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SELECTION AND CHARACTERIZATION OF BOVINE AORTIC ENDOTHELIAL CELLS

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SUMMARY

This paper reports techniques for isolation, selection and long-term passage of bovine aortic endothelium (BAE). A [^{3}H]thymidine-selection technique was developed to limit overgrowth of cultures by contaminating smooth-muscle cells. The resulting cultures could be passaged for a replicative life span of 35 to 40 doublings and maintained a stable, normal karyotype throughout this period. Despite the fact that these cultures reached a stable monolayer with density-inhibited growth state, postconfluent cells showed focal areas of a second growth pattern called "sprouting." This was seen only when cultures were maintained at high densities for periods of 1 to 2 weeks. Ultrastructural analysis, as well as immunofluorescence studies with markers for endothelial cells (factor VIII) and smooth-muscle cells (actin), indicates that this phenomenon is not due to overgrowth of a residual population of smooth-muscle cells, but may represent a second growth pattern of the endothelial cells themselves.

Key words: thymidine selection; endothelium; contact inhibition; replicative life span.

INTRODUCTION

Isolation and culture of endothelial cells from human umbilical vein and from aortas of several nonprimate species have become routine procedures (1-4). Endothelial cells grow in culture as a strongly contact-inhibited monolayer and retain much of the morphology characteristic of endothelium in vivo (5, 6). These cells, in primary or early passage, have been used to identify a variety of biochemical functions that are specific to the endothelial cells and of obvious importance to our understanding of the normal and pathological functions of the vessel wall (7-10). In comparison, very little has been done to characterize endothelial cell strains in the long-term culture. Such characterized cell strains of a normal diploid cell with these growth properties would be particularly useful for studies of contact inhibition, wound healing and in vitro senescence. This paper describes techniques for isolation and long-term culture of bovine aortic endothelium (BAE).

MATERIALS AND METHODS

Primary isolation. Segments of descending thoracic aorta, 20 to 30 cm in length, were obtained from cows at a local meat-processing plant immediately after death by gunshot wounds to the head. In the routine procedure of this plant, the whole process from death to the obtaining of the piece of vessel took no more than 10 min. The segments were rinsed briefly in phosphate-buffered saline to remove free blood and then placed in a sterile, stainless-steel container with a humidified atmosphere maintained by a few pieces of wet gauze. The segments then were taken to our laboratory where the tissue was processed within 2 hr.

Culture techniques were adapted from the procedures introduced by Gimbrone (1) and Jaffe, Hayer and Nochman (2). Intercostal branches were tied and the caudad end of the vessel was cross-clamped. A 50-cc syringe was modified by cutting off the closed end. The cut end, now open, was heated in a gas flame in order to provide a lip. The modified syringe was inserted in the craniad end of the vessel, fastened in place with sutures and suspended from a ring stand. The vessel lumen was filled with approximately 50 cc warm collagenase solution (1 mg per ml) dissolved in phosphate-buffered saline at 37° C with calcium (0.15 mg per ml) and magnesium (0.10 mg per ml) (collagenase, CLSII, Worthington Biochemical Corp.). Incubation was done at room temperature. At 5 min, the preparation was agitated gently by tilting the ring stand from side to side, and the enzyme solution was removed and replaced

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with fresh solution. This was repeated every 2 to 5 min for a maximum of 30 min. The collected solutions from each time point were individually centrifuged in a small table-top centrifuge at 1000 rpm for 5 min. The supernate was discarded, and the pellet was resuspended in complete tissue-culture medium and transferred to a 35-mm Falcon tissue-culture dishes containing 2.5 ml medium. The tissue-culture medium consisted of Waymouth's medium containing 30% heat-inactivated (56° C for 30 min) fetal bovine serum (GIBCO, Santa Clara, Calif.); 2.5 mmol glutamine; 10,000 U per 100 ml penicillin; 10 μg per 100 ml streptomycin; and sodium bicarbonate. The medium was replaced every 3 days.

At time of passage, the medium was removed from cells and the dishes were rinsed with several ml EDTA buffer solution (0.54 mm EDTA, buffered to pH 7.4 with Tris-HCl) to cover the whole surface of the tissue-culture dish. In 3 to 4 min, when cells began to come off, the solution was agitated gently with a pipette and transferred to a centrifuge. The tissue-culture dish then was rinsed with Waymouth's medium lacking serum, and this also was transferred to the same centrifuge tube. The tube was spun at 1000 rpm for 5 min; the supernate was discarded; the cell pellet was suspended in complete tissue-culture medium; and the cells were transferred to a tissue-culture dish. Cell numbers were estimated using aliquots of cells counted with a hemacytometer.

Thymidine selection. Cells from primary culture were passaged into flasks (Corning 25-cm^2 tissue-culture flasks) at an initial density of 4.0×10^4 cells per cm^2. These passaged cultures were always chosen from confluent primary cultures apparently free of smooth-muscle cells. However, when the passaged cultures were kept at confluence (approximately 10^5 cells per cm^2) for more than 1 to 2 weeks, in all cases a second growth pattern appeared, overgrew the culture, and eventually replaced the normal "cobblestone" pattern of the endothelium with the "hills and valleys" characteristic of smooth-muscle cells (11). Cultures to be thymidine-treated were allowed to grow to the point where the endothelium was confluent. That is, there were no free spaces between cells, and overgrowth of the second cell type had begun to occur in a number of focal areas (Fig. 1). At that point, the medium was removed and cells were treated as follows.

The medium was removed and 2.0 ml fresh tissue-culture medium containing 2.0 μCi per ml [^{3}H]thymidine (New England Nuclear Corp; NET-027Z high specific activity, 50 to 60 Ci per mmol) was added to cells. Treatment was for 24 hr in a 37° C CO_2 incubator. After the 24-hr treatment, the [^{3}H]thymidine-containing medium was removed; the cells were rinsed three times with Hanks' balanced salt solution (BSS); and fresh medium was added without isotope. Three days later, cells were treated a second time with 2.0 μCi per ml [^{3}H]thymidine for 24 hr, washed with BSS, and fresh medium was again added. Three days later, cells were passaged and transferred to another 25-cm^2 flask.

The results of this protocol will be presented in some detail in the Results section. However, for purposes of continuity with the remainder of Materials and Methods section, a brief review is presented here. Following passage, the treated cultures showed three cell types: (a) small, rounded cells either single or in clumps resembling the endothelium of a confluent monolayer; (b) large, flat cells with extensive cytoplasm; and (c) large, strap- or fusiform-shaped cells. The latter cell type was usually seen only as single cells and did not increase in number. In time the large, flat cells decreased in number. The small, rounded cell type increased in number and in time overgrew the culture.

Estimation of replicative life-span, serial passages. The initial passage after thymidine was treated as the first passage. Subsequently, each culture was passaged after sufficient time to double the cell number. Cells were maintained at a constant, initial plating density of approximately 9.0×10^5 cells per 25-cm^2 flask (3.6×10^4 cells per cm^2) with two flasks being prepared from the one flask of each previous passage. At early passages, this required approximately 4 days to double the original cell number. As passage number increased beyond 18, the interval increased to 7 to 8 days. In most cases, at each passage 1 aliquot of cells was continued in culture and the remaining cells were frozen. After 18 passages, each culture was passaged every 9 to 10 days allowing time for a 4-fold increase in cell number and plating of four flasks. In this way we were able to increase the number of replications per week. Cultures were passaged at this schedule until cells lost their ability to replicate. Cells not used for the in vitro aging either were frozen (see below) or used in other experiments.

