Serial Propagation of Human Endothelial Cells In Vitro

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ABSTRACT Human umbilical vein (HUV) endothelial cells were grown for 15 to 21 passages at a split ratio of 1:5 (at least 27 population doublings) on a human fibronectin (HFN) matrix in Medium 199 supplemented with fetal bovine serum (FBS) and endothelial-cell growth factor (ECGF). This system also permitted the growth of HUV endothelial cells at cell densities as low as 1.25 cells/cm^2. In addition to delaying the premature senescence of HUV endothelial cells, ECGF also reduced the serum requirement for low-density HUV endothelial-cell growth; 2.5% serum and ECGF yields half-maximum growth as compared to high serum controls. Significant HUV endothelial-cell growth was also observed in medium supplemented with either ovine hypophysectomized (HYPOX) serum, plasma-derived serum (PDS), or HYPOX-PDS in the presence of ECGF, suggesting that neither the pituitary nor the platelet contributes to HUV endothelial-cell growth.

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In recent years major advances in understanding vascular endothelial-cell growth and function have been possible due to the perfection of techniques to isolate, grow, and identify these cells in vitro. Bovine (1, 3, 5, 11, 22, 26) and porcine (14, 31) aortic endothelial cells are widely studied due to the relative ease with which they can be propagated in long-term culture. Although endothelial cells from human pulmonary arteries and veins have been grown in culture (19, 28), umbilical veins are the most readily available source of human large-vessel endothelium. Methods for the isolation and primary culture of these cells are well established (8, 9, 18). However, attempts to subculture the human umbilical-vein endothelial cell through multiple passages have met with limited success. Occasionally, up to 10 to 19 passages of HUV endothelial cells grown on plastic dishes in 20% fetal bovine serum may be accomplished (12, 18) but, in general, these cells cannot be grown reproducibly for more than 2 to 3 passages (8, 9, 29). Although useful information can be gained from the study of primary cultures, extended subculturing facilitates the collection of large quantities of cells for biochemical studies and is necessary to study cell growth properties and requirements, as well as cellular aging in vitro.

We have previously identified a human endothelial-cell mitogen from extracts of bovine hypothalamus prepared at a neutral pH (20). The neural-derived endothelial-cell growth factor (ECGF) is an acid- and heat-labile protein, physically and chemically distinct from fibroblast growth factor (10), possessing a molecular weight of ~ 75,000 (20). The major attribute of ECGF is its ability to stimulate quiescent HUV endothelial cells to grow in culture (20). The addition of ECGF to low-seed-density cultures of HUV endothelial cells in fetal bovine serum results in a significant increase in endothelial cell growth compared to that achieved with serum alone (20). In the present paper we report that the use of ECGF and fibronectin sustained the replicative capacity of HUV endothelial cells in long-term culture.

MATERIALS AND METHODS

Preparation of Reagents

Human fibronectin (HFN) was purified from human Cohn fraction I (Revlon Health Care Group, Tuckahoe, N. Y.) by the method of Engvall and Ruoslahti (6). The HFN preparation migrated as a single band on nonreduced 5% acrylamide SDS-PAGE (SDS-polyacrylamide gel electrophoresis) gels and was stored at −20°C. The ECGF was prepared from bovine hypothalamus as previously described (20). Briefly, bovine hypothalamus (Pel-Freeze Biologicals, Rogers, Ark.) was homogenized for 3 min in 0.15 M NaCl at 4°C (1 liter/1.25 kg of tissue). The homogenate was extracted at a neutral pH and low ionic strength for 1 h, centrifuged, and lipid was removed by extraction with 0.4% streptomycin sulfate (Gibco Laboratories, Grand Island Biological Co., Grand Island, N. Y.) for 18 h at 4°C. The extract was centrifuged and the supernatant fluid stored as a lyophilized powder at 4°C.

Fetal bovine serum (FBS) and other cell culture media and reagents were purchased from Gibco Laboratories (Grand Island, N. Y.). Blood from normal and hypophysectomized (HYPOX), random-bred Suffolk sheep was used for the preparation of ovine whole-blood serum (WBS). Ovine citrated plasma, obtained by venous puncture from normal and hypophysectomized sheep (14 d post-HYPOX), was used for the preparation of normal and HYPOX plasma-derived serum (PDS) using the method of Ross et al. (27). Trace amounts of plasma and platelet-derived cationic mitogens were removed by passing the PDS, HYPOX serum, and HYPOX-PDS through a carboxymethyl Sephadex A-50 (Pharmacia Fine Chemicals, Piscataway, N. J.) column as recommended (27). All sera were stored frozen at −20°C. The ovine PDS, HYPOX serum, and HYPOX-PDS were not capable of supporting the growth of rat abdominal-aorta smooth-muscle

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(RAA-SM) cells in culture. Although control ovine WBS did permit the proliferation of RAA-SM cells, the rate of RAA-SM cell growth was significantly better in FBS.

