Organizational Behavior of Human Umbilical Vein Endothelial Cells

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ABSTRACT Culture conditions that favor rapid multiplication of human umbilical vein endothelial cells (HUV-EC) also support long-term serial propagation of the cells. This is routinely achieved when HUV-EC are grown in Medium 199 (M-199) supplemented with fetal bovine serum (FBS) and endothelial cell growth factor (ECGF), on a human fibronectin (HFN) matrix. The HUV-EC can shift from a proliferative to an organized state when the in vitro conditions are changed from those favoring low density proliferation to those supporting high density survival. When ECGF and HFN are omitted, cultures fail to achieve confluence beyond the first or second passage: the preconfluent cultures organize into tubular structures after 4–6 wk. Some tubes become grossly visible and float in the culture medium, remaining tethered to the plastic dish at either end of the tube. On an ultrastructural level, the tubes consist of cells, held together by junctional complexes, arranged so as to form a lumen. The smallest lumens are formed by one cell folding over to form a junction with itself. The cells contain Weibel-Palade bodies and factor VIII-related antigen. The lumens contain granular, fibrillar and amorphous debris. Predigesting the HFN matrix with trypsin (10 min, 37°C) or plasmin significantly accelerates tube formation. Thrombin and plasminogen activator had no apparent effect. Disruption of the largest tubes with trypsin/EDTA permits the cells to revert to a proliferative state if plated on HFN, in M-199, FBS, and ECGF. These observations indicate that culture conditions that do not favor proliferation permit attainment of a state of nonterminal differentiation (organization) by the endothelial cell. Furthermore, proteolytic modification of the HFN matrix may play an important role in endothelial organization.

Normal human diploid cells in culture require serum as a source of hormones and growth factors for cell division (13). The human umbilical vein endothelial cell (HUV-EC) is a fastidious cell with only limited proliferative potential in serum-supplemented medium (11, 22). Since serum supplies hormones and growth factors for cell growth in vitro (13, 21, 38), it can be argued that serum is deficient in the requisite hormone(s) responsible for HUV-EC growth. This approach to HUV-EC growth control has resulted in the identification and characterization of an endothelial cell growth factor (ECGF), partially purified from bovine hypothalamus (20), that stimulates the growth of HUV-EC in vitro (20, 22). In the presence of a human fibronectin (HFN) matrix, ECGF permits the growth of HUV-EC at clonal cell densities, reduces the serum requirement for HUV-EC growth, and permits the serial propagation of the HUV-EC (22). One can presently achieve at least 34 cumulative population doublings in an environment composed of a HFN matrix and medium 199 supplemented with ECGF and fetal bovine serum (22).

In the absence of HFN and ECGF, HUV-EC do not proliferate beyond 2 or 3 passages. However, under these conditions, the cell population actively organizes into three-dimensional tubular structures. Our observations suggest that this organizational behavior, most readily exhibited by HUV-EC cultures maintained under conditions not favoring rapid proliferation, may represent nonterminal differentiation of the HUV-EC population in vitro.

MATERIALS AND METHODS

Preparation of Reagents

Human fibronectin (HFN) was prepared from human Cohn fraction I (Revlon Health Care Group, Tuckahoe, NY) as previously described (9). SDS PAGE of the purified HFN preparation revealed a single band (5% acrylamide), while SDS

PAGE under reducing conditions generated two HFN chains. The endothelial cell growth factor (ECGF) was prepared from bovine hypothalamus (Pel-Freeze Biologicals, Rogers, AR) as previously described (20).

All cell culture reagents, including fetal bovine serum (FBS) and Medium 199, were obtained from Gibco Laboratories (Grand Island Biological Co., Grand Island, NY). Human alpha-thrombin and plasminogen were kind gifts of Dr. D. Bing (Center for Blood Research, Boston, MA). Human kidney plasminogen activator (55,000 dalton, urokinaselike form) was kindly supplied by Dr. Rydichi Naito (Green Cross Corp., Osaka, Japan). Goat anti-human urokinase (UK) antiserum was provided by Dr. F. Booyse (Michael Reese Research Foundation, Chicago, IL). Fluorescein-conjugated rabbit anti-human factor VIII-related antigen (f-VIII:Ag) antiserum was purchased from Atlantic Antibodies (Scarborough, ME) and rabbit anti-human HFN antiserum was obtained from Collaborative Research Inc. (Waltham, MA).

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

Cultures of primary HUV-EC established in the absence of a HFN matrix or ECGF supplementation were the generous gift of Dr. M. Gimbrone (Harvard Medical School, Boston, MA). The cells were harvested by treatment with 0.05% trypsin–0.02% EDTA, the trypsin inactivated with 1.25 mg soybean trypsin inhibitor (Sigma Chemical Co., St. Louis, MO) and plated on a HFN-coated cell culture dish (10 $\mu g/cm^2$) in Medium 199 supplemented with 20% FBS and 150 μg ECGF/ml. The HUV-EC were routinely grown and passaged in this system for at least 34 cumulative population doublings. At various passage levels, HUV-EC were collected and stored frozen in liquid nitrogen in the growth medium containing 10% glycerol.

Immunofluorescence and Transmission Electron Microscopy

Immunofluorescent staining of HUV-EC for endothelial cell-derived antigens was performed on cell monolayers that were fixed in methanol for 5 min at −20°C and rinsed with phosphate buffered saline (PBS). The cells were incubated with either the appropriate fluorescein-conjugated or nonconjugated antibody (FITC-Anti-f-VIII:Ag, 1:40 dilution; Anti-HFN, 1:20 dilution; Anti-UK, 1:20 dilution) for 45 min at 25°C. In the case of the non-FITC-conjugated antibodies, the specimens were washed three times with PBS and the appropriate FITC-conjugated second antibody added (Atlantic Antibodies) for 45 min at 25°C. The specimens were again washed three times with PBS and mounted with buffered glycerol under glass cover slips. The cells were examined with a reflected Nikon epiilumination system. Human umbilical cord frozen sections and normal human melanocytes served as positive and negative controls.

Evaluation of the HUV-EC by electron microscopy was performed by washing the cell culture dish and fixing the specimen in the cell culture dish with 2.5% gluteraldehyde in 100 mM cacodylate buffer, pH 7.4. Specimens for transmission electron microscopy were fixed for 1 h in the fixative buffer, postfixed in 10% osmium tetroxide buffered with cacodylate and then stained en bloc with 1% uranyl acetate for 30 min. The samples were dehydrated through ethanol, embedded in Epon-812 and 1-μm thick sections prepared and stained with toluidine blue. Thin sections were stained with uranyl acetate and lead citrate and examined with a Phillips 300 electron microscope (31).

