METHOD OF CULTURING CARDIOMYOCYTES FROM HUMAN PEDIATRIC VENTRICULAR MYOCARDIUM

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SUMMARY: This study describes a method of isolating, culturing, purifying, subculturing, and identifying noncontracting ventricular myocytes grown from myocardium removed from tetralogy of Fallot patients during corrective cardiac surgery. Minced myocardium is digested in trypsin and collagenase. The isolated cells are cultured in Iscove's modified Dulbecco's medium containing 10% fetal bovine serum and 0.1 mmol/liter β-mercaptoethanol. The cardiomyocytes are purified by dilutional cloning. Subcuturing is done by trypsin digestion and transferring of the cells to new culture dishes containing the above culture medium. The myocardial contractile proteins (actin, sarcomeric myosin, human ventricular myosin heavy chain, and human ventricular myosin light chain 1) are identified in the cultured cells by immunofluorescence antibody staining. Disorganized myofibrils and intercalated disks are visible by transmission electron microscopy. The ratio of the activities of creatine kinase MB isozyme to total creatine kinase of cultured cardiomyocytes is similar to that reported in fresh myocardium. Although significant phenotypic modulation does occur, the cultured cardiomyocytes promise to be useful in the study of normal and abnormal aspects of the biochemistry and molecular biology of the heart.

Key words: human ventricular cardiomyocyte isolation; culturing; identification.

I. INTRODUCTION

Since freshly isolated adult cardiomyocytes were first prepared in 1960 (12), studies (15,19) show that some aspects of their metabolism and electrophysiology can mimic the in situ cardiomyocyte. However, problems with yield, viability, calcium intolerance, and preparation damage make these cells difficult to use in research studies. In addition, the technology for long-term subcuturing of adult cardiomyocytes is difficult. Although Nag et al. (16) were able to maintain adult rat heart myocytes for as long as 1.5 mo., their technique does not passage these cells.

In this report we present a method to isolate and to passage human ventricular cardiomyocytes obtained from a pediatric myocardial biopsy for as long as 5 mo. This method can also be used to culture cardiomyocytes from neonatal and adult rat, adult dog, and adult human myocardial ventricles.

II. MATERIALS

A. Equipment

1. Inverted microscope, Diaphot-TMD, Nikon
2. Epi-fluorescence equipment "TMD-EF" with a B-2A blue filter for the inverted microscope Diaphot-TMD
3. Hemacytometer, Bright-Line, Reichert Scientific
4. Tri-gas automatic, dual chamber water-jacketed incubator, model 3327, Forma, Caltec Scientific
5. pH Meter, model 40, Beckman
6. Spectrophotometer, DU-40
7. Waterbath shaker, model-WB-10, Elmeco
8. Table-top centrifuge, Hettich Zentrifugen, Diamed Lab
9. Liquid scintillation counter, 1219 Rack Beta, LKB Wallar, Fisher
10. Electronic cell counter, Coulter Electronics Inc.
11. Water purification system, Milli-Q no. 2D-20-11584 Millipore
12. Electron microscope, Phillips 201
13. Scissors (08-940-5), tweezers (13-812-42)
14. Biological safety cabinet, model 1104, Forma
15. Balance, PC-180, Mettler
16. Eppendorf centrifuge, no 5415, Brinkmann
17. COBAS-FARA analyzer, Hoffmann-LaRoche
18. Cryostat, no 12-625-150, Histostat
19. Freeze dryer, Labconco

B. Glassware

1. Pasteur pipettes, 23 cm disposable, no 13-678-20F
2. Polypropylene sterile centrifuge tubes, 50 ml disposable, no 14-950-49A Falcon; scintillation vials, no 03-337-15, micro centrifuge tubes, no 05-402-15