Cell freezing. A portion of the cells at most passages was frozen for later use. The freezing and thawing procedures consisted of the following: Cells were removed from the dish with tryp-

sin/EDTA, centrifuged and resuspended in complete tissue-culture medium containing 10% dimethylsulfoxide. This preparation then was transferred to Pro-Vial plastic freezing vials (1.0 ml freezing medium and approximately 3×10^6 cells per vial) and put on ice. Vials were suspended in ethyl alcohol in an insulated beaker surrounded by dry ice in 95% ethanol. This gives a cooling rate of 1° C per min. When the ethanol reached −40° C, vials were placed on storage canes and in liquid-nitrogen cannisters for permanent storage. Vials to be thawed were removed from the nitrogen tank and placed in a small beaker with lukewarm H_2O at a level high enough to just cover cells. Just at the point where cells had thawed, the preparation was transferred with a pasteur pipette to centrifuge tubes containing complete tissue-culture medium and centrifuged at 1000 rpm for 5 min. The supernate then was removed; the pellet was resuspended in fresh medium; and the cells were transferred to 25-cm^2 tissue-culture flasks. Approximate survival, based on counts of attached cells at 5 hr, was 50%. This number dropped with increasing passage number.

Karyotyping. Karyotyping was conducted by Dr. Holger Hoehn of the Cytogenetics Laboratory within this department by means of conventional techniques (12). In brief, cells at an appropriate passage level were thawed, brought to confluency, replated at 10,000 cells per cm^2, allowed to proliferate, arrested in mitosis with colcemide, and then processed for karyotyping. Ten to 20 mitotic figures were photographed for each culture, numbers of chromosomes were counted, and karyotypes were prepared.

Determination of saturation density. The principal objective of this experiment was to determine the saturation density of each strain and the effect of confluence on cell replication. In order to accomplish this, cells were plated at a confluent density with initial concentration of 1×10^5 cells per cm^2 in Falcon multiwell tissue-culture dishes (22-mm diameter). At this density, cells are already confluent; that is, there are few areas of the dish not covered by the cell layer. Nonetheless, cells are not yet at their maximum density and proliferation is still occurring. For autoradiography, round plastic cover slips were dropped in wells before plating. All cells were maintained in complete tissue-culture medium (0.5 ml per multiwell). Every 3 days, replicate wells were given a 4-hr pulse of [^{3}H]thymidine at a concentration of 1.0 μCi per ml (New England Nuclear Corp.; NET 027, 6.7 Ci per mmol). After the labeling interval, cells were rinsed three times in Hanks' basic salt solution. The cover slips then were removed from wells and stored in formaldehyde fixative. Before autoradiography, the cover slips were rinsed three times with water. Autoradiography was performed by conventional methods (13): cover slips were dipped in NTB-2 emulsion (Eastman Kodak), exposed for 2 weeks, developed by conventional methods, and then stained with hematoxylin.

Cloning technique. Cells were plated at 125, 250, 500, 1000 and 2000 cells per 35-mm dish. The following day cultures were observed for cell concentrations; however, only dishes in which the cells were widely dispersed and attached singly were used for cloning. This required a density of less than 1000 cells per dish. At densities of 125 and 250 cells per 35-mm dish, cells attached singly. Isolated cells were identified and marked on the underside of the culture dish using a diamond scribe. About 1 week after plating, small clusters of 8 to 10 cells were found at the sites of these single cells. The medium then was drawn off. The cells were rinsed with versene buffer. A 7-mm diameter stainless-steel cylinder, dipped at one end in sterile silicon lubricant, was dropped over the individual colony, and 3 to 4 drops of trypsin EDTA solution (as above) were put into the cylinder. After several minutes, the contents of each cylinder were transferred to a 35-mm dish containing 2.5 ml complete tissue-culture medium. Out of the initial colonies plated, less than 50% attached and showed continued cell growth. These cells were initially vacuolated and spread out, and a great deal of debris was present. However, 2 to 3 weeks after cloning, about 50% of these cultures were confluent and could be further passaged. Other dishes contained fewer cells and those cells had the appearance of senescent cultures (see Results). These cells could not be further passaged.

Immunofluorescence. For factor VIII immunofluorescence, cultures were grown on square glass cover slips in 35-mm tissue-culture dishes. These were rinsed with phosphate-buffered saline (PBS) and fixed in acetone for 10 min at 0° C. Selected areas of the cover slips then were overlaid with rabbit antibody globulin to human factor VIII (AHF) associated protein (Behring Diagnostics). The cover slips were placed in a wet chamber and incubated at 37° C for 30 min. Next they were rinsed in PBS for 45 min changing the PBS every 15 minutes. The cover slips were dried, and fluorescein-conjugated goat antibody globulin to

rabbit immunoglobulins (Behring Diagnostics) (diluted 1:1) was put on test areas. Next specimens were incubated in a humid chamber at 37° C another 30 min and again rinsed in phosphate-buffered saline for another 45 min. Cover slips were dried and mounted face down with buffered glycerol on glass slides. In all cases, control areas of the same cover slip were stained with nonspecific rabbit antibody before being counterstained with the fluorescein-conjugated antibody.

An attempt also was made to look at immunofluorescence to factor VIII in mixed culture. Bovine aortic smooth-muscle cells were obtained by allowing smooth-muscle cells to overgrow an endothelial culture that had not been subjected to tritiated thymidine. These cells were plated in a 1:1 mixture with bovine endothelial cells at a density near confluence. Cells were allowed to attach and remain in culture for 4 days. At that point, endothelial cells and smooth-muscle cells were both present in clusters and in apparent juxtaposition to one another. These mixed cultures were stained as described above.

We were able also to obtain a small amount of antibody to smooth-muscle actin from Ute Gröschel-Stewart (14). Again, cells were grown on cover slips in 35-mm tissue-culture dishes. These were rinsed in two changes of phosphate-buffered saline (PBS) fixed in 2% formaldehyde for 20 min at room temperature. Then the cover slips were rinsed twice again with PBS, placed in acetone at 15° C for 10 min, air-dried and stored in a desiccator. The specific antibody, a rabbit gamma globulin to chicken gizzard smooth-muscle actin, was applied for 30 min in a moist chamber. Slides were subsequently counterstained with fluorescein-conjugated goat-anti-rabbit immunoglobulin as described above.

Electron microscopy. Cultures to be studied for electron microscopy were fixed by replacing the tissue-culture medium with an aldehyde fixative composed of 1% paraformaldehyde and 2% glutaraldehyde in 0.1 M sodium cocodylate buffer. After a minimum of 1 hr, the fixative was replaced and cultures were stored in 0.2 M sodium cacodylate buffer. After a minimum of 1 hr, the fixative was replaced and cultures were stored in 0.2 M sodium cacodylate containing 5% dextrose for 24 to 48 hr. Tissue was subsequently fixed in 1% osmium tetroxide, dehydrated in graduated alcohols and embedded in Epon. Sections were made at right angles to the culture dish surface and stained with uranyl acetate and lead citrate.

RESULTS

Primary cultures. Cells obtained after incubation in the enzyme solution for short periods (5 to 10 min) were routinely free of obvious smooth-muscle contamination. In contrast, cells obtained after incubation in the enzyme solution in excess of 15 min routinely showed a mixture of cell types and were rapidly overgrown by a multilayered fusiform cell population with the characteristic "hills and valleys" described for smooth-muscle cells in culture (11, 15). These cultures either were discarded or in some cases passaged as a source of intimal smooth-muscle cells.