RESULTS

Organizational Behavior of HUV-EC In Vitro

The use of HFN and ECGF significantly delays the pre-

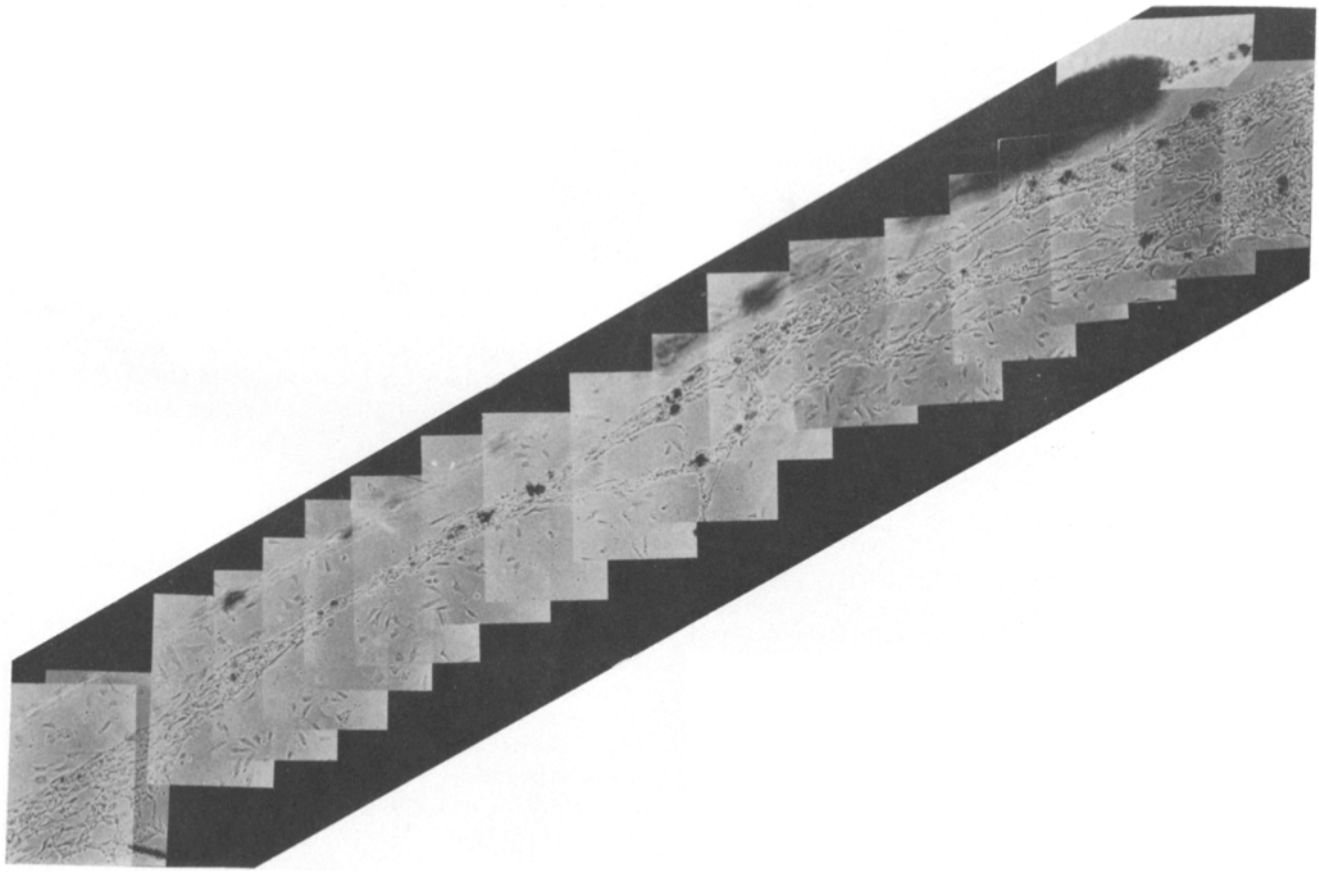

FIGURE 1 Phase-contrast photomicrograph of organized HUV-EC population. Primary cultures of HUV-EC were digested with trypsin and split into plastic cell culture dishes at a 1:5 split ratio. The culture was supplemented with Medium 199 containing 20% FBS. The culture was fed every 2–3 d for a period of ~2 mo. At this time, white, bulbous treads were macroscopically visible. Overlapping phase-contrast photomicrographs were taken (× 250). A linear collage of the phase-contrast photomicrograph is presented. Note the complex weblike structures attached to the surface of the culture dish and the large threadlike structure (not focused) that is floating longitudinally above the surface of the dish and is attached at one end of the cell culture dish. Also note the dramatic change in diameter and the presence of a dark luminal interior in the floating tubular structure.

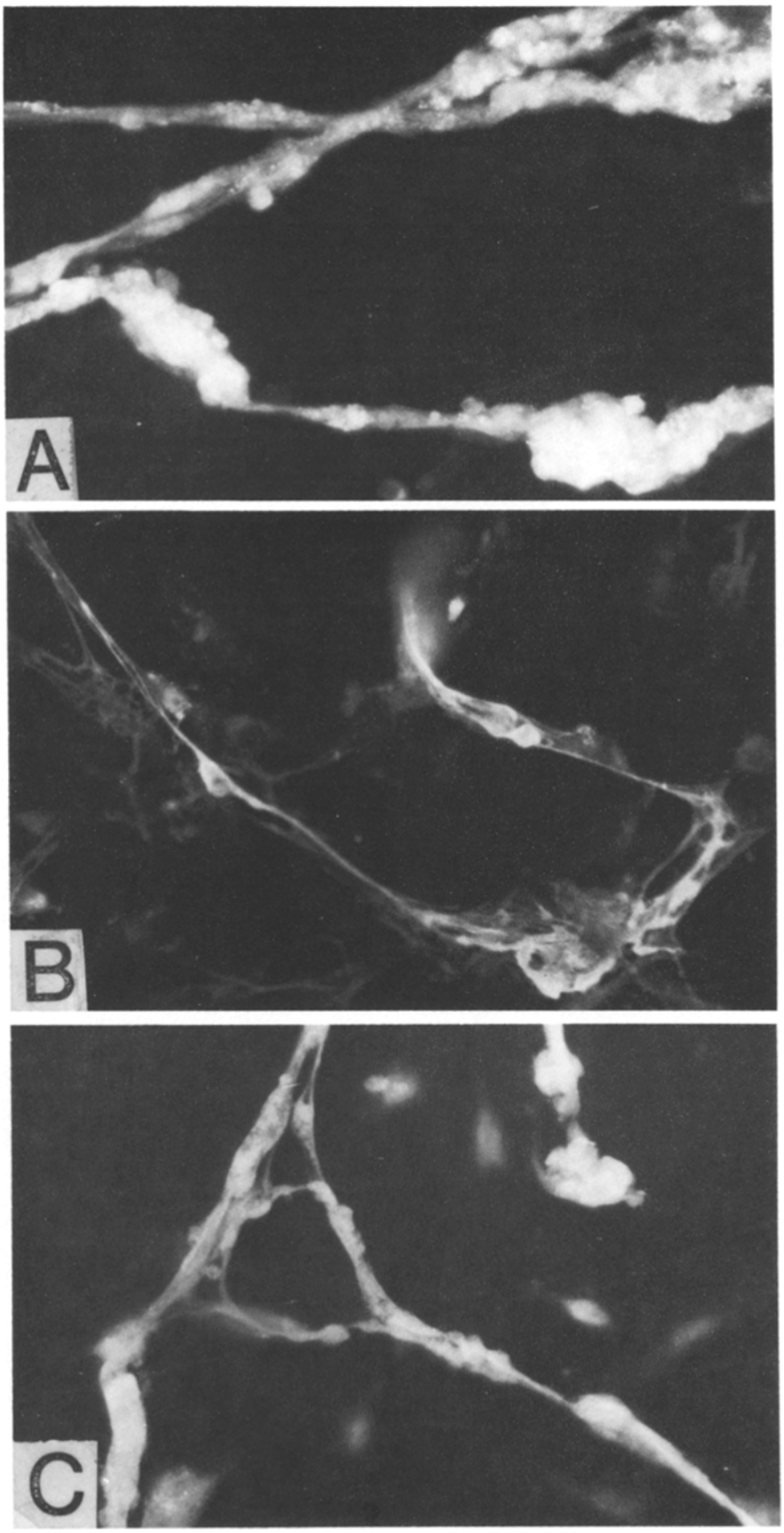

FIGURE 2 Immunofluorescence of the HUV-EC organized structures with anti-human factor VIII:Ag, plasminogen activator and fibronectin. The weblike tubular HUV-EC-derived array was stained for the human f-VIII:Ag, plasminogen activator and fibronectin as described in Materials and Methods: (*A*) organized HUV-EC stained with anti-human f-VIII:Ag, (*B*) anti-human fibronectin, and (*C*) anti-human kidney plasminogen activator. × 250.