Preparation and Maintenance of Human Umbilical Vein (HUV) Endothelial Cells In Vitro

Primary cultures of HUV endothelial cells grown in the absence of an HFN matrix or ECGF supplementation were obtained from the laboratory of Dr. M. Gimbrone (8). These primary cultures were harvested and plated on cell-culture dishes coated with 10 μg HFN/cm^2 cell culture-dish surface area (7) and grown in a medium comprised of Medium 199 containing 20% FBS, 100 μg ECGF/ml, penicillin G (10 U/ml), streptomycin (1 μg/ml), and fungizone (5 μg/ml). The cultures were fed every 2–3 d until confluent. At confluence, the cells were harvested by treatment with 0.05% trypsin-0.02% EDTA, the trypsin was inactivated by the addition of 1.25 mg of soybean trypsin inhibitor (Sigma Chemical Co., St. Louis, Mo.), and the cells were routinely passaged at a constant 1:5 split ratio. The endothelial cells at various passages were stored in liquid nitrogen in the growth medium containing 10% glycerol.

Estimation of Replicative and Functional Capacity of HUV Endothelial Cells

For two separate stains of HUV endothelial cells, an aliquot of cell suspension at each harvest was used to count trypan-blue-excluding cells in a hemocytometer. The number of population doublings (PD) occurring between passages was calculated according to the equation: $PD = \log_2 (C_H/C_S)$ where C_H is the number of viable cells at harvest and C_S is the number of cells seeded. The sum of all previous population doublings determined the cumulative population doubling level (CPDL) at each passage. The population doubling time (PDT) was derived using the time interval between cell seeding and harvest divided by the number of PD for that passage.

Samples of lyophilized, conditioned medium from confluent cultures at passages 1 through 13 of one strain of HUV endothelial cells were assayed by radioimmunoassay for human Factor VIII: antigen (fVIII:AGN) by Dr. L. Hoyer (16). Immunofluorescent staining of the HUV endothelial cells for fVIII:AGN was performed on confluent cultures grown in 35-mm dishes. The medium was aspirated, and the cells were washed with phosphate-buffered saline (PBS), fixed in methanol for 5 min at −20°C, and rinsed with PBS. The cells were incubated with a 1:40 (vol/vol) dilution of fluorescein-conjugated rabbit anti-human fVIII: AGN (Atlantic Antibodies, Westbrook, Maine) for 45 min. The dishes were washed three times with PBS, rinsed briefly with distilled water, and mounted with buffered glycerol under glass cover slips. The cells were examined with a reflected Zeiss II epiillumination system. Rat abdominal-aorta smooth-muscle cells (36), rat promegakaryoblasts (35), rat bone marrow (cytocentrifuge slides), and human umbilical cord frozen sections served as positive and negative controls for the immunofluorescence studies.

HUV Endothelial-Cell Attachment Assay

HUV endothelial cells were seeded in Medium 199 at a density of 1.25×10^4 cells/cm^2 in duplicate 35-mm cell culture dishes that had been precoated with various concentrations of HFN. After 10 min at 25°C, the medium was aspirated, and Medium 199 containing 20% FBS and 100 μg/ml ECGF was added to each culture dish. The cells were incubated at 37°C for 2 h, at which time the culture dishes were washed twice with Medium 199, the cells were harvested with trypsin-EDTA, and duplicate hemocytometer cell counts were obtained. Cell culture dishes that were not treated with HFN served as controls.

HUV Endothelial-Cell Growth Assays

All endothelial-cell growth assays were performed with HUV endothelial-cell stock cultures between passages 3 and 11. To ascertain the activity of ECGF, as a function of the concentration of various sera, we seeded cells at a density of 1.25×10^3 cells/cm^2 in Medium 199 containing 100 μg/ml ECGF in 35-mm cell culture dishes coated with 10 μg/cm^2 HFN. Cells plated in Medium 199 containing 20% FBS with or without a standard preparation of ECGF served as controls. The cell culture dishes were fed every 2–3 d with the appropriate supplements, and the assay was terminated after 10 d in culture. The cells were harvested by trypsin-EDTA treatment, and duplicate hemocytometer counts were obtained. Results are reported directly as the mean number of viable endothelial cells per dish ± SD.

