FUNCTIONAL HUMAN HEPATOCYTES:
ISOLATION FROM SMALL LIVER BIOPSY SAMPLES AND PRIMARY CULTIVATION
WITH LIVER-SPECIFIC FUNCTIONS

Hwan-Mook KIM, Sang-Bae HAN, Byung-Hwa HYUN,
Chang-Joon AHN*, Young-Nam CHA**, Kyu-Shik JEONG and Goo-Taeg OH

Korea Research Institute of Bioscience and Biotechnology,
Taejon City, 305-333, Korea
*Catholic Medical College,
Taejon, 301-010, Korea
**Medical College, Inha University,
Inchon, 402-75, Korea

(Received July 20, 1995; Accepted September 8, 1995)

ABSTRACT — Morphologically and functionally intact human hepatocytes were isolated from small liver biopsy samples weighing about 1–2 g by initial digestion with collagenase followed by repeated digestions with trypsin. The usual yield of hepatocytes was greater than $1 \times 10^7$ cells per g of liver sample and cell viability, as judged by dye exclusion test, was routinely over 90%. The isolated human hepatocytes showed intact morphology under scanning electron microscope. Formation of membrane protrusions upon phalloidin addition demonstrated that the actin in isolated hepatocytes was maintained with its structural integrity. The cultured human hepatocytes retained a variety of liver-specific functions which were similarly exhibited by rat hepatocytes isolated using the same procedure. The cultured human hepatocytes exhibited a specific cytochrome P-450 related enzyme activity, and active amino acid uptake that increased upon addition of hormones like glucagon and dexamethasone. Additionally, the cultured human hepatocytes synthesized DNA actively and, human serum albumin, and was found to be responsive to modulation by growth modulating hormones, cytokines and hepatotoxic agents. Based on the profile of activity described above, the presently established conditions for isolation and culturing of human hepatocytes demonstrate that functional liver cells can be obtained from small biopsied liver samples.

KEY WORDS: Human liver, Non-perfusion hepatocyte, Morphology, Toxicology.

INTRODUCTION

To conduct pharmacological and toxicological studies with human hepatocytes, successful isolation and culturing of functionally intact hepatocytes that maintain their liver-specific characters is essential. Since the introduction of the hepatocyte isolation technique using collagenase perfusion method by Berry and Friends (1969), intact whole livers of rats and mice have been the most common sources of primary hepatocytes.
Because methods for preservation and shipment of hepatocytes over long distances have not been adequately proven to be reliable, some laboratories wanting to work with human hepatocytes must resort to use of resected tissues from nearby hospitals as a source material. In those cases, only small pieces of human liver tissue weighing about 1 to 2 g are available after biopsies conducted for diagnostic purposes. To overcome these limitations, some laboratories have applied mechanical manipulations such as scraping hepatic tissue with a syringe needle, and perfusion methods by using a silicon catheters (Trevisan et al., 1982; Lau et al., 1991a and 1991b, 1992; Lechon et al., 1990). The former approach results in a low yield of human hepatocytes which exhibit a low plating efficiency in culture and could not be maintained for long enough durations to be employed for virological-, immunological-, pharmacological- and toxicological studies. The later one also caused a limitation of liver samples that only a wedge biopsied sample could be used for partial perfusion.

Thus, in the present study, we have attempted to isolate intact and functional human hepatocytes from small liver biopsy samples weighing about 1 to 2 g by a non-perfusion method. These biopsied human liver tissue samples were obtained from local hospitals upon permission from volunteer patients. Biopsied samples used in the study could be defined as within a normal functional range through all experiments. In the present study, the liver tissues were thinly sliced, digested with collagenase in the absence of Ca$^{2+}$ and Mg$^{2+}$, filtered, and repeatedly digested with trypsin, and then, harvested. The yield and viability of harvested human hepatocytes prepared by the methods described herein were superior to those obtained in previous attempts (Gripon et al., 1988; Ismail et al., 1991; Lau et al., 1991a) using other approaches. The freshly isolated human hepatocytes resulting from our attempt were morphologically and functionally intact and did overcome the limitation of samples. These human hepatocytes had high adhering efficiency to collagen coated culture plates and could be cultured as a monolayer for over 12 days. Properties of these human hepatocytes were comparable to those of rat hepatocytes isolated from intact rat liver prepared by the collagenase perfusion method in the previous report (Dickins and Peterson, 1980). After establishing the conditions for isolation of intact and functional hepatocytes and then for culturing the primary hepatocytes, these cultured human hepatocytes were tested for various liver-specific physiological parameters to demonstrate their usefulness in pharmacological- and toxicological studies.

