Isolation and Culture of Pulmonary Endothelial Cells

by Una S. Ryan*

Methods for isolation, identification and culture of pulmonary endothelial cells are now routine. In the past, methods of isolation have used proteolytic enzymes to detach cells; thereafter, traditional methods for cell passaging have used trypsin/EDTA mixtures. Cells isolated and passaged using proteolytic enzymes have been useful in establishing the field and in verifying certain endothelial properties. However, there is a growing awareness of the role of endothelial cells in processing vasoactive substances, in responding to hormones and other agonists and in cell–cell interactions with other cell types of the vascular wall, with blood cells and with cellular products. Consequently, a new requirement has arisen for cells *in vitro* that maintain the differentiated properties of their counterparts *in vivo*. The deleterious effects of trypsin and other proteolytic enzymes commonly used in cell culture on surface structures of endothelial cells such as enzymes, receptors and junctional proteins, as well as on extracellular layers such as the glycocalyx or "endothelial fuzz," have led to the development of methods that avoid use of proteolytic enzymes at both the isolation step and during subsequent subculture. This chapter describes traditional methods for isolating pulmonary endothelial cells but emphasizes newer approaches using mechanical harvest and scale-up using microcarriers. The new methods allow maintenance of long-term, large-scale cultures of cells that retain the full complement of surface properties and that maintain the cobblestone monolayer morphology and differentiated functional properties. Methods for identification of isolated cells are therefore also considered as methods for validation of cultures during their *in vitro* lifespan.

Introduction

The pulmonary endothelium is ideally situated as a strategic command post for regulating the entry of vasoactive substances into the systemic circulation (1–3). Endothelial cells of the lungs are now known to conduct a wide variety of specific metabolic activities important in the pulmonary processing of vasoactive substances. For example, enzymes, inhibitors, receptors and transport systems of endothelial cells can determine the levels of biogenic amines, kinins, angiotensins and prostaglandins entering the circulation (1,4). In this way the pulmonary endothelium plays a major role in the responses of target organs including the lungs themselves.

Many of the original studies implicating a role for the pulmonary endothelium were indirect, e.g., isolated perfused lung preparations (5,6). One step which was critical to our understanding of specific metabolic activities of endothelial cells was the ability to isolate pure lines of pulmonary endothelial cells and to propagate them in culture. First, it is pertinent to consider the anatomical features which characterize the pulmonary vasculature.

Anatomical Considerations

Endothelium of the lungs is of the continuous type (3,7). From earlier studies (5,6,8) it appeared likely that the site of the major processing of vasoactive substances was likely to occur at the level of the smallest vessels where circulating substrates would have their greatest exposure to lung cells. However, it is hard to imagine from conventional cross-sections at the level of the alveolar capillary unit (Fig. 1) that endothelial cells could play an active role in metabolizing bloodborne substrates. As seen in Figure 2, even with the resolving power of the electron microscope, endothelium in transverse section is extremely thin with few apparent cytoplasmic organelles. It is not until the extensiveness of the pulmonary capillary bed is taken into account, together with the strategic position of the lungs in the circulation, that one can appreciate the vast surface area available for processing. Even so it is hard to envisage how these cells, invisible except by electron microscopy, could be made amenable to harvest and culture.

Fortunately, the pulmonary artery of large animals can be dissected out and the endothelial layer can be removed by enzymatic digestion or by mechanical means. Although there are many similarities of the cell type in different locations it must be remembered that there are structural and probably functional differences

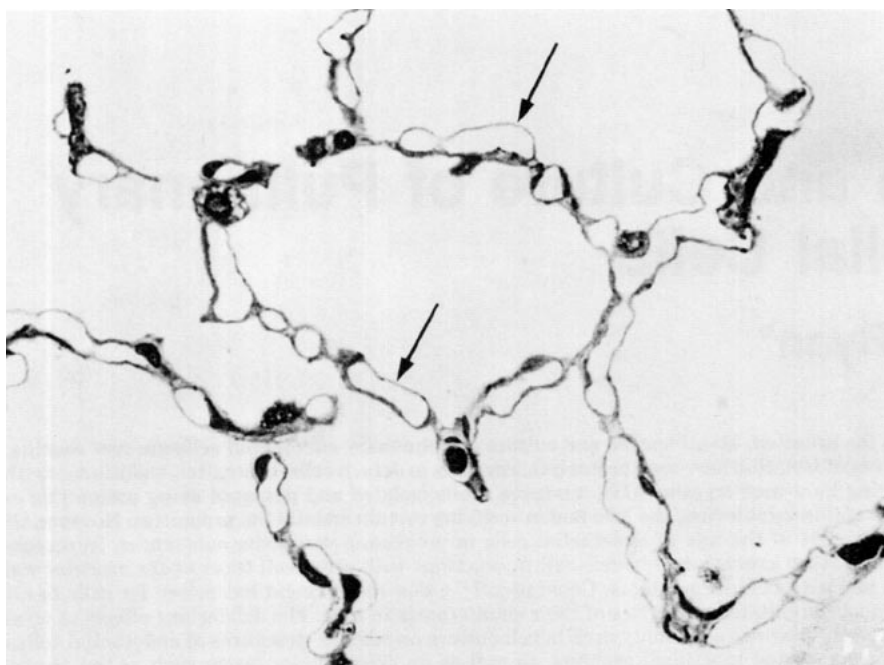*Department of Medicine (D-58), University of Miami School of Medicine, P. O. Box 016960, Miami, FL 33125.

FIGURE 1. Light micrograph of a rat lung which had been perfused free of blood. The alveolar–capillary unit (arrows) appears as a simple membrane and the cellular nature of the barrier cannot be resolved (compare Fig. 2). ×650.

between endothelial cells derived from vessels of different organs, different regions of each vascular bed and different species. For example, more projections, Weibel-Palade Bodies and fibrils occur in endothelium of large vessels than in those of the microvasculature (1,3) (Figs. 2 and 5). Furthermore, the junctional organization differs at different levels of the vascular tree and gap junctions are absent between capillary endothelial cells.

To a certain extent, developments and improvements in both isolation and culture procedures are still ongoing but it is true to say that the establishment of pulmonary endothelial cultures is now routine and that the techniques are reproducible and functioning in a number of laboratories for a wide variety of applications.