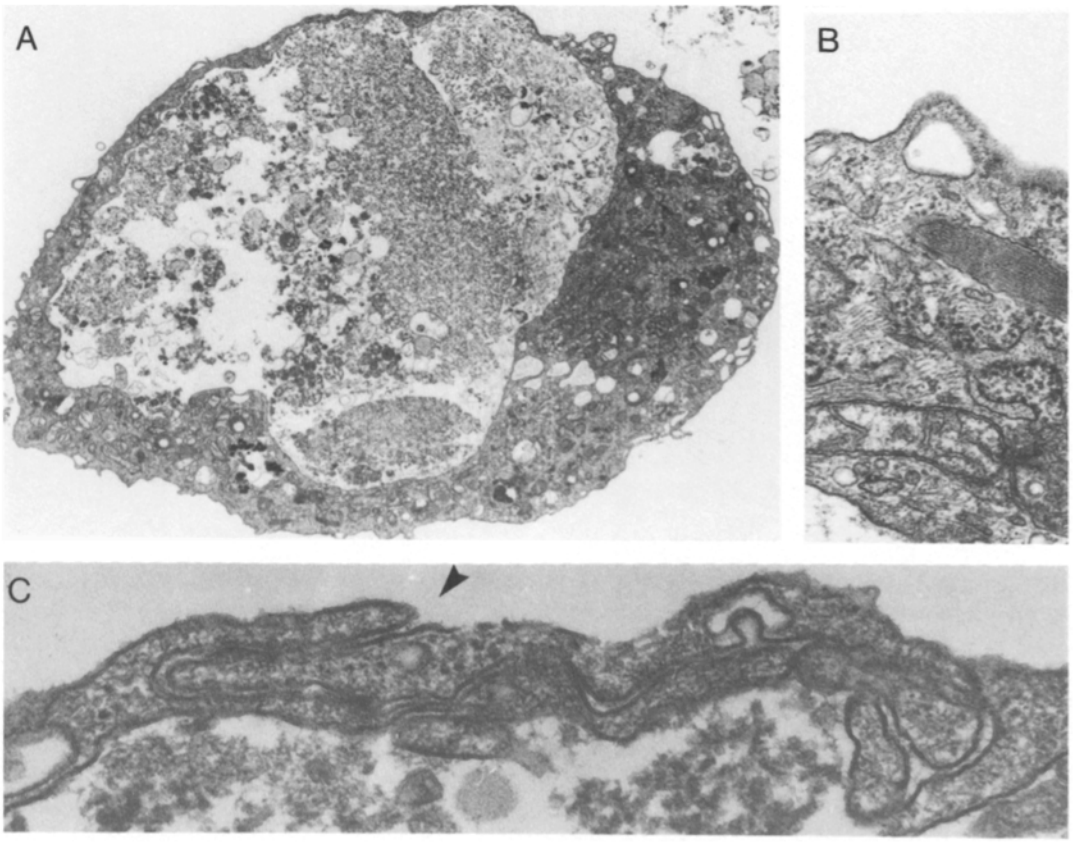

FIGURE 3 Transmission electron microscopy of the HUV-EC organized structures. The tubular array was processed for transmission electron microscopy as described in Materials and Methods. (*A*) Cross-section through the weblike HUV-EC organized network. Note that the lumen is composed of a single HUV-EC and the luminar space is composed of amorphous and fibrillar debris. × 2,800. (*B*) Higher magnification of the tubular structure shown in (*A*). × 60,000. Note the presence of distinct Weibel-Palade body. (*C*) Higher magnification of the tubular structure observed in (*A*). × 40,000. Note the interdigitating junctions (arrow).

mature senescence of HUV-EC populations in vitro. In the absence of HFN and ECGF, confluent HUV-EC primary monolayers could be passaged at least once at a 1:5 split ratio. However, the cells routinely failed to achieve confluence after the second passage. Instead, the preconfluent cells began to migrate into clusters and, after ~4–6 wk in culture, had formed a complex network of tubular structures (Fig. 1). Under phase-contrast microscopy, the network was arranged as branching strands that varied in diameter and appeared randomly distributed throughout the culture dish. Large areas of the culture dish did not contain a cobblestonelike monolayer of HUV-EC. The weblike network appeared to contain a lumen, as indicated by the accumulation of amorphous debris within these structures. After ~7–8 wk in culture, the tubular array became large enough to be grossly visible as white, threadlike strands. In some cases, individual strands partially detached from the surface of the culture dish and floated in the medium remaining attached to the culture dish at either one or both ends of the structure (Fig. 1). These large tubelike structures also varied in diameter and contained much debris within their lumens.

Endothelial Nature of the Organized Structures

The weblike network was assessed for endothelial cell character by immunofluorescent staining. As shown in Fig. 2*A*, the tubular structures stained positive with anti-human f-VIII:Ag. We were also able to determine that the change in HUV-EC morphology from the conventional cobblestone pattern to a spindle-shaped pattern (Fig. 5*A* and *B*) occurred without the loss of the human f-VIII:Ag marker. The tubular structures also stained positive with antiserum to human fibronectin and human urokinaselike plasminogen activator (Fig. 2*B* and *C*).

The endothelial nature of the tubular structure was confirmed by transmission electron microscopy (Fig. 3). Examination of the surface-attached tubular element by transmission electron microscopy revealed that the circumference of the lumen was formed by a single HUV-EC (Fig. 3*A*). The border of the endothelial cell was held together by a junction composed of interdigitating extensions of the cytoplasm (Fig. 3*C*). The presence of Weibel-Palade bodies in the cytoplasm of the tubular structures was also noted (Fig. 3*B*). Large amounts of amorphous and granular debris are also clearly visible in the channel area of the tube (Fig. 3*A* and *C*).

The Reversible Nature of HUV-EC Organization

To determine whether the HUV-EC present as organized structures are capable of proliferating in vitro, the floating multicellular macroscopic tubular structures were mechanically removed from the cell culture dish and washed three times with Dulbecco's phosphate-buffered saline. The intact structure was digested with trypsin-EDTA and the single-cell suspension plated onto a HFN matrix in the presence of 20% FBS and ECGF (150 μg/ml). The HUV-EC processed in this manner grew to confluence and were positive for human f-VIII:Ag (Fig. 4*A* and *B*). We also observed that the HUV-ECs could be grown from the tubelike structure as an explant under cell culture conditions optimized for HUV-EC attachment and growth.

