Chapter 15

Primary Cultures of Rat Hepatocytes

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1. Introduction

With the advent in 1969 of the collagenase-perfusion technique for the high-yield preparation of isolated, differentiated hepatocytes (*1*), easy establishment of primary cultures of hepatocytes was made possible. Since then, this experimental system has been increasingly used in many research fields.

Hepatocytes are anchorage-dependent cells, and cultures can be established as confluent, nonproliferating monolayers. This experimental system is superior to other in vitro liver preparations because of a longevity of several weeks, as compared to a few hours for perfused liver or suspensions of isolated hepatocytes, making studies of cellular processes that occur within a time scale of hours or days feasible. Another advantage of primary hepatocyte culture, as compared to freshly isolated suspensions, may be that deficiencies of the latter, resulting from damage of the cell membrane by the collagenase treatment, are resolved during the early stages of culture (*2–4*).

Examples of topics that have been studied in primary cultures of hepatocytes are DNA-synthesis (*5,6*), transcriptional and translational events including hormone effects (*7–9*), the regulation of hepatic metabolism, (*10,11*), secretory processes, (*12–14*), metabolism and effects of xenobiotics

such as ethanol and drugs (*15–17*), and hepatotoxicity, including carcinogenic and mutagenic effects (*18*). Hepatocyte cultures have also been used as an activating system in the *Salmonella* mutagenicity test (*19*).

One important consideration when using primary cultures of hepatocytes is the phenotypic stability of the cells. Early work with hepatocyte cultures demonstrated changes in characteristic functions of differentiated hepatocytes, such as declining activity of liver-specific enzymes (*2–4*) and cytochrome P-450 content (*3,15,20,21*), increased production of α-fetoprotein (*22*), and enhanced response to β-adrenergic agents (*4,23,24*). Although much work has been devoted to the establishment of phenotypic stable cultures, many reports appear without adequate characterization of the culture system actually used. This aspect will be discussed in more detail in section 4.

In the following sections, a standard procedure for the preparation of primary monolayer cultures of hepatocytes from adult rats will be described, together with some methods for the preparation of samples for analysis. Methods for preparation of hepatocyte cultures from neonatal and postnatal rats and from other species, and conditions for proliferating hepatocyte cultures will not be described in detail, but will be discussed briefly in section 4.

2. Materials

1. Adult Wistar rats, weighing 200–230 g and starved for 16 h. Animals are housed at 21°C with alternating 12-h cycles of light (7 AM–7 PM) and darkness.
2. A solution of collagen prepared from rat tails. Six to seven tails are immersed in ethanol (70%) to ensure sterility. Under sterile conditions, the tails are skinned, and the four ligaments are pulled off and transferred to a sterile flask with 1 L of acetic acid (0.1%). After stirring for 48 h at 4°C, the suspension is centrifuged (10,000*g* for 30 min) and the supernatant is decanted. The collagen solution is kept at 4°C and can be used for at least 1 mo. Coating of Petri dishes is normally performed the day before isolation of the cells. A collagen solution of 1.0–1.5 mL is evenly distributed on the dish. Immediately before plating, the dish is tilted, the solution is carefully sucked off, and the dish is used without further treatment.
3. The composition of the standard hepatocyte culture medium is given in Table 1. Stock solutions, 50x concentrated, of solutions 1, 3a, 3b, 3c, and 4 can be prepared in water. Preparation of stock solution of folic

Table 1
Hepatocyte Culture Medium, HCM, Standard

Vitamins and glutathione	μM	mg/L
L-Ascorbic acid	99.4	17.5
D-Biotin	4.1	1.0
Choline. HCl	1790.4	250.0
Inositol	10.0	1.8
Nicotinamide	8.2	1.0
Panthothenate, Ca	2.1	1.0
Pyridoxine. HCl	4.9	1.0
Thiamine. HCl	29.6	10.0
Vitamin B_{12}	0.15	0.2
Glutathione, reduced	48.8	15.0
Folic acid and riboflavin	**μM**	**mg/L**
Folic acid	2.3	1.0
Riboflavin	2.7	1.0
Inorganic salts	**mM**	**mg/L**
$CaCl_2 . 2H_2O$	0.82	120.0
$MgSO_4 . 7H_2O$	1.99	490.0
NaCl	72.33	4225.2
KCl	2.01	150.0
$Na_2HPO_4 . 2H_2O$	2.11	376.0
KH_2PO_4	0.59	80.0
$NaHCO_3$	25.00	2100.0
Carbohydrates		
D-Glucose, H_2O	25.00	4955.0
Amino acids	**mM**	**mg/L**
L-Alanine	1.80	160.4
L-Asparagine	1.50	225.2
L-Arginine	3.00	522.6
L-Aspartic acid	0.46	61.2
L-Cysteine	0.57	69.1
L-Glutamic acid	0.80	117.7
Glycine	4.00	300.3
L-Histidine	0.51	79.2
L-Isoleucine	0.81	106.3
L-Leucine	1.50	196.8
L-Lysine, HCl	2.45	447.4