MATERIALS AND METHODS

Materials: Human recombinant epidermal growth factor (EGF), transforming growth factor-beta (TGF-β), and interleukin-1 (IL-1) were obtained from Genzyme Co. (Cambridge, USA). Insulin and phalloidin were purchased from Boehringer-Mannheim (Mannheim, Germany). Hydrocortisone and other chemicals were from Sigma Co. Ltd. (St. Louis, USA). Culture media (Waymouth's and Williams E) and Hank's balanced salt solution (HBSS) were made by Gibco BRL, (Gaithersburg, USA). [Methyl-3H]thymidine was obtained from Dupont-New England Nuclear, (Boston, USA). All other analytical reagents were used the highest grade commercially available.

Liver biopsy samples: Human liver tissue samples were obtained from local hospitals after permission by about 50 volunteer patients. Biopsied samples used in the study could be defined as within a normal functional range. In general, we could obtain only small sized human liver tissue samples usually weighing about 1 to 2 g and samples were taken from different sites of liver. In the case of animal experiments, studies were performed on 200–250 g male Sprague-Dawley rats. Rats were provided with food (Purina rodent chow) and water ad libitum, generally eating from lights out at 6 p.m. to lights on 6 a.m. Liver samples were aseptically dissected after cervical dislocation.

Isolation of hepatocytes: The freshly biopsied human liver tissues immersed in cold Hanks’ Balanced Salt Solution (HBSS) were transferred to the laboratory within 1 hr and were washed with cold HBSS to remove the contaminating blood. The washed human liver tissue was then cut with a sharp surgical blade into thin slices (1–2 mm thick) and washed 3 times with cold
HBSS which did not contain Ca\(^{2+}\) or Mg\(^{2+}\) ions. The washed liver slices were transferred into a 100 ml bottle and then digested with collagenase (20 ml, 300 units/ml in Ca\(^{2+}\) and Mg\(^{2+}\) free HBSS) for 20 min in a shaking water bath set at 37\(^{\circ}\)C. After allowing the tissue to settle, the supernatant was discarded. The remaining liver tissue was transferred to 50 ml conical tube containing 20 ml of trypsin solution (0.25% trypsin powder in saline) and digested for 10 min at 37\(^{\circ}\)C with intermittent vortexing. The trypsin digest was then filtered through a 210 \(\mu\)m nylon mesh filter and mixed with 20 ml of cold HBSS containing 10% heat-inactivated fetal calf serum (FCS) in order to stop further trypic digestion. Subsequently, the mixture was centrifuged at 50 \(\times\) g for 4 min, supernatant discarded, and the cell pellet was resuspended in a 5 ml of HBSS containing 10% FCS. Such trypic digestion and harvesting cycles were repeated for 5 times. After the final harvesting, the hepatocytes were washed 3 times by centrifugation in cold HBSS containing 10% FCS. Cell viability and number were determined by trypan blue exclusion test. The usual yield of hepatocytes was greater than 1 \(\times\) 10\(^7\) cells per g of biopsied human liver tissue and the usual viability was over 90%. For comparison, rat hepatocytes were also isolated by above procedure.

Primary culture of human hepatocytes: The final pellet of human hepatocytes was suspended in the serum free modified Waymouth’s medium (Decad et al., 1977) to the cell concentration of 6 \(\times\) 10\(^5\) cells/ml and seeded onto plastic microplate precoated with Vitrogen-100 (Celtrix, CA, USA). Rat hepatocytes were plated in the same way but the cell concentration was adjusted to 4 \(\times\) 10\(^5\) cells/ml. In some cases, where specific experimental design demanded, hydrocortisone and/or insulin was omitted from the Waymouth’s complete medium. After a 4 hr attachment period at 37\(^{\circ}\)C in a humidified atmosphere of 5% CO\(_2\) and 95% air, the cells were refed with fresh culture medium every 24 hr thereafter.

Morphology of hepatocytes: Freshly isolated as well as the cultured primary hepatocytes were viewed under inverted phase contrast microscope to check their morphology. Alternatively, the hepatocytes were prefixed with phosphate buffered saline containing 2.5% glutaraldehyde and 2% paraformaldehyde and then fixed with 1% osmium tetroxide. The dehydrated hepatocyte samples were then examined with scanning electron microscope (Jeol JSM-35C) at 15 kV (Sattler et al., 1978; Kawamoto et al., 1980).