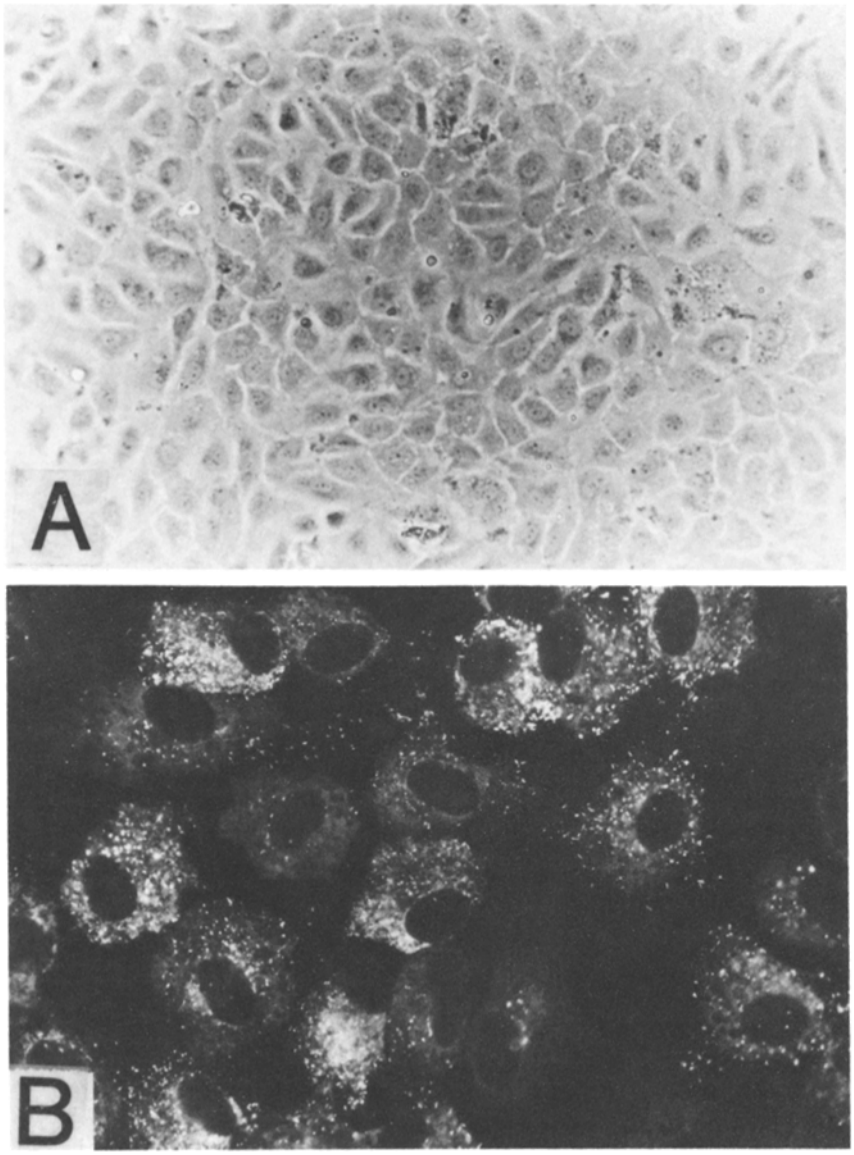

FIGURE 4 Phase-contrast morphology and anti-human factor VIII:Ag immunofluorescence of HUV-EC derived from the organized structure. A floating, macroscopic tubular structure was removed from the organized HUV-EC population. The tube was washed with Medium 199 and digested with 0.05% trypsin–0.02% EDTA for 10 min at 37°C. The trypsin was inactivated with soybean trypsin inhibitor (250 μg/ml) and a single-cell suspension created from the tubular structure. The tube-derived single-cell suspension was plated on a HFN matrix (10 μg/cm^2) and the culture fed 20% FBS and ECGF (150 μg/ml). (*A*) Phase-contrast morphology of the monolayer derived from the floating tubular structure. Note the presence of the classic large vessel endothelial cell morphology × 200. (*B*) Immunofluorescence of the tube-derived monolayer with anti-human factor VIII:Ag. × 1,000.

Are Confluent HUV-EC Cultures Capable of Organization?

HUV-EC cultures were grown on a plastic surface in the presence of 20% FBS and 150 μg/ml ECGF. After confluence, ECGF was removed from the growth medium and the cultures were fed Medium 199 containing 20% FBS every 2–3 d for a period of 2 mo. We observed, after 1–2 wk, the presence of spindle-shaped HUV-EC which appeared to be migrating out of the typical endothelial cell cobblestone pattern (Fig. 5*A*). This event was called Stage I behavior. After 2–3 wk in culture, a majority of the HUV-EC became migratory and the culture had lost its cobblestone pattern (Fig. 5*B*). At that time, the migrating HUV-EC aggregated around debris that had accumulated on the surfaces of the aligned cells (Fig. 5*B*). These events were termed Stage II behavior. After 3–4 wk in culture, the migratory HUV-EC formed a channel of aligned endothelial cells with debris at the end of the structure (Fig. 5*C*). At that time, the presence of a small lumen became visible in the center of the channel of the aligned endothelial cells (Fig. 5*D*, Stage III behavior). After 5–6 wk in culture, the HUV-EC organized structure became weblike with an exaggerated honeycomb pattern (Fig. 1, Stage IV). Large areas of the cell culture dish were free of endothelial cells. The HUV-EC tubular structures increased in diameter so that after ~7–8 wk in culture, the tubes became grossly visible (Fig. 5*E*). These debris-containing structures lifted off the surface of the cell culture dish, even in those cases in which branch points were apparent (Fig. 5*F*). This event was termed Stage V behavior. The morphological changes during the Stage I to Stage V transition are summarized in Table I.

The morphological events leading to HUV-EC tube formation (Stage I through Stage V) also occurred in confluent HUV-EC cultures grown on a HFN matrix in the presence or absence of ECGF. Under these conditions, HUV-EC tube formation occurred only after a delay of ~2–3 wk when compared to HFN-free, ECGF-free, control cultures.

The Role of Extracellular Matrix in HUV-EC Organization

Because (*a*) the addition of ECGF delayed but did not prevent HUV-EC organization and (*b*) the morphological events leading to HUV endothelial cell alignment appeared to be primarily migratory, the role of the HUV-EC extracellular matrix was examined as a possible mediator of HUV-EC

