LONG-TERM CULTURE OF HUMAN ENDOTHELIAL CELLS

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SUMMARY

Human umbilical vein endothelial cells can be grown in vitro for 28 passages in Medium 199 supplemented with newborn bovine serum and a partially purified growth factor derived from bovine brain. Newborn bovine serum is superior to fetal bovine serum for the proliferation of human umbilical vein endothelial cells seeded at low density in the presence of the growth factor. The endothelial cells, which can be passaged every 7 to 10 d at a 1-to-5 split ratio, retain their morphological and biochemical characteristics. The proliferation of cells seeded at low density (10^3/cm^2) is proportional to the concentration of the growth factor present in the medium. The growth factor, which has an isoelectric point between 5.0 and 5.5, can support cell proliferation at reduced serum concentrations; half-maximal growth is achieved in medium containing the growth factor and 3% serum. The brain endothelial cell growth factor does not stimulate DNA synthesis significantly in cultures of human skin fibroblasts.

Key words: human endothelial cells; growth factor.

INTRODUCTION

The vascular endothelium forms a selective barrier between blood and underlying vascular cells and is important in the regulation of hemostasis and blood vessel permeability. Alterations in endothelial cell structure and function may be important in wound healing (1), thrombosis (2,3), atherosclerosis (4-6), and neoplasia (7,8), and factors involved in the regulation of cell proliferation are important in understanding endothelial cell function in disease.

Although human umbilical vein endothelial cells have been subcultured successfully (9-15), they have exhibited limited proliferative potential in vitro and have required high plating densities at successive subcultivation (10-12,14-15). Long-term cultures of human and bovine endothelial cells (>10 passages) have been established. In the presence of thrombin, Gospodarowicz et al. (16,17) have demonstrated that fibroblast growth factor (FGF) can supplement serum to support human and bovine endothelial cell proliferation for a large number of passages. Several investigators have shown that FGF was not required for the propagation of bovine endothelial cells (18-20). Folkman et al. (21) have established long-term cultures of human capillary endothelial cells using tumor conditioned medium and endothelial cell growth supplement. Maciag et al. (22,23) have isolated a growth factor (termed endothelial cell growth factor) from bovine brain that permits the growth of human umbilical vein endothelial cells for 15 to 21 passages on a human fibronectin matrix. Ocular tissue also contains a growth factor capable of stimulating vascular and corneal endothelial cells (24,25).

We have identified and partially purified an endothelial cell growth factor(s) from bovine brain. The factor(s) is a potent endothelial cell mitogen and is biologically active in the nanogram range. The proliferative life span of human endothelial cells was approximately 58 cumulative population doublings. The factor(s), which has an isoelectric point between 5.0 to 5.5, is not a potent human skin fibroblast mitogen. Endothelial cell proliferation was significantly lower in cell growth assays when fetal bovine serum (FBS) was used in place of newborn bovine serum (NBS). The growth factor reduces the serum requirements for endothelial cell proliferation and supports the growth of cells at low seed densities.
MATERIALS AND METHODS

