# Selective Propagation of Retinal Pericytes in Mixed Microvascular Cell Cultures Using L-Leucine-Methyl Ester

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**ABSTRACT**

*Endothelial cell (EC) propagation has been simplified by developing cell-specific selection criteria. Methods commonly used for selectively isolating EC include:* (i) *differential sieving of disaggregated tissue,* (ii) *differential plating of cells on extracellular matrices,* (iii) *lectin affinity isolation of cell populations and* (iv) *fluorescence-activated cell sorting of cells labeled with a carbocyanine dye of acetylated low-density lipoprotein (DiI-Ac-LDL). Few criteria for selectively propagating pericytes (PC) are currently available. Nonspecific esterases exhibit a high degree of multiplicity when compared with other mammalian isozymes and may be suitable for the identification and selective propagation of cells of the microvasculature. Evaluation of esterase isotype expression in PC and EC by zymography indicates PC contain α-naphthyl acetate and α-naphthyl butyrate hydrolyzing esterases as well as dipeptidyl peptidase I, while EC only contain α-naphthyl acetate esterase. The cytotoxic response of PC and EC to various amino acid esters is assessed by monitoring vital dye uptake and by light microscopy. Several amino acid esters are cytotoxic to both cell types, whereas 50 mM L-leucine methyl ester (L-Leu OMe) is toxic to EC but not to PC. This amino acid ester is also toxic to mesothelial and retinal pigmented epithelial cells, other common contaminants of PC cultures. Analysis of protein composition by two-dimensional gel electrophoresis indicates that L-Leu OMe does not stimulate expression of stress response proteins in PC. Thus, L-Leu OMe can be utilized to cultivate PC selectively from mixed cell populations.*

## INTRODUCTION

The microvasculature consists of two predominant cell types: pericytes (PC) and endothelial cells (EC) (21). EC form the micro-vessel patent tube structure, whereas PC are intimately associated with the extra-luminal surface of EC. The function of PC has yet to be definitively determined; however, they may be multi-potent progenitors to other mesenchymal cells (e.g., smooth muscle, adipocytes, osteoblasts and macrophages) and functionally may control micro-vessel blood flow, regulate new capillary growth and/or modify vascular permeability (9,21). Since both cell types express α-smooth muscle actin (9,21), PC are often considered to be the microvascular equivalent of large-vessel smooth muscle cells.

In culture, PC grow slowly as small clusters and fail to form confluent, contact-inhibited monolayers (6). PC are characterized as being polymorphic, irregularly shaped and elongated. Because of their close association with EC, it is difficult to obtain significant numbers of PC totally free of contaminating EC (9). No cell-specific marker for PC has yet been defined. As a result, PC are identified based upon morphological criteria and exclusion of other potentially contaminating cell types using a battery of specific markers. PC cultured from retina are most likely to be contaminated with EC and/or retinal pigmented epithelial cells (6). EC are commonly identified based upon uptake of acetylated low-density lipoprotein (DiI-Ac-LDL), expression of angiotensin-converting enzyme and immunoreactivity to factor VIII antibody (20,21). Retinal pigmented epithelial cells are identified based upon expression of simple epithelial cytokeratins, cellular retinaldehyde-binding protein and black lipofuscin granules (12,24).

Nonspecific esterases are commonly used as cytochemical markers in the classification of acute monocytic and granulocytic leukemias but are not routinely used as diagnostic markers for cells of the vascular wall (28). The esterases exhibit a high degree of multiplicity when compared with other mammalian isozyme systems, with more than 80 electrophoretically defined isoforms of chromosome 8 esterases existing in the house mouse (25). Esterases would thus appear to be suitable for cell identification in the vasculature. However, the physiological substrates of esterases are generally unknown, while artificial substrates and inhibitors discriminate poorly between esterases. Also, few esterases have been purified to homogeneity, thus immunochemical methods to monitor them are scarce.

As esterase isotype diversity suggests, the capacity of different cell types to metabolize simple esters differs substantially. This has been most complete-

ly investigated in myeloid cells using various amino acid and peptide methyl esters (13,14,22,23). This study demonstrates that PC contain dipeptidyl peptidase I (DPPI) as well as α-naphthyl acetate and α-naphthyl butyrate hydrolyzing esterases, while EC only contain α-naphthyl acetate esterase. Evaluation of nine different amino acid esters indicates that EC and PC differ with respect to their capacity to metabolize L-leucine methyl ester (L-Leu OMe). Experiments with pure cultures of bovine retinal PC and bovine pulmonary artery EC indicate that L-Leu OMe is toxic to EC but not to PC. Other contaminating epithelial cell types, such as mesothelial and retinal pigmented epithelial cells are also eradicated by L-Leu OMe. Mixed cultures of bovine retinal PC and microvascular EC are readily made homogenous for PC by brief application of L-Leu OMe. Evaluation of PC stress-response proteins indicates that the cells are not damaged by application of the amino acid ester. Differences in amino acid ester sensitivity between PC and EC permits the rapid chemical selection of homogenous PC populations from mixed cultures and should facilitate the study of PC physiology, biochemistry and function in diverse tissues.