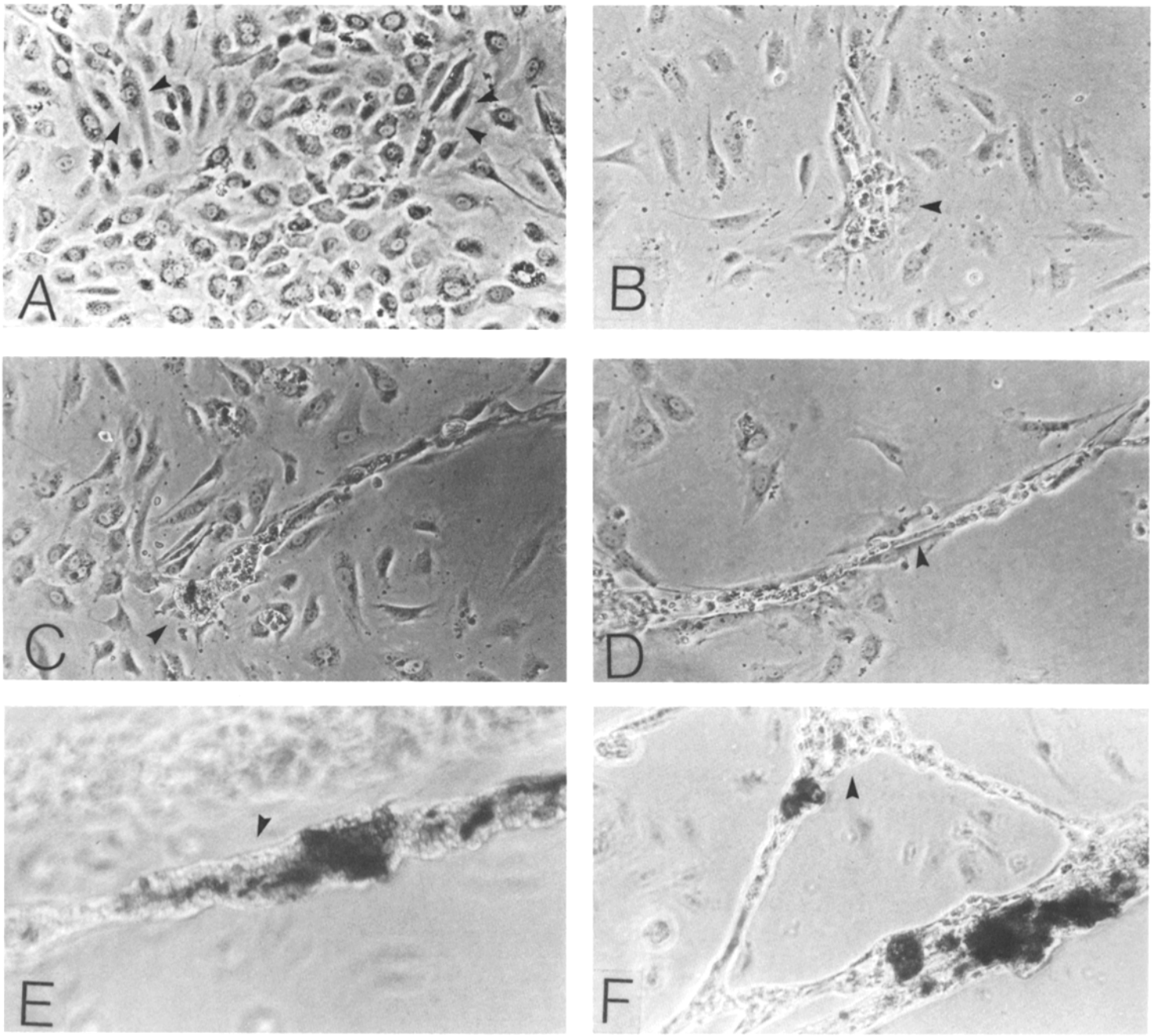

FIGURE 5 Organizational behavior of a confluent monolayer of HUV-EC. HUV-EC (Passage 5) were grown to confluence in Medium 199 containing 20% FBS and ECGF (150 μg/ml) on a HFN matrix (10 μg/cm^2). After confluence (3.5 × 10^4 HUV-EC/cm^2), the culture was fed every 2–3 d with Medium 199 and 20% FBS. The organizational process was monitored over a period of 2 mo. The magnification of the phase-contrast photomicrographs is × 250. (*A*) Appearance of the HUV-EC monolayer 1–2 wk after the removal of ECGF. Note the appearance of migratory HUV-EC (arrows) indicative of Stage I behavior. (*B*) Appearance of the HUV-EC monolayer after 2–3 wk. Note the loss of the cobblestone morphology and the appearance of predominant migratory cells. This morphology is termed Stage II behavior. Also note the appearance of aligned cells with the accumulation of debris on the cell surface. (*C*) HUV-EC monolayer 3–4 wk after ECGF withdrawal. Note the formation of a channel and the appearance of a lumen. This morphology is termed Stage III behavior. Note also that the debris associated with Stage II morphology is still present. The number of HUV-EC present at this time was estimated to be 2.7 × 10^4 HUV-EC per cm^2. This represents approximately a 20% loss of HUV-EC over 25 d in culture in the absence of ECGF. (*D*) Appearance of the HUV-EC monolayer after 5–6 wk. Note the formation of an exaggerated weblike structure on the surface of the dish and the loss of single endothelial cells around the honeycomb pattern. This morphology is indicative of Stage IV behavior. (*E* and *F*) HUV-EC monolayer 7–8 wk in culture after the removal of ECGF. Note the appearance of macroscopic tubular forms floating in the dish and the presence of debris inside the tubular structure. The appearance of the threadlike structures is termed Stage V behavior. Also note the appearance of floating tubes complete with branch points. The honeycomb pattern is still visible on the surface of the cell dish but is not in focus. Compare the morphology shown in Fig. 1 (Stage IV and V behavior) with these transitional changes.

TABLE I

Summary of the Morphological Events During the Organization of the HUV-EC In Vitro

Stage	Time	Morphological changes
	wk	
I	2	Increased incidence of migratory human factor VIII:Ag positive HUV-EC.
II	3	Aggregation of migratory cells to form small channels. Appearance of accumulated cell surface debris is apparent.
III	4	Alignment of HUV-EC with growth of the channel. Appearance of visible lumen.
IV	5-6	Development of an organized weblike pattern with an exaggerated honeycomb pattern.
V	6	Elongation of macroscopic floating tubular structures attached to the surface of the dish at one or both ends.

See Figs. 1 and 5 for morphological correlates during the transitional changes.

organization. Confluent monolayers of HUV-EC were digested with 0.25% trypsin for 10 min at 37°C. The cells were removed from the culture dishes and the trypsin inhibited with 2.5-mg soybean trypsin inhibitor. The cells were recycled back into the trypsin-treated cell culture dishes at a split ratio of 1:5 and fed Medium 199 and 20% FBS. The endothelial cells responded to trypsin digestion of the HUV-EC-derived extracellular matrix by organizing into Stage III structures after ~1 wk in culture, representing a fourfold decrease in the time ordinarily required to generate Stage III behavior. This phenomena occurred in approximately 6 out of 10 cases.

A similar response was also observed using a trypsin-digested HFN matrix. Purified HFN was plated onto the surface of cell culture dishes at a concentration of 10 $\mu g/cm^2$. The matrix was digested with 0.25% trypsin for 10 min at 37°C and the trypsin inhibited with 2.5-mg soybean trypsin inhibitor/ml. We observed the same fourfold decrease in the time required to generate Stage III behavior (Fig. 6*E*). This phenomenon occurred with approximately the same frequency as observed for the trypsin-digested HUV-EC-derived extracellular matrix.

Because these results suggested that proteolytic modification of HFN by trypsin is important in increasing the rate of HUV-EC organization, other more physiologically relevant proteases were assessed for their ability to achieve the same effects as trypsin. Replicate HFN-coated cell culture dishes were treated (37°C, 1 h) with alpha-thrombin, plasminogen, plasminogen activator, or plasmin. HUV-EC plated on a HFN matrix altered with plasminogen activator or plasmin formed tubular structures at a markedly increased rate (Fig. 6*A*, *B*, *C*, *D*, and *E*). In contrast, alpha-thrombin and plasminogen did not augment the rate of HUV-EC organization on a HFN matrix. Furthermore, plasminogen activator and plasmin had no effect on HUV-EC tube formation in the absence of HFN.