(continued)

Table 1 (*continued*)

Amino Acids	mM	mg/L
L-Methionine	0.50	74.6
L-Phenylalanine	1.50	247.8
L-Proline	3.00	345.3
L-Serine	1.00	105.1
L-Threonine	0.99	117.9
L-Tyrosine	1.20	217.4
L-Tryptophan	0.69	140.9
L-Valine	1.00	117.2
L-Glutamine	4.00	584.5
Antibiotics, pH-indicator		
Penicillin G	–	60.0
Streptomycin sulfate	–	100.0
Phenol red	–	10.0

acid and riboflavin requires the addition of NaOH. Preparation of stock solution of amino acids (minus glutamine) requires addition of 150-mL conc. HCl. Portions of 20 mL are stored at –18°C and can be used for at least half a year.

Preparation of 1L of HCM:

a. Dissolve 20 mL each of solutions 1, 2, and 5 in 500 mL of water. Adjust pH to 7.4 with NaOH (about 25 mmol).
b. Add 20 mL of solutions 3a, 3b, 3c, and 4.
c. Add $NaHCO_3$, L-glutamine, phenol red, and antibiotics as indicated in Table 1.
d. The solution is bubbled with O_2/CO_2 (19:1, vol/vol) for 30 min and pH is adjusted to pH 7.4 if necessary.
e. Add water to 1 L.

4. Stock solutions of oleate (250 m*M*), palmitate (175 m*M*), plus linoleate (75 m*M*) are prepared by dissolving the fatty acids in dimethyl sulfoxide. Appropriate volumes are filled in ampules, sealed under N_2, and stored at –18°C.
5. HCM with albumin and fatty acids: Prepare 1 L of HCM as described above, and then heat the medium to 37°C. Add, with stirring, 10 g of fatty acid-free bovine albumin, obtained commercially or prepared according to ref. 25. Complete solubilization takes 1–2 h. To the warm albumin-containing medium add slowly, with stirring, 1.0 mL of the stock solution of fatty acids. Continue stirring at 37°C for 2–3 h and afterwards at 4°C until the next day. Centrifuge the medium in sterile tubes (10,000*g* for 30 min).

Other hydrophobic compounds may be solubilized in dimethyl sulfoxide. Care should be taken to avoid high concentrations of dimethyl sulfoxide in the medium, as concentrations of 1–2% have been reported to have their own effects on cultures of hepatocytes (*26,27*).

6. Pentothal-Natrium: 0.5 g is dissolved in 10 mL of sterile water before use.
7. Hibitane: A 0.5% solution in water is prepared before use.
8. Iodide solution containing 50 mg of iodine and 35 mg of potassium iodide/mL of ethanol.
9. Trypan blue: A solution is prepared as described in Chapter 14 (*28*). Portions are stored at –18°C.
10. Penicillin and streptomycin.
11. Dexamethasone is obtained as Decadron (4 mg/mL) from Merck, Sharp & Dome, Haarlem, Nederlands and kept at 4°C.
12. Crystalline insulin and glucagon. Solutions are prepared in 40 m*M* phosphate buffer, pH 7.4 with 1% human albumin after dissolving the desired amounts of insulin and glucagon in a small amount of diluted HCl and NaOH, respectively. Aliquots can be stored at –18°C for at least 1 yr. The water used for preparation of all solutions should be of high quality as, e.g., triple distilled water.
13. During the isolation of the hepatocytes, sterile technique is necessary. Glassware, instruments for surgery, tubings, and so on, should be autoclaved. Alternatively, washing with ethanol (70%) is advisable. All solutions and media are filtered through 0.45 μm filters. Before use, media are brought to room temperature and bubbled with sterile O_2/CO_2 (19:1, vol/vol) if pH is too high. After isolation of the cells, all work is performed in a laminar flow bench.