## MATERIALS AND METHODS

### Isolation and Cultivation of Vascular Cell Types

**Bovine retinal PC and micro-vessel EC.** Bovine retinal pericytes are isolated as previously described (6,10,11). Briefly, the adventitia surrounding the eyes are removed using scissors and forceps. The sclera is punctured about 5-mm posterior to the limbus and cut around the globe. The vitreous humor is then removed. Using a probe, the retina is gently scraped off the back of the eye, and the attachment to the optic nerve is severed after the entire retina is free. Each retina is washed 3× in phosphate-buffered saline (PBS) to remove loose pigmented epithelial cells (black and gray areas). Any remaining black and gray material on the retina is removed by teasing it away from the retinal tissue, while rinsing periodically with PBS. Then the retinas are finely minced using scalpels, incubated in 0.1% Collagenase II (Worthington Enzymes, Freehold, NJ, USA)/0.1% bovine serum albumin (BSA; ICN Pharmaceuticals, Costa Mesa, CA, USA) in PBS and agitated for 30 min at room temperature. The solution is repeatedly pipetted up and down to break up clumps of cells and filtered through a 100-µm nylon mesh (Tetko, Briarcliff Manor, NY, USA). The filtrate is centrifuged at 450× *g* for 6 min, and then the supernatant is removed. The pellet is resuspended in media containing 10% bovine calf serum (BCS; HyClone Laboratories, Logan, UT, USA), 2 mM L-glutamine (Life Technologies, Gaithersburg, MD, USA), 1% Fungizone (Life Technologies) and 10 µg/mL ciprofloxacin (Miles, Kankakee, IL, USA) in Dulbecco's modified Eagle medium (DMEM; Life Technologies). Cells are then seeded into 6-well plates and incubated for 24 h at 37°C in a humidified 5% $CO_2$ incubator. After the 24-h incubation, plates are gently tipped back and forth to loosen surface debris. The media are then removed, and fresh media are added. Media are replaced 2× per week thereafter. After 2 weeks, Fungizone and ciprofloxacin are replaced with 1% antibiotic-antimycotic (Sigma Chemical, St. Louis, MO, USA).

Micro-vessel EC are isolated from calf retinas by homogenizing the retinas and passing the material through 183- and 53-µm nylon meshes (Tetko) as previously described (6,10,11). Material is digested with 0.1% collagenase/dispase (Boehringer Mannheim, Indianapolis, IN, USA) in PBS for 30 min at 37°C. Material is then resuspended in media containing 15% fetal calf serum (FCS; HyClone Laboratories), 1% antibiotic-antimycotic, 30 µg/mL heparin (Sigma Chemical) and 50 µg/mL endothelial cell-growth supplement (Collaborative Biomedical Products, Bedford, MA, USA) in DMEM D-valine modification (Sigma Chemical) supplemented with minimum essential medium (MEM) Vitamins and Amino Acids (Life Technologies). Material is seeded onto tissue culture plates coated with 1.5% gelatin (Sigma Chemical) and kept at 37°C in a humidified 5% $CO_2$ incubator. Media are replaced 2× per week.

**Bovine pulmonary artery EC.** Bovine pulmonary artery EC are isolated as previously described (26). Briefly, the adventitia surrounding the artery is trimmed and cut into cross sections. Each artery is then rinsed twice in PBS containing 0.1% collagenase II and 2% antibiotic antimycotic. Arteries are cut in a longitudinal direction and laid flat, lumen-side-up. Gently, the luminal surface is scraped using a scalpel blade. Cells collected on the blade are rinsed into a Petri dish containing 0.1% collagenase. The cells are incubated at 37°C in a humidified 5% $CO_2$ incubator for 15 min. After the incubation, the solution in the Petri dish is pelleted at 450× *g* for 5 min. The supernatant is then removed, the pellet is resuspended in culture media (DMEM containing 20% FCS, penicillin (100 U/mL), streptomycin (100 µg/mL), heparin (30 µg/mL) and L-glutamine (2 mM), and cells are seeded into a gelatinized 6-well plate. The culture media are changed 24 h later and every 3 days thereafter.

**Human omentum mesothelial and microvascular EC.** Omental fat is obtained aseptically from patients undergoing abdominal surgery. All patients were advised of procedures and potential risks by the surgeon in accordance with the institutional guidelines of the Massachusetts General Hospital, Boston, and gave informed consent. EC and mesothelial cells are isolated from human omental tissue as described (3–5). Briefly, segments of omental fat tissue with intact peritoneum are enzymatically digested in 0.1% collagenase in calcium-magnesium-free Hank's balanced salt solution (HBSS) (Life Technologies) to release mesothelial cells. Mesothelial cells in the collagenase mixture are recovered by centrifugation, resuspended in Medium 199 (Life Technologies) containing 10% FCS and seeded onto tissue culture plastic pre-coated with 1% gelatin in water. Residual tissue from the mesothelial cell isolation is subsequently used for isolation of microvascular EC. Omental tissue is fixed in 70% ethanol solution followed by extensive washing in PBS to eliminate remaining mesothelial cells. The omental tissue is minced and digested with 0.2% collagenase in calcium-magnesium-free HBSS with 4% BSA and 0.1% soybean

trypsin inhibitor for 15 min at 37°C with agitation. The digested tissue is then filtered through a 215-µm nylon mesh to remove undigested tissue. The filtrate is allowed to stand so that the adipocytes float to the surface. The medium below the adipocytes containing microvascular fragments and single cells are collected and centrifuged at 450× *g* for 4 min. The cell pellet is resuspended in Medium 199 supplemented with 10% FCS and filtered through a 20-µm nylon mesh. Microvascular fragments retained by the mesh are washed, recentrifuged and resuspended in Medium 199 containing 10% FCS. The cell suspension is then gently layered on 5% BSA in PBS, and the EC aggregates are allowed to separate from the residual single cells. After 10 min, the top 10 mL of the BSA cushion are removed and discarded. The remaining BSA cushion is centrifuged, resuspended in Medium 199 containing 10% FCS and plated in 25-$cm^2$ culture flasks at 1000 cells/flask. Mesothelial and microvascular EC cultures are maintained at 37°C in a humidified 5% $CO_2$ incubator. The culture media are replaced 24 h later and 3 three days thereafter.