DISCUSSION

The luminal lining of blood vessels consists of a monotonous continuum of nonthrombogenic endothelial cells that possess a low mitotic index in vivo (1, 41). Disruption of the endothelial continuum with proteolytic enzymes in a selective manner provides access to the endothelial cell for cultivation in vitro (10). The introduction of the proteolytically removed endothelial cell population into an environment consisting of a HFN matrix in Medium 199 supplemented with FBS and ECGF maximizes the proliferative potential of the endothelial cell culture (22). Such an environment provides the requisite attachment and mitogenic stimuli for the serial propagation of normal and genetically variant HUV-EC in vitro (2, 22). Repeated exposure of the HUV-EC to this environment results in the proliferative senescence of the endothelial cell population after approximately 34–45 cumulative population doublings (22). Conventional HUV-EC culture in the absence of HFN and ECGF leads to premature senescence of the HUV-EC population after approximately 3–5 cumulative population doublings (2, 10, 11, 29). Cultivation of the conventional HUV-EC cultures in Medium 199 and FBS for a period of 4–6 wk results in the appearance of organized HUV-EC structures. We have characterized the organizational process of HUV-EC behavior to represent a process of nonterminal HUV-EC differentiation.

The organizational behavior of HUV-EC can be observed under a variety of circumstances, that include (*a*) removal of ECGF from a confluent monolayer, (*b*) plating HUV-EC populations in the absence of HFN and ECGF, and (*c*) providing requisite matrix-derived signals for HUV-EC organization. In all instances, HUV-EC organization occurs in an environment in which the proliferative potential of the HUV-EC population is minimized. Under these conditions HUV-EC tube formation occurs relatively rapidly in preconfluent cultures. The addition of either an intact HFN matrix or ECGF to the culture environment did not prevent, but significantly delayed the process of HUV-EC organization. The cells involved in tube development were endothelial cells and not smooth muscle cell contaminants, as the tubular structures contained the factor VIII:Ag and Weibel-Palade bodies, two markers that are specific for endothelial cells (15, 36). Furthermore, cells derived from tubular structures by either trypsin digestion or tube explant methods grew to form a classic cobblestone endothelial cell monolayer and contained factor VIII:Ag.

The ability of the endothelial cells that were organized into tubular structures to revert to a proliferative state under conditions that were optimal for HUV-EC growth suggests that these organized cells were in a state of nonterminal differentiation. This concept is described in model form in Fig. 7. The reciprocal relationship between HUV-EC growth and organization is a concept that has been described in other differentiating cellular systems including the smooth muscle cell (3) and megakaryocyte (37). The ability of the HUV-EC to organize independently of cumulative population doublings under culture conditions that minimize proliferation emphasizes the reciprocity between growth and organization. It will be of interest to determine whether the process of organizational behavior influences HUV-EC senescence in vitro.

The process of HUV-EC organization has been divided into five stages. It is apparent from phase-contrast and time-lapse photomicrographs (Kadish, J., and T. Maciag, unpublished observations) that the initial event in the organizational process involves migration of the endothelial cell (Stage I). The migratory HUV-EC aggregate, accumulate cell-surface debris (Stage II) and align in a manner conducive to lumen formation (Stage III). Although the mechanism of lumen formation is not presently clear, the appearance of luminal debris suggests that the aligned HUV-EC use the cell-surface debris and neighboring cells as a scaffolding, such that podia are extended over the debris to form single cell junctions. The appearance of basement membranelike material in the lumen of these three-dimensional structures also suggests that if a nonthrombogenic surface is expressed by the HUV-EC organized structures, it

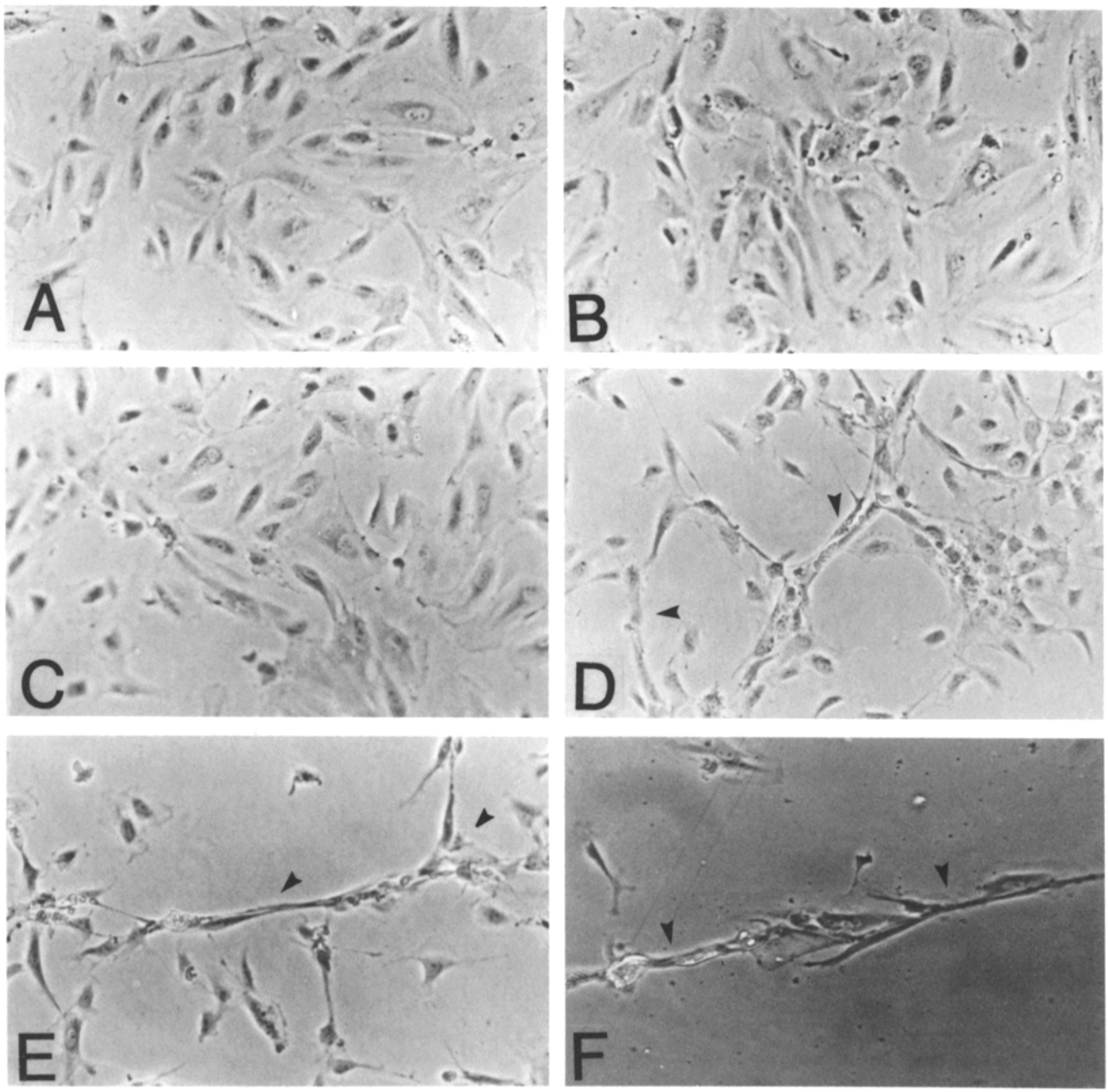

FIGURE 6 Enhancement of HUV-EC organizational behavior by proteolytic modification of fibronectin. Plastic cell culture dishes were coated with purified HFN (10 $\mu g/cm^2$). Duplicate dishes ($n = 4$) were treated with a variety of proteolytic enzymes in Dulbecco's phosphate buffered saline. The proteolytic enzymes included trypsin (1.0 $\mu g/ml$), human kidney plasminogen activator (1.0 $\mu g/ml$), human alpha-thrombin (5 $\mu g/ml$), and human plasminogen (1.8 $\mu g/ml$). The enzymes were exposed to the HFN-coated surface for 1 h at 37°C (trypsin, 10 min exposure). HFN-free cell culture dishes and protease-treated HFN-free cell culture dishes served as controls. HUV-EC (passage 6) were added to each dish at a seed density of 2×10^4 cells/cm^2 and the cultures fed 10% FBS and Medium 199 every 2–3 d. The cultures were assessed for Stage III behavior in a blind fashion after 1 wk in culture and phase-contrast photomicrographs (× 250) taken. (*A*) HFN: protease-free control, (*B*) HFN and plasminogen, (*C*) HFN and alpha-thrombin, (*D*) HFN and plasminogen activator, (*E*) HFN and plasmin (plasminogen activator plus plasminogen), and (*F*) HFN and trypsin. Note the appearance of Stage III behavior (arrows) in *D*, *E*, and *F*. The HFN-free controls (not shown) were negative.

