Capillary Endothelial Cell Cultures: Phenotypic Modulation by Matrix Components

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ABSTRACT Capillary endothelial cells of rat epididymal fat pad were isolated and cultured in media conditioned by bovine aortic endothelial cells and substrata consisting of interstitial or basement membrane collagens. When these cells were grown on interstitial collagens they underwent proliferation, formed a continuous cell layer and, if cultured for long periods of time, formed occasional tubelike structures. In contrast, when these cells were grown on basement membrane collagens, they did not proliferate but did aggregate and form tubelike structures at early culture times. In addition, cells grown on basement membrane substrata expressed more basement membrane constituents as compared with cells grown on interstitial matrices when assayed by immunoperoxidase methods and quantitated by enzyme-linked immunosorbent inhibition assays.

Furthermore, when cells were grown on either side of washed, acellular amnionic membranes their phenotypes were markedly different. On the basement membrane surface they adhered, spread, and formed tubelike structures but did not migrate through the basement membrane. In contrast, when seeded on the stromal surface, these cells were observed to proliferate and migrate into the stromal aspect of the amnion and ultimately formed tubelike structures at high cell densities at longer culture periods (21 d).

Thus, connective tissue components play important roles in regulating the phenotypic expression of capillary endothelial cells in vitro, and similar roles of the collagenous components of the extracellular matrix may exist in vivo following injury and during angiogenesis. Furthermore, the culture systems outlined here may be of use in the further study of differentiated, organized capillary endothelial cells in culture.

Capillary endothelial cells in vivo are surrounded by and rest on basement membranes composed of several extracellular matrix components (10, 24, 28, 37). These cells are normally quiescent and are constrained by their investing basement membranes. Following injury or in response to other stimuli these cells somehow escape the constraints of the basement membrane, undergo several phenotypic changes, migrate through and proliferate in the interstitium, and ultimately form a new capillary network complete with investing basement membranes (2). Thus, throughout the reparative process capillary endothelial cells are in intimate contact with matrix components. Therefore it is not unreasonable to suggest that the matrix composition and possibly organization in the microenvironment surrounding such cells may play important roles in directing the dynamic responses of capillary endothelial cells following injury.

With the development of isolation and culture techniques it becomes possible to study capillary endothelial cells in vitro (8, 9, 18, 39, 40). This advance has allowed for a systematic approach in which many variables can be controlled, with the ultimate objective being to mimic as closely as possible the in vivo condition. Folkman et al. (8, 9) and other investigators (41) have made it possible to study angiogenesis in culture by developing culture techniques and conditions in which angiogenesis can occur. In several of these studies a gelatin substratum and tumor conditioned media were found to be necessary for the continued growth, passage, and expression of in vitro angiogenesis of capillary endothelial cells (1, 8, 9, 41).

The purpose of this work is to determine what roles (if any) specific extracellular matrix components have in the control of proliferation of cultured capillary endothelial cells, ability

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to exhibit angiogenesis, and matrix molecule synthetic ability. Cultured capillary endothelial cells from rat epididymal fat pads were plated on petri dishes coated with heterologous interstitial collagens (types I and III), basement membrane collagen (type IV or types IV and V) and on the interstitial and basement membrane surfaces of human amnionic membranes. Such cultures exhibited marked differences in morphology, proliferation rate, ability to undergo angiogenesis, and matrix biosynthetic profiles, depending upon the composition of the substratum.

MATERIALS AND METHODS

Cells: Rat capillary endothelial cells were isolated from epididymal fat pads as described by Wagner and Matthews (39), with several modifications. Briefly, the epididymal fat pads from adult rats (400 g) were removed and pinned out flat on dental Byte wax sheets in petri dishes containing sterile HEPES Buffer (pH 7.4; containing 140 mM NaCl, 10 mM HEPES, 10 mM KCl, 0.1 mM $CaCl_2$, 0.2 mM $MgCl_2$, with 11 g/liter $NaHCO_3$ and 5.0 g/liter glucose). Fat free of large vessels was removed, minced, and placed in sterile 50-ml screw cap Erlenmayer flasks (2 pads/flask) containing 10 ml of HEPES buffer and collagenase (Sigma Chemical Co., St. Louis, MO; type II) 5 mg/ml and bovine serum albumin (BSA), 5 mg/ml. The flasks were incubated for 30 min at 37°C with gentle agitation. At this time the contents of the flask have the appearance of a pinkish slurry. This slurry is made 10% with respect to BSA (Sigma Chemical Co. Fx 5, fatty acid free) and centrifuged at 200 *g* for 7 min in 15-cm^3 disposable, sterile conical tubes (Corning Glass Works, Corning, NY). The pellet is resuspended in HEPES buffer containing 10% BSA and spun down, as described above, two times. The resultant pellet is resuspended in 45% Percoll (Pharmacia Fine Chemicals, Piscataway, NJ) (9 ml of Percoll, 1 ml of 10× HEPES buffer and 10 ml of HEPES buffer containing 10% BSA), and centrifuged at 15,000 *g* for 20 min at 4°C in a SS 34 fixed-angle rotor (Sorvall). The tufts of capillary endothelial cells are in a milky off-white layer beneath the lymphocytes and above a translucent layer containing larger cells and above a translucent layer containing larger vessel fragments. The tufts of capillary endothelial cells were washed twice by pelleting them by centrifugation in HEPES-BSA buffer 200 *g* for 3 min. The tufts were then plated on 60-mm petri dishes coated with type I/III collagen ([100 μg/dish] by the methods of Elsdale and Bark [7]) in media (Medium 199 E [Gibco, Grand Island, NY]) containing 20% heat-inactivated fetal calf serum, penicillin (100 U/ml), streptomycin (100 U/ml), and glutamine mixed one to one with the same media conditioned for 48 h by incubation with confluent bovine aortic endothelial cell cultures (22). Cultures were incubated in a 5% CO_2, 95% air humidified atmosphere at 37°C. After passage number 10, the cells also grew well in nonconditioned media (data not shown).

Cells were passaged at confluence (every 7 d) using 0.02% trypsin (Worthington Biochemical Corp., Freehold, NJ) in Ca^{++}- and Mg^{++}-free phosphate-buffered saline (PBS) containing 0.01% EDTA to dissociate the cells. Cells were plated at a 1:10 split ratio. Criteria for endothelial origin of these cells included angiotensin-converting enzyme activity: 3.31 ± 0.011 nmol/h/10^6 cells as compared with 2.83 ± 0.0 nmol/h/10^6 cells freshly isolated (6) and fluorescence after incubation with anti-factor VIII (Calbiochem-Behring Corp., American Hoechst Corp., La Jolla, CA [17] [Fig. 1]).

Collagens and Antibodies: The heterologous collagens used for coating culture dishes (collagen types I, III, IV, and V) were prepared from human placenta as previously described (19, 23). Antibodies to rat type I and III collagens, murine Engelbreth-Holm-Swarm (EHS) tumor IV and EHS laminin and antibodies to human placental membrane type V collagen were prepared and assayed as previously described (23, 24, 28, 29). Affinity-purified antibodies to rat type I and III collagens reacted equally well against rat and mouse antigens (28) but showed no cross reactivity against human types I and III collagens used in coating dishes (28, and summary report of National Cancer Institute contract NO-1-CB-842555-37). The EHS tumor type IV collagen antibody was selected for its ability to react with rat and mouse antigens but not against the pepsinized human placental type IV collagen used in coating dishes and in enzyme-linked immunosorbent (ELISA) assays (unpublished observations). Since our type V collagen antibodies react equally well with human and rodent antigen, type V collagen was omitted from the coating buffers in immunostaining and ELISA assay quantitation experiments and was found to have no effect on the studies described. All primary antibodies were at stock concentrations of 0.5 mg/ml in PBS. All primary antibodies were used at concentrations of 25 μg/ml in fluorescence and peroxidase staining assays. Controls included omission of primary antibody, nonimmune rabbit immunoglobulin (Ig) G and omission of secondary antibody where applicable. Tetramethyl rhodamine-coupled goat anti-rabbit IgG was purchased from Cappel Laboratories (Cochranville, PA). Peroxidase-conjugated sheep anti-rabbit IgG, and biotinylated sheep anti-rabbit IgG, were prepared as described previously (27).