Mixed function oxidase activity: The activity of ethoxyresorufin O-deethylase (EROD) in both the freshly harvested hepatocytes as well as the cultured hepatocytes harvested at 1, 2, 3, and 4 days was measured as described previously (Lubert et al., 1985; Blank et al., 1987). Also, to determine whether the EROD activity could be enhanced by a well known inducer, 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) (final concentration 10 nM) dissolved in dimethylsulfoxide (DMSO), was added to the culture medium on days 1, 2, and 3. On day 2, 3, and 4, cells were harvested and, the EROD activity was measured and the EROD activity was also measured and compared with those of the control values. Briefly, the assay buffer used for determination of EROD activity in the homogenized cell preparations consisted of 0.1M potassium phosphate (pH 7.5), 2 mg/ml bovine serum albumin, 10 \(\mu\)M dicoumarol, 5 mM glucose 6-phosphate, 20 units/ml of glucose 6-phosphate dehydrogenase and 0.5 mM NADPH. The rate of forming resorufin by the homogenized cell suspensions upon adding 2.5 \(\mu\)M ethoxyresorufin was quantitated using a Hitachi model 650-10S spectrofluorimeter at an excitation and emission wavelengths of 550 nm and 585 nm, respectively.

Hormonal stimulation of amino acid uptake and metabolism: Inducibility of \(\alpha\)-aminoisobutyric acid (AIB) transport as well as the tyrosine amino-transferase (TAT) activity with hormones like glucagon and/or dexamethasone was measured according to the methods described earlier. The induction of AIB transport was done in 2 steps, first by the addition of 1.0 \(\mu\)M dexamethasone at 24 hr after initial plating and secondly by the addition of 0.2 \(\mu\)M glucagon at 42 hr to the primary hepatocyte culture. The rates of \(^{14}\)C]AIB uptake were measured at 48 hr according to the previously published method (Kletzien et al., 1976; Pariza et al., 1976). The activity of TAT was induced by the addition of 10 \(\mu\)M dexamethasone at 24 hr after initial plating (Bonney et al., 1974). At 48 hr, cells were harvested in homogenizing buffer (0.2 M potas-
sium phosphate, 10 mM $\alpha$-ketoglutarate, 0.04 mM pyridoxal phosphate, 1 mM EDTA, pH 7.3) and disrupted by repeated freezing and thawing. The activities of TAT were monitored by the method of Diamondstone (1966).

**Albumin synthesis**: The amount of human albumin produced and secreted by the cultured human hepatocytes was monitored by the ELISA technique utilizing the human serum albumin as the standard antigen (Ochiya et al., 1989). For this assay, hepatocytes were cultured in the William’s E medium instead of the modified Waymouth’s medium to avoid the interference caused by the bovine serum albumin in the ELISA test procedure. The culture medium was sampled at 24 hr intervals during cultivation and used to determine the rate of human serum albumin synthesis in the ELISA determinations. The sampled culture medium was diluted with carbonate buffer (50 mM, pH 9.6) containing 3 mM Na$_2$CO$_3$ and incubated in 96 well microplate for 2 hr at 37°C. After blocking with 3% casein in TBS (Tris buffered saline, 100 mM Tris, 150 mM NaCl, pH 7.4), mouse monoclonal antibody against human serum albumin (Sigma) was added and then incubated for 2 hr. After washing with TBS-0.05% Tween 20, horseradish peroxidase-conjugated goat anti-mouse IgG antibody (diluted 1:2000 with 0.3% casein-TBS, Sigma) was added to the plates and incubated at 37°C for 2 hr. After washing, bound enzyme activity was measured with 0.4 mg/ml o-phenylenediamine (Sigma) in 25 mM citric acid/50 mM phosphate buffer, pH 5.0, containing 0.00012% H$_2$O$_2$ as substrate. The enzyme reaction was stopped with 2 M H$_2$SO$_4$ and the OD$_{490}$ was read in an ELISA reader (model Emax, Molecular Devices).

**Effect of hormones and cytokines on DNA synthesis**: The rate of incorporation of [methyl-$^3$H]thymidine into cellular DNA was determined to assess the rate of DNA synthesis. Cultured hepatocytes were pulsed with [methyl-$^3$H]thymidine (1.0 Ci/ml, 6.7 Ci/mmole) for 2 hr before harvesting according to the method described by Nakamura et al. (1984) and by Miyazaki et al. (1992), and the cellular macromolecules were precipitated with the same volume of 10% trichloroacetic acid (TCA). The TCA precipitated materials were then washed 3 times with 5% TCA by centrifugation. The final pellet was resuspended and solubilized in 1.0 ml of 0.2 N NaOH and the solubilized aliquots were then used for measuring the radioactivity and the DNA amount. Radioactivity was determined by liquid scintillation counting (LS 6000TA, Beckman) and the DNA content was determined after labelling with Hoechst 33258 by using a spectrofluorometer (Model 650-108S, Hitachi).