may indeed be on the outside surface of the tube (Stemerman, M. B., and T. Maciag, unpublished observations). The organizational process continues to develop a weblike structure (Stage IV). The continuity of the structure can be demonstrated by injecting fluorescein dye into the lumen under nitrogen pressure, through a micropipette. The dye can be observed to be restricted to the lumen, suggesting functional integrity of the endothelial cell junctions noted in the transmission electron micrographs (Clivden, J., G. A. Hoover, R. Weinstein, and T. Maciag, unpublished observations). This capillarylike continuum further develops until macroscopically distinct white bulbous threadlike structures anchored at either one or both ends of the cell culture dish, are readily ascertained (Stage V). It is of interest to note that the HUV-EC grown from explanted threads morphologically resemble the tightly compacted cobblestone pattern unique to the large vessel endothelium (29).

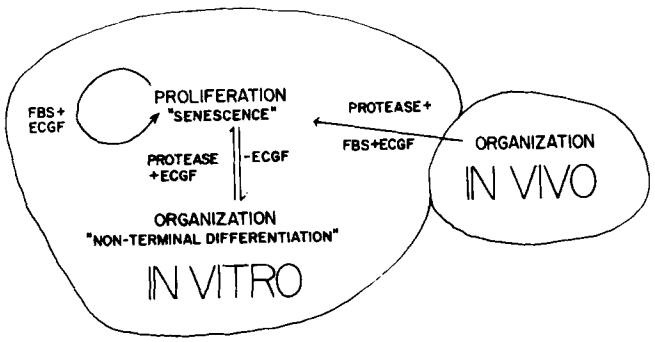

FIGURE 7 Model summarizing the reciprocity between HUV-EC proliferation and organization. The endothelium in vivo exists as a monolayer of nonproliferative cells. Digestion of this monolayer with proteolytic enzymes and the introduction of the HUV-EC population to a growth factor-rich environment (*FBS + ECGF*) results in cell proliferation. Repetitious subcultivation in the proliferative environment results in HUV-EC senescence. However, the HUV-EC population can be induced to form complicated three-dimensional structures at any point in the in vitro aging process by removal of HFN and ECGF after the subculture. Similar results can also be obtained with confluent HUV-EC cultures. The organized structures represent a form of HUV-EC nonterminal differentiation because the organized population can revert into a proliferative compartment by treatment with proteolytic enzymes and the introduction of a growth-stimulating environment.

These studies confirm and extend the observations of Folkman and Haudenschild (8) concerning capillary-derived endothelial cell behavior in vitro. The structures formed by the HUV-EC organizational process are morphologically consistent with the three-dimensional structures that have been derived from confluent cultures of human capillary endothelial cells (8). In addition, our observations concerning the transition from Stage I to Stage IV development agree with the morphological events associated with capillary angiogenesis in vitro (8).

We have attempted to elucidate the mechanism responsible for the process of HUV-EC organization. Our data strongly suggest that the rate of transition from Stage I to Stage IV morphology is strongly influenced by proteolytic modification of the extracellular matrix. It is also apparent from these studies that the extracellular matrix is a source of proteolytically modified HFN as a mediator of this process because trypsin and plasmin-modified HFN substitute for the HUV-EC extracellular matrix and increase the rate of the Stage I to Stage III organizational transition.

The ability of plasmin to initiate these events via HFN modification represents an observation that is consistent with mechanisms proposed for normal and tumor angiogenesis. It is established that HFN provides an efficient attachment surface for mammalian cells in culture (9, 21, 27, 38), including the HUV-EC (22). Plasminogen activator is a serine protease that has been implicated in a wide variety of developmental and pathophysiological systems including embryogenesis (33), ovulation (32), wound healing (18), tissue remodeling (12), tumor invasiveness (34) and growth (17, 24), and the migratory behavior of mammalian cells such as the macrophage (12). In addition, purified plasminogen activator and heparin as well as fibrin and fibrin digestion products have been shown to markedly stimulate angiogenesis in vivo (4, 6, 7, 25, 27). Furthermore, HFN and proteolytically modified HFN are chemotactic for mammalian cells in vitro (30, 35) and HFN possess a binding site for heparin (23, 27). The suggestion that fibrin provides a scaffold for cell growth (39) is an observation consistent with the report that HFN is covalently cross-linked to fibrin by factor-XIII (23) and that fibrin deposition and fibrinolysis are events important to wound healing (28) and tumor growth (6, 7, 14, 25, 26, 40). In addition, the HUV-EC is capable of synthesizing plasminogen activator (19) and HFN (16). It is therefore not surprising that delayed HUV-EC organization occurs in vitro in a serum-supplemented environment, because serum is a potential source of plasminogen. The enhancement of HUV-EC tube formation by a HFN-dependent fibrinolytic system strongly argues that the HUV-EC organizational process involves the activation of latent matrix-derived signals by specific proteolytic enzyme systems. It is of interest to note that plasmin-derived fragments of HFN contain transformation-enhancing activity in chicken embryo fibroblasts infected with Rous sarcoma virus (5).

The HUV-EC organized structure represents an alternative model for the study of endothelial cell development and tumor angiogenesis in vitro (8). Although it has been suggested that ECGF and tumor-conditioned medium are required for capillary angiogenesis in culture (8), our studies demonstrate that HUV-EC organization routinely occurs in an environment that limits HUV-EC growth. It is possible that the addition of tumor-conditioned medium to the endothelial cell culture may increase the rate of HUV-EC organization in a manner similar to the effect observed with trypsin and plasmin-modified HFN.