Avidin DH and biotinylated horseradish peroxidase were purchased from Vector Laboratories (Burlinghame, CA).

Cell Counting: Counting on dishes was performed on methanol-fixed, hematoxylin-stained cultures with a calibrated ocular eyepiece. Counting of cell suspensions was accomplished with a standard hemocytometer.

Amnion Membranes: Acellular amnionic membranes were prepared as described by Liotta et al. (17). Membranes were held in 1-cm diameter rings made from the upper tube bodies and caps of Sarstedt tubes (58.490 and 65.790). Cells were cultured on the stromal side or the basement membrane side of these membranes.

Electron Microscopy: Cell cultures were prepared for electron microscopy by standard procedures (22). Cultures were fixed in 1% glutaraldehyde in PBS for 1 h at 4°C, washed in arsenate buffer, dehydrated, and embedded in Epon. Grids were examined on a Philips EM 300 electron microscope at 80 KV. Photographs were taken on Kodak EM film 4489.

Light Microscopy: Examination of living cultures was performed on Olympus IMT inverted binocular tissue culture scope equipped with bright field, phase contrast, and Hoffman interference optics. Photographs were taken using an Olympus IMT inverted microscope and PM 10 microprocessor with Kodak technical pan film.

Fluorescence Microscopy: Examination of fixed labeled cultures was performed on a Zeiss 14 binocular microscope equipped with epiillumination and standard fluorescein and rhodamine excitation and emission fiter sets. Photographs were taken on Ektachrome ASA 400 film (27).

Peroxidase Labeling of Cultures: Culture dishes were washed gently with PBS, fixed with absolute methanol for 10 min, and air dried. Dishes were then hydrated with PBS and incubated with primary antibodies and using the avidin-biotin peroxidase complex detection system.

Collagen Coating of Petri Dishes: 60-mm^2 petri dishes (Costar 3060 [Data Packaging, Cambridge, MA] or Falcon 3006 liner plates [Falcon Labware, Oxnard, CA]) were coated with 100 μg each of pepsin-soluble human collagen types I and III in a 1:1 ratio or IV and V in a 1:1 ratio or IV according to the methods of Elsdale and Bard (7). Coated dishes were washed four times with media and incubated with media in the incubator for 2 h before cell plating.

Biosynthetic Labeling of Cultured Cells and Quantitative Immunoprecipitation Assays: Biosynthetic labeling of these cells and manipulation of labeled products were performed as previously described (22). Briefly, two 75-cm^2 flasks of confluent cells grown were incubated with 2 mCi each of [H^3]glycine and [H^3]proline (New England Nuclear, Boston, MA) in the above-mentioned media plus 100 μg/ml ascorbate and 100 μg/ml β-aminoproprionitrile. After 24 hs the medium was decanted and saved with one PBS wash, and the cell layers were scraped off and solubilized in 1 mM acetic acid. Insoluble material was solubilized with pepsin (100 μg/ml). Autoradiograms of media and cell layer fractions before and after pepsin treatment revealed a predominance of type I collagen and type III collagens with only small amounts of type V collagen noted. In addition, using collagenase and pepsin sensitivities as general markers for the collagenous and noncollagenous nature of synthesized proteins, we found that 30% of biosynthetically-labeled protein was collagenous in nature (data not shown). These data are in agreement with previously published reports of the synthetic profiles of capillary endothelial cells grown on plastic (30).

To quantitate each type of collagen synthesized, we made serial dilutions of antibodies (50 μg initial concentration) and we added constant amounts of media and cell layer fractions. After a 24-h incubation, secondary antibody was added and after another 24-h incubation the material was centrifuged and precipitates and supernatants were counted. From these data, the relative amounts of each collagen type present were determined as previously described (22). Controls included nonimmune and preimmune sera controls. These data were used to test the validity and reproducibility of the following quantitative ELISA inhibition assay.

Quantitative ELISA Inhibition Assays: Newly synthesized collagens were assayed by a sensitive, quantitative ELISA inhibition assay using a biotin-avidin-biotinyl peroxidase detection system previously described in detail (21). This approach is possible because of the species-specificity of the antibodies used in that the heterologous coating collagen(s) are not detected (28, summary report of National Cancer Institute contract NO-1-CB-842666-37), allowing for the measurement of only rat collagens, i.e., those synthesized by the cultured cells. Briefly, 96-well flat-bottom polyvinyl chloride microtiter plates (Cooke Engineering, Alexandria, VA) were coated with specific collagens at 100 ng/well. In separate, uncoated plates, constant dilutions of antibodies (anti-rat I

1:160; anti-rat III 1:2,500; anti-EHS IV 1:1,000; anti-human V 1:500) were incubated with serial dilutions of known inhibitors and media and cell layer samples (solubilized in 2 M guanidine HCl, 50 mM Tris HCl, pH 7.5). This mixture was then transferred to the specific coated plate, incubated, decanted, and the plate washed. Biotinylated secondary antibody was then applied, incubated, and the plates were washed. Avidin DH-biotinylated peroxidase complex was then added to the plates, incubated, and the plates were washed. Substrate (*O*-phenylene-diamine, Sigma Chemical Co.) was added and the reaction stopped with 2 N H_2SO_4 after 10 min. Plates were read on a Titertek micro ELISA reader at 492 nm. Data were processed on a dedicated Apple II plus microprocessor using linear regression fit analyses. All assays were done in duplicate at three concentrations of the fractions to be tested (1:5, 1:10, 1:50). At these dilutions and subsequent serial dilutions, the guanidine was observed to have no effect on known inhibitor profiles.

The amount of the various collagens synthesized by the cells on the various substrata were determined and corrected to milligram per microgram of DNA. DNA was determined by the method of Hill and Whatley (13), which uses mithramycin.

The amounts of the various collagens synthesized were then expressed as percentages of the total collagen sum in the final comparisons of the effects of substrata (Table I *a*, *b*, *c*).

RESULTS

Adhesion and Growth of Capillary Endothelial Cells on Connective Tissue Components

The use of collagen coatings composed of the interstitial collagen types I and III allow for the rapid adhesion and spreading of tufts of the epididymal fat pad endothelial cells (data not shown). Upon culturing such tufts, the endothelial cells were observed to grow and eventually cover the entire surface of the culture vessel. These cells could be passaged regularly on collagen-coated dishes. Cultured cells stained uniformly with antisera to Factor VIII antigen (Fig. 1) and expressed levels of angiotensin-converting enzyme comparable with levels obtained from freshly isolated, noncultured capillary endothelial cells: 3.31 nmol/h/10^6 cells for cultured cells vs. 2.83 nmol/h/10^6 cells for freshly isolated cells. When cells were plated on uncoated plastic petri dishes the cells also grew but at a much slower rate and had a "spindle" morphology and never attained confluence (data not shown).

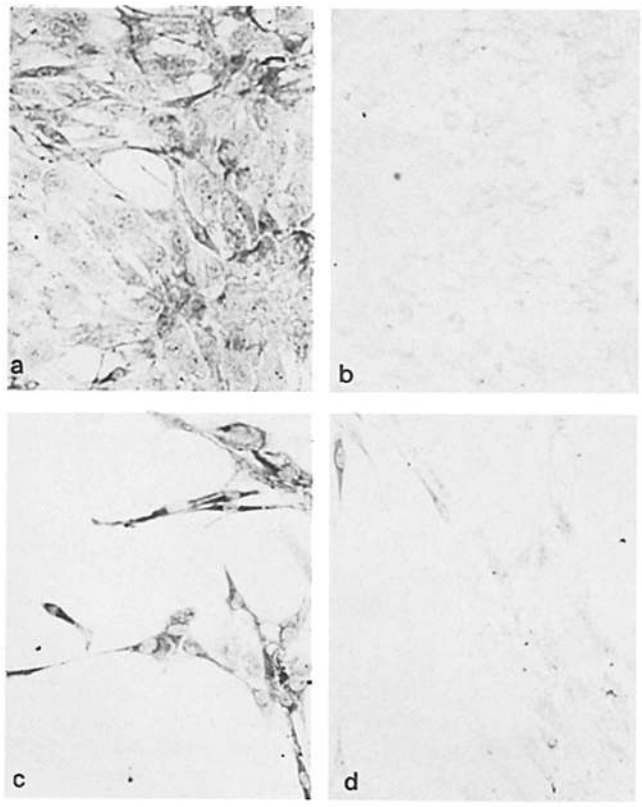

FIGURE 1 Capillary endothelial cell cultures labeled with anti-Factor VIII antigen. Labeling was performed on collagen types I/III and IV/V-coated dishes using an avidin-biotinyl peroxidase detection system. The cultures were counterstained lightly with hematoxylin. Nonimmune serum was used as a control (*b* and *d*). (*a*) Cells grown on collagen types I/III-coated dishes labeled with anti-Factor VIII antigen. Note the black reaction product over the cell cytoplasm. (*b*) Cells grown on collagen types I/III-coated dishes labeled with nonimmune sera. No labeling is noted. (c) Cells grown on collagen types IV/V-coated dishes labeled with anti-Factor VIII antigen. Note the black reaction product in cytoplasmic areas. (*d*) Cells grown on collagen types IV/V-coated dishes labeled with nonimmune sera. No reaction product is observed. × 200.