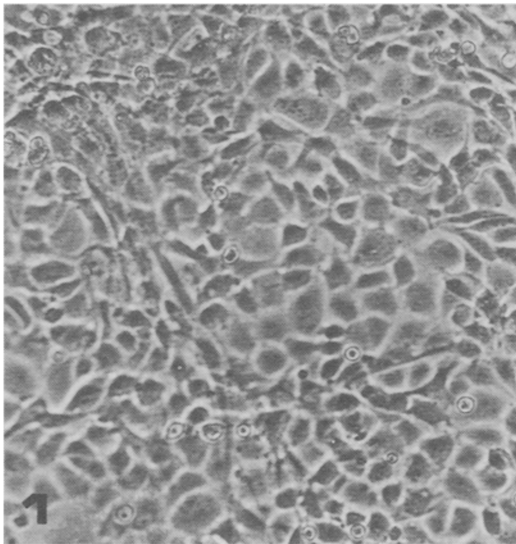
Cultures obtained after short incubation times could be passaged several times without apparent overgrowth by smooth muscle. However, this was true only when cultures were maintained and passaged at relatively low densities (less than 1×10^6 cells per cm^2). At this density, cells were confluent; however, growth did not cease (Fig. 7). If the cultures were allowed to continue growth until they reached a stable, saturation density, within 2 weeks they began to show focal areas of growth of fusiform cells (Fig. 1) which continued to grow again replacing the endothelium with the hill-and-valley growth pattern characteristic of smooth-muscle cells.

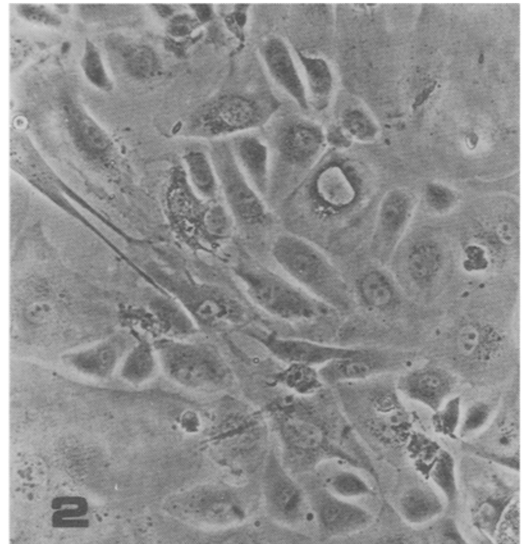
Thymidine selection. We reasoned that this late overgrowth was due to a subpopulation of cells that retained high growth potential after the point at which growth of the endothelium became inhibited by cell density. If this were true, then post-confluent endothelial cells should be protected from agents that are specific for the cell cycle whereas the actively growing subpopulation should be sensitive to these agents. This appeared to be the case. Following the thymidine-selection protocol given above, no further overgrowth occurred nor was there any obvious change in morphology or cell density over periods up to 1 week. Once the cultures were passaged, however, it was clear that the thymidine treatment had affected both cell types (Fig. 2). The smooth-muscle cells were proportionally greatly decreased in cell number. Moreover, they usually appeared as single, isolated cells or as small clusters of cells that did not increase further in number (Fig. 3). At the same time, the predominant cell type was also abnormal. Many cells showed a large volume of flattened cytoplasm, an appearance also seen in cultures at late passages (see below). In addition, the cultures contained amorphous debris and many cells were vacuolated. Despite cytopathic effects

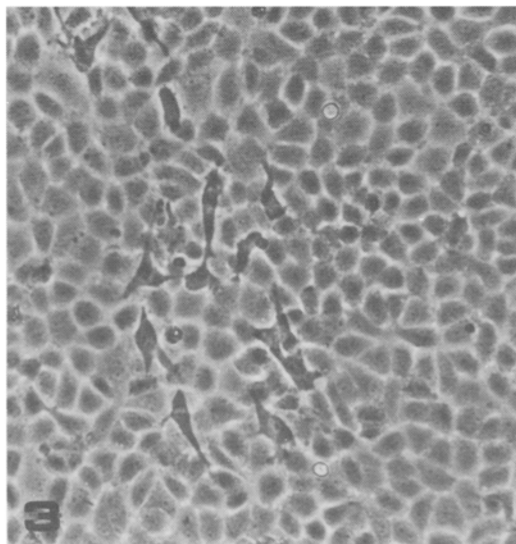
in some cells, many other endothelial cells appeared as normal, small cells forming focal areas with the characteristic confluent, cobblestone structure. Within 1 to 2 weeks (Figs. 3, 4), this pattern became predominant.

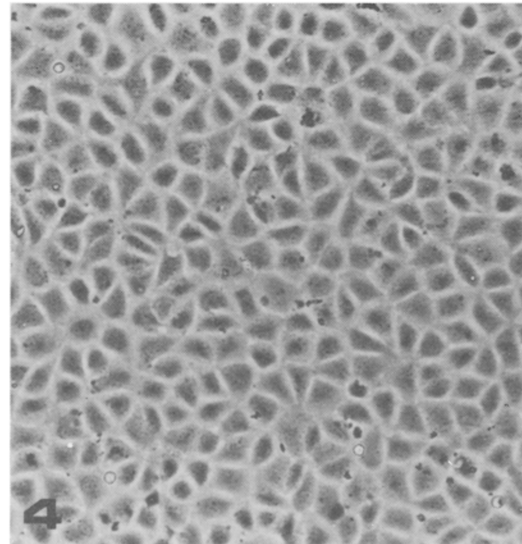
In subsequent passages, the cells appeared as homogeneous monolayers of small regularly shaped cells (Figs. 4, 5). We have maintained treated cultures at confluent densities for as long as 2 months without smooth-muscle overgrowth. However, a second pattern of growth and possibly a second cell type did appear in some postconfluent cultures. The identity of this cell will be discussed below. At no time did the second cell type overgrow and replace the endothelial monolayer (Fig. 6).

Growth properties. In order to study the inhibition of endothelial growth at confluent densities, cells were plated at densities sufficient to produce confluence but still less than maximum density. Cells plated at densities of approximately 2×10^5 cells per 22-mm multiwell tissue-culture plate (1×10^5 cells per cm^2) reached a maximum density of approximately 1×10^6 cells per 22-mm multiwell plate (5×10^5 cells per cm^2) within 1 week. This density was stable for at least 2 weeks (Fig. 7*a*). During the interval of stationary density, thymidine index declined to values less than 1% (Fig.



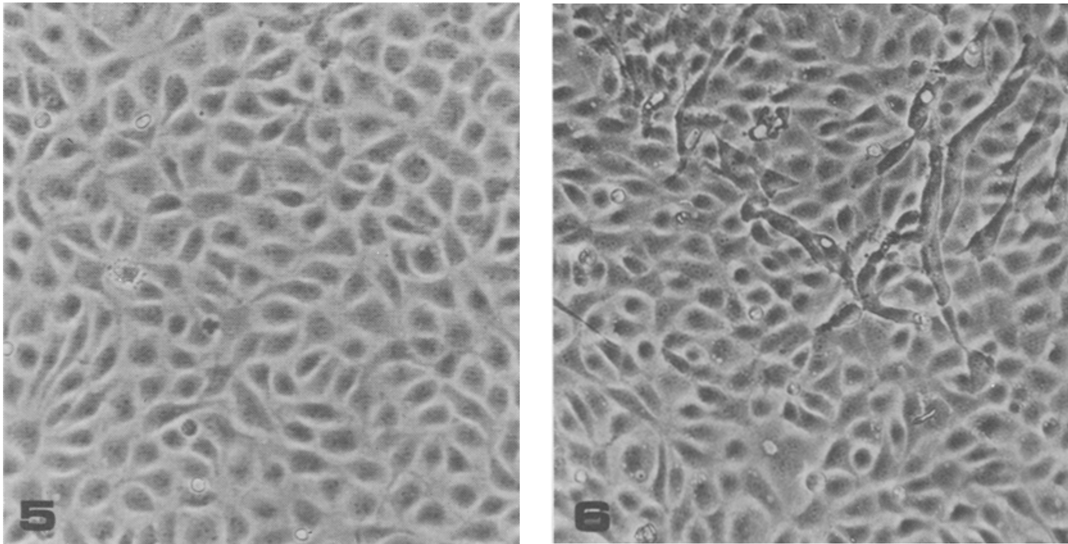






7b). This is of some interest since it is a value comparable to the thymidine index of endothelium in vivo (13).