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REFERENCES

1. Aursnes, I. 1974. Increased permeability of capillaries to protein during thrombocytopenia: an experimental study in the rabbit. *Microvasc. Res.* 7:283–295.
2. Booyse, F. M., A. J. Quarfoot, J. Chediak, M. B. Stemerman, and T. Maciag. 1981. Characterization and properties of cultured human von Willebrand umbilical vein endothelial cells. *Blood.* 58:788–796.
3. Chamley-Campbell, J. H., G. R. Campbell, and R. Ross. 1981. Phenotype-dependent response of cultured aortic smooth muscle to serum mitogens. *J. Cell Biol.* 89:379–383.
4. Clark, C. R., and E. L. Clark. 1939. Microscopic observations on the growth of blood capillaries in the living mammal. *Am. J. Anat.* 64:251–301.
5. DePetro, G., S. Barlati, T. Vartio, and A. Vaheri. 1981. Transformation-enhancing activity of gelatin-binding fragments of fibronectin. *Proc. Natl. Acad. Sci. U. S. A.* 78:4965–4969.
6. Dvorak, H. F., A. M. Dvorak, E. J. Manseau, L. Wiberg, and W. H. Churchill. 1979. Fibrin gel investment associated with Line 1 and Line 10 solid tumor growth, angiogenesis and fibroplasia in guinea pigs. Role of cellular immunity, myofibroblasts, microvascular damage and infarction in Line 1 tumor regression. *J. Natl. Cancer Inst.* 62:1459–1472.
7. Dvorak, H. F., S. C. Quay, N. S. Orenstein, A. M. Dvorak, P. Hahn, A. M. Bitzer, and A. C. Carvalho. 1981. Tumor shedding and coagulation. *Science (Wash. D. C.).* 212:923–924.
8. Folkman, J., and C. Haudenschild. 1980. Angiogenesis in vitro. *Nature (Lond.).* 288:551–556.
9. Gilchrest, B. A., R. E. Nemore, and T. Maciag. 1980. Growth of human keratinocytes on fibronectin-coated plates. Cell Biol. Int. Rep. 4:1009–1016.
10. Gimbrone, M. A., Jr. 1976. Culture of vascular endothelium. *Prog. Hemostasis Thromb.* 3:1–28.
11. Gimbrone, M. A., Jr., R. S. Cotran, and J. Folkman. 1974. Human vascular endothelial cells in culture: growth and DNA synthesis. *J. Cell Biol.* 60:673–684.
12. Gordon, S., J. C. Unkeless, and Z. A. Cohn. 1974. Induction of macrophage plasminogen activator by endothelium stimulation and phagocytosis. *J. Exp. Med.* 140:995–1010.
13. Hayashi, I., and G. H. Sato. 1976. Replacement of serum by hormones permits growth of cells in a defined medium. *Nature (Lond.).* 259:132–134.
14. Hiramoto, R., J. Bernecky, J. Jurandowski, and D. Pressman. 1960. Fibrin in human tumors. *Cancer Res.* 20:592–593.
15. Hoyer, L. W., R. P. DeLos Santos, and J. R. Hoyer. 1973. Antihemophilic factor antigen: localization in endothelial cells by immunofluorescent microscopy. *J. Clin. Invest.* 52:2737–2744.
16. Jaffe, E. A., and D. F. Mosher. 1978. Synthesis of fibronectin by cultured human

endothelial cells. *J. Exp. Med.* 147:1779–1785.
17. Jones, P. A., W. E. Lang, and W. F. Benedict. 1975. Fibrinolytic activity in a human fibrosarcoma cell line and evidence for the induction of plasminogen activator secretion during tumor formation. *Cell.* 6:245–252.
18. Kwaan, H. C., and T. Astrup. 1965. Fibrinolytic activity in thrombosed veins. *Circ. Res.* 17:477–491.
19. Loskutoff, D. J., and T. S. Edgington. 1977. Synthesis of a fibrinolytic activator and inhibitor by endothelial cells. *Proc. Natl. Acad. Sci. U. S. A.* 74:3903–3907.
20. Maciag, T., J. Cerundolo, S. Ilsley, P. R. Kelley, and R. Forand. 1979. An endothelial cell growth factor from bovine hypothalamus: identification and partial characterization. *Proc. Natl. Acad. Sci. U. S. A.* 76:5674–5678.
21. Maciag, T., R. E. Nemore, R. Weinstein, and B. A. Gilchrist. 1981. An endocrine approach to the control of epidermal growth: serum-free cultivation of human keratinocytes. *Science (Wash. D. C.).* 211:1452–1454.
22. Maciag, T., G. A. Hoover, M. B. Stemerman, and R. Weinstein. 1981. Serial propagation of human endothelial cells in vitro. *J. Cell Biol.* 91:420–426.
23. Mosher, D. F., and L. T. Furcht. 1981. Fibronectin: review of its structure and possible functions. *J. Invest. Dermatol.* 77:175–180.
24. Newcomb, E. W., S. C. Silverstein, and S. Silagi. 1978. Malignant mouse melanoma cells do not form tumors when mixed with cells of a non-malignant subclone: relationships between plasminogen activator expression by the tumor cells and the host's immune response. *J. Cell. Physiol.* 95:169–178.
25. O'Meara, R. A. Q., and R. D. Jackson. 1958. Cytological observations on carcinoma. *Ir. J. Med. Sci.* 391:327–328.
26. O'Meara, R. A. Q. 1958. Coagulative properties of cancers. *Ir. J. Med. Sci.* 394:474–479.
27. Pearlstein, E., L. Gold, and A. Garcia-Pardo. 1980. Fibronectin: a review of its structure and biological activity. *Mol. Cell Biochem.* 29:103–123.
28. Schoefl, G. I. 1963. Studies on inflammation III. Growing capillaries: their structure and permeability. *Virchows Arch. Pathol. Anat. Physiol. K. M.* 337:97–141.
29. Schwartz, S. M. 1978. Selection and characterization of bovine aortic endothelial cells. *In Vitro (Rockville).* 14:966–980.
30. Seppa, H. E. J., K. M. Varmada, S. T. Seppa, M. H. Silver, H. K. Kleinman, and E. Schiffman. 1981. The cell binding fragment of fibronectin is chemotactic for fibroblasts. Cell Biol. Intern. Rep. 5:813–819.
31. Stemerman, M. B. 1978. Thrombogenesis of the rabbit arterial plaque. An electron microscopic study. *Am. J. Pathol.* 73:7–26.
32. Strickland, S., and W. H. Beers. 1976. Studies on the role of plasminogen activator in ovulation. *J. Biol. Chem.* 251:5694–5702.
33. Strickland, S., E. Reich, and M. I. Sherman. 1976. Plasminogen activator in early embryogenesis: enzyme production by trophoblast and parietal endoderm. *Cell.* 9:231–240.
34. Unkeless, J. C., A. Tobia, L. Ossowski, J. P. Quigley, D. B. Rifkin, and E. Reich. 1973. An enzymatic function associated with transformation of fibroblasts by oncogenic viruses. I. Chick embryo fibroblast cultures transformed by avian RNA tumor viruses. *J. Exp. Med.* 137:85–112.
35. Vartio, T., H. Seppa, and A. Vaheri. 1981. Susceptibility of soluble and matrix fibronectins to degradation by tissue proteinases, mast cell chymase and cathespin G. *J. Biol. Chem.* 256:471–477.
36. Weibel, E. R., and G. E. Palade. 1964. New cytoplasmic components in arterial endothelia. *J. Cell Biol.* 23:101–112.
37. Weinstein, R., M. B. Stemerman, D. E. MacIntyre, H. N. Steinberg, and T. Maciag. 1981. The morphological and biochemical characterization of a line of rat promegakaryoblasts. *Blood.* 58:110–121.
38. Weinstein, R., M. B. Stemerman, and T. Maciag. 1981. Hormonal requirements for growth of arterial smooth muscle cells in vitro: an endocrine approach to atherosclerosis. *Science (Wash. D. C.)* 212:818–820.
39. White, J. F., and M. S. Parshley. 1951. Growth in vitro of blood vessels from bone marrow of adult chickens. *J. Anat.* 89:321–345.
40. Wood, S. 1958. Pathogenesis of metastasis formation observed in vivo in the rabbit ear chamber. *Arch. Pathol.* 66:550–568.
41. Wright, H. P. 1972. Mitosis patterns in aortic endothelium. *Atherosclerosis.* 15:93–107.