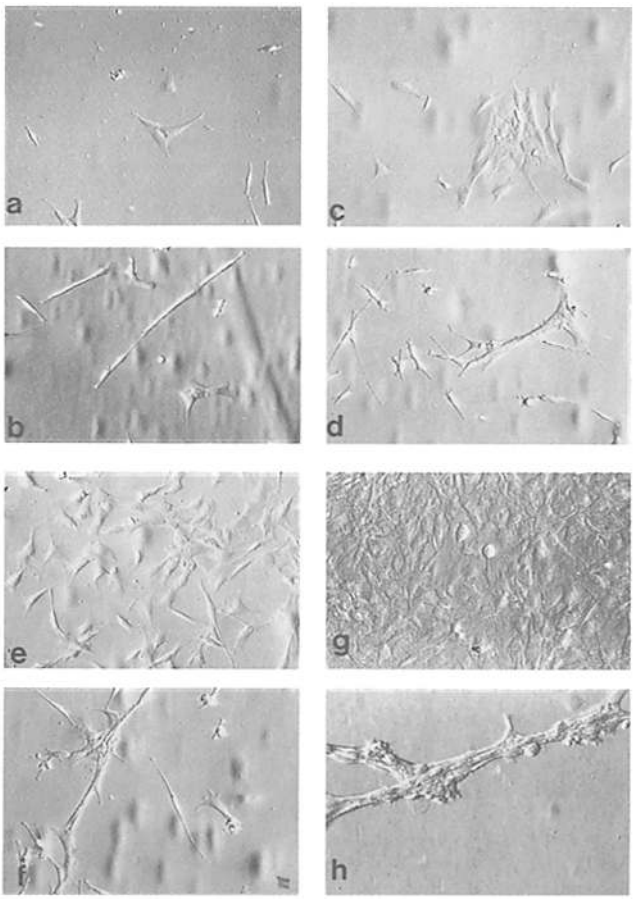

FIGURE 2 Comparison of capillary endothelial cells plated and grown on type I/III collagen-coated dishes vs. type IV/V collagen-coated dishes. (*a* and *b*) 12 h. At this time the cells on type I/III collagen (*a*) are variable in shape, ranging from roughly polygonal to fusiform and spindlelike. In contrast the cells grown on type IV/V collagen (*b*) are spindle shaped for the most part. (*c* and *d*) 24 h. At this time cells grown on type I/III collagen (c) are forming nests of roughly polygonal to fusiform cells by proliferation, while the cells grown on type IV/V collagen (*d*) are aggregating to form elongated tubelike structures. (*e* and *f*) 48 h. At this time cells grown on the interstitial collagens (*e*) are evenly dispersed over the surface in increasing cell numbers having variable shapes from fusiform to spindlelike, while cells grown on the basement membrane collagens (*f*) are aggregated in tubelike structures. (*g* and *h*) 96 h. At this time the cells grown on the interstitial collagens (*g*) have formed a continuous covering over the surface, while the cells grown on the basement membrane collagens (*h*) have formed highly organized tubelike structures with branch points Hoffman interference microscopy. × 100.

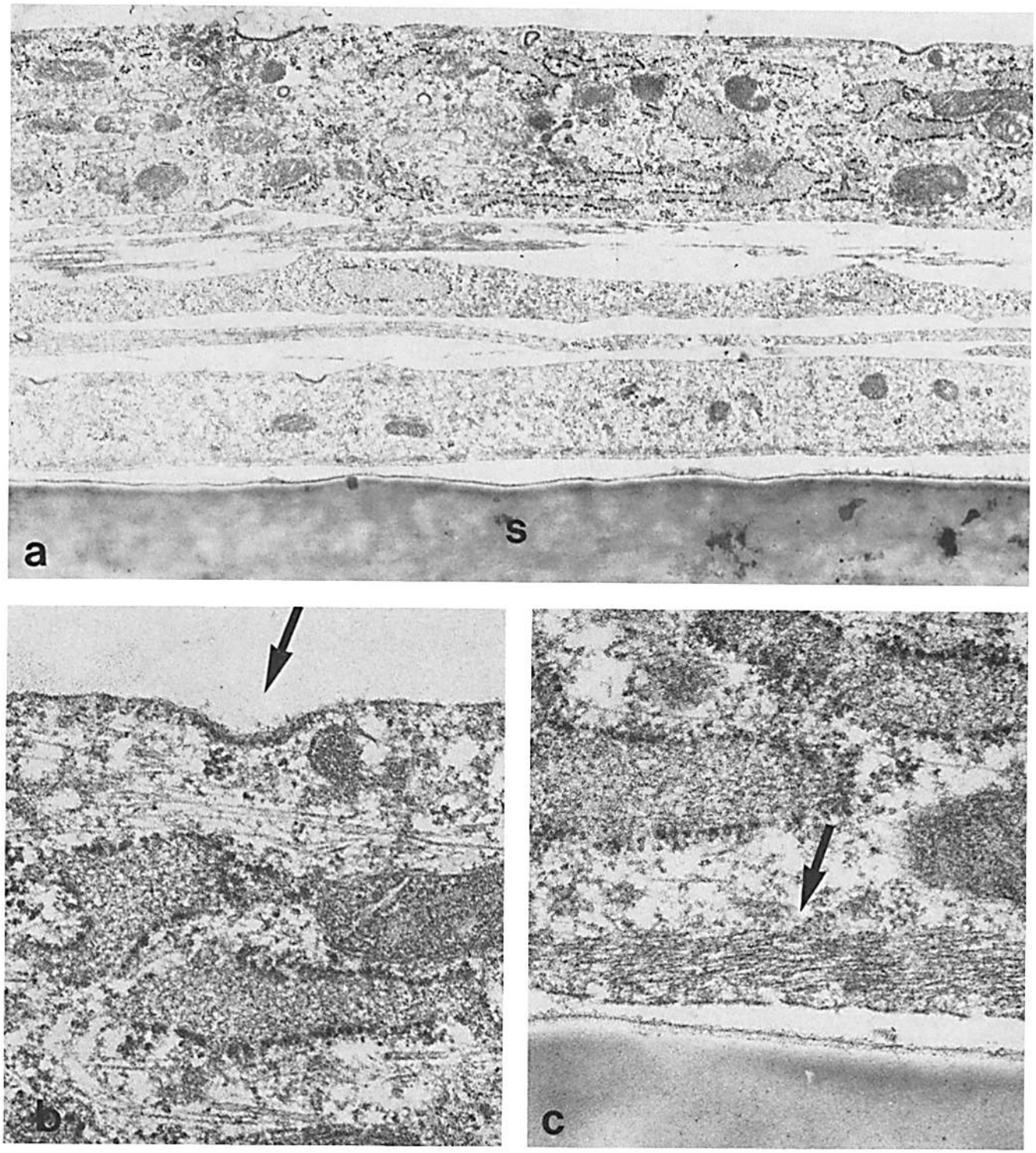

FIGURE 3 Cross sectioning analysis of capillary endothelial cells grown on interstitial collagens as observed in Fig. 2g. (a) Low power view of thin sections of cells reveals a flattened, spread morphology. Cells are noted to be three to four cells in depth and have organized filamentous arrays on their basal surfaces (surfaces abutting the coated liners) and occasional coated pits on their apical surfaces. *S*, coated liner of Falcon petri dish × 1,100. (*b*) Higher power micrographs reveal coated pits (arrow) on apical surfaces of the cells. × 15,600. (c) Other fields reveal filaments (arrow) in close opposition to the cytoplasmic aspect of the plasma membrane in organized arrays running parallel to the liner surface. × 15,600.