3. Methods

1. Rats, usually starved for 16 h, are anesthesized with Pentothal-Natrium (10 mg/100 g rat), administered intraperitoneally. Care is taken to avoid noise and harmful distress. The rat is immobilized on the dorsal side. Before surgical procedures, the ventral side of the animal is washed thoroughly with Hibitane solution. A midline incision through the skin of the abdominal cavity is performed. The skin is pulled aside and the underlying tissue is washed with iodine solution before the abdominal cavity is opened. Cannulation of portal and caval veins, perfusion of the liver, incubation, and filtration of dispersed cells are performed as described in Chapter 14.

2. After filtration of the dispersed liver, the cells obtained are transferred to two 50-mL centrifuge tubes with caps, and centrifuged at $20g_{max}$ for 1 min.
3. The supernatant is discharged, and the cell pellets are gently suspended in a few mL of HCM. Additional medium is added to bring the volume in the tubes to about 30 mL, and the cells are thoroughly mixed by inverting the tubes several times. The cells are centrifuged as described above.
4. The cell pellets are resuspended in a few mL of HCM, and the suspension is transferred to a graduated 15-mL centrifuge tube. Sufficient medium is added to bring the volume to about 12 mL, and the cells are mixed again before centrifugation at $30g_{max}$ for 2 min. The amount of packed cells varies from preparation to preparation and is between 2 and 5 mL.
5. Immediately after centrifugation, the packed cells are suspended and diluted to 20x the packed volume with HCM.
6. To 0.1 mL of the diluted cell suspension, 0.1 mL of Trypan blue solution and 0.8 mL of Krebs-Henseleit buffer are added. After mixing, an aliquot is immediately transferred to a counting chamber, and the number of stained and unstained cells counted. Only cell preparations in which more than 85% of the cells exclude vital dye should be used for culturing. Normally, the hepatocytes from starved rats at this stage of preparation appear spherical with a distinct cell border in the light microscope.
7. The stock solution of cells is diluted with HCM and horse serum. Dexamethasone and insulin are added to final concentrations of 10% (vol/vol), 1 μM and 0.1 μM, respectively. The diluted cell suspension should contain 0.55 million cells/mL. Normally, the yield from a 200 g starved rat is between 250 and 350 million hepatocytes, which is sufficient for 100–150 culture dishes (60 mm).
8. Cell suspension of 4.5 or 3.0 mL is pipeted into 60- and 35-mm Petri dishes, respectively, equivalent to application of 120,000 cells/cm^2. If multidishes with 24 chambers are used, the cell suspension is further diluted to 0.15 million cells/mL. To each chamber is added 1.5 mL cell suspension, equivalent to 120,000 cells/cm^2. Since hepatocytes sediment rapidly, it is necessary to swirl the suspended cells frequently during pipeting.
9. After plating, the cells are incubated at 37°C with atmospheric air/CO_2 (19:1, vol/vol) for 3 h.
10. At that time, the medium is sucked off in order to eliminate loose cells. 3.0 or 2.0 mL HCM with albumin, fatty acids, dexamethasone (0.1 μM

or 1 μ*M*), insulin (0.1 μ*M* or 0.01 μ*M*), and glucagon (0.1 n*M*) are added to 60- and 35-mm Petri dishes, respectively. To multidishes are added 0.5 mL of the same medium to each well. The cultures are normally changed every second day. With the medium used, the interval between changes can be prolonged to 3 d. When medium is changed, the dishes are tilted slightly. The medium is sucked off by using, e.g., a sterile Pasteur pipete connected to a suction device. Replacement of medium should be done cautiously to avoid mechanical damage of the monolayer.

11. Preparation of samples for analyses:
    a. Dishes to be used for analyses are initially placed on ice. The medium is collected and stored at –18°C or –70°C until analysis. The dishes are afterward tilted and residual medium is sucked off.
    b. For determination of enzyme activities and DNA, the cells are homogenized in 1.5 mL (60-mm dish) glycyl-glycine buffer (glycyl-glycine, 25 m*M*, KCl, 150 m*M*, $MgSO_4$, 5 m*M*, EDTA, 5 m*M*, dithiothreitol, 1 m*M*, and defatted albumin, 0.2%, pH 7.5). Homogenization by ultrasonication (40 W for 10 s) is performed in the dishes without previous scraping. Unless analysis is performed immediately, homogenates should be frozen at –70°C.
    c. For determination of the content of cytochrome P-450, medium is removed as described and 0.8 mL (60-mm Petri dish) phosphate buffer (phosphate, 0.1*M*, EDTA, 1 m*M*, dithiothreitol, 1 m*M*, glycerol, 20%, and Lubrol PX, 2%) is added to the dishes. The cells are scraped with a rubber policeman, transferred to vials, and frozen at –70°C.
    d. For determination of metabolites, 0.2 mL perchloric acid (70%) is added to a 60-mm dish with 3 mL medium. The dishes are swirled and allowed to stand on ice for 10 min. All material is transferred to a centrifuge tube and centrifuged. An aliquot of the supernatant is neutralized and stored at –70°C until analysis.