### Non-denaturing Gel Electrophoresis and Zymography

Confluent EC and PC monolayers are trypsinized, and cells are recovered by centrifugation. Cell lysates are homogenized in two packed cell volumes of 0.2% Triton® X-100, disrupted with a probe sonicator (three bursts, 20% power) and microcentrifuged for 2 min to pellet particulate matter. Approximately 100 µg of total protein are loaded per lane. A 4%–20% non-denaturing gradient gel is used to resolve the esterases according to manufacturer's instructions (Phast™ gels; Amersham Pharmacia Biotech, Piscataway, NJ, USA). To preserve esterase activity, buffer bars must not contain sodium dodecyl sulfate (SDS). After the electrophoretic separation, gels are incubated in 100 mM Tris-base, pH 7.4 for 10 min. The esterase substrate and dye are prepared during the incubation period. Ten milligrams of α-naphthyl acetate or butyrate are prepared in 200 µL Triton X-100/acetone (1:1 wt/wt). This solution is added to 2.50 mL of 20 mM Tris-base, pH 7.4 buffer, and then the substrate solution is added to 17.5 mL of 20 mM Tris-base, pH 7.4, 10 mg Fast Blue BB salt (Sigma Chemical). The final substrate/dye solution is added to the gels, and then the gels are incubated in the dark on a shaker (50 rpm) for 40 min and washed with distilled water. A complex is generated between the azo moiety of the dye and the hydrolysis product generated by the cleavage of the naphthyl ester by the esterase. Esterase bands appear as dark blue-black bands. Finally, the gels are placed in 2% glycerol (Sigma Chemical) for 30 min or overnight and then air-dried.

DPPI activity is detected using an enzyme overlay membrane technique. Cellulose diacetate membranes impregnated with the dipeptide glycine-arginine coupled to 7-amino-4-trifluoromethyl-coumarin (AFC) are obtained from Enzyme Systems Products (Livermore, CA, USA). The substrate-containing membranes are briefly dipped into distilled water and placed onto the surface of the non-denaturing gel without trapping air bubbles. The membrane and gel are incubated at 37°C in a humidified 5% $CO_2$ incubator for 40–60 min. After the incubation period, the membrane is removed from the surface of the gel, washed for 1–2 min with 10% glycerol and then air-dried. DPPI activity is viewed using a UV light box at 365 nm (3 UV Transilluminator; UVP, Upland, CA, USA). The enzyme activity appears as green fluorescent bands. Fluorescent images are acquired using a BioImage® charge-coupled device (CCD)-based computerized image analysis workstation as previously described (BioImage, Ann Arbor, MI, USA) (15).

### Two-Dimensional Electrophoresis

Whole cell lysates are evaluated by two-dimensional (2-D) gel electrophoresis to determine whether L-Leu-OMe induces stress-response protein expression in PC. Cell lysate proteins are extracted according to the procedure for mammalian cells given in the Investigator 2-D Electrophoresis System Manual (ESA, Chelmsford, MA, USA) (17). Approximately 50 µg protein are applied per gel, and 2-D electrophoresis is performed as described previously (17). After electrophoresis, gels are silver-stained as previously described (17).

### Cell Toxicity to Amino Acid Esters

Cells are grown in 6-well plates until confluent, then trypsinized and plated in 96-well plates. PC require a more extended period of trypsinization to detach them from plates (8–10 min) than do EC (3–5 min). Cells are exposed to the following amino acid esters: *(i)* L-Phenylalanine OMe, *(ii)* L-Glutamine $(OMe)_2$, *(iii)* L-Leucyl-Leucine OMe, *(iv)* L-Valine OMe, *(v)* L-Leucine OMe, *(vi)* D-Phenylalanine OMe, *(vii)* L-Leucine OEt, *(viii)* L-Valine OEt and *(ix)* D, L- Valine OMe (Sigma Chemical). Final ester concentrations evaluated are 0.5, 5, 12.5, 25, 35 and 50 mM. All amino acid esters are prepared as 20× stock solutions in water. Ten microliters of stock ester solution are added to each well containing 190 µL of culture media (10% BCS, DMEM, 1% antibiotic antimycotic and 2 mM of L-glutamine). The cells are then incubated in the amino acid esters for 1 h, each well is aspirated, washed with PBS, aspirated again and 150 µL of culture media are added. The cells are allowed to recover for 24 h at 37°C in a humidified 5% $CO_2$ incubator.

After the recovery period, cell viability is monitored using a vital dye uptake assay (7). The culture media are aspirated, cells are washed with PBS and 100 µL of (3-[4,5- dimethyl-thiozol-2-yl]-2,5-diphenyltetrazolium bromide (MTT, 1 mg/mL; Sigma Chemical, in 10% BCS) are added. The cells are incubated in MTT for 1 h, the solution is removed and cells are washed with PBS. Then, 100 µL of dimethyl sulfoxide (DMSO) (Sigma Chemical) are added to each well. The plate is gently agitated on a rotary shaker for 30 min to solubilize the colored formazan product formed in the cells. The absorbance at 540 nm of the formazan product is read using a 96-well plate reader (Fisher Scientific, Pittsburgh, PA, USA).