When cells grown on interstitial collagen coatings or uncoated plastic are cultured for 2–4 wk, occasional "tubelike" structures form that have many branch points (20). Such formations have been observed by others (8, 18) and are thought to represent in vitro "angiogenesis" or vessel formation; the organized, differentiated state of endothelial cells. When such cultures are labeled with antibodies to connective tissue components, the tubelike structures label specifically with types IV and V collagen and laminin (all components of basement membranes) (20). In light of these observations and the intimate physical association that exists between basement membrane components and endothelial cells in vivo (10, 24, 28),

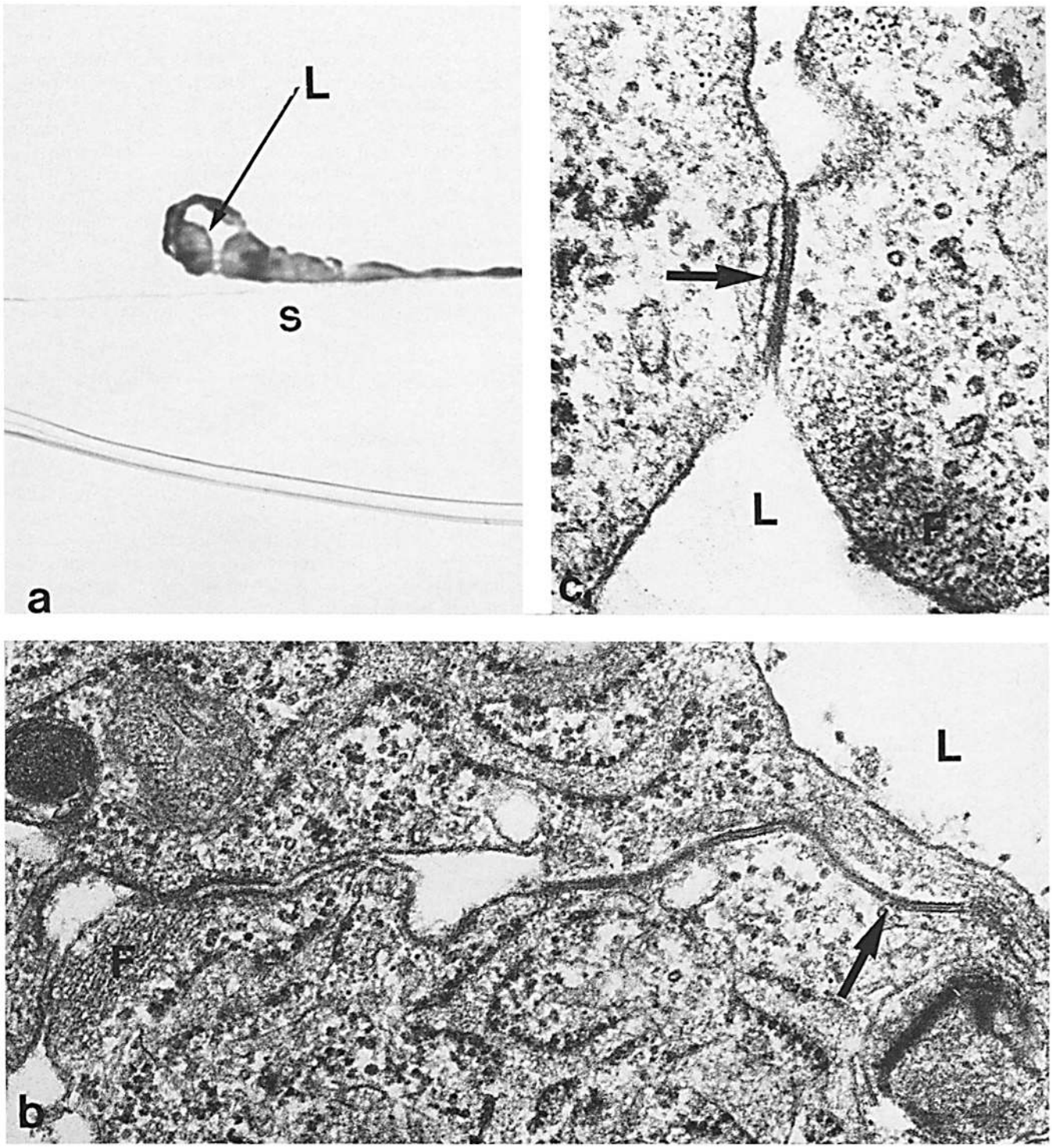

FIGURE 4 Cross sectioning analysis of capillary endothelial cells grown on basement membrane collagens for 96 h, as observed in Fig. 2*h*. Low-power views of thin sections reveal (*a*) cells which have formed a tube with a lumen (*L*) formed by apposition of cell processes. *S*, coated liner of Falcon petri dish. × 1,100. (*b*) Higher power micrographs reveal abutting cell processes that are noted to join by junctional processes (arrows) having dense membranous plaques × 19,500. (*c*) Organized cytoplasmic arrays of filaments (*F*) adjacent to the junctional complexes (arrow) are also noted. × 26,000.

we reasoned that matrix components may have profound effects on the biology of cultured capillary endothelial cells.

Morphology of Capillary Endothelial Cells Grown on Various Extracellular Matrix Components

When cultured capillary endothelial cells are plated on petri dishes coated with heterologous (human) interstitial types I and III collagen they adhere, spread, and assume variable morphologies from roughly polygonal to fusiform. They proliferate and eventually form a confluent cell layer (Fig. 2*g*). In contrast, capillary endothelial cells grown on petri dishes coated with heterologous (human) basement membrane collagens IV and V adhere, spread, and have a very elongated, spindlelike morphology. There is not a great deal of proliferation and the cells aggregate and eventually form branched

tubelike structures (Fig. 2*h*). A comparison of cells grown on heterologous interstitial and basement membrane collagenous substrata is illustrated in Fig. 2,*a–h*. In such experiments tubelike structures were observed after 96 h in culture on basement membrane collagens. When examined with the electron microscope, capillary endothelial cells grown on type I/III collagens appear as flattened cells approximately one to three cells in thickness exhibiting organized cytoplasmic filamentous arrays, occasional coated pits, and abundant rough endoplasmic reticulum. No evidence of tube formation was observed (Fig. 3). In contrast, cells grown on type IV/V collagens were observed to form lumina having junctional complexes linking two adjacent cells or cell processes of single cells (Fig. 4).

Proliferative Stimuli of Various Connective Tissue Components on Capillary Endothelial Cells

To determine the effects (if any) of the substratum on cell proliferation, we plated capillary endothelial cells onto petri dishes coated with heterologous types I/III or IV/V collagens and counted the cell number at various time intervals. When 5.0×10^4 cells were plated onto 60-mm^2 petri dishes, approximately equal numbers of cells adhered to the different collagen substrata $\sim 2.0 \times 10^4$ cells/dish. However, upon incubation for several days (five) the cells plated on the interstitial collagen substrata (I/III) underwent marked proliferation (5.0×10^4 cells/dish) as compared with the cells plated on the basement membrane collagen substrata (IV/V) (2.6×10^4 cells/dish). This is illustrated in Fig. 5*a*. Representative high-power fields of cells grown on collagen types I/III (Fig. 5*c*) and collagen types IV/V (Fig. 5*b*) are shown for comparison. Seven determinations were made on each collagen type. Standard deviations are represented by markings on the bars of the graph.