4. Notes

1. Results should preferably be related to DNA, and the DNA content/culture dish should be reported. The common practice of using protein content as a measure of cell number may lead to erroneous results, since the protein content/cell may vary with culture conditions. Fur-

thermore, accurate determination of the cellular protein content is incompatible with albumin- and serum-containing media.

2. Cell death may be evaluated by determination of the DNA-content of the monolayer, since dead cells detach from the substratum and are removed by medium changes. Cell disintegration (cell death or membrane damage) may be quantitated by determination of enzyme leakage to the culture medium. Lactate dehydrogenase is well suited for this purpose because of its high activity and cytosolic localization, but other enzymes may also be used, e.g., transaminases and the liver-specific argininosuccinate lyase. Uptake of Trypan blue by cells in culture is a poor criterion for cell survival, since dead cells detach from the substratum. Light microscopic observations are difficult to quantitate. Counting of cells is possible but cumbersome.
3. Several parameters can and should be applied to evaluate the metabolic competence and the maintenance of hepatocyte-specific properties during the culture period. It is worth mentioning that the majority of hepatocyte culture experiments have been carried out for a few days only, and that the choice of parameters of course has to depend on the purpose of the actual experiments. Some useful parameters are described in the following:
   a. Hepatocyte-specific enzymes (*see also* Chapter 14, this volume). Glucokinase, pyruvate kinase (L-form), and urea-cycle enzymes are hepatocyte specific and should ideally be maintained at least at the same activity/cell as in vivo.
   b. ATP-level. The level of ATP reflects cell integrity and the metabolic condition of the cells, and ought to be no less than 2.5 μmol/g cells (*29,30*), corresponding to 1 μmol ATP/mg DNA.
   c. Rate of metabolic pathways. Gluconeogenesis (*10,11,31,32*), glycogen synthesis (*33,34*), urea synthesis (*35,36*), fatty acid oxidation (*16*), and esterification (*12,16,37*), ethanol metabolism (*38,39,40*), and protein synthesis (*4*) are examples of integrated metabolic pathways that require the interaction of intracellular compartments, and that have been shown to proceed at acceptable rates in hepatocyte cultures.
   d. Rate of secretory processes. Hepatocytes in vivo secrete a number of proteins, which are not produced by other cell types. Examples are albumin, acute-phase proteins, and some lipoproteins. Albumin secretion is often used as a criterion for the maintenance of specific characteristics. It is, however, not the most sensitive liver-specific parameter, since other properties

are lost before albumin secretion starts to decrease (*36*). Cultured hepatocytes produce and secrete bile acids (*13*), however, there are some indications that these are secreted at a much lower rate than in vivo as the cultures age, and that primary cultures of hepatocytes may represent a sort of cholestatic state (*41*).

e. Response to hormones. Primary cultures of hepatocytes have been shown to respond to glucocorticoids (*4,11,36,40,42,43*), insulin (*4,36,40,44*), glucagon (*11,35,36*), α-adrenergic agonists (*4,23*), triiodothyronine (*45,46*), and growth hormone (*45,47–50*), indicating that receptors for these hormones are present and that a functional receptor-coupling exist in the cells. Conversely, the estrogen receptor has been reported almost to disappear within 24 h of culture (*49*).

4. Hepatocytes in primary culture survive better, when plated on some sort of substratum rather than directly on tissue culture polystyrene plastic. On tissue culture plastic, the cells do not spread, and the typical monolayer of polygonal cells with sharply delineated cell borders is not established (*51*). Furthermore, the cells detach rapidly from naked plastic (*52*). Primaria plastic (Falcon, Becton Dickinson) has been claimed suitable for hepatocyte cultures without further treatment (*12*). Satisfactory results have been obtained with plastic or glass surfaces coated with rat tail collagen (*36,51*), with floating collagen gels (*53*) or with collagen-gels supported by nylon mesh (*15*). Pure substances, e.g., Con A, collagen I, collagen IV, fibronectin, and laminin have also been successfully used as substrata (*9,51,52,54,55*), although they in some respects, e.g., transcription of liver-specific genes, appear inferior to rat tail collagen (*9*). Extracts of extracellular components, so-called biomatrix, have been reported to have advantages over other substrata (*52,56*); however, little comparative work has been carried out. Cocultures of hepatocytes and a rat liver epithelial cell line (*57*), the latter of which may be considered as a special case of substratum for the hepatocytes, appear to be superior to hepatocytes cultured on plain plastic. No systematic comparison of such cocultures and hepatocyte cultures on collagen-coated plastic has been reported.
5. Commercially available tissue culture media (*see* Appendix) may be used for hepatocyte cultures, the most commonly used being Leibovitz L15, Dulbecco's Modified Eagle's Medium, Williams E, Waymouth 752/1, RPMI 1640, and Ham F-12, or mixtures thereof. A num-

