A STANDARD PROCEDURE FOR CULTIVATING HUMAN DIPLOID FIBROBLASTLIKE CELLS TO STUDY CELLULAR AGING

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SUMMARY: Human diploid cultures are widely used for the study of aging. We have developed quantitative cell culture procedures that include optimization of culture conditions and determination of replicative age by direct cell count. Using these procedures we are able to determine reproducibly the stage in the replicative life history of diploid cell cultures.

Key words: aging; diploid cells; population doubling level; life span; cell culture.

I. INTRODUCTION

It has been well documented that human diploid fibroblastlike cultures, derived from donors ranging from the fetal to the adult stage of development, have a limited proliferative capacity in vitro (1,2). This finite replicative life span is characterized by a period of rapid proliferation followed by a decline in the rate of proliferation, after which the cultures can no longer be propagated (1-3). This decline in proliferative capacity has been interpreted as an expression of aging at the cellular level (1-3). Proliferative rate and the stage in the life history of a culture can be monitored best by well controlled and reproducible cell culture procedures and by recording the cumulative number of actual population doublings (CPDL). This paper describes the standard subcultivation procedures we use for the study of aging in normal human diploid fibroblastlike cell cultures.

II. MATERIALS

A. Growth medium components

Auto-Pow minimal essential medium Eagle, modified, with Earle’s salts, without glutamine, without sodium bicarbonate, No. 11-100-22 Flow

Sodium bicarbonate, 7.5% solution; No. 17-163 M.A. Bioproducts

L-Glutamine, 200 mM; sterile in 100-ml quantities, No. 16-801-49

Fetal bovine serum; sterile in 100-ml quantities, No. 29-101-49

Vitamins for basal medium Eagle (BME), 100X; sterile in 100-ml quantities, No. 16-004-49

B. Dissociation medium components

10x Trypsin solution, 2.5% in Hanks’ saline without Ca++ and Mg++; sterile in 100-ml quantities, No. 16-893-49

NaCl, No. 3632-1 Baker

KCl, No. 3040-1

NaH2PO4·H2O, No. 3818-1

Glucose (dextrose), No. 4893-2

MEM amino acids (50X solution), No. 16-011-49

MEM vitamins (100X solution), No. 16-014-49

Phenol red (0.5% solution), No. 16-900-49

C. Plastic tissue culture items

Tissue culture flasks, T75, No. 3024 Falcon

Other plasticware may be used if designated as tissue culture quality (special growth surface treatment)

D. Glassware

Sterile cotton-plugged Mohr pipettes (1-, 2-, 5-, 10-ml)

Prescription bottles, 32-oz, screw capped

Sterile 100-ml serum bottles, screw capped

Sterile 25-, 50-ml graduated cylinders

Sterile 250-ml Erlenmeyer flasks

Beakers, 250-ml

Sterile screw-cap test tubes (120 × 50 mm)

E. Equipment

Laminar flow hood. Sterilgard hood, Model No. VBM 400

Coulter counter, Model ZB Coulter

Incubator, e.g., Forma Model No. 7172 Forma

F. Miscellaneous items

70% Ethanol

Isoton (azide-free), No. 75-467
Special gas mixture; 95% air, 5% CO₂, Ohio
Sterile cotton-plugged CaCl₂ drying tubes
Nalgene filter unit 0.20 μm, 500 ml, No. 09-740-25A Fisher Scientific

III. PROCEDURES

A. Storage of medium components
All should be prepared sterile.
1. L-Glutamine: purchase in 100-ml bottles and store frozen (−20 °C). At first thawing, prepare 20 aliquots (5 ml each) in screw-cap test tubes and refreeze. Thaw immediately before use.
2. 10X Trypsin solution: keep frozen (−20 °C); aliquot at first thawing as for L-glutamine.
3. Sodium bicarbonate, 7.5% solution: keep refrigerated (4 °C) after opening.
4. Vitamins (BME, 100X): store frozen (−20 °C) and protect from direct light.
5. Fetal bovine serum: store frozen (−20 °C); once thawed, do not refreeze. Test each lot (before purchase) by determining proliferation rate and harvest density for three consecutive weeks.

B. Preparation of incomplete medium (1 liter)
1. To 854 ml deionized H₂O, add 9.4 g of Auto-Pow MEM Powder and 10 ml BME vitamins. Mix thoroughly.
2. Dispense 432 ml of incomplete modified MEM into each 1 liter bottle.
3. Screw the caps on loosely, apply autoclave tape to each bottle and autoclave for 15 min at 121 °C.
4. When the sterilization cycle is finished, remove the bottles from the autoclave quickly, as prolonged heat destroys some medium components. With the caps still loose, place the vessels in the laminar flow hood and allow to cool to room temperature.
5. When cooling is complete, tighten the caps and store the vessels at 4 °C in the dark.

C. Preparation of complete medium with 10% vol/vol fetal bovine serum.
1. This medium should be prepared fresh with each use, but may be stored at 4 °C for up to
1 wk. If stored longer, add 1 ml more of L-glutamine (200 mM)/100 ml medium.
2. To a 500-ml bottle containing 432 ml of incomplete medium, add in the following order:
   a. 7.5% Sodium bicarbonate (13 ml)
   b. Fetal bovine serum (50 ml)
   c. 200 mM L-Glutamine (5 ml)

D. Preparation of Ca⁺², Mg⁺²-free MEM
1. To 900 ml distilled water, add in the order listed:
   a. NaCl, 6800 mg
   b. KCl, 400 mg
   c. NaH₂PO₄·H₂O, 140 mg
   d. Glucose, 1000 mg
   e. MEM amino acids, 50X, 20 ml
   f. MEM vitamins, 100X, 10 ml
   g. Phenol red, 0.5% solution, 10 ml
2. Dilute to 1 liter and filter sterilize (0.20 μm pore size), with house vacuum.

E. Preparation of trypsin solution (0.25%)
1. To 40 ml of MEM without Ca⁺² or Mg⁺², add 5 ml of 7.5% sodium bicarbonate.
2. Add 5 ml of sterile 2.5% trypsin. Final pH is 7.4. Prepare fresh.

F. Trypsinization
1. Pour off the medium into a beaker by decanting the fluid from the side opposite the cell growth surface.
2. Rinse the cell sheet twice with 4 ml of the trypsin solution.
3. Aspirate the excess trypsin using a 1-ml pipette.
4. Add enough trypsin to just cover the cell sheet. (For a T75 flask, 2 ml is sufficient.)
5. Incubate the flask for approximately 15 min at 37 °C. Check to see if the cells are detaching. The cells will round up as they lift off the growth surface. The process may be speeded up by gently tapping the sides of the flask. Do not splatter the cells against the top and sides of the flask, or errors in cell number will be introduced.