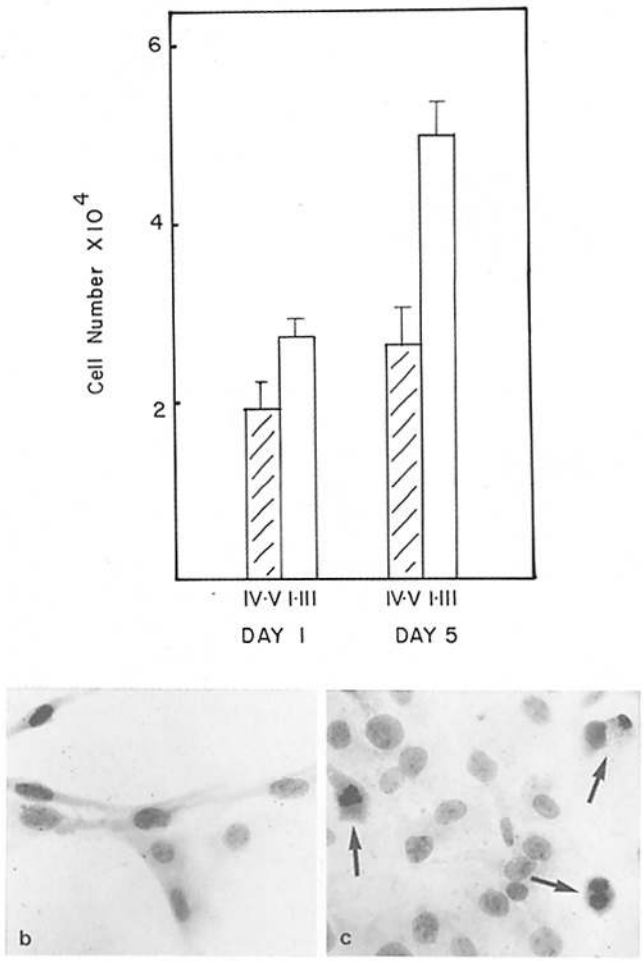

FIGURE 5 (*a*) Comparison of the proliferation rates of capillary endothelial cells grown on basement membrane collagens IV/V vs. interstitial collagens I/III. Note the marked increase in cell number noted on day 5 of the cells grown on interstitial collagens. Lines above the bars represent the standard deviations. (*b* and *c*) Representative micrographs of cells grown on types IV/V collagen (*b*) and types I/III collagen (*c*) revealing organized tubelike structures having no mitotic figures (*b*) vs. a more dense cell covering with several mitotic figures per high-power field (arrows). × 400.

Connective Tissue Macromolecule Expression by Capillary Endothelial Cells Grown on Various Substrata

MORPHOLOGICAL DETERMINATIONS: Since connective tissue components have been shown to be important in modulating (in part) the synthetic profiles in a variety of cell culture systems (12, 15), the effects of growing capillary endothelial cells on heterologous interstitial or basement membrane collagen substrata on matrix biosynthesis were studied. When cells were grown on heterologous interstitial collagen and labeled with species-specific antibodies to the various connective tissue components, there was uniform cytoplasmic and matrix labeling with antibodies to type I and III collagens. Faint, lacy, matrix-associated labeling was observed with antibodies to type V collagen, and only faint cytoplasmic labeling in perinuclear regions was observed using antibodies to type IV collagen (Fig. 6, *a–e*). In contrast, when cells grown on heterologous basement membrane collagen type IV were labeled, types I and III collagen were found to have uniform cytoplasmic localizations. Labeling with species-specific antibodies to type IV collagen revealed intense uniform cytoplasmic and matrix-associated labeling especially in areas of tube formation. Similar findings were observed when antibodies to type V collagen were used (Fig. 6,*g–k*).

When cells grown on interstitial or basement membrane collagens were labeled with antibodies to laminin, faint uniform cytoplasmic labeling was noted in cultures grown on interstitial collagens, while intense matrix-associated labeling was noted in areas of tube formation in cultures grown on basement membrane collagen (Fig. 6,*f* and *l*).

IMMUNOCHEMICAL DETERMINATIONS: To quantitate the differences observed in the immunoperoxidase labeled cell cultures, we performed quantitative immunoprecipitation of biosynthetically-labeled collagens and quantitative ELISA inhibition assays of the collagens synthesized.

The collagens synthesized by these capillary endothelial cells when grown on plastic substratum reveal a predominance of interstitial collagens, with type I being the greatest component (Table I *A*, *B*, and *C*). The data are consistent whether the immunoprecipitation method or the ELISA assay is used, revealing a good correlation between the two methods. Therefore, for assay of the collagens synthesized by the capillary endothelial cells grown on collagen types I/III or IV/V or IV coated dishes, the ELISA assay was used. In comparing the relative amounts of collagens produced by cells grown on plastic to those grown on collagen-coated dishes, several striking differences are noted: (*a*) The percentage of interstitial

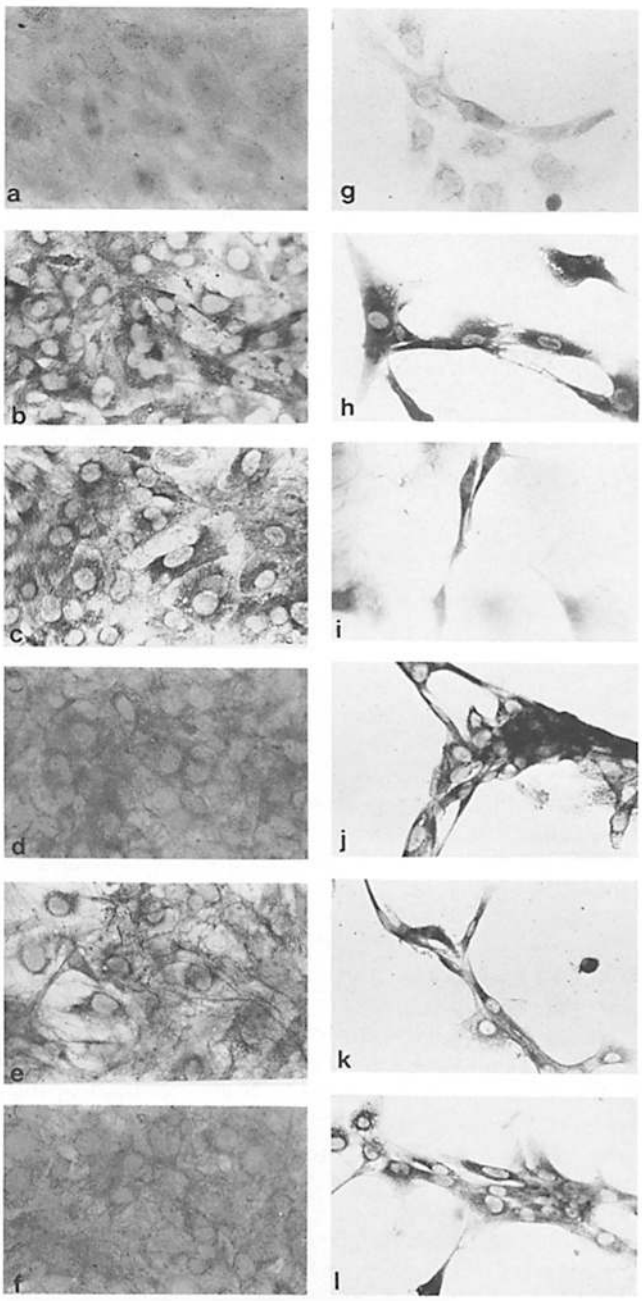

FIGURE 6 Immunoperoxidase labeling of capillary endothelial cells grown on heterologous interstitial collagen types I/III (*a–f*) and heterologous basement membrane collagen type IV (*g–l*) using species-specific collagen antibodies. (*a* and *g*) Negative controls incubated with normal rabbit IgG and sheep anti-rabbit IgG coupled with horseradish peroxidase. (*b* and *h*) Cells labeled with rat type I antibody. Note the uniform intracellular and matrix staining of the cell layer grown on interstitial collagens and the cellular labeling on cells grown on basement membrane collagen. (*c* and *i*) Cells labeled with rat type III antibody. The labeling patterns are identical to those observed for the previous antibody (rat type I antibody). (*d* and *j*) Cells labeled with EHS tumor type IV antibody. Note the faint perinuclear labeling of the cell layer grown in interstitial collagens in contrast with the intense cellular and matrix labeling in the tubelike structures formed by cells grown on basement membrane substrata. (*e* and *k*) Cells labeled with human type V antibody. Note the lacy matrix labeling of the cell layer grown on interstitial collagens in contrast with the intense cellular matrix labeling in the tubelike structures formed by cells grown on basement membrane substrata. (*f* and *l*) Cells labeled with EHS tumor laminin antibody. The labeling patterns are identical to those observed for the type IV antibody, i.e., faint cytoplasmic labeling on interstitium-grown cells vs. intense cytoplasmic and matrix labeling on basement membrane-grown cells. × 400.