To a large extent the methods of choice depend on the final goals to which the cultures are dedicated. For example some studies require fresh isolates which resemble as closely as possible the *in vivo* state, others require vast numbers of cells and depend on large-scale, long-term production of endothelium. Furthermore, concerns with respect to species and the size of vessel from which the endothelium is derived must be taken into account.

Basically, the methods for *in vitro* cultivation of endothelial cells can be grouped as follows: (1) isolation and harvest following perfusion with proteolytic enzymes: (a) to obtain endothelium from large vessels of large animals and (b) to obtain endothelium from small vessels of small animals (this latter technique is adaptable for obtaining endothelial cells from a lobe of lung, including human lung obtained by open biopsy); (2)

culture and subculture by routine state-of-the art methods; (3) isolation and harvest avoiding the use of proteolytic enzymes; (4) long-term, large-scale culture avoiding the use of enzymes.

Whichever method or combination of methods is chosen, endothelial cells, in common with all cells maintained *in vitro*, must be characterized by rigorous criteria. First, they must be identified once they are no longer in the vessel and second they must be assessed for the maintenance of differentiated characteristics after long periods in culture. As a result of studies originally aimed at improving understanding of functional properties of the pulmonary endothelium (9,10), a number of morphological and biochemical characteristics of endothelial cells in culture are now known and together form a spectrum of tests for identification and assessment of the quality and purity of pulmonary endothelial cultures (11,12). Many of these properties are shared by endothelial cells from other sites (13). In addition, these tests have provided a standard against which the relative health of the endothelial cells under given culture conditions, or in response to experimental maneuvers or toxic factors, can be assessed.

Isolation of Endothelial Cells from Pulmonary Artery by Collagenase Digestion

Full details of methods for isolation of endothelial cells from the pulmonary artery of large animals (such as cow or pig) have been described (10,12). Segments of bovine pulmonary artery are obtained as fresh as
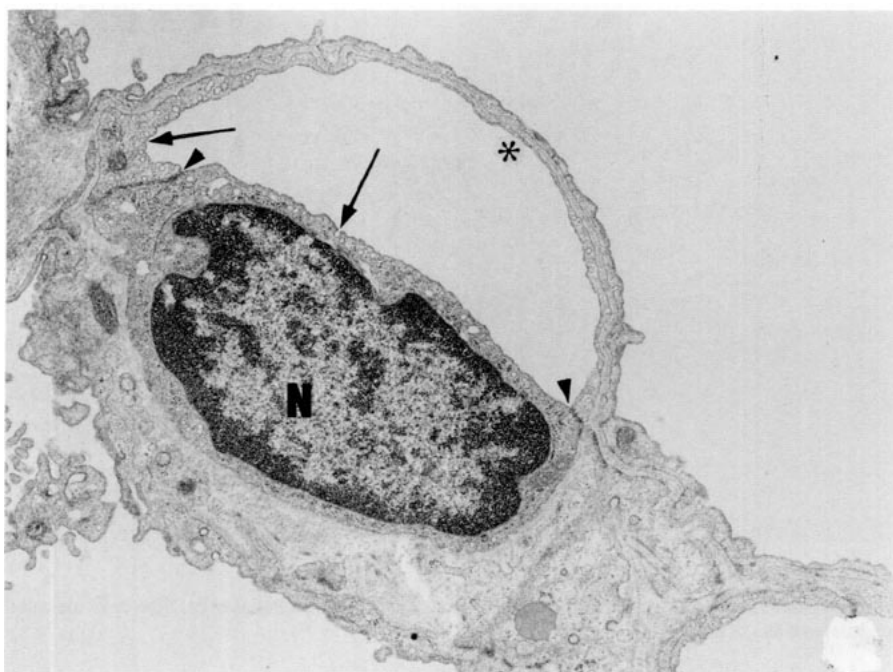
FIGURE 2. Electron micrograph from the alveolar–capillary level of a blood-free rat lung. The capillary endothelial cell is extremely thin in regions other than the nucleus (N). In transverse section the endothelial cell appears as barely more than two opposing cell membranes in some areas (asterisk) and in others the most prominent subcellular specializations are the large numbers of caveolae, many of which are on the luminal plasma membrane. Endothelial tight junctions are indicated by arrowheads. × 10,000.

possible from a slaughterhouse. The vessels are rinsed in sterile Dulbecco's phosphate-buffered saline (PBS) with calcium and magnesium (Gibco, Grand Island, NY), pH 7.4, containing 200 U/mL penicillin, 200 µ/mL streptomycin, and 325 µg/mL fungizone. Cells are obtained by an adaptation of the method of Maruyama (14), similar to the modifications of Jaffe et al. (15) and Gimbrone et al. (16).

Each pulmonary artery is separated from the heart and aortic tissue and stripped of extraneous fatty and connective tissue. The artery is then washed in two changes of Dulbecco's PBS with 3× antibiotic concentration. The artery is then placed in a large beaker of cold Dulbecco's PBS with 3× antibiotics on ice and placed in the cold room for 2 hr to reduce the likelihood of bacterial contamination. The arteries are removed individually and trimmed of any extra tissue and heart fragments. The end of the vessel where it branches into the left and right pulmonary arteries is tied off using a surgeon's knot and 0 surgical silk (or a large hemostat). The artery is drained of any remaining PBS and then filled with an 0.25% solution of collagenase (CLS II) in Dulbecco's PBS at 37°C. The other end of each artery is then clamped by using a large hemostat. The artery is then suspended and covered in a large beaker of PBS (prewarmed to 37°C) and placed in a shaker water bath at slow speed for 25 min. The artery is removed, blotted on sterile tissue, and then suspended by the ligature over a conical centrifuge tube. The clamp is opened and the enzyme–cell suspension is collected in

the tube. Each artery is rinsed once with Dulbecco's PBS and the rinse collected in the same tube. Tubes are capped and centrifuged at 1000g for 10 min at 4°C. The supernatant is discarded and the pellet washed two times with cold medium 199 (without FBS). The final pellet is then resuspended in 2.1 mL medium 199 containing 0.1 mL/100 mL Biostat 100. A 0.1 mL portion of suspension is counted in a hemocytometer to determine seeding density. We have found seeding densities of 1.5 to 3 × 10^5 per T25 flask in a total of 5 mL of medium to be satisfactory. However, hemocytometer counts of the original seed are not accurate since endothelial cells are best seeded as small clumps rather than as monodispersions (Fig. 3) (11,12). The flasks are capped loosely and placed in a CO$_2$ incubator at 37°C (5% CO$_2$). Flasks reach confluency within 14 days. Medium is changed every 2 days.

