PREPARATION OF PRIMARY MONOLAYER CULTURES OF ADULT RAT HEPATOCYTES

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SUMMARY: We describe in detail the technique of hepatocyte isolation and establishment of primary cell cultures of adult rat hepatocytes. These cultures contain hormonally responsive hepatocytes that retain many adult characteristics under completely serum-free conditions. The cells retain a normal morphology and do not exhibit fetal characteristics during a 4 d culture period.

Key words: hepatocyte isolation; hepatocyte primary culture; serum-free media.

I. INTRODUCTION

The technique of hepatocyte isolation described by Berry and Friend (1) has provided the basis for most isolation procedures reported in the literature. Although modified extensively, a complete description from perfusate buffers to serum-free culture medium, which maintain viable, hormonally responsive adult rat hepatocytes in primary culture, is not available to those interested in using this technique. Hepatocyte isolation and maintenance of primary cell culture can be entirely serum-free. Under the conditions described in this report, the hepatocytes retain many adult characteristics during a 5 d culture period and do not demonstrate an increase in the fetal enzyme gama glutamyl transpeptidase (GGTP). Cytochrome *p*-450 content is maintained during culture and this system remains responsive to phenobarbital induction (2, and unpublished data). The culture medium represents a composition of multiple reports in the literature describing modifications that enhance/increase hepatocyte viability and metabolic characteristics. Although many substrates have been found suitable for hepatocyte cell culture (3), collagen or serum fibronectin coated plastic, or both, remain the most widely used. The procedures in this report describe in detail the isolation and culturing techniques for adult rat hepatocytes that may also prove useful for hepatocytes from other species.

II. MATERIALS

A. Equipment

Incubator, water jacketed and humidified, Fisher[1]
Inverted microscope, phase contrast microscope[1]
Hemocytometer[1]
Water bath[1]
Centrifuge, low speed and large volume capabilities[1]
Tissue culture hood (Labconco 11000)[1]
Perfusion pump (Masterflex), Cole Palmer[2]
Cannula, Longdwel catheter No. 6710, Becton Dickinson[1]

B. Culture medium and chemicals

Waymouth's 752/1, Hanks', Swim's S-77, powdered formulas, K. C. Biological[3]
Antibiotics (gentamycin, amphotericin B), Sigma[4]
L-Amino acids[4]
Pyruvic acid (sodium salt), U.S. Biochemicals[5]
Bovine serum albumin (Fraction V)[5]
Collagenase (CLS II, Type II), Millipore[6]
Hormones (insulin, dexamethasone, glucagon, estradiol, and testosterone)[4]
HEPES, Research Organics[7]
Aminolevulinic acid (ALA) and heme, Porphyrin Products[8]
Calf skin collagen, Chemicon[9]

C. Glassware and plastics

Sterivex-GS filter units[6]
Tissue culture dishes (100 × 20 and 60 × 15 mm sizes)[1]
Centrifuge tubes, 50 ml siliconized glass[1]
Pipettes, Pasteur and serological (sterile)[1]

D. Animals

Adult Fisher F-344 (well-defined life span and associated pathologies), Harlan Sprague Dawley[10]

0271-8057/82/0169-0173 $1.50/0

III. PROCEDURE

A. Media preparations

1. Serum-free Waymouth's 752/1 medium (Table 1)

   a. Add dry powder to 800 ml distilled water.

   b. Taking into consideration the composition of the powdered medium, add according to Table 1 so as to contain the following: 5 m*M* asparagine, 5 m*M* glutamine, 2.5 m*M* lysine, 2 m*M* tyrosine, 0.2 m*M* alanine, 0.2 m*M* serine, 20 m*M* pyruvate, 2.5 m*M* bicarbonate, 30 m*M* HEPES, 0.1 mg/ml BSA, 0.4 m*M* ascorbate, 0.2 m*M* ALA, 25 μg/ml gentamycin and 1 μg/ml amphotericin B. (Dissolve tyrosine in KOH solution before addition).

   c. Add nutritional and hormonal supplement via an ethanol/propylene glycol carrier (2 ml) that contains the following: 10 μl DL-tocopherol acetate, 10 μl oleic acid, 10 μl linoleic acid, 10^{-8} *M* glucagon, 10^{-7} *M* testosterone, 10^{-7} *M* Beta-17-estradiol, 10^{-7} *M* dexamethasone, and 10^{-7} *M* insulin.

   d. Adjust the pH to 7.6, quantity sufficient to 1 liter and sterilize by filtration (Sterivex-GS unit for 2 liters).

   e. Check postfiltration that the osmolality is near 400 mOSM.

   f. Store in amber bottles at 4° C for no more than 1 wk.