### Selection of Retinal PC in Mixed Cultures

Bovine retinal micro-vessel EC and PC are readily distinguished from one another using the EC-specific marker

acetylated low-density lipoprotein 1,1′-dioctadecyl -3,3,3′,3′-tetramethylindocarbocyanine perchlorate (DiI-Ac-LDL) (Sigma Chemical). In a first passage, mixed culture of retinal microvessel EC and PC, three drops of DiI-Ac-LDL are added into three wells of a 6-well plate. The other three wells are not treated. The cells are incubated at 37°C for 4 h. Black and white photographs are taken of all six wells with a Minolta X-700, 35-mm camera mounted onto a Zeiss IM 35 microscope (Carl Zeiss, Thornwood, NY, USA). Then, 50 mM L-Leu OMe is added into the three wells containing DiI-Ac-LDL, and cells are placed at 37°C in a humidified 5% $CO_2$ incubator and incubated for 1 h. Media are then aspirated from all 6 wells, cells are washed with PBS and fresh culture media are added to each well. The cells are then allowed to recover in culture for 21 h. After the recovery period, three drops of DiI-Ac-LDL are added into the three untreated wells, and the cells are incubated for another 4 h. Black and white photographs are again taken of all 6 wells (three treated with L-Leu OMe and three untreated).

## RESULTS

### Esterase Expression in PC and EC

Expression of nonspecific esterases in PC and EC is determined by zymography. Based upon molecular weight standards, EC contain two esterase bands with approximate molecular weights of 130 and 200 kDa, using α-naphthyl acetate as substrate (Figure 1A). A single esterase band of 115 kDa

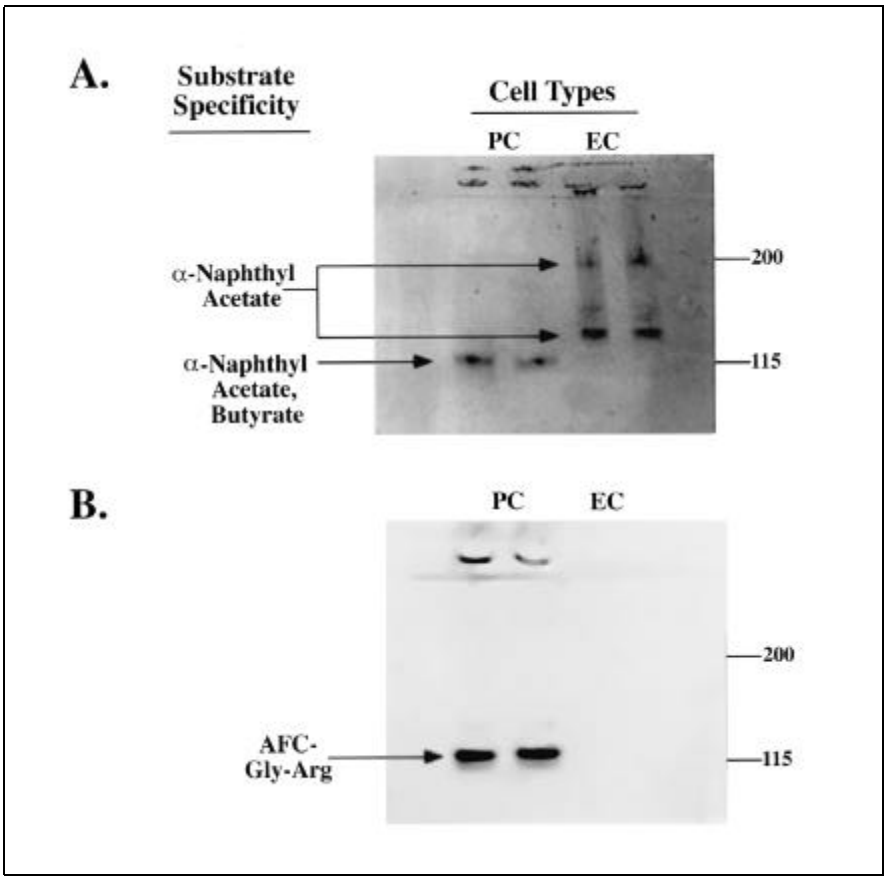


**Figure 1. Zymographic analysis of esterase expression in retinal PC and pulmonary artery EC.** All lanes are run in duplicate. (A) Nonspecific esterase profiles. Gels are incubated in α-naphthyl acetate or α-naphthyl butyrate, and esterases are visualized with Fast Blue BB. PC esterase cleaves both substrates, while EC esterases only cleave the acetate ester. (B) DPPI profiles. The enzyme is visualized using a membrane overlay assay and the substrate AFC-Gly-Arg. Fluorescent bands indicate that DPPI is present in PC. Grayscale values are inverted in this image to facilitate visualization of the peptidase bands.