Bovine brains were supplied by Max Insel Cohen, Inc., Livingston, NJ. [Methyl-14C]thymidine (80.3 Ci/mmol) and Omnifluor scintillant were obtained from New England Nuclear, Boston, MA. Ammonium sulfate, ultra pure, was purchased from Schwartz Mann, Orangeburg, PA. N-2-Hydroxyethylpiperazine-N-2-ethanesulfonic acid (HEPES) was from Calbiochem-Behring Corp., La Jolla, CA. Collagenase Type I (Cl. histolyticum) and trypsin (bovine pancreas 242 U/mg) were obtained from Worthington Millipore Corp., Freehold, NJ. Gelatin (swine skin) Type IV, bovine serum albumin, dithiothreitol (DTT), collagenase Type II, hyaluronidase Type III, and soybean trypsin inhibitor were from Sigma Chemical Co., St. Louis, MO. Tissue culture supplies: Medium 199, Dulbecco’s Modified Eagle’s Medium (DME), NBS, FBS, L-glutamine, and trypsin-EDTA (1X) were purchased from Grand Island Biological Company, Grand Island, NY; tissue culture dishes and conical centrifuge tubes (15, 50 ml) were from Falcon Products, Los Angeles, CA. Luer-lok cannulas were obtained from Becton Dickinson, Rutherford, NJ, and nylon ties from Extracorporeal Medical Specialties, King of Prussia, PA. Highly purified human α-thrombin was the generous gift of Dr. John Fenton, Division of Laboratories and Research, New York State Department of Health, Albany, NY. CM-Sephadex-C-50 and Sephadex G-75 were purchased from Pharmacia Fine Chemicals, Division of Pharmacia, Inc., Piscataway, NJ. Bio-Rad protein assay was from Bio-Rad Laboratories, Rockville Centre, NY. Dialysis tubing (mol wt cut off 3500) was from Arthur H. Thomas Comp., Philadelphia, PA. Bovine serum albumin, molecular mass of 65,400 daltons, was obtained from Sigma. Rabbit muscle aldolase subunit and lysozyme, molecular mass of 40,000 and 14,400 daltons, respectively, were obtained from Millipore Corporation, Freehold, NJ. The ampholytes for isoelectric focusing were obtained from either LKB, Stockholm-Bromma, Sweden or Serva, Feinbiochemica, Heidelberg. All other chemicals were of the highest grade available.

Preparation of Reagents

Collagenase (0.1 or 0.2% wt/vol) was added to HEPES buffer (0.8 g NaCl, 0.03 g KCl, 0.238 g HEPES, 0.2 g glucose/100 ml buffer). The latter was centrifuged for 10 min at 26,000 x g at 4° C, in a Sorval SS-34 rotor and the supernatant fluid collected. Both collagenase solutions were adjusted to pH 7.55. Bovine serum albumin:EDTA (0.02%:0.5% wt/vol), gelatin (0.2% wt/vol in H2O) and phosphate buffered saline (0.14 M NaCl, 6.5 mM Na2HPO4, 2.68 mM KCl, 1.47 mM KH2PO4, 0.5 mM MgCl2) were prepared. All reagents were sterilized before use. Omnifluor scintillant was prepared by adding 4.0 g Omnifluor to 1 liter scintanalyzed toluene.

Preparation of Gelatin-Coated Dishes

Initially, gelatin (0.2% wt/vol) was warmed to 37° C, added to tissue culture dishes, and incubated at 4° C overnight. Excellent results, however, can be obtained by adding gelatin to tissue culture dishes and allowing the dishes to incubate between 10 min and 2 h at room temperature. Gelatin was removed by aspiration just before cell plating.

Tissue Culture

Human umbilical vein endothelial cells were routinely grown in Medium 199, supplemented with heat-inactivated 20% NBS, 1.6 mM L-glutamine, 15 mM HEPES, 300 U/ml penicillin, 70 to 90 μg/ml streptomycin, 10 μg/ml Fungizone, 5 μg/ml of the partially purified endothelial cell growth factor, and 2.9 U/ml thrombin. The NBS was heat inactivated at 56° C for 30 min. All cells were grown in a humidified atmosphere, 95% air:5% CO2. Human endothelial cells were fed three times per week and passed every 7 to 10 d. At confluence, cells were washed once with HEPES buffer, incubated in a solution containing BSA:EDTA (0.5%:0.02%) and collagenase (1:1) for 10 min at 37° C to harvest cells, collected by centrifugation, and plated at a 1-to-4 or 1-to-5 split ratio. Human skin fibroblasts were grown in DME containing 10% FBS, 100 U/ml penicillin, 130 μg/ml streptomycin, and 2 μg/ml Fungizone. Cells were fed three times per week and were split once a week with trypsin:EDTA at a 1-to-4 split ratio.

Isolation of Human Skin Fibroblasts

Newborn foreskins were washed three times with DME containing 1,000 U/ml penicillin, and the fat removed. Foreskins were minced in DME containing 1 mg/ml collagenase and 10 μg/ml hyaluronidase and the suspension incubated for 3 h, 37° C, on a rocker. Cells were collected by centrifugation at 1,000 rpm for 10 min, the supernatant fluid removed, and cells plated in DME.