Isolation of Endothelial Cells from Small Vessels of the Lungs by Perfusion with Collagenase

Techniques for the isolation of endothelium from the microvasculature of the lungs of small laboratory animals by retrograde perfusion with collagenase have been described in detail (11) the rabbit lungs being used as an example. For contrast, a similar technique for obtaining endothelial cells from rat lungs by forward perfusion with collagenase is described here. This
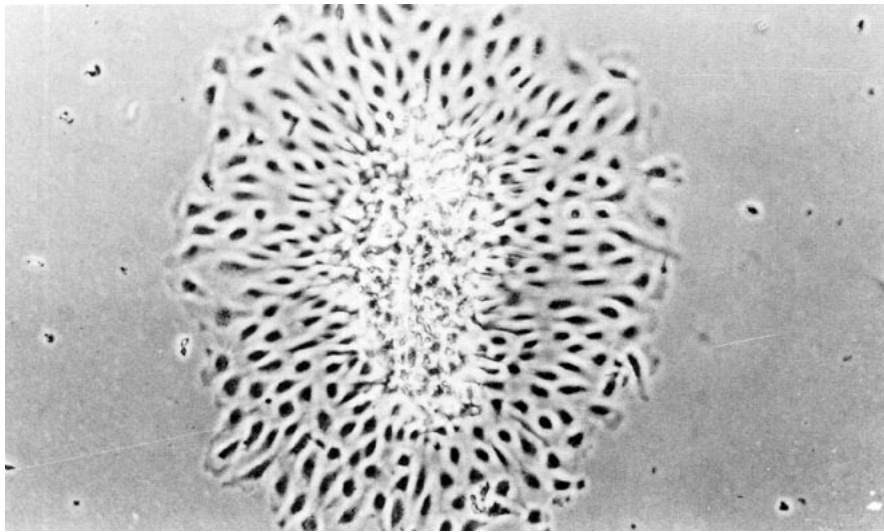
FIGURE 3. Phase contrast micrograph of an initial isolate of bovine pulmonary artery endothelial cells. The cells are seeded as small clumps or sheets rather than as monodispersion. ×200.

technique has the advantage that it is readily adaptable for use with a lobe of lung of a larger animal such as pig (10). In both cases, the principle is the same: the lungs are perfused blood-free with physiological saline followed by perfusion with an enzyme mixture to loosen the endothelial cells which are then collected via the vasculature. In all cases where cells are collected via the vasculature, retrograde perfusion at the enzyme step tends to increase the yield. Otherwise loosened cells may tend to be sieved by the capillaries.

Young female Sprague Dawley rats (80–100 g) are used. The rats are anesthetized with an intraperitoneal injection of sodium pentobarbital, 50 mg/kg body weight. A tracheostomy is performed, and the lungs are ventilated mechanically with a Harvard respirator (80 strokes/min, 2 mL tidal volume). Heparin, 500 units in 0.5 mL, is injected into the left femoral vein. The chest and abdominal cavities are opened with a midline incision and the rib cage is removed taking care not to cut major blood vessels. The pulmonary artery and aorta are exposed and ligatures are placed around each vessel. An 18–gauge Teflon catheter is inserted into the pulmonary artery and secured with a ligature. The lungs are pumped with HEPES Dulbecco phosphate-buffered saline (Process and Instruments Corp. Model 4) at 4 mL/min. As soon as the catheter is secured in the pulmonary artery, the aorta is cut at the level of the diaphragm. A second 18–gauge Teflon catheter is inserted in the ascending aorta, and the tip is advanced through the aortic valves into the left ventricle. The catheter is secured with a ligature. The aortic catheter is attached to a large drain tube (approx. 1/8 in. internal diameter) and the drain tube is positioned such that its outlet is approximately 10 cm below the level of the heart. The effluent is collected into a beaker. After 10 min of continuous pumping, the effluent is essentially free of blood. The lungs are then pumped with medium 199 containing 0.25% collagenase (Worthington CLS2, pH 7.4) and 4 g-% of bovine serum albumin (BSA). The effluent is collected into a series of 10–mL conical centrifuge tubes (ca. 10 mL of effluent/tube). Each of the tubes contains medium 199 with 10% fetal bovine serum (FBS). The collection is stopped when the lungs become grossly edematous (ca. 30 min), at which time the drain flow ceases.

The effluent samples are centrifuged at 4°C, 50g for 5 min to sediment any contaminating red blood cells. The supernatants are transferred to clean centrifuge tubes and then centrifuged at 1000g at 4°C for 10 min. The supernatant after the second centrifugation is removed by aspiration. The pellets are then washed three times with fresh medium 199 plus 10% FBS. Each pellet is resuspended, in 1 mL of the same solution. When the pellet is evenly suspended, a 0.1 mL sample is placed into each well of a Falcon eight-well dish and the remaining suspension is counted in a hemocytometer. Each sample is then diluted with 1 mL of medium 199 plus 10% FBS.

Each Falcon eight-well dish is incubated for 1 hr at 37°C in an atmosphere of 5% CO_2 and air. At the end of the incubation, the medium is removed by aspiration and is replaced with 2 mL of medium 199 plus 10% FBS plus antibiotics (Biostat 100, 0.1 mL/100 mL medium). The cells which attached during the first incubation are incubated again and medium is changed every 72 hr. When the cells reach confluency (6 to 14 days, depending on seeding density), they are removed by trypsinization and seeded into 25 cm^2 culture flasks.

Culture and Subculture of Pulmonary Endothelial Cells

With either of the methods of harvest described above, endothelial cells grow to form confluent monolayers within 14 days. After an initial medium change 24 hr after seeding, the medium (M199 + 10% FBS) is changed weekly. The approximate density at confluence is 3×10^6 per T25 flask.