Replicative life span. Cultures were passaged after thymidine treatment as described in Materials and Methods and either at a 1:2 or a 1:4 ratio. One strain developed large numbers of large, vacuolated and flattened cells and would not divide after the 10th passage. The four other strains studied showed a consistent pattern, ceasing to divide after 25 to 30 passages, which corresponded in terms of total cell doublings to a maximum value of 35 to 40 (Table 1). This estimate does not take into account variations in the effects



FIGS. 1-6. Phase photomicrographs of endothelial cells before and after selection.

FIG. 1. Primary culture with smooth-muscle contamination. This plate shows a primary culture of endothelium in a confluent monolayer with overgrowth of a second cell type. If allowed to continue growing, this second cell will become predominant, filling the dish with a "hills-and-valley" growth pattern. At this stage of contamination, cultures were treated by the thymidine protocol. ×175.

FIG. 2. Secondary culture, 2 days following thymidine protocol and passage. This plate shows a culture in the initial passage following thymidine selection. While the thymidine protocol has little obvious effect on cells at the time it is applied, following passage the cells show a variety of cytopathic effects. The most common appearance, as seen here, is of large flattened cells. Similar cells are seen at later passages in the purified cultures (see Figs. 10, 11). ×175.

FIG. 3. Secondary culture, 2 weeks following thymidine protocol and passage. After about 1 week in the initial passage following thymidine selection, the majority of cells recover a normal cobblestone appearance. A few spindle-shaped cells are seen in both sparse and dense cultures at this stage. ×175.

FIG. 4. Tenth passage after thymidine protocol. At later passages, endothelial cells in confluent densities at and below the maximum saturation density appear as a homogeneous monolayer resembling a cobblestone pavement. ×175.

FIG. 5. Fourteenth passage after thymidine protocol, strain C. One culture, strain C, showed a somewhat distinctive shape. While still limited to a monolayer, the cells in this culture were shaped more like teardrops than cobblestones. This culture also showed a small number of sprouting cells. ×175.

FIG. 6. Ninth passage after thymidine protocol, strain D. When cultures were maintained for more than 1 or 2 weeks at postconfluent densities, a second growth pattern appeared. This consisted of elongate, spindle-shaped cells located beneath the monolayer and often aligned end to end to form a mycelial structure. This growth pattern was restricted to focal areas and did not overgrow the cultures. ×175.

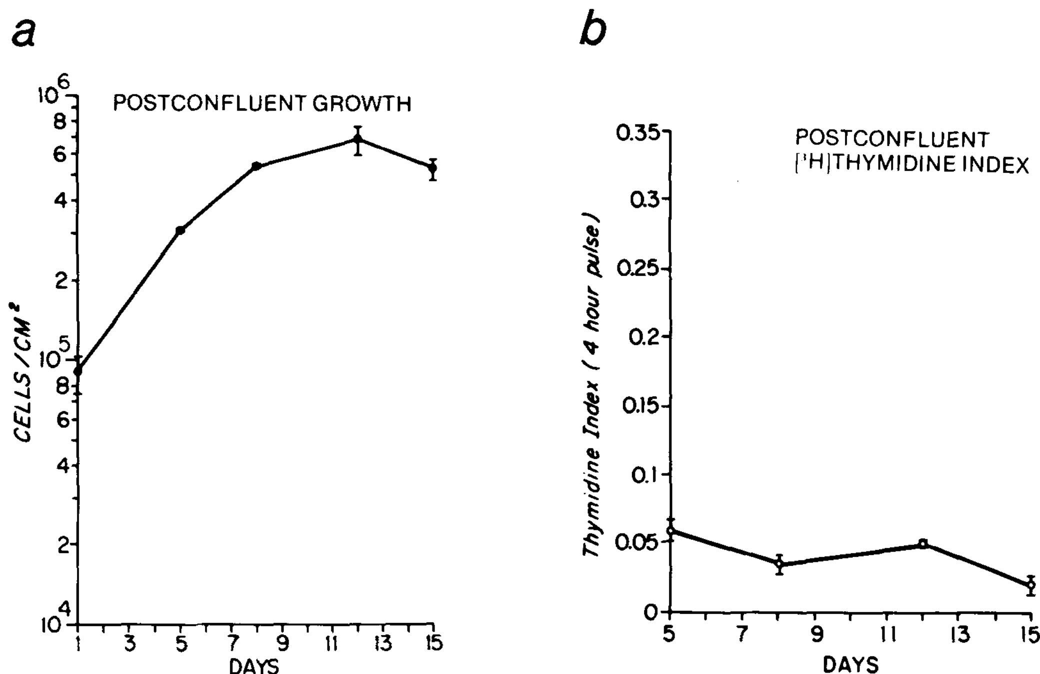


FIG. 7. *a*, Endothelial growth at confluent density; *b*, endothelial replication (thymidine index) at confluent density. Ordinate represents percent labeled cells.

of trypsin at passage, plating efficiencies, or the number of doublings required to overcome the initial toxic effects of tritiated thymidine.

There were two principal changes observed as a function of passage number. First, at higher passages, increasing numbers of flattened cells with extensive cytoplasm were observed (Figs. 10, 11). At earlier passages, these cells were often seen but gradually disappeared as the culture came to confluence. We could not tell whether this was due to a change in morphology of individual cells or it was due to loss of cells from the dish. At later passages, these cells became the predominant cell type, and cells in the culture would no longer divide. Second, the rate of doubling decreased with

TABLE 1

ENDOTHELIAL CELL CULTURES

Strain	Passage No.	Doublings[a]
A	10	10
B[b]	26	32
C[b]	29	38
D	31	42
E	32	44

[a] Based on passaging at a 2:1 ratio until the 20th passage, followed by passaging at a 4:1 ratio until cells attained senescence.

[b] Strains B and C showed extensive evidence of "sprouting" growth pattern.

age. Early-passage cells doubled every 3 to 4 days. By passage 18, this increased to 7 days. Despite the change in growth properties, these cells did not alter their karyotype with increased age. Chromosome number remained at the normal bovine value of 60 (16), and the karyotypes were unaltered when passage 5 was compared with passage 20.

Ultrastructure. Endothelial cells from early passages showed an abundance of organelles, including mitochondria, phagosomes, Golgi apparatus, cytoplasmic filaments, and rough endoplasmic reticulum (Figs. 8, 9). As described by Blose and Chacko (17), the cells contained large numbers of intermediate (100 Å diameter) filaments and smaller amounts of thin filaments (60 to 80 Å diameter). However, dense bodies were rare except at the cell periphery. Weibell-Palade bodies were not seen. In contrast, the typical senescent cell showed an extremely attenuated cytoplasm (Figs. 10, 11) with few organelles.