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C. Chemicals and solutions
1. Phosphate buffered saline (PBS) composition: NaCl 136.9 mM, KCl 2.7 mM, Na₂HPO₄ 8.1 mM, KH₂PO₄ 1.5 mM. The solution is adjusted to yield a final pH of 7.3 with 100 mM HCl or 100 mM NaOH and sterilized by filtration.
2. Enzyme digestive solution: 0.2% trypsin (Difco 0153-60-2¹), 0.1% collagenase (type II, 34243, Worthington Biochemical Corp.), 0.5 mM EDTA and 0.02% glucose in PBS (pH 7.3). Sterilized by filtration.
4. Cell culture medium: IMDM containing 10% FBS, 0.1 mmol/ml ~-mercaptoethanol, NaCl 0.153-60-2¹, 0.5 mM EDTA, and sterilized by filtration.
5. Subculture enzyme digestive solution: 0.05% trypsin in PBS with 0.5 mM EDTA and 0.02% glucose in PBS (pH 7.3). Sterilized by filtration.
7. Monoclonal antibody MF-20 anti-myosin, no 5006-1380, developmental studies hybridoma bank ¹⁸.
9. Affinity-purified polyclonal anti-human ventricular myosin light chain 1 antibody (HVMLC1), obtained from Dr. G. Jackowski, Department of Clinical Biochemistry, University of Toronto.
10. Rabbit anti-mouse IgG and sheep anti-rabbit IgG conjugated with fluorescein isothiocyanate (FITC), ICN 65-171; 60-617-1²⁰.
12. CK assay reagent: ADP 2 mmol/liter, AMP 5 mmol/liter, NADP 2 mmol/liter, N-acetylcyesteine 20 mmol/liter, diadenosine pentaphosphate 10 mmol/liter, hexokinase 2.5 U/liter, G6P-dehydrogenase 1.5 U/ml, imidazole buffer 0.1 mol/liter (pH 6.7), glucose 20 mmol/liter, EDTA 2 mmol/liter, MgCl₂ 10 mmol/liter, creatine phosphate 30 mmol/liter.
13. Isomune-CK Kit, 43379, Roche²²
14. [H]Thymidine, Amersham SJ.204; TRK.120²³
15. Hydrocount LSC cocktail, Baker²⁴
16. Phosphate buffered glycerine solution (1:1) glycerol:PBS. Adjusted to yield pH 8.0 to 8.6 with 100 mM HCl or 100 mM NaOH.
17. OCT compound solution¹³
18. Tris-hydrochloride buffer: 2.4 g tris dissolved in distilled water and adjust pH to 7.8 with 36% HCl.
19. Saline: 0.85 g NaCl in 100 ml distilled water. All chemicals from BDH²⁵

III. PROCEDURE
A. Cell isolation and purification procedure
1. Ventricular myocardial biopsies, 5 to 20 mg, were obtained from 2- to 14-y-old tetralogy of Fallot patients undergoing corrective cardiac surgery. Permission was obtained from the hospital's Human Experimentation Committee.
2. Transfer the biopsies from the operating room to the tissue culture room under sterile conditions in cell culture medium.
3. Wash the biopsy with PBS. Remove connective tissue and mince the myocardium minced into small pieces. Transfer the tissue to a tube containing 5 ml digestive enzyme solution and dissociate at 37 ° C with continuous agitation for 5 min in a waterbath shaker.
4. Collect the supernatant in the neutral medium and process the tissue twice more. After combining the supernatants, centrifuge the sample at 581 g for 5 min at room temperature.
5. Resuspend the pellet in 5 ml cell culture medium. Count cells in a hemacytometer. Seed cells in cell culture dishes and culture at 37 ° C and 5% CO₂.
6. Use the dilution cloning method for cardiac myocyte purification (8). When the cells are seeded at a low density of 50 to 100 cells per dish, the viable cells often form individual colonies after about 2 wks in culture. At this time, pick up a cardiomyocyte colony by a sterile Pasteur pipette and transfer to a new cell culture dish with 10 ml fresh cell culture medium. Culture purified cardiomyocytes at 37 ° C and 5% CO₂. After a few days, necrose the few fibroblasts transferred with the cardiomyocyte colony by slicing through the cell with the point of a sterile needle. If too many fibroblasts are carried over, repick the clone for further purification.
7. Use subculture enzyme digestive solution to dissociate cells for subculture. Incubate cells with 2 ml enzyme solution for 5 min at 37 ° C and add 28 ml cell culture medium. Mix cells and transfer to three new cell culture dishes. Culture cells at 37 ° C and 5% CO₂.

B. Cell identification procedure
1. Antibodies against actin: Use Gown's method (11) for staining actin with minor modifications.
   a. Culture cardiomyocytes in chamber/slide for 3 to 4 days.
   b. Wash cells with PBS 3 times and fix onto the chamber/slide with 1 ml 100% cold methanol at −20 ° C for 15 min.
   c. Wash cells with PBS 3 times and dried by draining.
   d. Add antibody to the chamber/slide at concentration (10) of 1:1000 dilution with 1% bovine serum albumin (BSA) and 50 mM EDTA in PBS. Incubate cells with the antibody for 45 min at 37 ° C. Incubate control cells with PBS under the same conditions.
   e. Wash the cells with PBS for 15 min at room temperature with gentle shaking, 3 times.