The cells can be subcultured as follows. Flasks are washed for about 10 min with 5 mL of Ca^{2+} and Mg^{2+}-free Puck's saline at 37°C. The saline wash is aspirated off and the cells are incubated with 0.05% trypsin with 0.02% EDTA in Ca^{2+} and Mg^{2+}-free Puck's saline (5 mL at 37°C). The cells are monitored on an inverted microscope to determine the optimal length of time of exposure to the trypsin–EDTA solution. We have found that after 3 min the small, polygonal endothelial cells begin to lift off while the larger, flatter smooth muscle cells still adhere to the flask. With a minimum of agitation, the enzyme mixture containing cells is transferred, by using a 5-mL pipet, to 15-mL conical centrifuge tubes. The cells are centrifuged at 750g for 10 min. The trypsin solution is removed by aspiration, and the pellet is resuspended in a small quantity of medium 199 without fetal bovine serum. The flasks are usually split two for one. Each new flask is seeded with approximately 10^5 cells. The doubling time for bovine pulmonary artery endothelial cells is about 20 hr. Flasks which are predominantly endothelial (less than 5% contamination with smooth muscle cells) can be purified by successive subculture, taking advantage of the difference in attachment and detachment rates of the two cell types. Trypsin preferentially detaches bovine endothelial cells; thus, short periods (2–3 min) of exposure to trypsin prior to transferring cells tend to leave smooth muscle cells adherent to the flasks. Furthermore, endothelial cells are the first to reattach. Hence, cells are seeded into new flasks and incubated for 1 hr at 37°C. Medium and any free-floating cells are removed by aspiration and replaced with fresh medium containing fetal bovine serum and antibiotics.

Isolation Avoiding the Use of Proteolytic Enzymes

Despite much recent progress in the culture of endothelial cells, few if any laboratories have succeeded in establishing long-term cultures of endothelial cells which maintain a cobblestone monolayer morphology, division rates and differentiated characteristics of endothelium. Gospodarowicz et al. (17) have succeeded in carrying cultures of bovine heart endothelial cells through a large number of passages by adding fibroblast growth factor (FGF). However, two serious problems have been encountered. First, the cells are virtually impossible to wean from FGF, an extremely expensive culture medium additive. Second, and perhaps most importantly, the cells tend to lose functional properties.

The factors responsible for *in vitro* aging of cells have not yet been determined, but repeated exposure to trypsin is likely to contribute to accelerated aging. Quite clearly, there are serious limitations to what can be learned from a line of cells in culture that do not retain characteristics of the same cells *in situ*. We have therefore begun to develop new techniques for the culture of pulmonary endothelial cells which avoid exposure to proteolytic enzymes at both the isolation step and during successive passages. In studies published recently (18) we have described techniques for the mechanical isolation of pulmonary artery endothelial cells by scraping with a scalpel.

Fresh plucks (heart and lungs with attached great vessels) are obtained from a slaughterhouse as before; veal or young calf material is preferable. The outside of the preparation is cleaned with a towel soaked in 70% ethyl alcohol. Pulmonary artery is dissected free from the pericardium and aorta and extraneous fatty and connective tissue is stripped away. The pulmonary artery is removed and placed in Puck's saline. If time allows, bacterial contamination is reduced if arteries are held in Puck's containing $3\times$ antibiotics for 1 hr in a cold room (4°C). Then, under a laminar flow hood, the pulmonary artery is slit open so that it lies flat and draped over the edge of a Petri dish. The luminal surface is scraped with a sterile scalpel (No. 11 blade) using light, single strokes, covering each area once only. The cells which build up on the scalpel blade are shaken off into a tube of Medium 199 containing antibiotics. The cells are sedimented at approximately 100g for 10 min at 4°C and the supernatant is decanted. The cells are resuspended in Medium 199 containing 10% FBS and centrifuged again. The pellet is pipetted several times to break up large clumps of cells and the cells are seeded into T25 flasks or 35 mm^2 Petri dishes.

Cells collected by mechanical harvest have been particularly useful for studies where the original polarity of the cells must be preserved such as studies of endothelial junctions. The morphology of the junctions is an important indication of the size of the vessel from which the cells were obtained. Figure 4 shows a replica of a freeze-fractured endothelial junction obtained from pulmonary artery and indicates a complex of tight and gap junctional elements similar to the arrangement *in vivo* (18).

However, initial isolates are not suitable for studies requiring large numbers of uniform cells. Scale-up of cultures implies growing the cells over many passages (see below).

Long-Term, Large-Scale Culture of Endothelium Avoiding the Use of Enzymes

Bovine pulmonary artery endothelial isolates that are harvested mechanically can be seeded onto culture
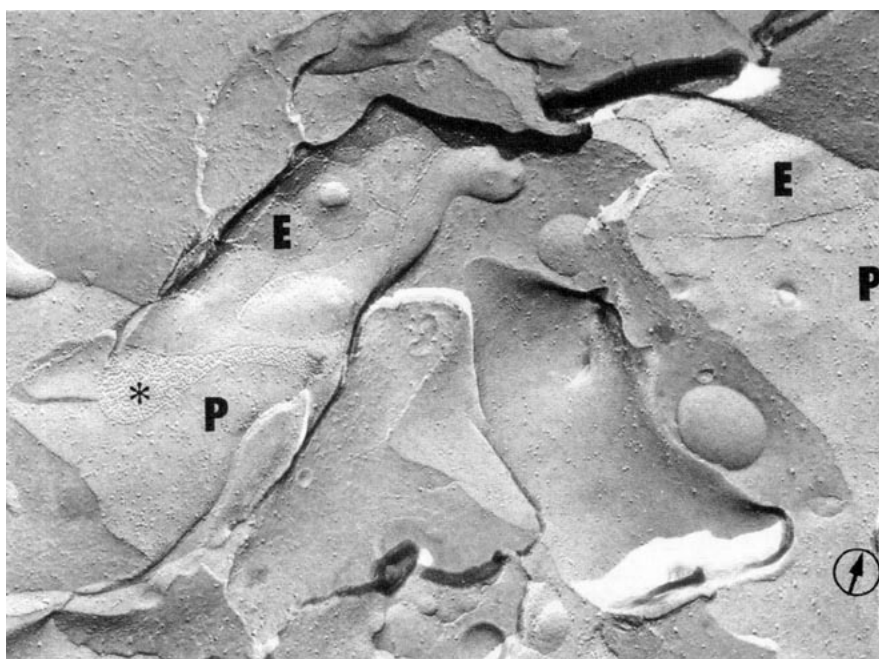
FIGURE 4. Replica of freeze-fractured endothelial isolate indicating tight junctions comprised of strands of discontinuous particles (arrows) on the E face and by barely perceptible ridges on the P face. The crest of the ridge may contain occasional particles but not in any regular linear array. Sometimes the ridge appears to extend into small irregular gap junctions. In other areas the gap junctions may be more extensive (*) and may flank the network of tight junctional strands. Reproduced with permission from Ryan et al. (17).