Human umbilical vein cells. The same technique was applied with less success to the human umbilical vein endothelium again obtained by the collagenase technique. A variety of doses of tritiated thymidine was used over a range from 0.8 μCi per ml to 2.0 μCi per ml, and the same protocol was followed. The initial results regardless of

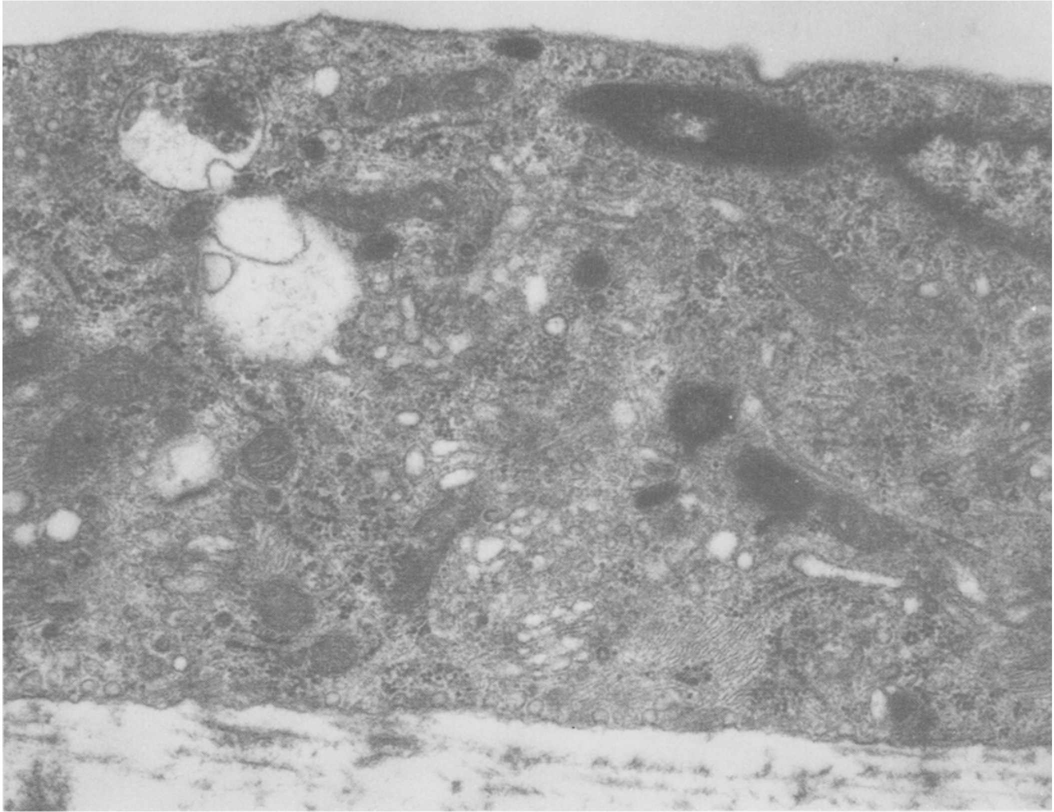


FIG. 8. Electron micrograph of strain D. The cytoplasm contains large numbers of 100 Å filaments. Sheets or strands of flocculent material located beneath the cell resemble normal basal lamina. Unlike the lumenal surface of a typical endothelial cells in vivo, the upper surface of this cell shows few caveolae. The basal surface of the cell shows large numbers of caveolae. This pattern of few caveolae on the upper surface but large numbers on the basal surface was commonly seen. Typically, as seen here, the cytoplasm of early-passage cells was characterized by large amounts or numbers of mitochondria, rough endoplasmic reticulum, Golgi, phagosomes and dense bodies. Weibell-Palade bodies were not seen.

dose were similar to our experience with BAE. Both types survived with continued growth only of the endothelium. However, the treated cells could be passaged no more than three times after thymidine; at that point, most of the cells had the appearance described above for senescent BAE.

Morphology of cells; sprouting. Although the results were otherwise consistent, three distinct morphological patterns were observed which varied with the cell strain. Strains A, D and E had a similar morphology consisting of a monotonous monolayer of small polygonal cells (Figs. 4, 5).

Strains B and C showed two patterns of growth. At low density (less than 3×10^4 cells per cm^2), the cells had a monotonous pattern similar to strain E. However, when maintained long enough to reach a saturation density, these cultures showed focal areas of growth (Figs. 6, 9) by cells in a second growth pattern. This growth pattern consisted of a one-cell-thick layer located beneath the confluent monolayer and comprised of fusiform cells connected end-to-end to form a mycelial or "sprouting" pattern. These cells never proliferated beyond a small number of cells, never formed more than a single layer beneath the monolayer, and did not overgrow the culture even when cultures were maintained for as long as 1 month. Similar foci were found rarely in strain E. An additional characteristic of late-passage cultures was the absence of this pattern in all strains.

Identity of cells in second growth pattern. The identity of the cells in this second growth pattern is obviously a matter of some concern. In essence, this is a genetic question. We wished to know whether cultures like B and C had two subpopulations, perhaps one descended from endothelium and one descended from smooth-muscle cells, or whether these cultures consisted of cells of a single parental type but with potential to grow in more than one pattern.

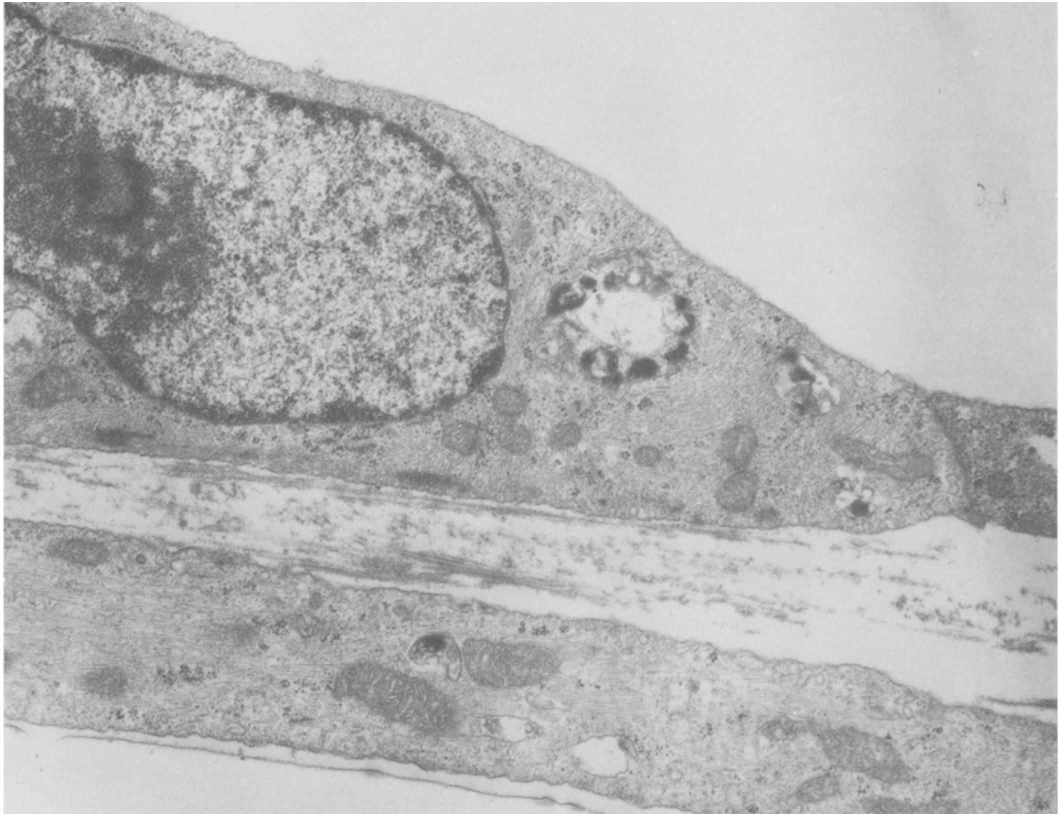


FIG. 9. Electron micrograph of strain B with sprouting. This micrograph shows two cells. The upper cell is part of the endothelial monolayer; the lower cell is part of the second growth pattern, that is the "sprouting pattern." Again, the endothelial cell contains large numbers of intermediate filaments. The basal portion of the cell shows a number of dense bodies as well as some thin filaments (60 to 80 Å diameter). This same pattern of dense bodies is often seen near the albumenal plasma membrane in aortic endothelium of the intact animal. The lower cell resembles the endothelial cell except by its position. Intermediate filaments are plentiful; thin filaments are scarce. No dense bodies are seen. Caveolae are common. As in Fig. 8, amorphous material located between the cells resembles basal lamina. ×17,000.