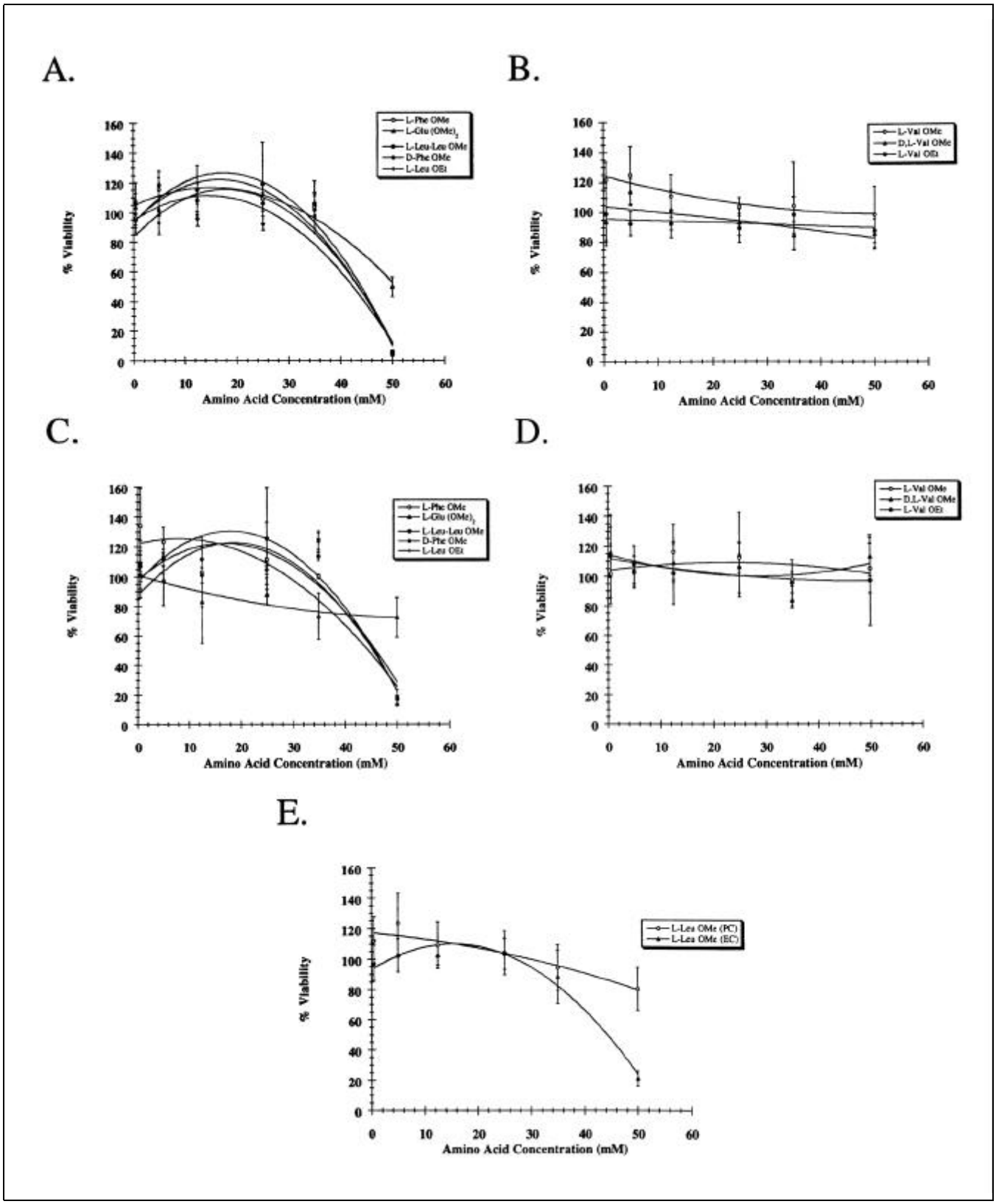


**Figure 2. The cytotoxic effects of nine different amino acid esters on bovine pulmonary artery EC and retinal microvessel PC.** Cells are incubated in each ester at the indicated concentrations for 1 h, then allowed to recover for 24 h ($n$ = 5). Cell viability is determined using the MTT assay. Of the nine amino acid esters evaluated, five are toxic to EC (A) and PC (C). Three amino acid esters are not toxic to EC (B) or PC (D). 50 mM L-Leu OMe is toxic to EC but not to PC (E). Error bars represent the standard deviation of the mean.

is obtained in PC using α-naphthyl acetate as substrate. Using α-naphthyl butyrate as substrate, no esterase bands are detected in EC, while a single esterase band of 115 kDa is observed in PC. DPP1 activity is measured using a membrane impregnated with the dipeptide glycine-arginine (Gly-Arg) conjugated to AFC. Comparison of EC and PC with respect to hydrolytic activity towards AFC-Gly-Arg indicates that PC contain an abundance of DPP1 comigrating with the 115-kDa esterase band, while EC lack the enzyme (Figure 1B). Thus, zymographic analysis of esterase isotypes in pulmonary artery EC and retinal PC indicates PC contain DPPI as well as α-naphthyl acetate and α-naphthyl butyrate hydrolyzing esterases, whereas EC only contain α-naphthyl acetate esterase. Esterase expression is similar in quiescent and proliferating cells. Differences in the expression of esterases suggest that a strategy to selectively propagate PC using cytotoxic esters is feasible.

**Amino Acid Ester Metabolism in PC and EC**

Cytotoxicity experiments are conducted on bovine pulmonary artery EC and retinal PC using nine different amino acid esters. Cells are incubated in media supplemented with different concentrations of esters ranging from 0.5 to 50 mM for 1 h, followed by a 24–48-h recovery period. Cytotoxicity is determined using an MTT-based assay (7). Fifty millimolar L-Leucyl-Leucine OMe, L-Leu OEt, D-Phenylalanine OMe, L-Phenylalanine OMe and L-Glutamate $(OMe)_2$ are toxic to both EC and PC (Figure 2, A and C). L-Glutamate $(OMe)_2$ is less toxic than the other amino acid esters in this group. Fifty millimolar D, L-Valine OMe, L-Valine OMe and L-Valine OEt are not toxic to either cell type (Figure 2, B and D). Only L-Leu OMe is found to be selectively toxic to pulmonary artery EC but not to retinal PC (Figure 2E). After 30-min exposure to 50 mM L-Leu OMe, EC appear noticeably smaller and phase bright compared to untreated cells. They no longer display "cobblestone" morphology, as cells retract away from one another. After 1 h, most cells detach from the tissue culture plate. L-Leu OMe is also evaluated in retinal PC and pulmonary artery EC cultures at a final concentration of 100 mM. This concentration of amino acid ester is toxic to both cell types. When a tenfold molar excess of L-Leu OMe is included in the α-naphthyl acetate esterase staining solution of the zymography gels, no inhibition of esterase activity is observed. This suggests that the α-naphthyl acetate esterase is not responsible for L-Leu OMe mediated cytotoxicity in EC.