TABLE 1

Hepatocyte Culture Medium

Compound	g/2 liters	g/liter
Asparagine	1.502	0.751
Glutamine	0.760	0.380
Leucine	0.556	0.278
Phenylalanine	0.560	0.280
Tyrosine (in KOH)	0.644	0.322
Alanine	0.036	0.018
Serine	0.050	0.025
Ascorbate	0.106	0.053
Amino levulinic acid	0.067	0.0335
Pyruvic acid (Na Salt)	4.400	2.200
Bovine serum albumin	0.200	0.100
Bicarbonate	0.420	0.210
HEPES	14.300	7.150
Gentamycin (50 mg/ml)	1.0 ml	0.5 ml
Amphotericin B (1 mg/ml)	2.0 ml	1.0 ml
Hormone mixture	2 Vials	1 Vial
pH 7.6	Osmolality (mOSM/kg)	400

TABLE 2

Liver Perfusion Buffer

Compound	g/500 ml	
KCl	0.200	
KH_2PO_4	0.030	
NaH_2PO_4	0.045	
$NaHCO_3$	0.210	
Ascorbic acid	0.035	
Pyruvate	0.550	
Glutamine	0.365	
Nicotinamide	0.061	
Dextrose	1.000	
NaCl	3.000	
HEPES	1.787	
Gentamycin (50 mg/ml)	0.25 ml	
Amphotericin B (1 mg/ml)	0.500 ml	
Phenol red	0.005	
pH 7.4	Osmolality (mOSM/kg)	300

2. Liver perfusion buffer (Table 2)

   a. Prepare as described and sterilize by filtration (0.2 μm).

   b. To 225 ml add 140 mg $ClCl_2$ (Solution A).

   c. To 225 ml add 48 mg EGTA (Solution B).

   d. Place both solutions into a 37° C water bath and bubble with 95% air:5% CO_2 for 1 h with no further bubbling required.

   e. Add 35 μl of insulin (40 U/ml) to both solutions just prior to use.

   f. Dissolve 100 to 130 mg collagenase into Solution A just prior to use.

3. Collagen coating of tissue culture dishes (100 × 20 mm size)

   a. Dissolve acid soluble collagen (calf skin type) in 0.1 *M* acetic acid (pH 4.2) to a concentration of 0.5 mg/ml.

   b. Stir in cold room overnight and then sterilize by filtration (0.45 μm).

   c. Add 600 μl of collagen solution to 4 ml of sterile Swim's S-77 medium, which contains no bicarbonate or HEPES; 25 μg/ml gentamycin; and 1 μg/ml amphotericin B (pH 7.4).

   d. Swirl to cover the bottom of the dish, leave at room temperature for 4 to 6 h and then place in 35° C incubator overnight.

   e. Aspirate off remaining solution and wash once with Swim's S-77 medium.

f. Add 9 ml of culture medium to each dish and return to incubator during the perfusion procedure.

g. Heme may be added to the culture medium in each dish to a final concentration of 1 μM from a stock solution dissolved in 0.1 M from a stock solution dissolved in 0.1 M NaOH containing 1 mg/ml BSA and stored in an amber bottle at 4° C.

B. Hepatocyte isolation procedure

1. Nonfasted animals are etherized or sedated with phenobarbital, the abdomen soaked with amphyl detergent, and the animal skinned with most abdominal hair removed to both sides of the animal. During this time perfusion buffer B is allowed to flow slowly through the catheter.

2. Placing the animal under the tissue culture hood, make an abdominal incision up to the sternum. Make sure that the liver lobes do not adhere to the abdominal wall. Following the lateral sides of the animal, completely open the body cavity to the rib cage on both sides. Move the viscera to the right exposing the portal vein. Be sure not to open the chest cavity at this time.

3. Using blunt-end forceps, place a suture under the portal vein, but do not tighten.

4. Reduce the flow of perfusion buffer B and insert the catheter carefully into the portal vein (both the needle and Teflon sheath) and start a slow flow of solution B immediately while tightening the suture around the cannulated portal vein. The tubing may also be secured to the basin by adhesive tape, if desired, for increased stability of the catheter.

5. As the liver blanches (usually in less than 45 s) remove the kidney on the left for a one-way perfusion of the liver. Open the chest cavity immediately and clamp off the supeior vena cava. Increase the flow of perfusate to 8 to 10 ml/min. The Teflon sheath may then be slid over the catheter needle to prevent piercing the portal vein during the perfusion.

6. Open the body cavity completely on the left side to enable the perfusate to flow from the animal. Carefully dissect away the intestines, spleen, and any fatty tissue around the liver.

7. After 175 to 200 ml of Solution B has passed through the liver add 200 ml of Solution A containing the collagenase and continue the perfusion until this solution is gone.