RESULTS

Attachment and Growth of HUV Endothelial Cells In Vitro

HUV endothelial cells attached to plastic cell culture dishes with a plating efficiency < 10%. If the cell culture dishes were coated with purified HFN, the plating efficiency of HUV endothelial-cell attachment increased to ~ 40 and 50% after a 10-min incubation. The relationship between HUV endothelial cell attachment and the concentration of HFN is shown in Fig. 1. These results demonstrate that 5–10 μg/cm^2 HFN are required for maximum HUV endothelial-cell attachment.

To ascertain whether HUV endothelial cells were capable of growth on an HFN matrix, we compared the growth of low-seed-density (10^3 cells/cm^2) cells plated on HFN-coated cell-culture dishes to the growth achieved on non-HFN-treated cell culture dishes (Fig. 2). We observed moderate endothelial cell growth on the HFN-coated cell-culture dishes in Medium 199 supplemented with 20% FBS. In contrast, the HFN-free cell culture dishes did not support the growth of low-seed-density HUV endothelial-cell cultures in the presence of 20% FBS. The addition of ECGF to cultures plated on an HFN matrix resulted in a significant increase in endothelial-cell growth (Fig. 2). Although substantial growth was observed in endothelial-cell cultures supplemented with 20% FBS and ECGF in the absence of HFN, no growth was observed in the absence of FBS or ECGF when HFN was omitted. Optimal HUV endothelial-cell growth was routinely achieved in Medium 199 supplemented with 20% FBS and 100 μg/ml ECGF on an HFN matrix (Fig. 2).

Growth of HUV Endothelial Cells at Clonal Cell Density

The HFN matrix also supported the growth of HUV endothelial cells at clonal-cell seed-densities (Figs. 3 and 4*A*). HUV endothelial cells were plated into cell culture dishes precoated with HFN (10 μg/cm^2) at cell densities of 125, 12.5, and 1.25 cells/cm^2. The cells were fed every 2–3 d with Medium 199 containing 20% FBS and 100 μg/ml ECGF. HUV endothelial-cell colonies become visible after ~ 2 wk in culture. After 28 d

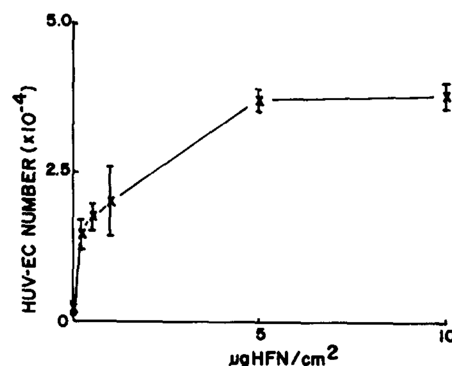

FIGURE 1 HUV endothelial cell attachment as a function of the concentration of human fibronectin (HFN). HUV endothelial cells (passage 5) were plated in a cell-attachment assay as described in Materials and Methods. The HFN matrix was prepared by adding the appropriate concentration of HFN to a 35-mm cell culture dish containing 1 ml of Dulbecco's PBS. The culture dish was allowed to adsorb the HFN for 10 min at room temperature, after which excess HFN was aspirated. The HFN-coated dishes were used immediately after preparation. The data are expressed as viable HUV endothelial-cell number ± SD.

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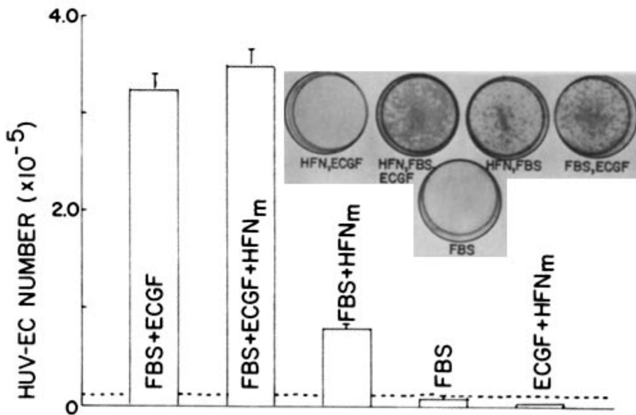

FIGURE 2 Effect of *HFN* and *ECGF* on *HUV* endothelial-cell growth. HUV endothelial cells (passage 9) were seeded at a density of 1.25 × 10^3 cells/cm^2 on cell culture dishes in the presence and absence of an HFN matrix (10 μg/cm^2). The cultures were fed with the appropriate medium (*ECGF*), 100 μg/ml, and 20% *FBS*, for a period of 10 d. The plates were washed twice with Medium 199 and either fixed with 10% formalin and stained with 0.1% aqueous crystal violet or the cells were treated with trypsin-EDTA and the mean viable cell number (± SD) was determined.