Experiments are performed with native, mixed cultures of PC and microvessel EC isolated from bovine retina (Figure 3, A and B). Cells are incubated with either 50 or 10 mM L-Leu OMe for 1 h. Cells are washed with PBS, and DiI-Ac-LDL is added for

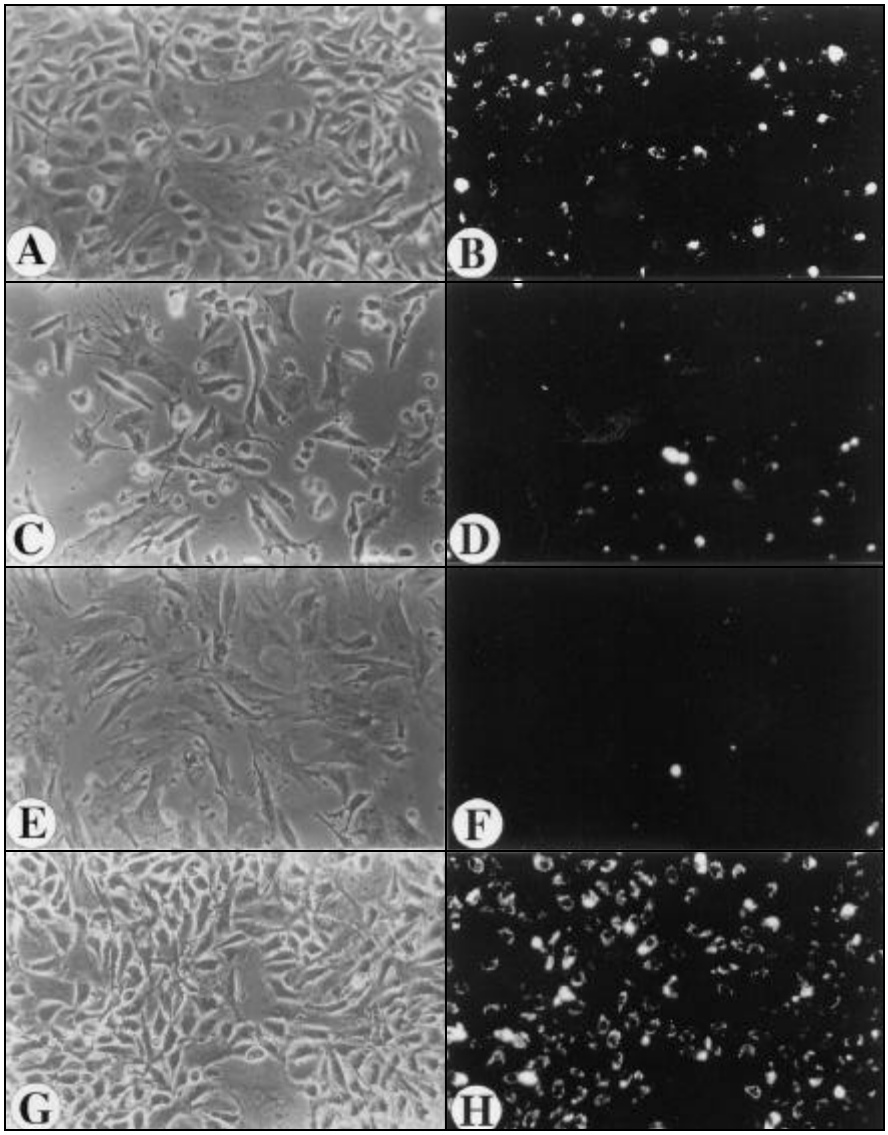


**Figure 3. Chemical selection of PC in mixed cultures.** (A) Phase-contrast photomicrograph of mixed cultures of retinal microvessel EC and PC. (B) Fluorescence photomicrograph of same cultures after labeling with Di-I-Ac-LDL for 4 h. (C) Mixed cultures exposed to 50 mM L-Leu-OMe for 1 h and allowed to recover for 1 h. (D) Same cultures labeled with 50 mM L-Leu-OMe for 1 h, allowed to recover for 1 h, followed by labeling with Di-I-Ac-LDL for 4 h. (E) Mixed cultures exposed to 50 mM L-Leu-OMe for 1 h, followed by 20-h recovery period. (F) Same cultures labeled with 50 mM L-Leu-OMe for 1 h, allowed to recover for 20 h, followed by labeling with Di-I-Ac-LDL for 4 h. (G) Mixed cultures exposed to 10 mM L-Leu-OMe for 1 h, followed by 20-h recovery, (H) Same cultures labeled with 10 mM L-Leu-OMe for 1 h, allowed to recover for 20 h, followed by labeling with Di-I-Ac-LDL for 4 h.

another 4 h. L-Leu OMe injures EC after a 1-h exposure period, and PC appear unaffected by the treatment (Figure 3, C and D). Although the DiI-Ac-LDL assay indicates that some EC are still attached to the culture dish after 1-h exposure to L-Leu OMe, these cells are rounded and phase bright. After a 24-h recovery period, only a few rounded EC remain in the culture dish, whereas PC remain unaffected (Figure 3, E and F). A similar exposure regimen using 10 mM L-Leu OMe is ineffective in eliminating contaminating EC from mixed cultures (Figure 3, G and H). Similar co-culture experiments were conducted using mixed cultures of retinal pigmented epithelial cells and retinal PC. Fifty millimolar L-Leu OMe is also found to be toxic to retinal pigmented epithelial cells using a 1-h incubation period, leaving only PC in the cultures (data not shown). Furthermore, 50 mM L-Leu-OMe is toxic to human omentum mesothelial cells and microvascular EC. Lowering the concentration of this amino acid ester to 25 mM allows for selective eradication of mesothelial cells, leaving microvascular EC intact (Figure 4).