flasks, maintained as monolayer cultures and passaged nonenzymatically by scraping with a rubber policeman followed by transfer to new flasks. This technique is time-consuming, and the cells tend to settle in clumps even if passed through a 30-gauge needle and reach confluence more slowly than cells passaged using trypsin/EDTA (18).

However, microcarriers, e.g., Bio-Carrier polyacrylamide beads (Biorad), Cytodex (Pharmacial), Biosilon (Nunc) or specially coated polystyrene microcarriers (19), provide a substratum suitable for obtaining high yields of endothelial cells in culture, that can be passaged with simplicity without using enzymes.

Spinner flasks are the vessels recommended for microcarrier culture by the manufacturer but roller bottles (Bellco) appear to be highly satisfactory provided they are rotated at the highest speed setting. Pulmonary artery endothelial cells can be seeded directly onto microcarriers from fresh isolates or from monolayer cultures. The cells attach to the beads rapidly, for example after 5 min, about 25 to 30% beads have one or more cells attached to them. After 10 min, about 50% beads have one or more cells attached and some have three or four cells. After 20 min, the cells spread out on the beads, and many division figures are apparent after 24 hr. Endothelial cells on beads have the cobblestone morphology characteristic of these cells in monolayer culture (Figs. 5 and 6).

Endothelial cells were found to attach to beads of all sizes; at confluence large beads (200 μm in diameter)

would carry 90 to 100 cells while smaller beads (100 μm in diameter) might have 40 to 50 cells attached.

The theoretical surface area for cell attachment using microcarriers is enormous: 2×10^9 cells per dry gram of Bio-Carriers (19). The concentration of beads per liter of culture medium can be varied considerably. We have found that, at confluence, 1×10^6 beads per 10 mL of medium yield about 2.2×10^7 cells (2.2×10^9). Cultures split 1:2 at this density replicate well. A corresponding 10 mL of medium would support approximately 3×10^6 endothelial cells in monolayer culture. The cells grow to confluence on each bead but at any time free cells can be found in suspension and colonization of fresh beads presumably occurs both from free cells in suspension and from other beads (Fig. 5) (17,20).

Microcarrier cultures can be initiated from fresh isolates, from a healthy monolayer culture in log phase or directly from blood vessels (21). The bead cultures are maintained in the same medium (M 199 with 10% FBS), with addition of 24 mM HEPES to compensate for the air atmosphere in the Bellco roller bottle incubator. The cultures are seeded into larger roller bottles (1 L, designed for the Bellco) or into smaller bottles of 100 mL or 500 mL capacity secured in a large cylindrical vessel. The bottles are coated with Siliclad to prevent sticking of beads or cells to the bottle walls. However, high yields of endothelial cells can be obtained if cells are allowed to replicate on the walls of unsiliconized roller bottles as well as on the beads.

Concentrations of cells and beads can be varied,

FIGURE 5. Bovine pulmonary artery endothelial cells in microcarrier culture. These cells were harvested mechanically and have never been exposed to proteolytic enzymes. They were seeded onto polyacrylamide beads 4 days previously and maintained in a roller culture. The cells are not yet confluent on each bead (compare Fig. 6). ×260.
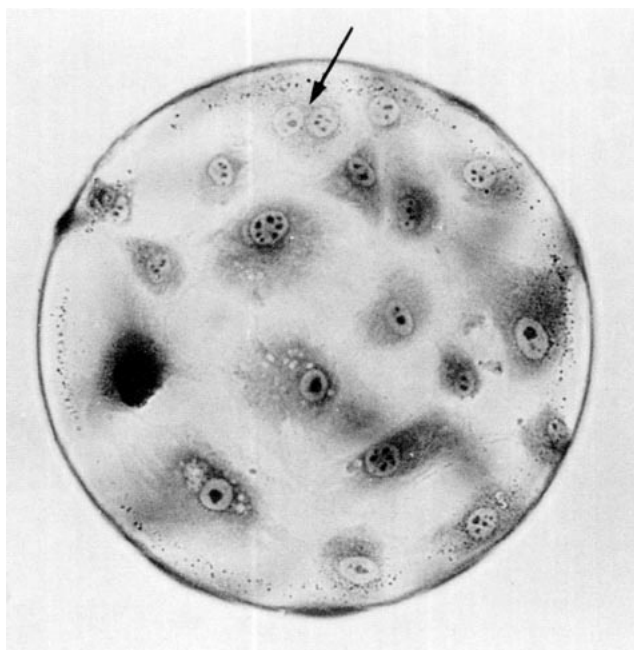

FIGURE 6. Polyacrylamide bead from a culture similar to that shown in Fig. 5 but approximately 8 days after seeding. The cells are almost confluent on each bead, cells in division can still be seen (arrow). ×260.

within wide limits. Primary cultures grow well if the cells are seeded in a 100 mL bottle, using 8.0 mL medium, 1.0 mL bead suspension and 1.0 mL cell suspension (approximately 1 × 10⁶ cells/mL). The bottle is swirled gently to mix ingredients, capped

tightly and placed in the roller apparatus at 37°C with rotation speed set at the maximum. The cells attach within 20 min, and division occurs after 24 hr. The medium is changed every 3 to 4 days.