**Effects of hepatotoxic agents and cytokines on DNA synthesis**: Stock solutions of TCDD and carbon tetrachloride (CCl$_4$) were prepared in DMSO and diluted in the culture media prior to additions to the cell culture. Ethanol (EtOH) was directly added to culture medium. Cytokines like EGF, TGF-β, and IL-1 were diluted directly with the culture medium. All agents were added to the culture medium at 4 hr after the initial plating and at every 24 hr medium change. The final concentration of DMSO was 0.1% of culture medium and it did not affect the DNA synthesis rate.

**Statistical Analysis**: The significance of the differences between the mean values was judged by Student’s $t$-test and the level of significance was set at $P \leqslant 0.05$.

**RESULTS**

The biopsied human liver tissues as received from the hospital showed a large sample to sample deviations in hardness, gross morphology and pathological conditions. Since the successful isolation and culture of human hepatocytes depend critically on the viability, yield and functional integrity, about 20% of liver tissue samples were not used for the cell isolation procedures. Of the remaining 80% of liver samples which were subjected to cell isolation procedure, after the final harvesting, the usual hepatocyte yield was different according to the conditions of samples, but was usually over $1 \times 10^7$ cells per g of liver sample used and the freshly isolated hepatocytes demonstrated cell viability which was greater than 90%. The freshly isolated hepatocytes showed an intact round morphology under an inverted phase contrast microscope (Photo. 1A). Also, the scanning electron microscopy demonstrated that the cell membranes were intact and the cells had many well-developed microvilli.
on their surfaces (Photo. 1B). Upon a 10 min exposure to phallloidin (10 μg/ml), a selective actin binding hepatotoxin, membrane protrusions resulting from phallloidin-actin aggregation appeared (Photo. 1C). This indicated that actin maintained its functional integrity through the vigorous and repeated trypsin digestion procedure. The isolated hepatocytes attached efficiently to the collagen-coated culture dish and spread to form a confluent monolayer and, under the present culture condition, the hepatocyte monolayer primary culture could be maintained for over 12 days without any significant changes in their gross morphology or cell types (Photo. 1D).

The freshly isolated human hepatocytes had cytochrome P-450 mediated mixed function oxidase activity like ethoxyresorufin EROD comparable to that found in the freshly isolated rat hepatocytes prepared by the standard collagenase perfusion method. Similar to the phenomenon found with rat hepatocytes prepared by collagenase perfusion method (Michalopoulos et al., 1976; Engelmann et al., 1985; Kim et al., 1988),...
during early phases of culture, the EROD activity of human hepatocytes declined continuously down to 50% of the initial level until 48 hr, and afterwards, the enzyme activity was maintained at the reduced 48 hr level for up to 96 hr of culture (Fig. 1). Upon addition of TCDD (10 nM), a well known inducer of cytochrome P-4501A1/A2, to the culture medium, the specific EROD activity increased markedly (Fig. 1). These results suggest that both the freshly isolated as well as the cultured human hepatocytes obtained by the current method might retain the drug metabolizing capacity and its inducibility.

To establish the functional status of the cultured human hepatocytes, hormone inducible amino acid uptake and metabolism, increased uptake of AIB and activity of TAT were determined following sequential glucagon and/or dexamethasone additions. Results shown in Table 1 indicate that the addition of glucagon enhances the AIB uptake and the combined addition of glucagon and dexamethasone increases the AIB uptake.

### Table 1. Hormonal induction of α-aminoisobutyric acid (AIB) uptake.

<table>
<thead>
<tr>
<th>Hepatocytes</th>
<th>Hormonal treatment</th>
<th>AIB uptake (nmol/mg protein/min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Human</td>
<td>none</td>
<td>0.82±0.14</td>
</tr>
<tr>
<td></td>
<td>glucagon (0.2 μM)</td>
<td>1.38±0.04*</td>
</tr>
<tr>
<td></td>
<td>glucagon (0.2 μM) + dexamethasone (1 μM)</td>
<td>4.43±0.15*</td>
</tr>
<tr>
<td>Rat</td>
<td>none</td>
<td>0.83±0.12</td>
</tr>
<tr>
<td></td>
<td>glucagon (0.2 μM)</td>
<td>1.30±0.06*</td>
</tr>
<tr>
<td></td>
<td>glucagon (0.2 μM) + dexamethasone (1 μM)</td>
<td>4.58±0.16*</td>
</tr>
</tbody>
</table>

Values are given as the means of 10 cultures (three plate/culture) ± S.D. An asterisk indicates P≤0.05 (t-test) when compared with untreated control.