### Expression of Stress Response Proteins in L-Leucine Methyl Ester-Treated PC

Though the MTT assay indicates that PC are not killed by exposure to 50 mM L-Leu-OMe, it is possible that they are injured. To rule out this possibility, PC stress response is monitored after exposure to the amino acid ester. Cells respond to a variety of environmental stresses by synthesizing a restricted collection of proteins aiding in adaptation to the hostile conditions. Stress response proteins may be expressed in situations of nutrient deprivation, exposure to elevated temperature, exposure to free radicals, exposure to chemicals, infection, inflammation, ischemia and autoimmune reactions (19,27). Though often named after specific stresses [heat-shock proteins (HSPs), glucose-regulated proteins (GRPs), oxygen-regulated proteins (ORPs) or hypoxia-associated proteins (HAPs)], the protein sets are often expressed under a wide variety of environmental conditions. PC are evaluated for upregulation of a number of stress-response proteins using 2-D gel electrophoresis. All proteins have previously been identified by computerized databasing and Edman-based protein sequencing as upregulated in vascular smooth muscle cells induced to undergo hyperplastic and hypertrophic growth (16). As shown in Figure 5, exposing PC to 50 mM L-Leu OMe for 1 h and allowing them to recover for 24 h in culture media does not result in increased expression of the common heat-shock proteins, cytoskeletal proteins, protein disulfide isomerases or calreticulin. Furthermore, no upregulation of stress response proteins is observed after 48 h in culture (data not shown).

## DISCUSSION

Zymographic analysis of EC and PC indicate that they differ with respect to their capability to metabolize simple esters (Figure 1). PC contain α-naphthyl acetate and α-naphthyl butyrate cleaving esterases as well as DPPI, while only α-naphthyl acetate cleaving esterases are detectable in EC. The substrate preference observed using artificial naphthyl esters suggests that the ester-binding site of the PC esterase is larger than its counterpart in EC. The difference in PC and EC ester metabolism suggests that cell-selection procedures can be devised to selectively propagate PC. Indeed, L-Leu OMe proves to be a valuable substrate in this respect.

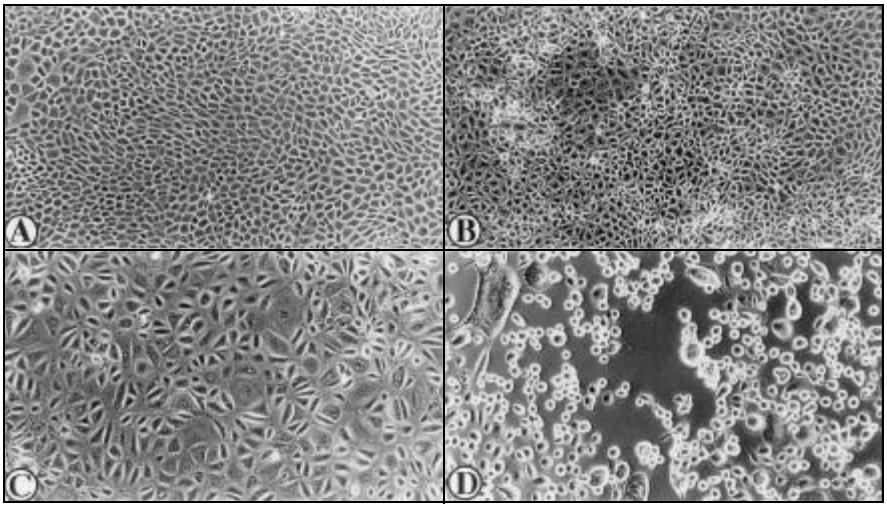


**Figure 4. Mesothelial cells are more sensitive than EC to L-Leu-OMe.** Phase-contrast photomicrographs of human omentum microvascular EC (A and B) and mesothelial cells (C and D). Panels A and C are control monolayers. Panels B and D are monolayers treated with 25 mM L-Leu OMe for 1 h. Mesothelial cells are more sensitive to the amino acid ester than EC. 50 mM L-Leu OMe is toxic to both cell types.

The specific esterases detected by zymographic analysis are not necessarily responsible for the differences in amino acid ester metabolism, but simply indicate that the cells differ in their capacity to metabolize esters. Though not pursued in this study, it should be possible to selectively eradicate PC from mixed EC/PC cultures using other substrates. A bulky ester toxin could be used that is only cleaved and activated by PC esterases. Alternatively, a toxin substrate could be devised that is only activated upon subsequent hydrolysis by DPPI.