Subculture. To subculture without using enzymes, the bead culture is evenly suspended and divided into two equal parts. Fresh medium and fresh beads are added. The cells rapidly colonize the new beads. This procedure can be repeated twice weekly. The split ratio can be as high as 1:10, 1 mL of cell-covered beads being used to seed a new flask.

Cells can be transferred from microcarrier cultures to flasks and vice versa at any stage. To transfer to a flask, 0.5 mL of an even suspension of cell-covered beads is placed in a 25 cm² flask, and 9.5 mL of medium is added. The cells "crawl" off the beads and establish a monolayer on the flask in 5 to 6 days (18,21). As the cells detach from the beads, most of the loose beads are removed when the medium is changed.

In order to transfer cells from flasks to microcarriers quickly without using enzymes, the endothelial monolayer is scraped with a rubber policeman, collected in a 3-mL syringe, passed through a 30-gauge needle to break up large clumps of cells, and seeded onto microcarriers as described for initial seeding of fresh isolates. Alternatively, microcarriers can be introduced into flasks containing confluent monolayers. Endothelial cells colonize the microcarriers which can then be transferred to flasks (21).

Sampling Microcarrier Cultures. The roller bottles are swirled to be sure that all the beads are in suspension. Under sterile conditions a sample of bead

suspension is withdrawn, no more than 5% by volume.

A 25 µL portion of an even suspension of a micro-carrier cultures is placed on a 35-mm gridded culture dish. The droplet is stained by mixing it with a small amount of methylene blue by means of a wooden applicator stick or toothpick. The drop is then covered with a 22-mm glass coverslip (see Figs. 5 and 6).

Since the beads are transparent, it is possible to focus through the bead and assess the total surface area. In this manner, it is possible to keep an accurate count of the total number of cells per milliliter culture.

Cloning. Unstained preparations may also be examined by phase contrast microscopy. At a seeding density of about one cell per two beads, cells are selected for cloning. By means of a small capillary tube (or micropipet) individual beads with one adherent cell are transferred to a microwell plate for cloning.

Removal of Cells from Beads by Vortexing. A number of procedures such as electron microscopy, counting in a hemocytometer, enzyme assays and subculture are sometimes more conveniently performed using cells removed and separated from beads. A quick, easy, reproducible and enzyme-free method for the removal of endothelial cells from Bio-Carriers is as follows: Plasticware or siliconized glassware is used throughout to prevent loss of cells or beads by sticking to the walls of vessels. An appropriate volume (generally 1–10 mL) of cell-bead suspension (thoroughly and evenly suspended) is withdrawn from the roller bottle under the laminar flow hood and placed in a centrifuge tube. The tube is vortexed (outside rim) for 2 min at a setting of 5. The suspension is passed through a 44 µm filter previously handcut from a roll of nylon monofilament mesh and fitted to a Swinex filter holder. The swinex holder is attached to a disposable syringe (3 mL). If necessary any beads adhering to the walls of the test tube are washed with fresh medium and filtered. The beads (80–200 µm, as supplied) are retained by the nylon mesh, and the endothelial cells pass through.

Identification of Endothelial Cells

Cells obtained by any of the above methods are readily identifiable as endothelial according to morphological and functional criteria (12). When examined in the inverted microscope by phase contrast microscopy, the cells grow as monolayers with a cobblestone appearance characteristic of endothelium (Fig. 7). When examined in the electron microscope, they contain all of the cellular organelles expected of pulmonary artery endothelial cells *in situ* (Figs. 8–10). The cells are routinely examined by electron microscopy of thin sections of monolayers still attached to the culture flasks (Fig. 9) (10,12). However, we have developed means of examining the cells in culture without removal from the culture flasks by additional techniques including freeze-fracture of monolayers, scanning electron microscopy, and examination of surface replicas (9, 22,23). Similarly endothelial cells on polystyrene micro-