The simplest approach to this question was to examine cloned cultures. Gospodarowicz and his colleagues reported that this provides an alternative method for establishing pure cultures. Cultures established by cloning showed a sprouting pattern (18). Parenthetically, the cells in Gospodarowicz's procedure were not exposed to tritiated thymidine; however, fibroblast growth factor (FGF) was used in order to facilitate clonal growth (19). The same phenomenon appeared in our study when thymidine-selected cultures were cloned. We did not use FGF. Strain B, at 20 passages after thymidine, was cloned. Twenty clones were plated of which seven grew and could be passaged further. All seven of these clones showed the sprouting growth pattern. Finally, strain C at 12 passages after thymidine was subjected to the thymidine-selection protocol a second time. The resulting cultures again showed the same two distinct growth patterns. Interestingly, the endothelial cells showed very little of the cytopathic effects usually seen in the first passage after thymidine.

The second approach to this problem was to apply available criteria for identifying endothelial cells. Factor VIII immunofluorescence, as introduced by Jaffe, Hayer and Nochman (2, 7), is perhaps the most useful of such techniques. There are no reported cases of endothelial cells being negative or of nonendothelial cells being positive. In our study, pure endothelial cells showed uniform fluorescence (Fig. 12). Moreover, we were able to coculture smooth-muscle cells with endothelial cells and show that only the endothelial cells fluoresced (Fig. 13). As shown in Fig. 14, the

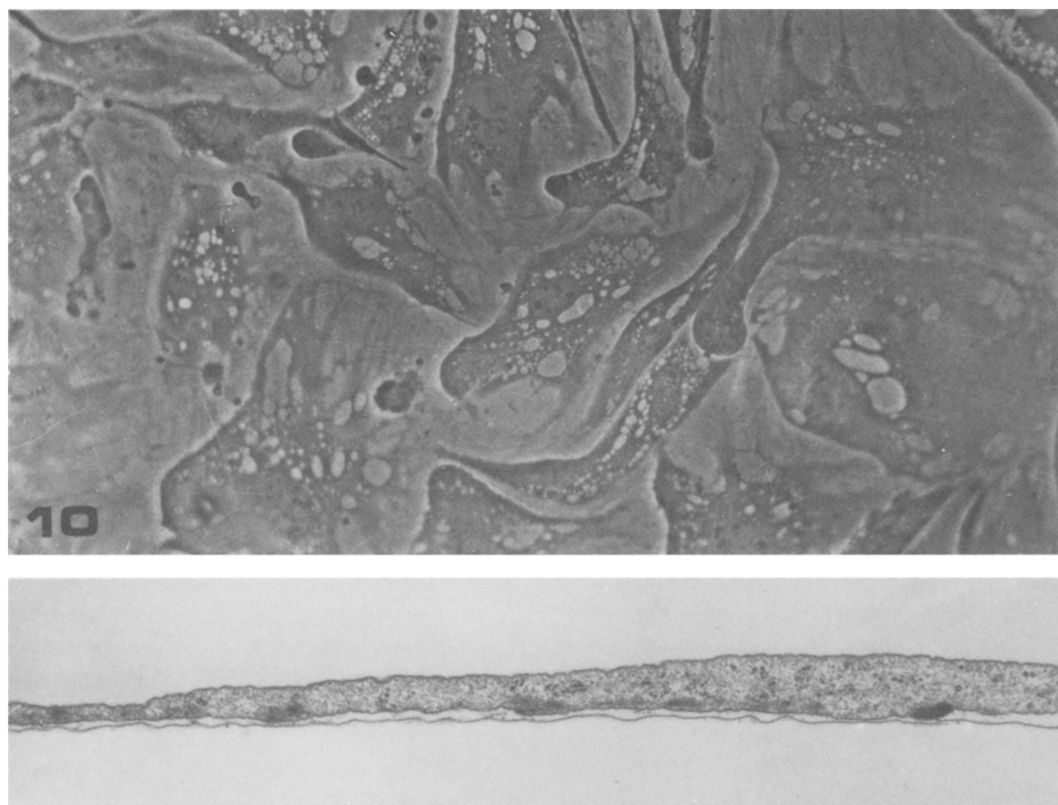


FIG. 10, 11. Senescent cells, strain B, passage 30. At late passage, the cells acquire a large veil-like perikaryon, resulting in a "fried-egg" appearance as seen in the phase microscope (Fig. 10). By electron microscopy, the cells show a cytoplasm containing much fewer organelles than at the earlier passages (Fig. 11). [Fig. 10, light micrograph (phase, ×500); Fig. 11, electron micrograph (×9500).]

sprouting growth pattern also shows positive fluorescence. This property was maintained after cultures were cloned.

Our third approach was to look for evidence of contaminating cells. Since the intima and media of the aorta are made up of endothelium and smooth muscle, the most likely contaminant of these cultures would be smooth-muscle cells. Julie Chamley and Gordon Campbell were visiting our institution at the time of these experiments and had with them an antibody to smooth-muscle actin prepared by Ute Gröschel-Stewart. This antibody has been reported to be specific for smooth-muscle cells (14, 15). They consented to stain cultures, and we provided them with three cultures: bovine smooth muscle, endothelium with little or no evidence of the sprouting pattern, and endothelium with extensive sprouting. Endothelial cultures showed a faint perinuclear staining which was also present in cultures stained with nonactin, hyperimmune serum (Fig. 15). Cells in sprouting areas showed the same perinuclear pattern with little or no staining of the remainder of the cytoplasm (Fig. 16). In contrast, smooth-muscle cultures showed a bright fibrillar pattern throughout the cytoplasm (Fig. 17).

