no sign of population change could be observed. The integrity of cytoplasmic membrane, cytoskeleton and intracellular organelle of isolated cells was proven by microscopic observation including electron microscopy and acute membrane protrusion formation induced by phalloidin. Functional integrities were examined on hormonal response, maintenance and induction of drug metabolizing capacity, hepatocyte-specific protein synthesis and DNA synthesis. Responses on steroid and proteineous hormones reflect an normal membrane receptor and nucleus gene turn-on process which were required for their actions. Amino acid (AIB) uptake and TAT is involved in cellular amino acid homeostasis. These two parameters showed that cultured hepatocytes contained a normal cellular nutritional metabolism. The fact that TAT was not induced by dexamethasone in human hepatocytes might reflect an species difference of TAT controlling mechanism. Most stringent characteristics of hepatocyte is that it contain drug metabolizing capacities and respond to many inducers. Human and rat hepatocytes cultured in present study kept intact Ah receptor in cytoplasm and Ah-dependent gene turn-on process in nucleus. The sharp decrease of drug metabolizing capacity in primary monolayer hepatocyte culture was an well-known phenomenon (Guzelian *et al.*, 1977; Michalopoulos *et al.*, 1976 and 1978). In present study, uninduced EROD activity was declined sharply at early time of culture but rebound to 0 time level at 4 days after plating. Two findings that uninduced EROD specific activity was increased in higher cell concentration and DNA synthesis was stimulated during culturing period, might confer an clue on this phenomenon as follows. Cell-cell contact was broken in initial culture period and hepatocytes was escaped from G_0 phase and entered growing cell cycle. In this state, the proportion of regenerated hepatocyte population was increased and caused an decrease of cytochrome P-450 and related enzyme activities. But after incubation for several days, cell-cell communication and cellular phase of growth might returned to 0 time level. This explanation remains to be elucidated. As mentioned above, primary hepatocytes culture could be applicable to the study on hepatocyte growth modulation. Ismail *et al.*, (1991) reported that DNA synthesis in cultured hepatocytes increased during

cultivation and showed a maximum at 72 hr in the presence of insulin and hydrocortisone. But the in present study, DNA synthesis increased continuously until 96 hr in rat hepatocytes and human hepatocytes maintained DNA synthetic capacity at 96 hr. The difference showed that DNA synthesis might be influenced by culture condition including medium components and intermediary metabolites (McGowan *et al.*, 1984).

In conclusion, human and rat hepatocytes could be successfully isolated from small size sample and cultured for a long time. Isolated and cultured hepatocytes were proven to retain the morphological and functionl integrities as judged by parameters presented in this study. Primary culture of hepatocytes is a good model for the studies such as medical research of liver, pharmacological and toxicological examination, new drug development and so on. Especially human hepatocytes culture might be an necessary *in vitro* tool for toxicological and pharmacological studies of human specific agents such as biological response modifiers. The culture technique presented in this study might increase the application of hepatocytes to various fields.

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