carriers can be examined by scanning and transmission electron microscopy (18). Thus, we are now able to recognize a variety of views of endothelial cells. In addition, our cultures possess functional aspects characteristic of endothelial cells. The cells possess angiotensin converting enzyme activity as demonstrated by using $[^{125}I]Tyr^8$-bradykinin as substrate (24) and a variety of synthetic substrates (25). In addition, the cells are reactive with antibodies to angiotensin converting enzyme, as can be shown with immunofluorescence microscopy and immunocytochemistry (9,10). Using the latter technique, we were able to localize angiotensin converting enzyme on the plasma membrane and caveolae of endothelial cells in culture, a result in accord with our previous demonstration of angiotensin converting enzyme on the luminal surface of pulmonary endothelial cells *in situ*. Bovine endothelial cells also react with antibodies to human factor VIII and α_2-macroglobulin, and this we have demonstrated by immunofluorescence (11,12). The lack of Fc receptors on uninjured pulmonary endothelial cells in culture (26) indicates that nonspecific binding of IgG need not be of concern in immunofluorescence studies.
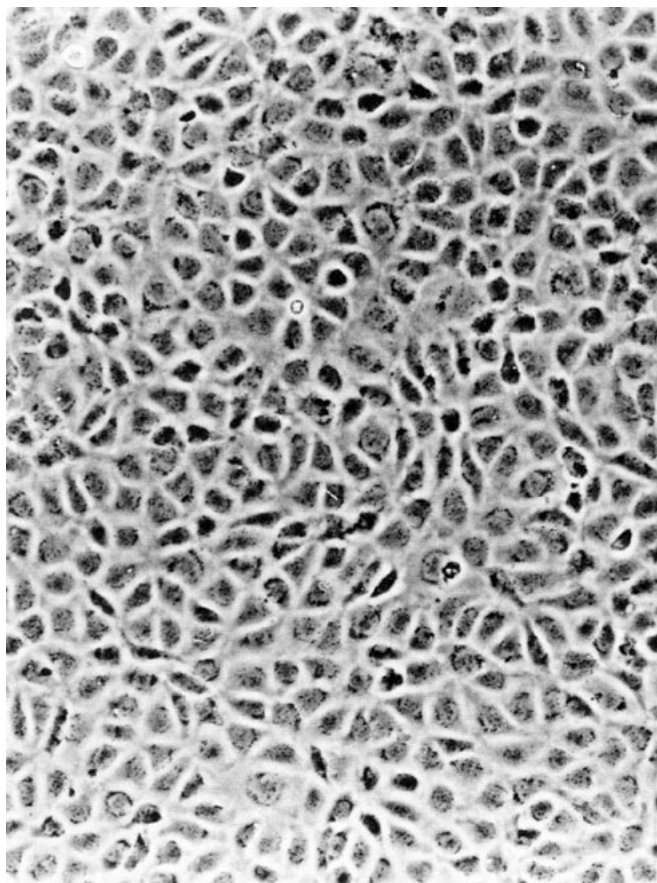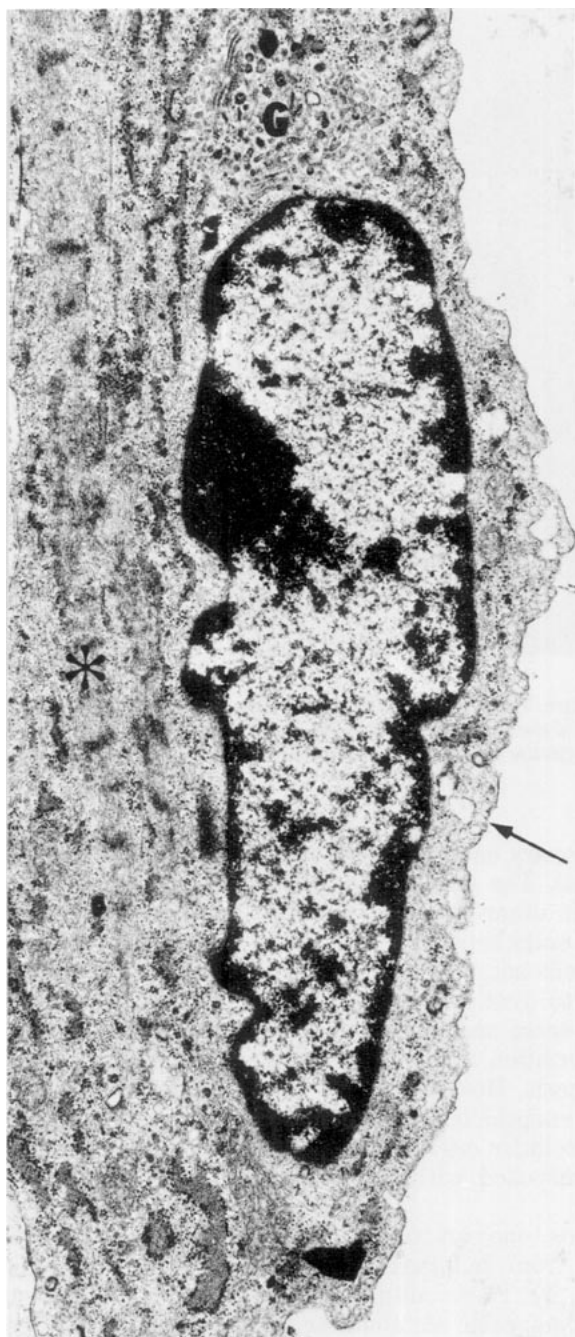

FIGURE 7.   Phase contrast micrograph of a confluent monolayer of bovine pulmonary artery endothelial cells in their 12th passage. The "cobblestone" morphology has become one of the hallmarks of a healthy endothelial culture. ×250.

FIGURE 8. Electron micrograph of bovine pulmonary artery endothelial cells freshly isolated by scraping with a scalpel. In addition to caveolae on the luminal surface (arrow) Golgi apparatus (G) is apparent near the nucleus, and fibrillar material is evident below the nucleus (asterisk), rough endoplasmic reticulum is distributed throughout the cytoplasm. ×20,000.
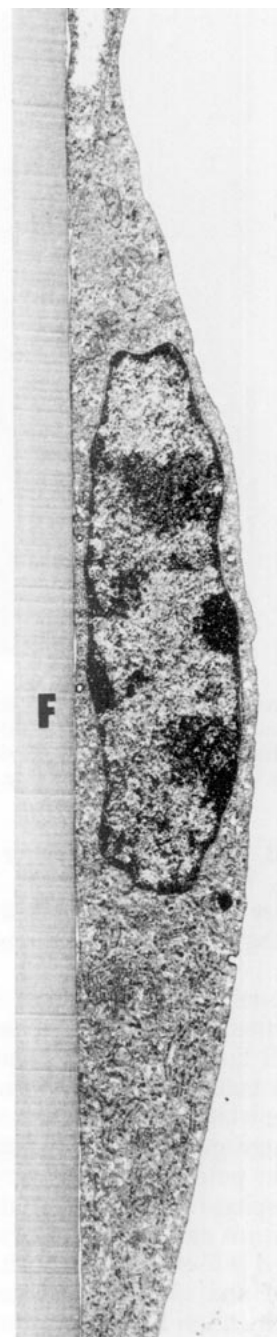
FIGURE 9. Low power electron micrograph of pulmonary artery endothelial cell in monolayer culture. Sections were cut without removal of the cells from the culture flask (F). The single layer of cells and the flattened morphology, except in the region of the nucleus, the caveolae along the upper surface and the intracellular organelles characteristic of the cell *in situ* (Fig. 8) persist in culture. ×7800.

Discussion

Pulmonary endothelial cells in culture have been used to demonstrate, *in vitro*, a number of other properties or lung functions, which are not at present used as criteria for identification of endothelial cells.