Finally, we examined these cultures by transmission electron microscopy. These images were no more definitive. The best electron-microscopic criterion for endothelial cells is the Weibell-Palade body. Although these structures have been reported in bovine endothelium (4), we were not able to identify the characteristic structure in either cell type in these cultures. This is in agreement with Gospodarowicz's observations of bovine endothelium in culture (19). Although both overlying cells and the undergrowth often contained large numbers of filaments, these were 100 Å diameter intermediate filaments which may be seen in either endothelium or smooth-muscle cells (15, 20) (Figs. 8, 9). Extensive accumulations of thin, 60 Å diameter, cytoplasmic dense bodies

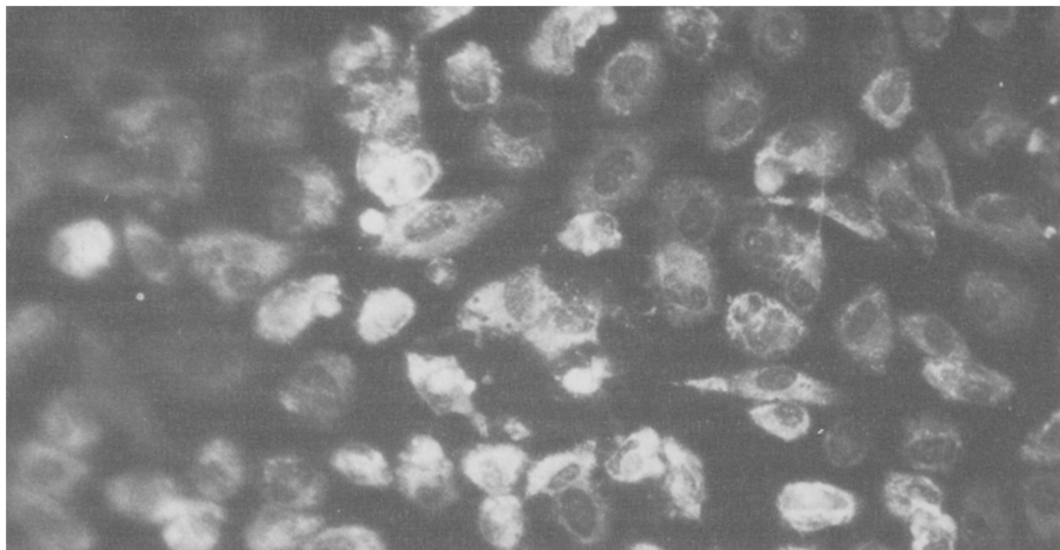


FIG. 12. Antifactor VIII immunofluorescence, strain D, endothelial cells. These cells show uniform, positive fluorescence. ×700.

and numerous surface caveolae, characteristic of smooth-muscle cells (11, 15, 20) were not seen. Other characteristics of the underlying cells were not sufficiently defined to allow differentiation of cell type. It is, however, important to point out that the undergrowth appeared only as a single cell layer separated from and beneath the overlying confluent endothelium. Multiple layers of these cells were never observed although cells did overlap one another. In some areas, individual cells in the monolayer appeared to extend down into the undergrowth suggesting that the sprouting cells may originate from the overlying monolayer.

DISCUSSION

In summary, the thymidine-selection technique provides one approach to the enrichment of density-inhibited cell types—in this case, the endothelium. These endothelial strains maintain

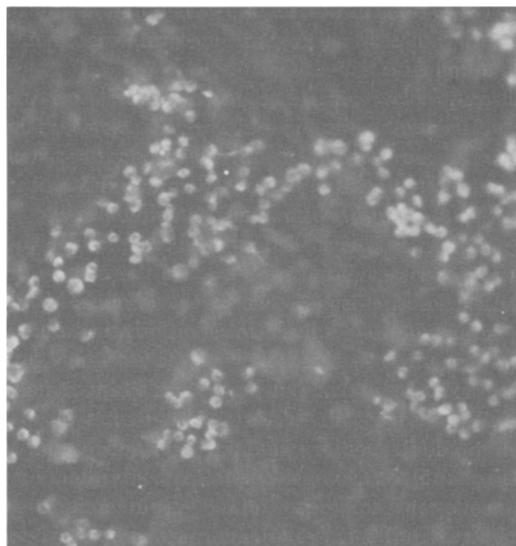


FIG. 13. Antifactor immunofluorescence, strain D, endothelial cells in mixed culture with smooth-muscle cells. Only the endothelial cells show fluorescence. ×65.

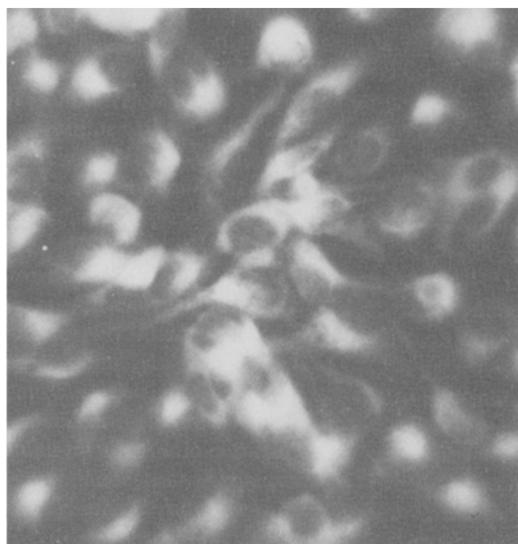


FIG. 14. Antifactor VIII immunofluorescence, strain B, endothelial cells, sprouting area. Sprouting cells show fluorescence. ×700.

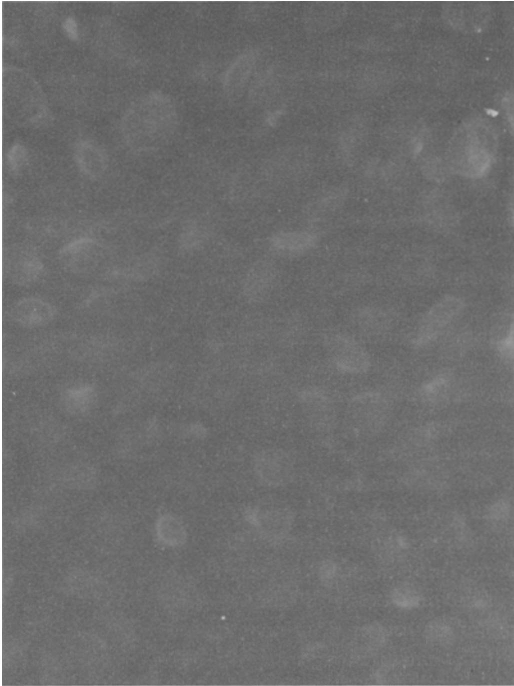


FIG. 15. Antismooth muscle actin immunofluorescence, strain D, endothelial cells. Cells show a faint perinuclear fluorescence. A similar pattern was seen with nonspecific serum also provided by Julie and Gordon Campbell. ×600.

FIG. 16. Antismootn muscle actin immunofluorescence, strain B, endothelial cells with sprouting. Again only a faint fluorescence is seen, primarily located near the nucleus. ×600.

constant karyotypic properties for their in vitro life span and senesce after 35 to 40 doublings. A second, perhaps contaminating, cell type is seen when cells are maintained at postconfluent densities for periods of 1 or 2 weeks.

Utilization of long-term cultures. These techniques were developed because we wished to have a consistent source of characterized cells with known properties. Equally important, the availability of cells of different passage levels makes it

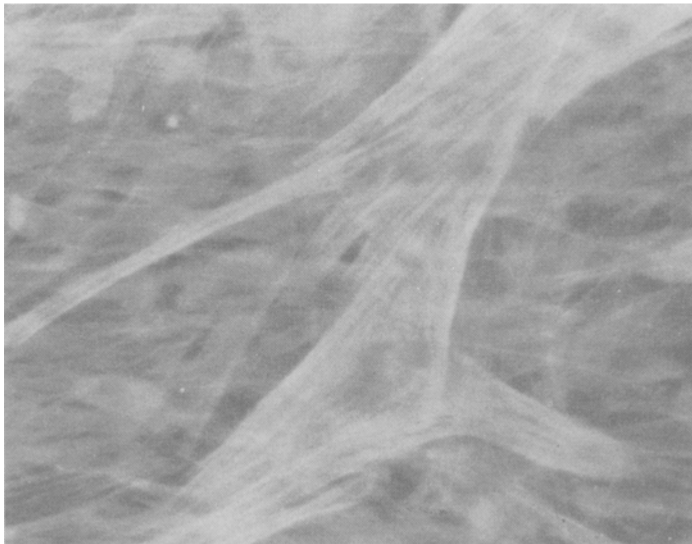


FIG. 17. Antismooth muscle actin immunofluorescence. Cells show uniform, positive fluorescence. ×600.

possible to study the effects of in vitro aging in a normal, diploid cell other than the fibroblast. The now-classic studies with fibroblasts established that cells in vitro replicate only a finite number of times and then undergo a change in morphology and loss of replicative ability (21). Moreover, the in vitro phenomenon parallels a loss of replicative capacity as a function of increasing age of the donor (22). The process of in vitro senescence is particularly interesting in view of the evidence that the focal areas of the endothelium in the intact animal have quite high cell turnover levels. For example, the aortic endothelium of the rat contains focal areas where 10% of the cells labeled with tritiated thymidine in 24 hr (23). If this represents the daily turnover rate, these cells must go through substantial numbers of generations during an animal's life span, and it is conceivable that as an animal ages, its endothelium may lose the capacity to maintain itself as a continuous cell layer.