collagens drops sharply from ~80 to ~50% while the relative amount of basement membrane collagens rises from ~10 to ~40% (Table I*C*). (*b*) Changes in the ratios of the interstitial collagen types I and III and of the interstitial collagen types IV and V are noted. In comparing cells grown on plastic, interstitial collagen substrata and basement membrane substrata, the I/III ratio is observed to drop from ~3:1 to 1.5:1 (Table I*C*). When the IV/V ratios are examined, the amount of type IV collagen relative to type V is observed to increase when going from plastic to interstitial collagen substrata to basement membrane collagen substrata, namely IV/V ratios of 1:5 to 1:3 to 1:2 (Table I*C*). (*c*) The interstitial collagens incorporated into the cell layer differ in cells grown on collagenous substrata (Table I*B*). Similar changes are also observed for the basement membrane collagens. Specifically, there is a marked relative increase in type IV collagen observed when cells grown on plastic are compared with those grown on collagenous substrata (Table I*B*). In addition, significant changes are observed in the collagen type IV/V ratios when comparisons of cells grown on interstitial vs. basement membrane collagenous substrata are made, namely increases in type IV relative to type V collagen in the latter (Table I*B*). (*d*) Analysis of the media collagen fractions reveals findings similar to those observed by others in several systems, namely that, of the interstitial collagens, type I is present in the greatest amount, presumably because of inefficient, slow processing, and that only small amounts of type IV collagen are detectable in the media fractions of "normal" cells in culture (Table I*A*).

Morphology of Capillary Endothelial Cells Grown on the Stromal (Interstitial) Aspect or the Basement Membrane Aspects of Amnionic Membranes

To more closely mimic in vivo basement membrane and interstitial stromal environments, capillary endothelial cells were plated on the basement membrane or stromal surfaces of acellular amnionic membranes as described by Liotta et al. (17). The acellular amnions are composed of a basement membrane structure lying on an interstitial stroma. This stroma is made up of a densely packed, interstitial collagen matrix consisting mostly of thin delicate fibrils underlying the basement membrane and a more loosely packed interstitial collagen matrix consisting of larger fibrils beneath the densely packed fibrillar region (see Fig. 9*a*). To determine the composition of the amnions, we performed immunofluorescence microscopy. By immunofluorescence labeling the basement membrane was found to be composed of collagen types IV and V and laminin. The stromal component was found to be composed of predominantly type III collagen present in the stromal area underlying the basement membrane and type I collagen present throughout the entire width of the stroma. No fibronectin was detected in these washed acellular amnions (Fig. 7, *a–e*).

Capillary endothelial cells plated on the basement membrane surface became adherent to and spread on the amnionic basement membrane. Endothelial cells eventually covered this surface, and "tube" formation was noted. At no time did the

TABLE I

Quantitative Immunoprecipitation/ELISA of Newly Synthesized Capillary Endothelial Cell Collagen Types

Substratum	Collagen types				Percent interstitial collagens	Percent basement membrane collagens	Ratios	
	I	III	IV	V			I/III	IV/V
A. Media collagens								
Plastic-Immppt.	71	23	—	6	94	6	3.1:1	—
Plastic-ELISA	71	22	—	7	93	7	3.2:1	—
I/III-ELISA	60	11	3	26	71	29	5.5:1	1:9
IV/V-ELISA	59	10	2	29	69	31	5.9:1	1:15
B. Cell layer collagens								
Plastic-Immppt.	60	20	—	20	80	20	3.0:1	—
Plastic-ELISA	59	25	4	12	84	16	2.4:1	1:3
I/III-ELISA	8	36	18	28	44	56	0.2:1	1:2
IV/V-ELISA	8	33	27	32	41	59	0.2:1	1:1
C. Total collagens								
Plastic-Immppt.	65	22	—	13	87	13	3:1	—
Plastic-ELISA	65	23	2	10	88	12	3:1	1:5
I/III-ELISA	34	24	10	32	58	42	1.5:1	1:3
IV/V-ELISA	34	22	15	29	55	45	1.5:1	1:2

Immppt, Quantitative immunoprecipitation method as described in reference 18; *ELISA*, Quantitative ELISA inhibition assay method as described in reference 23.

cells migrate through or invade the basement membrane (Fig. 8*a*). On the luminal surface of the tubelike structure, coated pits are observed as are coated vesicles and occasional plasmalemmal vesicles with stomatal diaphragms (Fig. 8, *b*, *c*, *d*). Beneath the luminal surface, organized filamentous arrays are noted in the adjacent cytoplasm (Fig. 8*d*). In areas of contact with the amnion basement membrane, several types of cell attachment are noted. In some areas there is close opposition of basal lamina and the cell surface (10–20 nm). At these points the plasmalemmal membrane is dense, and dense submembranous filamentous plaques are noted as are filamentous arrays in the adjacent cytoplasm in a plane parallel to the basal lamina (Fig. 8*e*). In other areas the attachment sites are different in that the cell is farther from the basal lamina (100 nm or greater). These sites are characterized by focal, loose, irregular, amorphous areas of matrix making contact with the cell surface. In these areas the cell surface exhibits dense submembranous filamentous plaques with intracellular filaments terminating at these areas (Fig. 8*f*). When small 1-mm^2 areas of the basement membrane were mechanically debrided with a pasture pipette and capillary endothelial cells were plated on such basement membranes, the endothelial cells were observed to migrate into the stromal area only through the debrided areas (data not shown). In contrast, capillary endothelial cells plated on the stromal surface were observed to migrate into the interstitial stroma but not through the basement membrane (Fig. 9*a*). If cells plated on the stromal surface were cultured for longer periods of time (21–30 d), the cells that had migrated into the stroma were occasionally observed to form tubelike structures, only in areas of high cell density (Fig. 9*b*). These structures were composed of interdigitating processes of one or two cells having a central lumen containing amorphous electron-dense debris. The cell processes were observed to be interdigitating, and attachment plates were sometimes noted (Fig. 9, *c*, *d*, and *e*).

DISCUSSION

A large body of evidence has been adduced supporting the notion that epithelial-mesenchymal interactions play important, if not pivotal, roles in morphogenesis, development, repair, and maintenance in many model systems (12, 15). In addition, specific roles have been assigned to selected matrix molecules and domains of these molecules in phenomena such as enhancement of cell attachment, spreading, proliferation, migration, and differentiation in vitro (12, 15, 26, 38). Specifically, various matrix components have been used to enhance proliferation and differentiation of large vessel and capillary endothelial cells in culture (5, 8, 11). Furthermore, in situ and in vitro studies of neovascularization following wounding suggest some degree of matrix-endothelial cell interaction as well (14).

In this study as in previous studies (8, 18, 20), long-term culture of capillary endothelial cells led to the formation of "tubelike" structures when the cells were grown on native or denatured interstitial collagen substrata. These tubelike structures label intensely with antibodies directed to basement membrane constituents, namely types IV and V collagen and laminin (20). When these capillary endothelial cells were plated on a basement membrane collagen substrata, "tube" formation was noted much earlier when compared with cells plated on interstitial collagen substrata (96 h vs. 14–21 d). This finding is compatible with the notion that the substrata, in part, affect the morphological phenotypic expression of capillary endothelial cells in culture. This finding of variable, endothelial cell morphologies is not unique to this culture system. Variable endothelial cell morphologies have been noted by several investigators. In in vivo preparations, arterial endothelial cells have been noted to have polygonal to fusiform shapes, depending upon the pressure they sense in a particular vascular bed (16). Others have observed and documented the so-called "high" endothelium seen in lymph node postcapillary venules, which have a cuboidal morphology. Endothelial cells of various vascular beds have also been shown to exhibit varied morphologies when regenerating after injury (16, 31). Similar findings have also been observed in culture where several morphologies have been documented including "sprout cells" and cells organizing to form tubes (4, 8, 16, 25). In addition, if one accepts the assumption that tube formation in capillary endothelial cells is a marker of