For example, pulmonary endothelial cells are capable of forming prostaglandins and related substances from 1-^{14}C-arachidonate. Using EM autoradiography of cells incubated with $[^3H]$-acetyl salicylate (a specific inhibitor of prostaglandin synthetase), we were able to show that the enzyme is situated on the endoplasmic reticulum
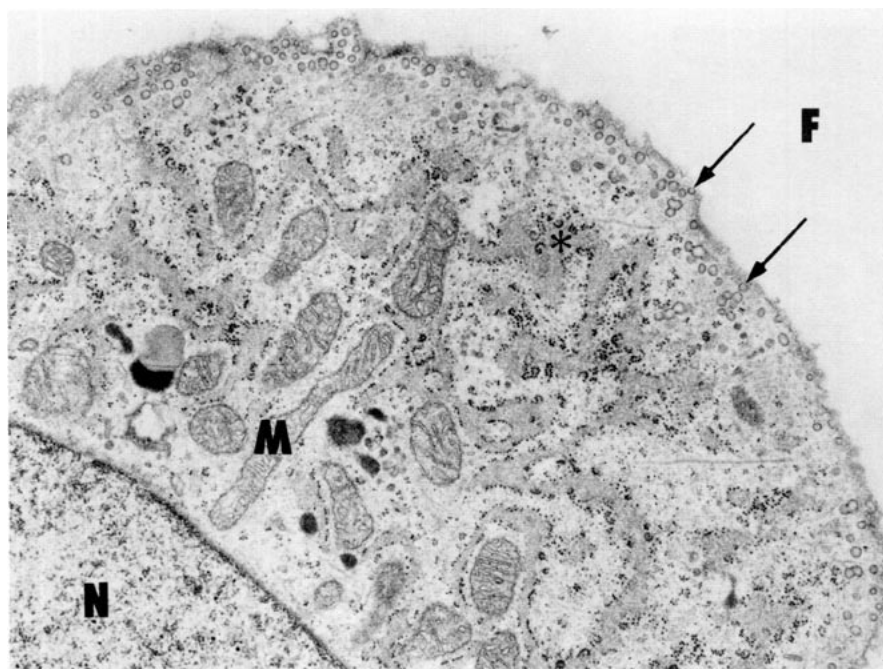
FIGURE 10. Electron micrograph of an *en face* section of an endothelial cell in culture. The plane of section grazes from the culture flask (F) to the nucleus (N). Large numbers of caveolae (arrows) are present at the periphery of the cell. Mitochondria (M) and obliquely sectioned cisternae of the rough endoplasmic reticulum (asterisk) indicate that endothelial cells in culture contain abundant intracellular organelles which are not shown to advantage in transverse sections (e.g., Fig. 9). ×14,000.

(11,12). PGE_2 and PGI_2 are the major metabolites. Synthesis of PGI_2 by endothelial cells has been described by a number of laboratories (27–29). It is likely that all or part of the PGI_2 formed by lungs arises from endothelial cells.

Intact blood vessel walls are known to have antithrombotic properties, thought to be associated with the endothelial cell lining layer. As mentioned above, endothelial cells in culture can synthesize prostacyclin (PGI_2), a substance which can prevent platelet aggregation and also disaggregate platelet clumps. The lungs can also degrade the potent, platelet aggregating agent adenosine-5'diphosphate (ADP) (30). Pulmonary endothelial cells in culture can rapidly degrade ADP. The first product formed is 5'-AMP, which is itself degraded to yield adenosine and inosine. Adenosine does not accumulate in the medium but is taken up by the cells and incorporated into ADP and then into ATP (31).

Thus, in addition to their ability to synthesize PGI_2, pulmonary endothelial cells can degrade ADP and together these properties may account for the capacity of endothelial cells to prevent or inhibit platelet aggregation.

It is now apparent that endothelial cells, especially those of the lungs play a key role in the regulation of the renin–angiotensin and kallikrein–kinin systems (1,32). A single enzyme: angiotensin converting enzyme or kininase II can inactivate bradykinin and convert angiotensin I to angiotensin II, this enzyme, which links the two systems, is situated on the luminal surface of

pulmonary endothelial cells *in situ* (1,10) and in culture (10,12). The enzyme probably occupies a similar position in other vascular beds (33). In the case of pulmonary endothelial cells, we have been able to show that the cells not only possess the enzyme but that they are able to synthesize it in culture (12). In this regard, transverse sections (Figs. 2, 8 and 9) do not give an appreciation of the intracellular machinery required for synthesis. However, *en face* sections (Fig. 10) illustrate that endothelial cells possess the full complement of intracellular organelles required for enzyme synthesis but disposed within the very flattened shape of the cells.

Thus, one can readily isolate and culture endothelial cells from pulmonary artery of, for example, cow (9,10,12). These cultures have provided useful information on specific metabolic activities of pulmonary endothelium and have the advantage that large quantities of cells can be obtained in original isolates. In addition, antigenic sites of bovine cells appear to cross-react with antibodies to human antisera. However, the vast preponderance of studies on metabolic functions of the lungs have been performed with small animals, in which it is virtually impossible to obtain an adequate number of cells from main-stem pulmonary artery. Furthermore, although we and others have shown that cells for main-stem pulmonary artery are capable of metabolizing bradykinin, angiotensin, ADP, adenosine, and biogenic amines, the general assumption is that the processing of such substances *in vivo* occurs most

prominently at the level of the microcirculation. New techniques of cell isolation by vascular perfusion allow use of small animals and therefore allow a more direct comparison with previous functional studies in which small animals, such as rats and rabbits, have been used (11,20).

To extend the scope of studies which can be undertaken of pulmonary endothelial functions, we have developed means of obtaining large numbers of endothelial cells in long-term culture which avoid repeated exposure to proteolytic enzymes. The factors involved in the aging process of cells in culture are not fully understood but repeated exposure to trypsin is likely to contribute to accelerated aging; loss of surface enzymes and alterations in size, shape and junctional contacts, together with progressively slower and asynchronous rates of division. To assess this last feature, we have examined our enzyme-free cell lines by time-lapse cinematography. Further, there is a high correlation of interdivision times and migration distances between daughter pairs (34). The cells are remarkably mobile and, by comparison with lung fibroblasts of high passage number (35) our cells show no indications of aging. The cobblestone morphology is preserved as are angiotensin converting enzyme, Factor VIII antigen and junctional contacts.

The bead cultures have the advantage of providing a system for the long-term, large-scale culture of pulmonary endothelial cells, features which to a large extent determine the scope of biochemical studies which can be undertaken. However, an additional advantage (which it is perhaps still too early to assess completely) is that a method now exists for culturing large numbers of endothelial cells which have never been exposed to exogenous proteolytic enzymes or growth factors and which retain the differentiated characteristics of primary cultures.

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