Thymidine selection. Unlike endothelial cells (5), smooth-muscle cells, the principal contaminant of endothelial cultures, grow to higher saturation densities and can be stimulated from a quiescent state by added growth factors (24). This difference in behavior appears to explain the overgrowth of endothelial cell cultures by smooth muscle. Primary or early-passage endothelial cultures will reach a monolayer with a stable, maximum density of approximately 0.5 to 1.0×10^6 cells per cm^2. In many cases, this monolayer appears homogeneous with no evidence of smooth-muscle cells. This is entirely adequate for most types of experiments, and there is little or no problem in maintaining and passaging the cells over reasonable time intervals. It is also possible, by careful selection of cultures, to achieve relative purity of endothelium. However, in our experience, if this layer is maintained at that cell density for times in excess of 7 to 14 days, at some point a second type will usually appear, proliferate and overgrow the culture with the characteristic growth pattern of smooth-muscle cells (Fig. 1). The most obvious interpretation is that small numbers of contaminating smooth-muscle cells are present and continue to grow beyond the point where endothelium is contact inhibited. This hypothesis suggested to us the possibility of selection for endothelial cells by treatment of postconfluent, SMC-contaminated cultures with an agent active only in the cell cycle. As presented in Results, when the [^{3}H]thymidine-treated cultures are passaged, the endothelial cells replicate preferentially, and even cultures that were previously badly contaminated can be cultured indefinitely without smooth-muscle overgrowth.

Cloning versus thymidine selection. Thymidine selection presents the potential disadvantage of incorporation of [^{3}H]TdR into endothelial cells with subsequent change in cell behavior. Indeed, as mentioned above, definite cytopathic effects were noted in endothelial cells in the initial passage following thymidine selection. The more obvious way to purify a cell is by cloning. This has been described previously by Buonassisi (10) and by Gospodarowicz and his colleagues (18, 19). Cloning, however, has some disadvantages. First, the sample size of a cloned population is, by definition, limited to a single cell. Second, unless endothelial cells are different from other cell types, the number of cells obtainable from a clone will be limited by the replicative life span of the cell. This is an important consideration since a minimum of 20 doublings is required to produce one million cells from a single cell. If cloned cells behave like our selected cells, this would severely limit the number of cells that could be produced. Furthermore, multiple-passaged cloned cells may well show acceleration of in vitro aging and thus may be a poor representation of the tissue of origin. Alternatively, if cloned cells do not age in vitro, then one must suspect spontaneous transformation since an indefinite, prolonged life span is characteristically seen in neoplastically transformed cells. The clonal line of rabbit endothelial cells described by Buonassisi (10) has now been passaged for several years and may fit this description.

Identity of cells. The usual criteria for contamination of endothelial cell cultures by other cell types include the presence of cells not having the characteristic homogeneous endothelial growth pattern and lacking factor VIII antigen (1, 2). The anatomy of the vessel wall and the technique of preparation make the smooth-muscle cell the most likely contaminating cell type. Factor VIII negative cells were not identified in these cultures. In contrast, smooth-muscle cells in both the contaminated primary cultures and in intentionally mixed cultures were factor VIII negative. The decision as to the purity of the cultures was complicated by the presence in three of five strains of a second growth pattern that has already been described by Gospodarowicz and Mecher (18). These cells are factor VIII positive (Fig. 15) and lack any distinguishing characteristics of smooth-muscle cells (11, 14, 15, 20). Furthermore, when

cultures were tested with antibody to smooth-muscle actin (15), smooth-muscle cells reacted positively, but endothelial cultures, including areas of sprouting, were negative (Figs. 15, 16). Gospodarowicz and Mecher (18) found the same growth pattern even in cloned cultures and observed that the sprouting pattern was factor VIII positive. We confirmed these observations, and again the cells with this second growth pattern retained factor VIII positive antigen.

Furthermore, we attempted to repeat the treatment of these cultures with tritiated thymidine. Upon passage following the selection procedure, the sprouting cells reappeared. These data have left us with two possibilities. If we accept the immunologic evidence, then the endothelium has two modes of growth in vitro. This is intriguing since the endothelium in vivo grows as solid cords as part of capillary formation in both wound-healing and embryogenesis. The "sprouting" pattern could represent the same phenomenon in vitro. Alternatively, it is important to remember that the selection process used here is selective for any cells with limited potential for postconfluent growth. This also might be expected to select for a subpopulation of a second cell type that is unable to overgrow the endothelium. The main reason for not believing this is the presence of factor VIII antigen in the "sprouting" cells and the reappearance of this pattern in cloned cells. However, it is important to point out that neither in the present study nor in Gospodarowicz's report were the clones derived from single cells in rigorous isolation. Both studies used dilute plating conditions in which single cells could be identified and the site of these cells cells marked on the culture dish. Clusters of cells arising at these sites were interpreted as clones; therefore the migration of contaminating cells into the site of a supposed clone was a possibility. At least in the Gospodarowicz study, this seems to be an unlikely problem given the degree of dilution of cells used in establishing the clones—5 cells per 15-cm dish.

Thus, although open to question, the available evidence supports the conclusion that under conditions of high cell density, endothelial cells can manifest a second growth pattern. The cells in this growth pattern contain little actin, are negative for factor VIII antigen, never acquire the hill-and-valley pattern characteristic of smooth muscle, and do not overgrow the endothelial monolayer.

Application of the thymidine-selection technique to other systems. One last point to be considered here is the comparison between the thymidine-selection technique and the procedures used by Todaro and Green (25) to establish the 3T3 mouse "fibroblast" lines. In their experiments, cells were transferred at a constant low density (3×10^5 cells per 5-cm petri dish, approximately 1.5×10^4 cells per cm^2) every 3 days. After a number of passages, this resulted in a slow-growing, contact-inhibited cell line—"3T3." The 3T3 cell line grows in a monolayer with morphologic similarities to the monolayer of endothelial cells. Indeed, a number of investigators have suggested that by repeatedly transferring cells to a low density, Todaro and Green effectively selected for a contact-inhibited cell type, perhaps an endothelial cell (26, 27). The only evidence for this endothelial hypothesis is the morphologic growth pattern and the formation of "vaso-formative sarcomas" when 3T3 is implanted in vivo (26). None of the biochemical markers cited in the Introduction, especially the characteristic factor VIII antigen or converting enzyme, has been localized to 3T3, nor has their absence been established. Nonetheless, it is important to point out that the technique used in the present study also is designed to select for a nongrowing or slowly growing population. Techniques similar to this might be of interest in fractionating mixed cell populations, including various fibroblast preparations, according to the growth properties of individual cell types.

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