8. During the perfusion, the liver will enlarge and soften. After completing the perfusion, carefully remove the liver one lobe at a time and place into the remaining 25 ml of the collagenase perfusion buffer.

9. Resuspend the liver by gentle aspiration with a large bore glass rod. A well-perfused liver should not have to be cut. Continue the incubation for 10 min at 37° C with occasional aspiration/agitation.

10. Gently resuspend the liver tissue and pore through 4 to 6 layers of sterile gauze to remove any large fragments. Dilute to 45 ml with Hanks' medium, and isolate hepatocytes by low speed centrifugation at 20° C (30 to 50 $\times g$ for 3 min).

11. Resuspend cell pellet gently into 25 ml Hanks' medium and filter through a sterile 60 μm pore nylon filter. Wash the filter with 20 ml of Hanks' medium and isolate the hepatocytes by low speed centrifugation. Repeat this step twice.

12. Resuspend the final cell pellet into the appropriate volume of culture medium to yield 8 to 12 $\times 10^6$ cells/ml (usually 60 to 80 ml). Determine the cell yield with a hemocytometer and viability by trypan blue dye exclusion.

C. Hepatocyte cell culture

1. Place 1 ml of the cell suspension into each culture dish and gently swirl to coat the surface with cells. Leave undisturbed for 1 h and gently swirl again.

2. Incubation may be performed in a humidified atmosphere of air (no CO_2) at 35° C.

3. After 6 to 12 h nonattached cells may be aspirated off and 10 ml of fresh medium added. By this time monolayer formation should be nearing completion and any further medium additions may be added (i.e. phenobarbital, chemical carcinogens, hormones, et cetera).

IV. DISCUSSION

Although expensive surgical operating equipment are available commercially, a plexiglass sheet, two test tube racks, and a wash

basin are suitable replacements. These may be placed under the ultraviolet hood the night before to ensure sterile conditions. The plexiglass operating board should be supported to a height that is comfortable. This board may be drilled with several holes to allow the perfusate to collect in the basin rather than around the animal. This hood contains a plug on both sides, which may be removed, and allows the perfusion tubing to be run conveniently from the adjacent water bath to the perfusion pump in the hood aseptically. By placing the pump under the hood, the heat from the pump and the lights provides a warm environment for the perfusion.

To initiate the perfusion, the cannula should be inserted into the portal vein with the perfusion buffer dripping off the tip of the needle. Once inserted into the vein, the flow should be increased to blanch the liver immediately and to ensure that no clots will form. By removing the kidney, the perfusate is easily directed away from the animal and flows through the holes in the operating board.

The perfusion technique provides cell yields of 400 to 700×10^6 cells/liver with viability exceeding 90%. Viability is greatly dependent upon the lot of collagenase used. Once a suitable lot is found from small quantities of several different lots, a larger quantity should be obtained, if possible. By severing the portal vein from the intestines, the possibility of retroperfusion and subsequent contamination is reduced. By carefully removing the intestines, spleen, and fatty tissues from around the liver, the liver can be more easily removed after it has swollen from the collagenase digestion.

The culture medium has been shown to provide viable, adultlike hepatocytes for up to 5 d in culture (2, and unpublished data). There is no need for serum at any time. The use of serum as a fibronectin source during the initial hours of culture can be eliminated by the use of serum-free spent media from fibroblast cultures. By culturing fibroblasts for 4 to 8 d under serum-free conditions, the spent medium is rich in fibronectin and can be used to assist in the plating of the hepatocytes. Although fibronectin is not usually needed with a collagen substrate, it is essential with uncoated plastic. The spent media can be used to pretreat the culture dishes as described by Seglen and Fossa (4).

The exogenous amino acid/pyruvate concentrations were determined based on Seglen's work (5,6). The nutritional/hormonal complex is a variation of the one described by Decad et al. (7). The addition of nicotinamide to the perfusate is as described by Paine et al. (8). The use of the additional ascorbic acid to both the culture medium and the perfusate is based on the observation by Omaye and Turnbull (9). The addition of ALA to the culture medium is based on the data from Paine and Hockin (10) and Guzelian and Bissell (11). In addition, we have recently shown that the addition of exogenous heme to the culture medium (1 μM) maintains cytochrome *p*-450 content through 48 h of culture at the level found in the freshly isolated hepatocytes (2). Data also suggest that the further addition of selenious acid (0.1 μM) to the culture medium enables phenobarbital mediated increases in cytochrome *p*-450 content to occur (12 and unpublished data).

Collagen coating of the culture dishes is a modification of the technique of Pariza as described by Doughterty et al. (13). The EGTA, Ca^{++}, and insulin concentrations of the perfusate are as described (13).

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