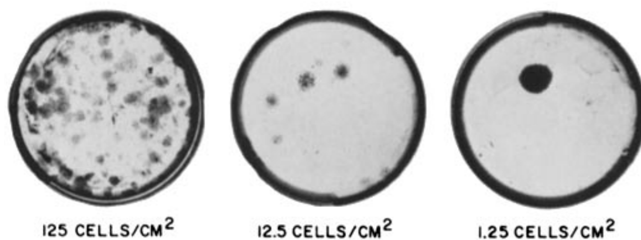

FIGURE 3 Growth of HUV endothelial cells at clonal cell density. Cell culture dishes were pretreated with 10 μg/cm^2 HFN. HUV endothelial cells (passage 10) were seeded in growth medium containing Medium 199, 20% FBS, and 100 μg ECGF/ml at seed densities of 125, 12.5, and 1.25 cells/cm^2. The cultures were fed every 2-3 d with the growth medium for a period of 28 d. At this time, the culture dishes were washed with Medium 199, fixed with 10% formalin, and stained with 0.1% aqueous crystal violet.

in culture, distinct colonies of various sizes could be observed (Fig. 3). It is important to note that HUV endothelial cells did not survive in control culture dishes that were neither coated with HFN nor supplemented with ECGF. Selected colonies were removed by treatment with trypsin-EDTA in cloning wells and transferred to plastic microtiter dishes pretreated with HFN. The seed density of the transfer was ~ 10^2 HUV endothelial cells per microtiter well. Although the endothelial cells survived the transfer and grew in Medium 199 supplemented with 20% FBS and 100 μg/ml ECGF, subsequent transfers of the confluent HUV endothelial-cell monolayer from the microtiter wells to 35-mm cell culture dishes (HFN-treated) were not successful.

The Long-term Growth of HUV Endothelial Cells

The use of HFN-coated cell culture dishes and ECGF supplementation in Medium 199 containing 20% FBS also permitted the long-term growth of HUV endothelial cells in vitro. We have routinely passaged these cells from primary isolates at a constant 1:5 split ratio for 15–21 times before the cultures became senescent and expired. Cultures grown without HFN and ECGF and passaged at this density ceased to grow beyond the second passage.

Within 7–8 d after seeding, the HUV endothelial cells had consistently formed a confluent epithelioid monolayer as observed by phase microscopy (Fig. 4 *B, C, D*). Cells within the monolayer possessed characteristic single ovoid nuclei with 2–3 prominent nucleoli and perinuclear granules. In mid to late passage cultures, the cells were more elongated and less densely packed at confluence (Fig. 4 *C*) than in early passages where cells appeared to have a smaller attachment area and formed a typical cobblestone pattern (Fig. 4 *B*). Giant, multinucleated cells with very broad and veil-like cytoplasm began to appear with a low incidence (one cell per 10^3 cells) at about passage 8 and gradually increased in number (one cell per 10^2 cells) in later passages (Fig. 4 *D*). At no time did we observe smooth musclelike cells in any of these cultures.

HUV endothelial cells from two strains grown from independent primary cultures completed between 2 and 3 PD at each passage from passage 2 to passage 15. The CPDL for one strain at passage 13 was 27 and for the other at passage 15 was 34. The density of cells at confluence showed a decreasing linear relationship with the CPDL (Fig. 5 *A*). The overall PDT did not increase significantly with CPDL (Fig. 5 *B*), remaining an average of 2.8 d ± 0.8 (SD) for the two strains of HUV endothelial cells.

The HUV endothelial cells were positive for fVIII:AGN by immunofluorescent staining throughout their lifespan in culture. However, in late passages there were qualitatively more cells with fewer grains per cell (Fig. 4 *F*) than at early passages where most cells were brightly stained (Fig. 4 *E*). The giant HUV endothelial cells also contained the fVIII:AGN (Fig. 4 *F*) at a qualitatively reduced level. The concentration of fVIII: AGN in the conditioned medium for one of the cell strains averaged 2.25 U/100 ml ± 1.09 (SD) for early passages (passage 1–7) and 1.80 U/100 ml ± 0.40 for later passages (passage 8–13). This represented a concentration of fVIII:AGN of 1.15 ± 0.48 U/100 ml per HUV endothelial cell in early passages and a similar level of 1.57 ± 0.39 U/100 ml per HUV endothelial cell for late passages.