### Table 2. Hormonal induction of tyrosine aminotransferase (TAT) activities.

<table>
<thead>
<tr>
<th>Hepatocytes</th>
<th>Hormonal treatment</th>
<th>TAT activity (mUnits/mg protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Human</td>
<td>none</td>
<td>23.30±6.34</td>
</tr>
<tr>
<td></td>
<td>dexamethasone (10 μM)</td>
<td>22.48±4.92</td>
</tr>
<tr>
<td>Rat</td>
<td>none</td>
<td>38.83±4.90</td>
</tr>
<tr>
<td></td>
<td>dexamethasone (10 μM)</td>
<td>281.91±16.6*</td>
</tr>
</tbody>
</table>

Values are given as the means of 10 cultures (three plate/culture) ± S.D. An asterisk indicates P≤0.05 (t-test) when compared with untreated control.
uptake further in a synergistic manner. Human hepatocytes show the same extent of inducibility with rat cultured hepatocytes. Results shown in Table 2 indicate that the addition of dexamethasone (10 μM) did not enhance the TAT activity of human hepatocytes. However, the same concentration of dexamethasone strongly induced the TAT activity of rat hepatocytes.

Albumin, a liver-specific protein, was also synthesized constitutively by the cultured human hepatocytes (Table 3). As was elucidated in the method section, to avoid the interference of bovine serum albumin present in the Waymouth’s medium during the ELISA assay employed for quantitation of the produced human albumin, human hepatocytes were cultured in the William’s E medium. In a morphological observation, cultured hepatocytes grown in the William’s E medium showed a slightly decreased spreading. Results shown in Table 3 indicate that the ability of human hepatocytes to synthesize albumin decreases slowly in a time dependent manner and this is thought to be caused by the altered culture medium. These results indicated that the cultured hepatocytes obtained in the present study are suitable for studies aimed at identifying agents that modulate human serum albumin synthesis.

The ability of cultured human hepatocytes to synthesize DNA as determined by the thymidine incorporation rate was affected by the various culture conditions used such as the volume of culture medium as well as the presence or absence of hormones and cytokines. Upon depletion of hydrocortisone and/or insulin from the complete Waymouth’s culture medium, the rate of thymidine uptake decreased significantly (Fig. 2A). Varying the volume of culture medium present in the culture well also affected the rate of thymidine uptake. Either increasing the volume of culture media or decreasing the cell to medium ratio resulted in a decrease in the rate of thymidine incorporation. The highest rate of DNA synthesis was observed with 1.0 ml of culture media per culture well. This phenomenon was observed regardless of whether the oxygen tension was increased or whether the cultures were rocked on a rocking platform (data not shown). The magnitude of the effect of altering the amount of culture medium on DNA synthesis was most obvious in the presence of complete medium and the effect was diminished when the hydrocortisone/insulin depleted medium was used. This is perhaps due to the limited rate of cell to cell interaction required for the synthesis of some unknown modulators but not due to lowered oxygen tension or to slow rate of oxygen transport. Perhaps, even with excess volume of the complete culture medium, opportunities for cell to cell interactions through chemical compounds may be diminished and this may have affected the rate of DNA synthesis in a negative manner. The presence of EGF significantly augmented the rate of thymidine uptake and the maximal effect was achieved at 20 ng/ml (Fig. 2B). The presence of TGF-β and IL-1 inhibited the DNA synthesis rate (Fig. 2C and Fig. 2D). While the TGF-β uniformly caused severe inhibition on the rate of DNA synthesis at all concentrations ranging from 2.5 to 20 ng/ml, IL-1 treatment produced a dose dependent inhibitory effect on the DNA synthesis at concentrations from 1.0 to 4.0 ng/ml.

As shown in Fig. 2, TCDD is a well known inducer of EROD mediated-function oxidase and, in support of this, TCDD (10 nM) treatment of hepatocytes significantly increased the DNA synthesis rate to the same degree as 10 ng/ml of EGF. However, when EGF was added in combination with TCDD, the co-treated EGF antagonized the stimulation of DNA synthesis caused by TCDD (Fig. 3A). The stimulatory effect of TCDD on hepatocyte DNA synthesis could be seen more clearly when hepatocytes were cultured in hydrocortisone-depleted medium.

### Table 3. Albumin synthesis in primary human hepatocyte culture. The amount of human albumin was determined by ELISA as designated in method section.