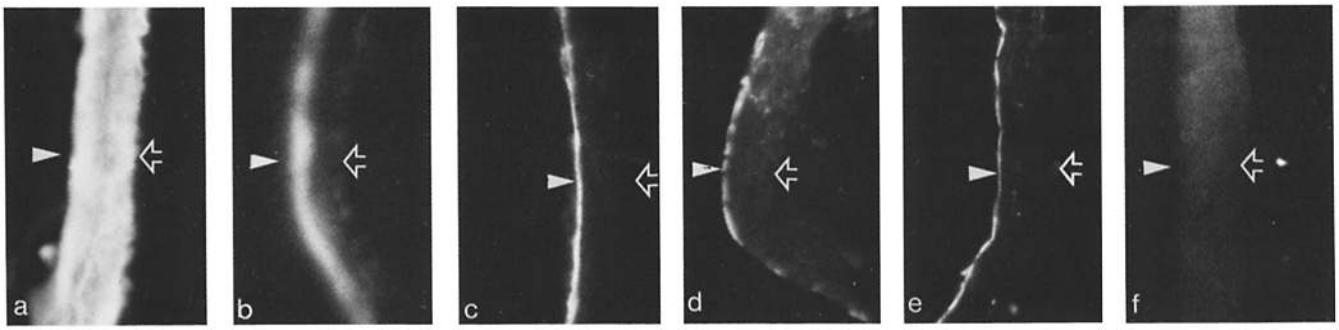

FIGURE 7 Immunofluorescence labeling of washed, acellular amnionic membrane. (a) Amnion labeled with antibodies to acid-soluble calf skin type I collagen reveals intense labeling of the entire width of the stromal compartment, sparing the basement membrane. (*b*) Amnion labeled with antibodies to pepsin soluble human placental type III collagen reveals intense labeling in the upper third of the stromal compartment, roughly demarking the densely packed collagen fibrillar area from the loosely packed collagen fibrillar area which is negative. (c) Amnion labeled with antibodies to acid-soluble EHS tumor type IV collagen reveals a smooth linear basement membrane labeling. (*d*) Amnion labeled with antibodies to pepsin-soluble human placental type V collagen reveal a granular linear labeling along the basement membrane and a faint irregular stromal fluorescence. (e) Amnion labeled with antibodies to neutral salt soluble EHS tumor laminin reveals a smooth linear basement membrane labeling. (*f*) Amnion labeled with antibodies to affinity-purified plasma fibronectin reveal no appreciable labeling. Solid arrowhead, basement membrane aspect of the amnion; open arrowhead, stromal aspect of the amnion. × 400.

the differentiated state (8, 18, 20), the matrix may also be implicated in providing a permissive or active stimulus in the process of differentiation and/or maintenance of the differentiated state (11, 36). In agreement with this concept is the finding that the composition of the substratum influences the proliferation rate of the cultured cells (Fig. 5). In a recent study by Maciag et al. (18) the proliferative state of umbilical vein endothelial cell cultures was related to the state of differentiation or organization of the cells. Thus, our findings of a high proliferative rate with no appreciable tube formation in cultures grown on interstitial collagen substrata and a low proliferative rate with tube formation in cultures grown on basement membrane collagen substrata are in agreement with this notion and implicate the substrata as one controlling factor.

In addition to the morphological data suggesting that collagen type influences differentiation, the findings of basement membrane components intimately associated with tube formation can also be construed as evidence for a differentiated state. There is an apparent discrepancy noted in matrix components present surrounding capillaries in situ (basement membranes composed of types IV and V collagen and laminin [24, 29]) when contrasted with the matrix components produced by capillary endothelial cells in culture (predominantly types I and III collagen [30]). Previously, we demonstrated that the basement membrane-associated collagen types IV and V as well as the glycoprotein laminin are present in intimate association with tubelike structures (20). In this study, cultures grown on interstitial collagen substrata synthesize predominantly interstitial collagens (which is in agreement with previous studies [30]) and are noted to express basement membrane constituents only in association with the occasional tube formations that occur only after extended culture periods (14–21 d) while other areas of these cultures express collagen types I and III predominantly. In contrast, cultures grown on basement membrane substrata express basement membrane constituents to a greater degree, and the tubelike structures that predominate from early time periods in culture (96 h) are enriched for basement membrane components. Thus, the connective tissue macromolecular biosynthetic profile of the cultured capillary endothelial cells appears to reflect and parallel the organizational (differentiation) state of the cultured cells. Previous studies have also documented variability in connective tissue synthetic profiles of cultured endothelia, lending support to the notion that there is no "specific endothelial cell collagen synthetic profile" but rather a variable synthetic response to media, factors, substratum, density, and other culture conditions (4, 25, 30).

Although the organizational (differentiational) state of the cultured capillary endothelial cells appears to be affected by the collagenous composition of the underlying substratum and although there is reasonable qualitative and quantitative agreement between morphology and matrix components present when compared with capillary beds in situ, the coated culture dishes can hardly be considered as intact biological substrata. Previous work by Liotta et al. (17) has demonstrated the usefulness of acellular amnionic membranes as a morphologically distinct and identifiable source for "intact" basement membrane and stromal substrata. Capillary endothelial cells grown on the basement membrane aspect of such amnions displayed differentiated behavior in that they form tubelike structures on the basement membrane surface at early time periods in culture. In addition, the cells forming these tubelike structures exhibited specialized morphological features on their luminal surfaces including coated pits, plasmalemmal vesicles with stomatal diaphragms, and organized intracellular filamentous arrays similar to those observed in previous in situ studies (32–35). Furthermore, abluminal cell-matrix attachment sites displayed specific diverse morphologies consistent with previously published reports on other mesenchymal cells in culture (3). In contrast, the cells grown on the stromal aspect of the amnion expressed a markedly different phenotype in that they appear to undergo considerable proliferation and migrate into the stromal substance, stopping only when contacting the underside of the basement membrane. Long-term culture of cells grown on the stromal aspect of the amnion allows the formation of occasional tubelike structures in the stroma only in areas of high cell density. This observation, while at first glance may appear to be contradictory to what has been found earlier in the paper, is consistent with