ber of modifications have been claimed to improve the performance of hepatocyte cultures. In the following, the various media components will be discussed shortly:

a. Serum. 1–10% is beneficial during the initial adhesion period of 2–4 h (*58*). At later time-points, serum addition has been reported to improve cell survival (*59*) but also to increase the level of liver-unspecific mRNA's (*60*). Satisfactory culture conditions can be established without serum addition, and since serum is not chemically defined, it should be avoided, except for the initial adhesion period.
b. Substrates. A high concentration of glucose (25 m*M*) has proven superior to a lower concentration (5 m*M*) as judged by the activity of alcohol dehydrogenase (*40*). By the same criterion, high amino acid concentrations (Table 1) are superior to lower concentrations (*40*). This effect of amino acids may be related to the depletion of several amino acids from the usual standard media within 24 h (*14, 55,61*). A high concentration of amino acids may enable medium change every second or third day, only. Substitution of arginine by ornithine may retard growth of nonparenchymal cells without affecting hepatocytes (*62*). However, growth of nonparenchymal cells is normally not a problem.
c. Fatty acids are not an essential component of media for hepatocyte cultures, at least not for short time cultures. They are, however, a quantitatively important substrate for the liver cells in vivo, and may be added to culture media as an albumin–fatty acid complex to attain physiological concentrations.
d. Micronutrients. Although a requirement for metal ions, vitamins, or other usual media components (inositol, choline, purine, and pyrimidine bases) has not been documented for hepatocyte cultures, these substances are often included as a matter of precaution. Ascorbate (*63*) and selenium (*64*) have been reported to improve the maintenance of cytochrome P-450.
e. Hormones. There is a general agreement that glucocorticoids significantly improve survival and performance of primary hepatocyte cultures. Dexamethasone is normally preferred over naturally occurring glucocorticoids because of its resistance to degradation (*65*). Insulin is also in many respects beneficial to the cultures (*4,7,12,14,36,44*), and the combined action of glucocorticoid and insulin is necessary for the expression of some hepatocyte-specific functions (*7,11,50,66*). It is therefore

advisable to add both hormones to cultures of hepatocytes. Insulin at a concentration of $10^{-8}M$ is rapidly degraded by hepatocytes with a half-life of about 8–16 h in primary cultures (unpublished results, *67*). A general effect of other hormones on confluent hepatocyte cultures has not been reported, although they respond to several hormones (*see* Note 3).

f. Other medium additions. Addition of 2% dimethyl sulfoxide to the medium has been reported to maintain albumin secretion in cultures for up to 40 d (*26*). Addition of glycosaminoglycans or proteoglycans to the medium has been shown to enhance the expression of liver-specific genes and to suppress the expression of tissue-unspecific genes (*9*). A number of medium additions have been recommended for maintaining the content of cytochrome P-450 in hepatocyte cultures, e.g., nicotinamide (*68*) metyrapone (*69*), heme (*64*), ascorbate (*63*), selenium (*64*), or high concentrations of dexamethasone (3). However, addition of these substances only partially prevents the decrease of the content of cytochrome P-450.

6. Hepatocytes prepared from fetal rat liver divide in primary culture, respond to several hormones including androgens and estrogens, and mature during culture to express adult hepatocyte characteristics (*70*). Hepatocytes from adult rat livers proliferate in primary culture, if the cells are plated at a low density (50,000 cells/cm^2), and if the medium contains glucocorticoid, insulin, and epidermal growth factor (*5,60*). Once confluency is reached, the cells stop dividing. Expression of hepatocyte characteristics in this preparation appears inversely proportional to the rate of cell division (*71*).
7. The preparation of primary cultures has been described using livers from a number of other species including humans (*72,73*), dogs (*73*), monkeys (*73*), mice (*74*), hamsters (*74*), guinea pigs (*73,74*), rabbits (*73,74*), chicken embryos (*75*), and fish (*76*).

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