<table>
<thead>
<tr>
<th>Culture time (hr)</th>
<th>Albumin synthesis (mg albumin/24 hr/mg protein)</th>
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<tbody>
<tr>
<td>4−24</td>
<td>1.88</td>
</tr>
<tr>
<td>24−48</td>
<td>1.54</td>
</tr>
<tr>
<td>48−72</td>
<td>1.21</td>
</tr>
<tr>
<td>72−96</td>
<td>0.98</td>
</tr>
</tbody>
</table>

Values are given as the means of 3 cultures (three plate per culture).
Fig. 2. Effects of hormones and cytokines on DNA synthesis of cultured human hepatocytes. Cells were maintained in culture for 72 hr. Medium was replenished daily and [methyl-\(^3\)H]thymidine was included for 2 hr before harvest to determine DNA synthesis. Values represent the means of 10 culture (three plate per each experiment) of ± S.D. An asterisk indicates P≤0.05 (\(t\)-test) when compared with each control. A, effects of hydrocortisone and insulin (a, complete Waymouth’s medium; b, hydrocortisone-depleted medium; c, hydrocortisone and insulin-depleted medium); B, epidermal growth factor (EGF); C, transforming growth factor-beta (TGF-\(\beta\)); D, interleukin-1 (IL-1).

When hepatocytes were cultured in hydrocortisone-depleted medium, in contrast to the result obtained in Fig. 3B where its presence increased the rate of DNA synthesis, the addition of EGF did not enhance the thymidine incorporation rate and completely abolished the TCDD dependent stimulation of DNA synthesis (Fig. 3B).

Results shown in Fig. 4A and 4B demonstrate the effect of nominally adding CCl\(_4\) and ethanol concentration on both the basal and EGF-stimulated thymidine incorporation rate. Although the presence of CCl\(_4\) did not suppress the basal thymidine incorporation rate into DNA, CCl\(_4\) suppressed the EGF-stimulated thymydine uptake rate in a dose dependent manner at doses ranging from 0.01 to 0.1 mM. The addition of ethanol, however, arrested both the basal and EGF-stimulated DNA synthesis at doses ranging from 85 to 170 mM. In both of these cases, while the DNA synthesis rate could be compromised by the additions of either CCl\(_4\) or ethanol, gross cytotoxicity was not observed in the microscopic examinations.
Fig. 3. Effects of TCDD on DNA synthesis of human hepatocytes cultured in complete Waymouth’s medium (A) and hydrocortisone-depleted medium (B). Cells were maintained in culture for 72 hr. Medium was replenished daily and [methyl-³H]thymidine was included for 2 hr before harvest to determine DNA synthesis. Values represent the means of 10 cultures (three plate per each experiment)±S.D. An asterisk indicates P=0.05 (t-test) when compared with untreated control.

A. untreated culture ; b. TCDD (10 nM) ; c. EGF (10 ng/ml) ; d. TCDD (10 nM) and EGF (10 ng/ml).

Fig. 4. Effects of carbon tetrachloride (CCL₄) and ethanol (EtOH) on DNA synthesis of human hepatocytes. Cells were maintained in culture for 72 hr. Medium was replenished daily and [methyl-³H]thymidine was included for 2 hr before harvest to determine DNA synthesis. Values represent the means of 10 cultures (three plate per each experiment)±S.D. An asterisk indicates P=0.05 (t-test) when compared with untreated control.

A. effect of CCL₄ (a. untreated culture ; b. CCL₄ (0.1 mM) ; c. EGF (10 ng/ml) ; d. CCL₄ (0.01 mM) and EGF (10 ng/ml)) ; e. CCL₄ (0.1 mM) and EGF (10 ng/ml). B. effect of EtOH (a. untreated culture ; b. EtOH (170 mM) ; c. EGF (10 ng/ml) ; d. EtOH (85 mM) and EGF (10 ng/ml) ; e. EtOH (170 mM) and EGF (10 ng/ml).
DISCUSSION

Since the early pioneering work of Berry and Friends (1969) on the isolation of hepatocytes from the collagenase perfused intact rat liver, the most common and widely used technique for isolation of hepatocytes has been the collagenase perfusion method. This original collagenase perfusion technique has been widely applied to intact whole liver from a variety of animal species including human. However, to perform the collagenase perfusion on human liver tissues, at least 20 to 30 g of liver tissue or a lobe with intact blood vessels is required (Gripón et al., 1988; Rijntjes et al., 1988; Ismail et al., 1991; Thornton et al., 1991). Lechon et al. (1990) reported that small size biopsy sample could be used for collagenase perfusion. However their method was applicable to only a wedge biopsied tissue which mostly retain intact blood vessels. By this reason, it has been extremely difficult to obtain human hepatocytes for routine hepatological studies. Accordingly, there has been a significant need for an efficient method to isolate hepatocytes from only small tissue samples. With recent advances made in histologic diagnostic techniques, only small biopsy samples are needed for clinical assessment of various liver diseases. However, these small biopsy preparations can not be perfused due to the disruption of the vasculature. Accordingly, a non-perfusion approach was developed for the isolation of intact functional hepatocytes and is described herein.