HUV Endothelial-Cell Growth in Various Sera

FBS titration curves were performed at a constant concen-

FIGURE 4 Phase-contrast photomicrographs and fVIII:AGN immunofluorescent staining of HUV endothelial cells. Frames *A*, *B*, *C*, and *D* are same magnification (×200). (*A*) Colony of HUV endothelial cells (passage 10) after 3 d in culture in Medium 199 containing 20% FBS and 100 μg ECGF/ml (1.25 × 10^2 cells/cm^2). (*B*) Confluent monolayer of HUV endothelial cells (passage 2). (*C*) Confluent monolayer of HUV endothelial cells (passage 8). Note the large cells (arrows). (*D*) Confluent monolayer of HUV endothelial cells (passage 18). Note the enlarged veil-like multinucleate cell (arrow) pushing against the tightly apposed polygonal cell. Frames *E* and *F* are same magnification. (×250). Human anti fVIII:AGN immunofluorescence was performed as described in Materials and Methods. (*E*) HUV endothelial cells (passage 8). (*F*) Large multinucleated HUV endothelial cell (passage 18). Note qualitative difference in staining intensity between frames *E* and *F*.

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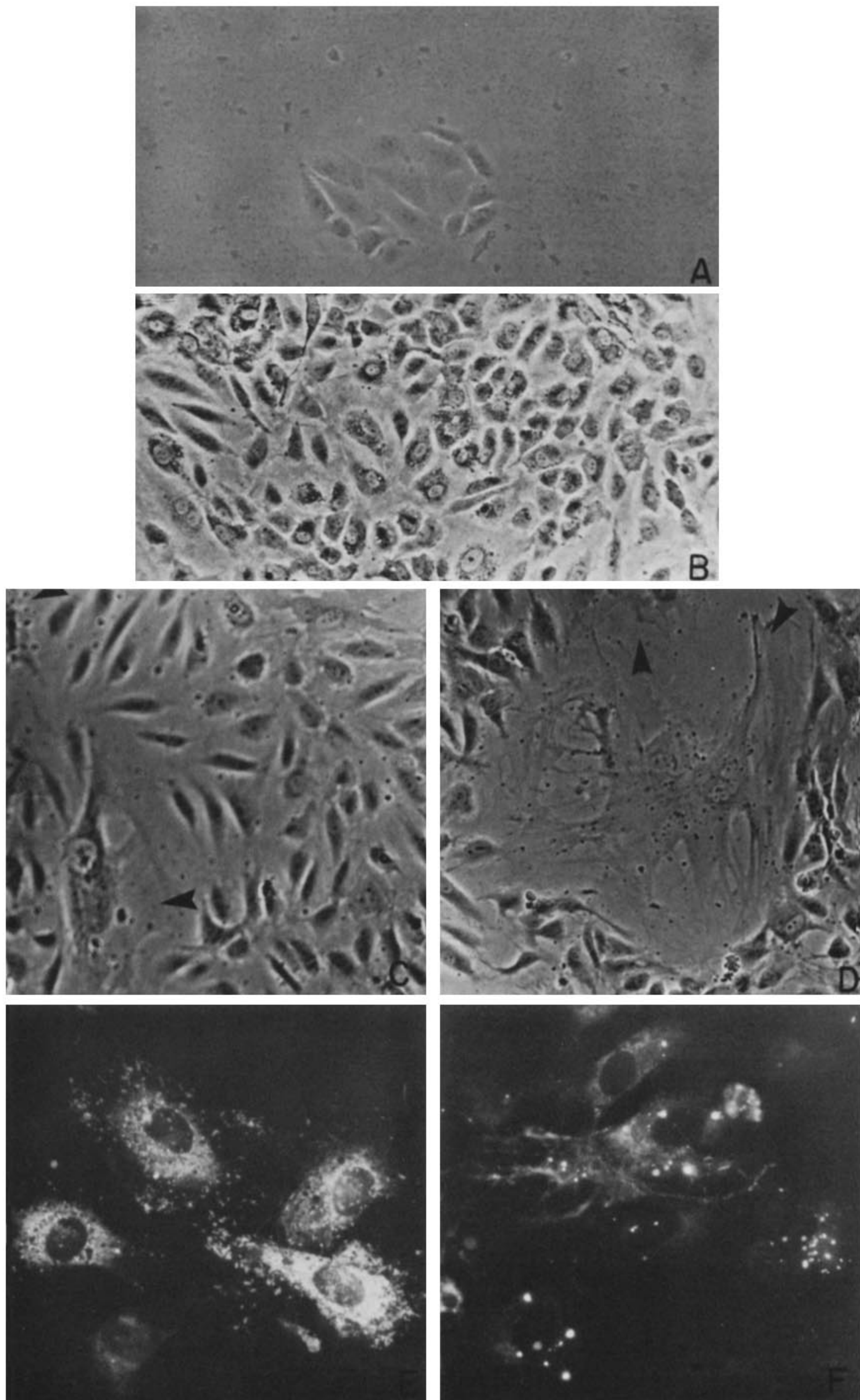