In co-culture, PC secrete the paracrine factor transforming growth factor-beta (TGF-β) that inhibits EC growth, while EC synthesize platelet-derived growth factor (PDGF) and heparin-binding epidermal growth factor (EGF), which promote PC growth (9,21). Consequently, in primary mixed cultures of PC and EC, PC eventually become the dominant cell type. Chemical selection of PC would thus appear to be unnecessary as, in time, cultures become relatively pure. It is well known, however, that smooth muscle cells quickly dedifferentiate in culture from a quiescent to a synthetic phenotype, accompanied by the loss of specialized properties (such as contractility) and the acquisition of

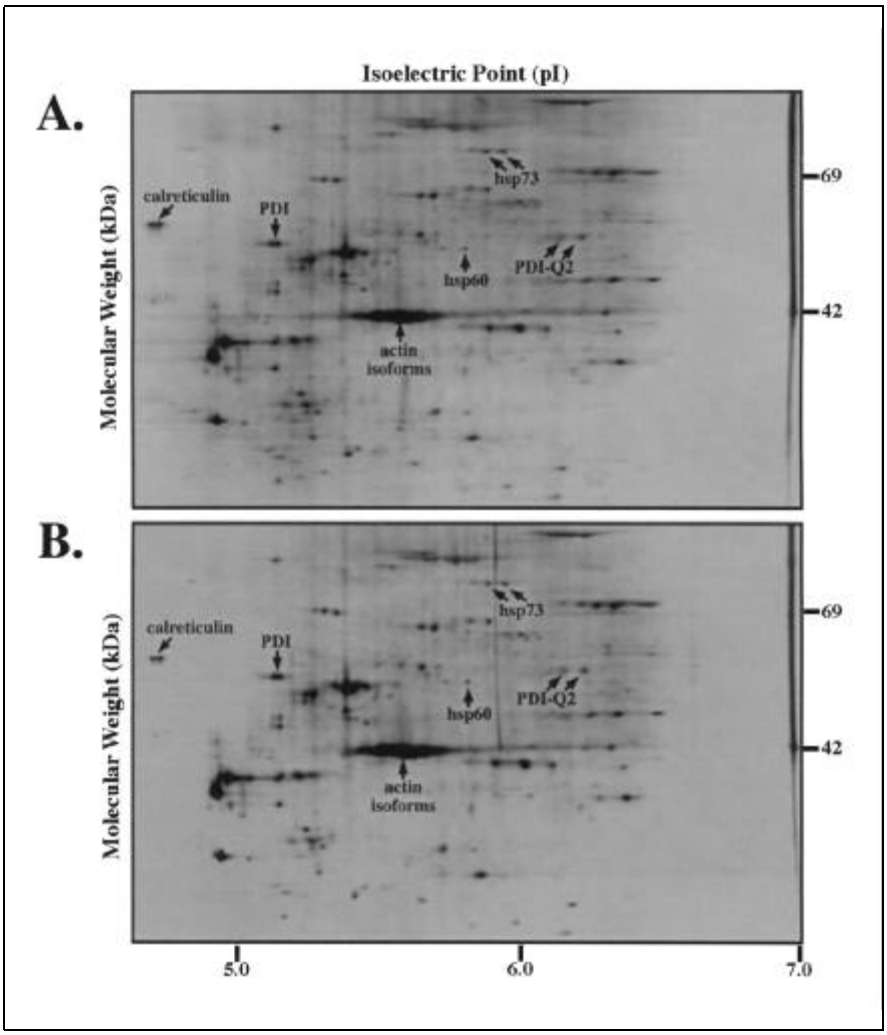


**Figure 5. Stress response protein expression in PC exposed to L-Leu-OMe.** (A) 2-D electrophoresis profile of protein expression in control PC. (B) 2-D electrophoresis profile from PC treated with 50 mM L-Leu-OMe for 1 h followed by continued culturing in standard media for 24 h. No increase in stress-response proteins arises from treatment with the amino acid ester. Similar results are obtained after extending the recovery period to 48 h. Acidic proteins are towards the left, basic proteins towards the right, high molecular weight proteins towards the top and low molecular weight proteins towards the bottom in the electrophoretic patterns.

proliferative properties (such as increased protein biosynthesis) (1,2,16). Similar phenotypic changes in cultured PC are observed in our laboratory (unpublished observations). Plating efficiency of trypsinized bovine retinal pericytes is quite poor, and as a result, only primary and first passage cells are commonly used in experiments (10,11). The chemical selection procedure is valuable because it reduces the period of time in tissue culture before experimentation, thus minimizing culture-induced changes in PC phenotype.

L-Leu-OMe is a less costly alternative to other rapid procedures that remove contaminating EC from mixed cultures, such as negative selection by fluorescence-activated cell sorting using DiI-Ac-LDL or fluorescently tagged Ulex europaeus agglutinin-1 (6). Retinal pigmented epithelial cells are also a contamination problem during PC isolation because they appear around the edges of the retina and must be carefully removed from the retinal tissue. Pigmented epithelial cells are commonly identified by the presence of black-lipofuscin granules in their cytoplasm. However, epithelial cells without pigment may be present and even those with pigment lose the granules in subsequent passages (6). The chemical selection procedure eliminates retinal pigmented epithelial cells and EC. By decreasing the concentration of L-Leu-OMe used, it is also possible to selectively propagate microvascular EC from mixed EC/mesothelial cell cultures (Figure 4). Mesothelial cells share numerous phenotypic properties with EC and are commonly encountered in omentum and pulmonary microvascular EC preparations (3–5).

The L-Leu OMe selection procedure highlights differences in the metabolic pathways of the cells of the microvasculature. In this context, it is interesting to note that EC may be selectively propagated in mixed cultures contaminated with fibroblasts by using D-valine rich media (18). Fibroblast growth is thought to be inhibited because these cells lack the D-amino-acid oxidase necessary to convert D-valine to L-valine. Differences in ester metabolism between EC and PC permit the chemical selection of homogenous PC populations from mixed cultures. The described chemical selection procedure should facilitate the study of PC physiology in a variety of tissues.

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