After the initial digestion with collagenase, the hepatocytes were isolated by repeated digestion with trypsin. Following the final harvesting, more than $1 \times 10^7$ cells were obtained per g of liver biopsy sample which exhibited greater than 90% viability as determined by dye exclusion method. These yields and viability were better than or comparable to those previously reported by others (Meis et al., 1986; Lau et al., 1991a and 1991b, 1992; Lechon et al., 1990) and under scanning electron microscopic observation, cells appeared intact with numerous microvilli and produced phalloidin-specific membrane protrusions. This indicates that the cytoskeleton of the hepatocytes was well preserved through the vigorous trypsin digestion employed in our procedure (Weiss et al., 1973; Lengsfeld et al., 1974). When these isolated human hepatocytes were placed in the collagen coated plate with Waymouth's medium, efficient anchorage dependent seeding and spreading were observed. Furthermore, the primary hepatocyte culture could be maintained for longer than 12 days without any signs of population change by fibroblasts or altered cell types. The amount of cellular protein was maintained during the entire period of monolayer culture period.

After establishing morphologically intact primary hepatocyte cultures, a variety of functional endpoints were employed to determine whether the isolated hepatocytes maintained the liver-specific cellular functions characteristic of this cell-type. The functional tests included measurements of cytochrome P-450 related mixed function oxidase activity and its inducibility in the presence of the well known inducer TCDD, hormone inducible amino acid uptake and metabolism, the ability to synthesize albumin, and also the ability to modulate the rate of DNA synthesis in response to hormones and cytokines. These test parameters reflected the functional integrity of cell membrane, subcellular organelles, and nucleus of the isolated and cultured human hepatocytes. In all of these tested parameters, the cultured human hepatocytes prepared by the present repeated tryptic digestions and culturing in Waymouth's medium conditions responded similarly to those of the cultured rat hepatocytes isolated by the standard collagenase perfusion and the present repeated trypsin digestion (Bonney et al., 1974; Pariza et al., 1976; Nakamura et al., 1986a and 1986b; Blank et al., 1987). There were, however, some minor differences in some of the responses measured when human and rat hepatocytes were compared. For example, while the addition of dexamethasone increased TAT activity in the cultured rat hepatocytes prepared by the current method, the TAT activity in the human hepatocytes was not increased by dexamethasone. These results suggest that species differences may exist in the induction of amino acid transport and metabolism by dexamethasone between rat and human hepatocytes.

Hormones, cytokines, and several hepatotoxic agents are known to affect the rate of DNA
synthesis in hepatocytes. For example, hydrocortisone and insulin are known to stimulate DNA synthesis rate in cultured rat hepatocytes (Leffert et al., 1977; Nakamura et al., 1984; Karasik and Kahn, 1988). As these effects have not been determined for the cultured human hepatocytes, it was confirmed in the present study. With the freshly isolated normal human hepatocytes, it was also reported that EGF stimulates and TGF-β inhibits DNA synthesis (Ismail et al., 1991). In the present study with cultured human hepatocytes, the recombinant human EGF and TGF-β are shown to be positive and negative regulators of DNA synthesis, respectively. Furthermore, recombinant human IL-1 was reported to be a potent negative regulator of rat hepatocytes (Andus et al., 1991) and this was also confirmed in the present studies with cultured human hepatocytes (Fig. 3D).