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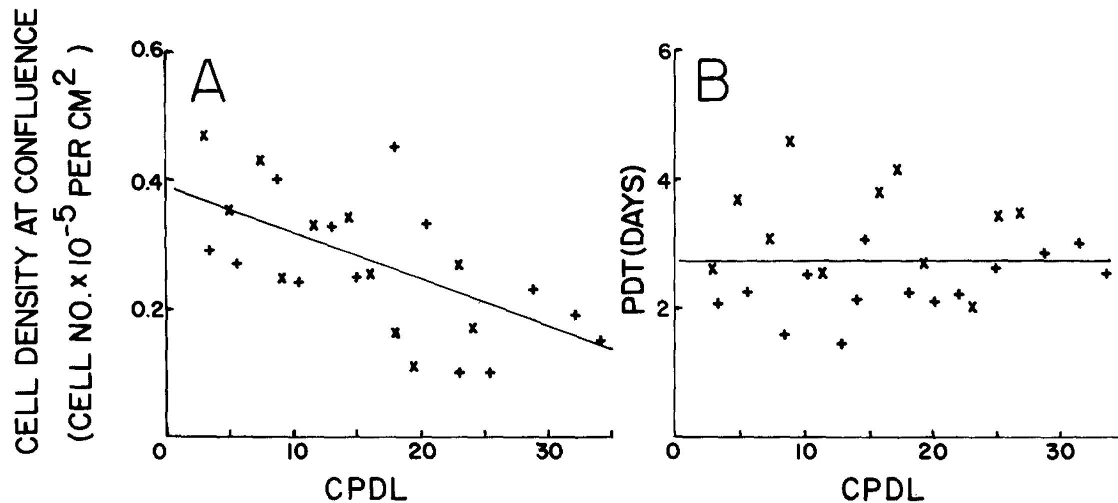

FIGURE 5 Growth characteristics of HUV endothelial cells in long-term culture. Stock cultures of two cell strains (+, X) were passaged at a constant split ratio of 1:5 as described. (*A*) Cell density at confluence *CPDL*. The data were fitted by the method of least squares to the equation: cell density at confluence = 0.39 (± 0.04) − 0.007 (± 0.002) *CPDL*. Numbers in brackets denote standard deviations of the coefficients. The F statistic associated with curve fit was 14.97, $P < .001$. (*B*) Population-doubling time (*PDT*) versus *CPDL*. The line fitted by the least squares method was: *PDT* = 2.68 (± 0.34) + 0.004 (± 0.018) *CPDL*. F = 0.05, $P < 0.05$.

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tration of ECGF on a HFN matrix to determine the relationship between HUV endothelial cell growth and the concentration of FBS. We observed considerable HUV endothelial-cell growth in FBS concentrations as low as 5% (vol/vol). Approximately half-maximum growth was obtained in 2.5% FBS (Fig. 6). Ovine WBS, PDS, and HYPOX serum also were capable of supporting HUV endothelial-cell growth in the presence of ECGF (Fig. 7). Although the cultures supplemented with various concentrations of serum without ECGF did survive, the amount of HUV endothelial-cell growth in these cultures was minimal.

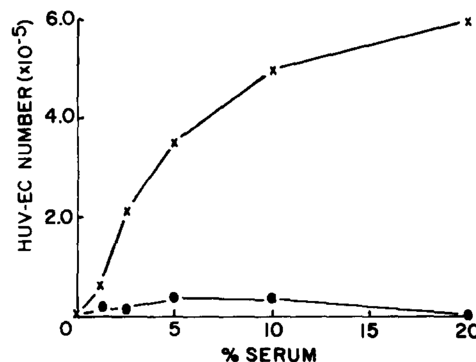

FIGURE 6 FBS titration curve of HUV endothelial-cell growth. HUV endothelial cells (passage 7) were plated on a HFN matrix (10 μg/cm^2) at a seed density of 1.25 × 10^3 cells/cm^2. The cultures were supplemented with various concentrations of FBS in the absence (●) and presence (X) of 100 μg/ml ECGF. The cultures were fed with the appropriate medium every 2-3 d for a total of 10 d. The cells were harvested by treatment with trypsin-EDTA and the number of viable cells was counted.

DISCUSSION

The difficulty in establishing long-term cultures of HUV endothelial cells grown on plastic substrates in medium containing 20% serum is well recognized. Using this traditional system, we were not able to cultivate these cells beyond the third passage. However, we have demonstrated that HUV endothelial cells can consistently propagate in long-term culture when grown on an HFN matrix in serum-supplemented medium containing ECGF. Under these conditions, the HUV endothelial cells attain at least 27 PD. A similar increase in the in vitro life span of cells by the addition of a growth factor has been achieved with human keratinocytes, which experience a significant delay in senescence when EGF is added to the culture system (23).

The decrease in the density of HUV endothelial cells in confluent cultures as a function of the CPDL is consistent with our qualitative observations by phase microscopy of a greater cell attachment area in later passages. These observations suggest that the HUV endothelial cells were either becoming broader or actually increasing in volume as a function of in vitro age. A decrease in confluent cell density with increasing age has also been demonstrated in bovine fetal aortic endothelial cells for which a measured increase in cell attachment area corresponds to larger cell volumes in older populations (26). The increasing frequency of extremely large HUV endothelial cells at later passages probably accounts for much of the decrease in confluent cell density in our older cultures.

Although the late-passage HUV endothelial cells maintained their morphological integrity, we have observed the appearance of large and morphologically distinct, fVIII:AGN -positive cells. We have interpreted these giant cells to be senescent HUV endothelial cells, as have other investigators observing both human and bovine endothelial cells in vitro (12, 29).

We did not observe an increase in PDT of the HUV endothelial-cell cultures measured through 34 PD, although confluent cell density decreased during this time. Similarly, we did not observe a change in the concentration of extracellular human fVIII:AGN from early-to late-passage HUV endothelial-cell cultures. However, the strains of HUV endothelial cells from which these measurements were taken had not yet achieved the end of their life spans. A more detailed study of cellular senescence of the HUV endothelial cells may indicate that their growth rate does decline at later stages, as does the doubling rate in adult (29) and fetal (26) bovine aortic endothelial-cell cultures. This suggestion is consistent with the observation of Rosen et al. (26) who demonstrated that a decrease in confluent culture density precedes a change in growth rate

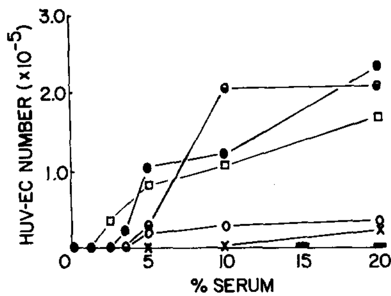

FIGURE 7 HUV endothelial-cell growth as a function of the concentration of ovine WBS, PDS, and HYPOX serum. HUV endothelial cells (passage 9) were plated on a HFN matrix (10 μg/cm^2) at a seed density of 1.25 × 10^3 cells/cm^2. The cultures were supplemented with the following reagents: increasing concentrations of ovine (PDS) in the absence (×) and presence of 100 μg/ml ECGF (●), ovine HYPOX WBS in the absence (○) and presence (◒) of 100 μg/ml ECGF, and ovine normal WBS in the absence (■) and presence (□) of 100 μg/ml ECGF. The cultures were harvested on day 10 of the growth assay, at which time the HUV endothelial-cell number was determined.

in bovine fetal aortic endothelial cells.