Several xenobiotics have been demonstrated to produce toxic effect on rat liver and hepatocytes. For example, TCDD is known to induce hepatomegally, to increase DNA synthesis, and in toxic doses, to cause hepatic necrosis in rats and rabbits. Consistent with these observations, Dickins et al. (1981) reported that TCDD (5 μg/kg) pretreatment of partially hepatectomized rat produced an increase in the rate of DNA synthesis which was above the normal synthetic rate observed in the recovering liver. Similarly, the results of the present study indicated that TCDD (10 nM) stimulated the DNA synthetic rate in the active human hepatocytes in primary culture condition. In studies with the cultured rat hepatocytes, addition of TCDD was reported to be relatively ineffective in enhancing DNA synthesis but when combined with EGF treatment, the added TCDD was found to be effective in increasing the DNA synthesis rate by acting as a co-mitogen with EGF. The maximum concentration required for their co-mitogenic action (3 × 10^{-12} M) was lower than that required for cytochrome P-450 induction (Schrenk et al., 1992; Wolfe et al., 1993). TCDD also showed mitogenic effects on mouse hepatocytes in primary culture. At 3 × 10^{-14} M, TCDD increased EGF-stimulated DNA synthesis approximately 1.4-fold in Ah<sup>a</sup> Ah<sup>b</sup> but not in Ah<sup>a</sup> Ah<sup>d</sup> cells at a plating density of 35,000 cells/cm<sup>2</sup>. In hepatocytes from Ah<sup>d</sup> Ah<sup>d</sup> mice, 3 × 10^{-12} M TCDD was required to elicit a similar co-mitogenic response. At a density of 10,000 cells/cm<sup>2</sup>, 3 × 10^{-12} M TCDD had a pronounced inhibitory effect on EGF-stimulated DNA synthesis in Ah<sup>a</sup> Ah<sup>b</sup> but not in Ah<sup>a</sup> Ah<sup>d</sup> cells (Schrenk et al., 1994). The results of present study conducted with human hepatocytes showed that high concentration (10 nM) of TCDD was mitogenic to EGF-untreated hepatocytes, but antagonized the EGF inducible increase in the DNA synthesis rate at a density of 12,000 cells/cm<sup>2</sup>. The concentration of TCDD was same with that required for cytochrome P-450 induction. These differences in TCDD mitogenic activity between human hepatocytes and rat and mouse hepatocytes may have been due to the differences in the concentration of TCDD and/or the medium components such as hydrocortisone and estradiol which was included as hormonal supplements in present study. The antagonistic effect of TCDD against EGF suggest that the human hepatocytes utilized in the present characterizations was derived from TCDD responsive patients.

Following a single injection of low amounts of CCl<sub>4</sub> to male mice and partial hepatectomized rat, hepatic DNA synthesis (as indicated by thymidine uptake) was not significantly affected at 24 hr (Nath et al., 1990). After a single acute administration of high dose (2.5 ml/kg) of CCl<sub>4</sub> to partially hepatectomized rats, thymidine incorporation as well as the percentage of labelled cells, highly stimulated by partial hepatectomy, were significantly decreased by high dose of CCl<sub>4</sub> (Kodavanti et al., 1989). However, the injection of high amount of CCl<sub>4</sub> to normal rat significantly suppressed hepatic DNA synthesis during 36 hr and thereafter DNA synthesis was gradually increased (Lindroos et al., 1991). In further studies using the same experimental model, Higuchi and Nakamura (1991) demonstrated that some cytokines like hepatocyte growth factor (HGF) produced by platelets were responsible for an increased rates of hepatocellular regeneration and DNA synthesis following partial hepatectomy and treatment with CCl<sub>4</sub>. In the present study using cultured human hepatocytes which were undergoing DNA synthesis, a high dose of CCl<sub>4</sub> suppressed only EGF-inducible DNA synthesis rate (Fig. 4A). These results may imply that an acute exposure of CCl<sub>4</sub> does not suppress the
growth of unprimed hepatocytes but elicited a suppression to the growth modulating effect of EGF in a selective manner in human hepatocytes (Fig. 2B).

In studies with cultured rat hepatocytes, it was demonstrated that addition of ethanol (85 to 170 mM) resulted in a substantial inhibition of the basal DNA synthesis rate (Carter and Wands, 1985, 1988; Henderson et al., 1989). Similarly, adding ethanol to the cultured human hepatocytes which are undergoing DNA synthesis has produced severe inhibition of both the basal and EGF-stimulated DNA synthesis rates (Fig. 4B). In contrast to these results, when ethanol was added to the actively proliferating mouse hepatoma cell lines (Hepa 1c1c7), the rates of DNA synthesis was stimulated instead of being inhibited (data not shown). Further studies are being conducted in the laboratory to delineate these differences in ethanol dependent responses on DNA synthesis rate between the normal and hepatoma cell cultures.

In conclusion, the present study has demonstrated that viable and intact human hepatocytes can be isolated with good yield from small biopsied human liver tissue samples by initial digestion with collagenase and then by repeated and vigorous trypsin digests. These studies further demonstrated that functional human hepatocytes could be cultured for over 12 days and cultured human hepatocytes retained all of the liver-specific functions. Lastly, our studies demonstrate the possibility that isolated human hepatocytes can be utilized as a model system for hepatological- and toxicological studies with a variety of agents, known to modulate liver function.

REFERENCES


Higuchi, O. and Nakamura, T. (1991) : Identification and change in the receptor for hepatocyte growth