The absence of HUV endothelial-cell growth at cell-seed-densities of ~ 10^3 cells/cm^2 in the presence of FBS without ECGF and the appearance of moderate endothelial-cell growth with ECGF suggest that ECGF is required for the growth and survival of HUV endothelial cells. In contrast, HUV endothelial cells at seed densities < 10^3 cells/cm^2 did not survive and grow in FBS- and ECGF-supplemented medium. These results suggest the requirement of attachment factor(s) for cell survival and growth at very low cell-seed-densities. We have identified one such attachment factor as plasma-derived HFN. Treatment of the cell culture dish with 10 μg/cm^2 HFN enabled HUV endothelial cells to plate with optimum efficiency. Although HFN is present in serum (15), the concentration of biologically active HFN is probably very low because coating the surface of the cell culture dish with serum does not abolish the HFN requirement for low cell-density HUV endothelial-cell attachment. Excellent HUV endothelial-cell growth, independent of seed density, was achieved in cultures supplemented with FBS and ECGF on an HFN matrix. On the other hand, only moderate HUV endothelial-cell growth was obtained at seed densities > 10^3 cells/cm^2 on HFN-coated cell culture dishes supplemented with FBS without ECGF. These data suggest that ECGF significantly contributes to HUV endothelial-cell growth and survival on an HFN matrix. Furthermore, these data are consistent with our ability to achieve serial propagation of the HUV endothelial cell in vitro.

The advantage of using an HFN matrix for HUV endothelial-cell growth in serum-supplemented medium containing ECGF is highlighted by the ability of such a culture system to support the growth of HUV endothelial cells at clonal seed densities (125–1.25 cells/cm^2). The observation that HUV endothelial cells failed to survive and grow at clonal seed densities in ECGF- and FBS-supplemented cultures lacking a HFN matrix suggests that ECGF was not acting as an attachment factor for HUV endothelial cells. These results are consistent with the observation that ECGF can stimulate the growth of relatively quiescent low-seed-density (10^3 cells/cm^2) HUV endothelial-cell populations (20).

The failure to achieve multiple passages of the HUV endothelial-cell colonies may be due either to a finite limit on the number of endothelial-cell PD or to the lack of additional factors required for HUV endothelial-cell survival and growth at very low seed densities. It is of interest that we have been able to routinely observe, by phase-contrast microscopy, HUV endothelial-cell colonies of various sizes suggesting that growth rates differ between individual colonies.

Numerous investigators have shown that serum supplies hormones and growth factors for mammalian cell growth in vitro (13, 17). The use of ECGF in low-density HUV endothelial-cell culture significantly reduced the FBS requirement for endothelial-cell growth suggesting that ECGF is acting in concert with existing hormones and growth factors present in FBS. The ability of ovine HYPOX serum and ECGF to support HUV endothelial-cell growth suggests that the pituitary-derived hormones do not significantly contribute to the HUV endothelial-cell growth-promoting capabilities of serum. These results are in agreement with the observation that substantial in vivo vascular endothelial-cell regrowth occurs in balloon-deendothelialized HYPOX rats (33). Similarly, the achievement of HUV endothelial-cell growth in either ovine PDS or HYPOX-PDS supplemented with ECGF and confirms other observations that platelet-derived growth factors are not required for endothelial cell growth (32, 34). Because HUV endothelial cells grew as a function of the concentration of either ovine PDS or HYPOX serum in the presence of ECGF, it is likely that (*a*) the hormones and growth factors that are supplied by serum for HUV endothelial-cell growth are common to FBS, ovine PDS, and HYPOX serum, and (*b*) the hormone components present in serum that are required by HUV endothelial cells for growth act in a synergistic manner with ECGF. The latter suggestion is consistent with the demonstration of hormonal synergy in a wide variety of mammalian cell culture systems grown in a serum-free, hormonally-defined environment (2, 21, 25).

The requirements for the in vitro growth of HUV endothelial cells appear to be different from the requirements for the vascular smooth-muscle cells. We have previously demonstrated that the vascular smooth-muscle cell requires platelet-derived growth factor and the somatomedins, insulin-like growth factor-I (24) and multiplication-stimulating activity (4) for optimal growth in a serum-free environment (36). Because the vascular smooth-muscle cell does not proliferate in HYPOX rats after in vivo balloon deendothelialization (33) and proliferates poorly in vitro in the presence of HYPOX-PDS (Maciag, T. and R. Weinstein, unpublished observation), the use of HYPOX-PDS may be valuable in the control of smooth-muscle-cell growth, a troublesome contaminant in vascular endothelial-cell culture.

Because ECGF and HFN permit the long-term cultivation of HUV endothelial cells and reduce the serum requirement for low-seed-density growth, one can presently study the biological effects of human endothelial cell-responsive hormones on human endothelial-cell growth and function. Further refinement of this system promises to facilitate the development of a completely defined serum-free system for the cultivation of human endothelial cells and lead to an understanding of those hormones responsible for human endothelial-cell growth. Although we have limited our approach to a study of human endothelial-cell-culture systems, the application of these results to endothelial cells from other species should be encouraged.

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