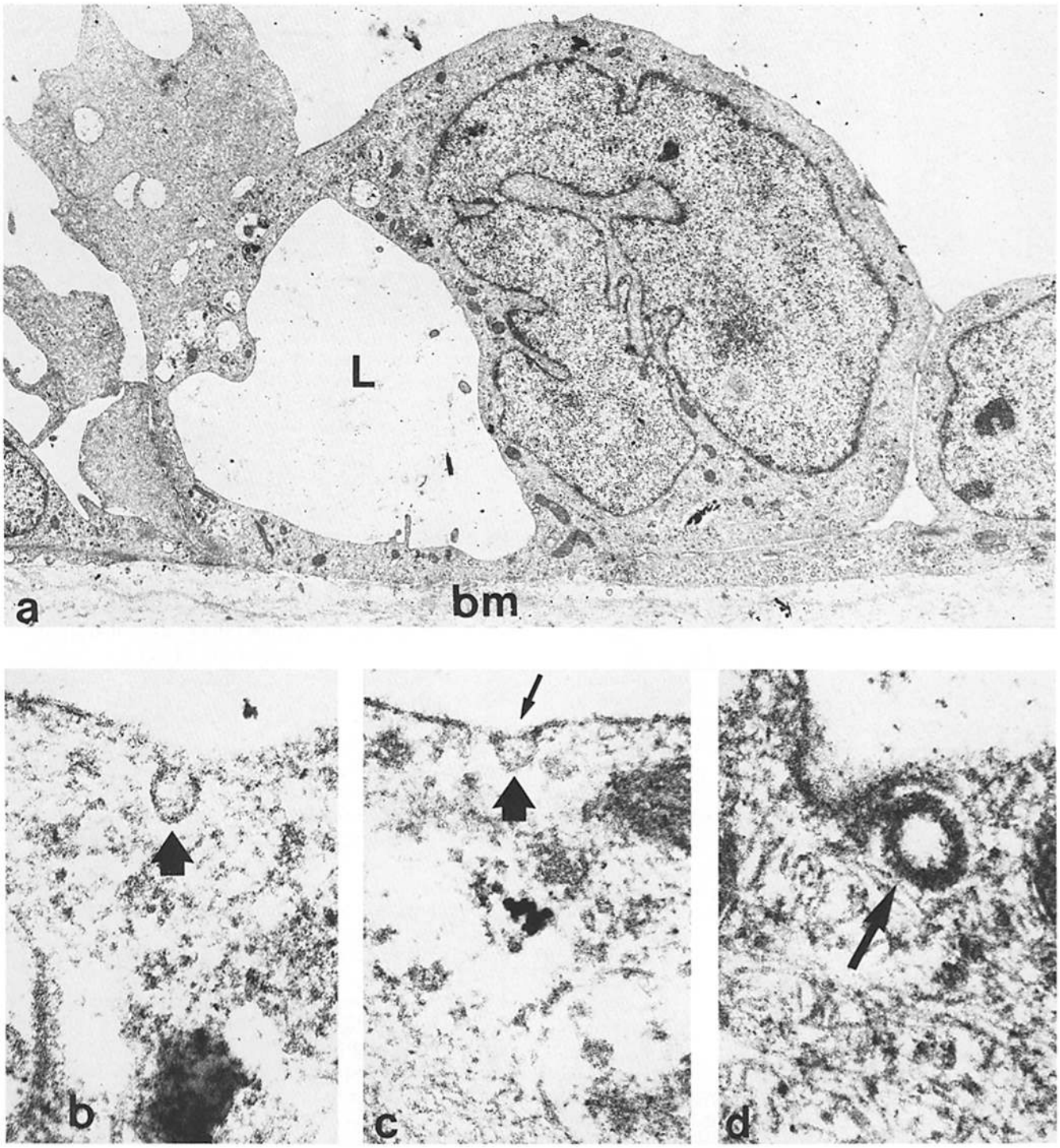

FIGURE 8 Cells grown on the basement membrane aspect of the amnion are noted to adhere to the basement membrane and form tubelike structures with lumina (*L*) early in the culture period (5 d). They never were observed to migrate through the basement membrane (*bm*). (*a*) Low-power electron micrograph of the cell forming the tubelike structure. Sections reveal a lumen (*L*) with coated pits on its surface and organized filamentous arrays and endocytic vesicles in adjacent cytoplasmic regions. × 3,100. (*b*, *c*, *d*, *e*, and *f*) Higher-power electron micrographs revealing details of luminal and abluminal structures noted in (*a*) including: (*b*) coated pits (large arrows) observed on the luminal surface; (*c*) occasional plasmalemmal vesicles (large arrow) have stomatal diaphragms (small arrow); (*d*) coated vesicles are also noted (arrow); (*e*) multiple, focal adhesion attachment sites (small arrows) are noted in areas of close contact (10–20 nm) with the underlying basement membrane and immediately above these areas, organized filamentous arrays are noted running in a plane parallel to the dense plasma membrane (large arrows); (*f*) occasionally contact with the basement membrane appeared to be far removed from the cell surface and mediated by loose amorphous matrix components (arrowhead). The plasma membrane at these points of attachment appears dense (small arrow) and arrays of microfilaments (large arrows) appear to terminate in these dense areas. × 24,000.

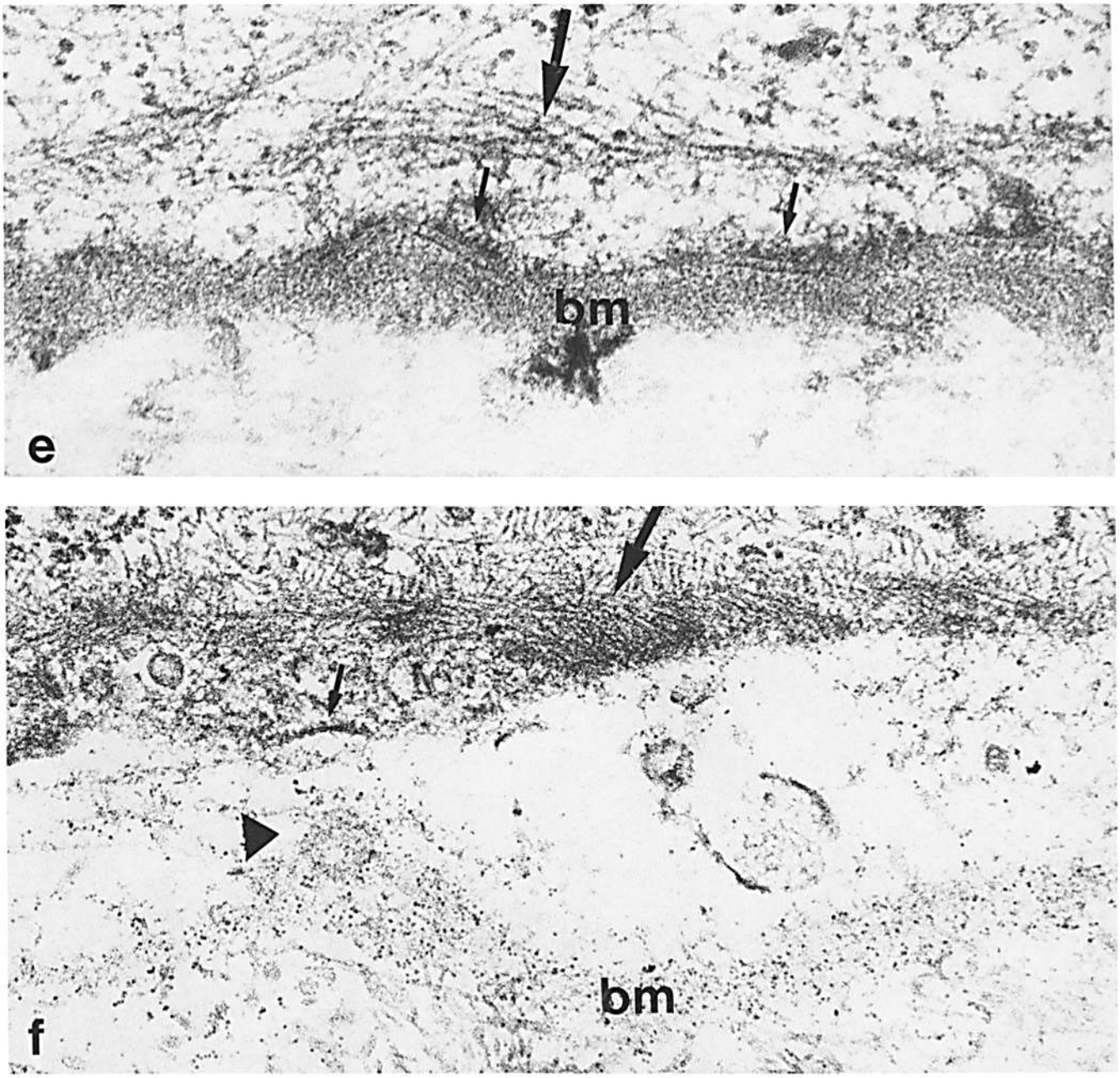

FIGURE 8

our notion of what events are taking place, namely, that the cells respond to the interstitial matrix by migrating into it and proliferating. Once at high density, they organize to form tubes. This event may be related to cell density or it is possible that the cells at high density synthesize and secrete enough basement membrane components (which are stabilized in the interstitial matrix) to affect alterations of cell attachment which lead to tube formation. This is consistent with a change or evolution to a more differentiated (organized) state with time and mimics the phenomenon of neovascularization following soft tissue injury in vivo.

The overall findings that interstitial collagens evoke a high proliferative rate and elicit a migratory response are consistent with the phenomena of capillary endothelial cell migration and proliferation observed in vivo after soft tissue injury when the endothelial cells are freed of constraints of their basement membrane and are exposed to the interstitium. The observations that the capillary endothelial cells have a low proliferative rate and associate to form tubelike structures when grown on basement membrane collagen are also in agreement with the in vivo findings of a low mitotic rate and a differentiated, organized phenotype of endothelial cells in normal capillaries or in the capillaries formed and maintained following injury.

Thus, the culture system described here represents a model for the study of capillary endothelial cell biology in which matrix components are seen to play important roles. This system may also serve as a way of modulating specific aspects of endothelial cell biology and as a method of preparing relatively large numbers of differentiated cells (tube-forming cells), thus making the study of luminal vs. abluminal surfaces and functions (polarity) possible in vitro.

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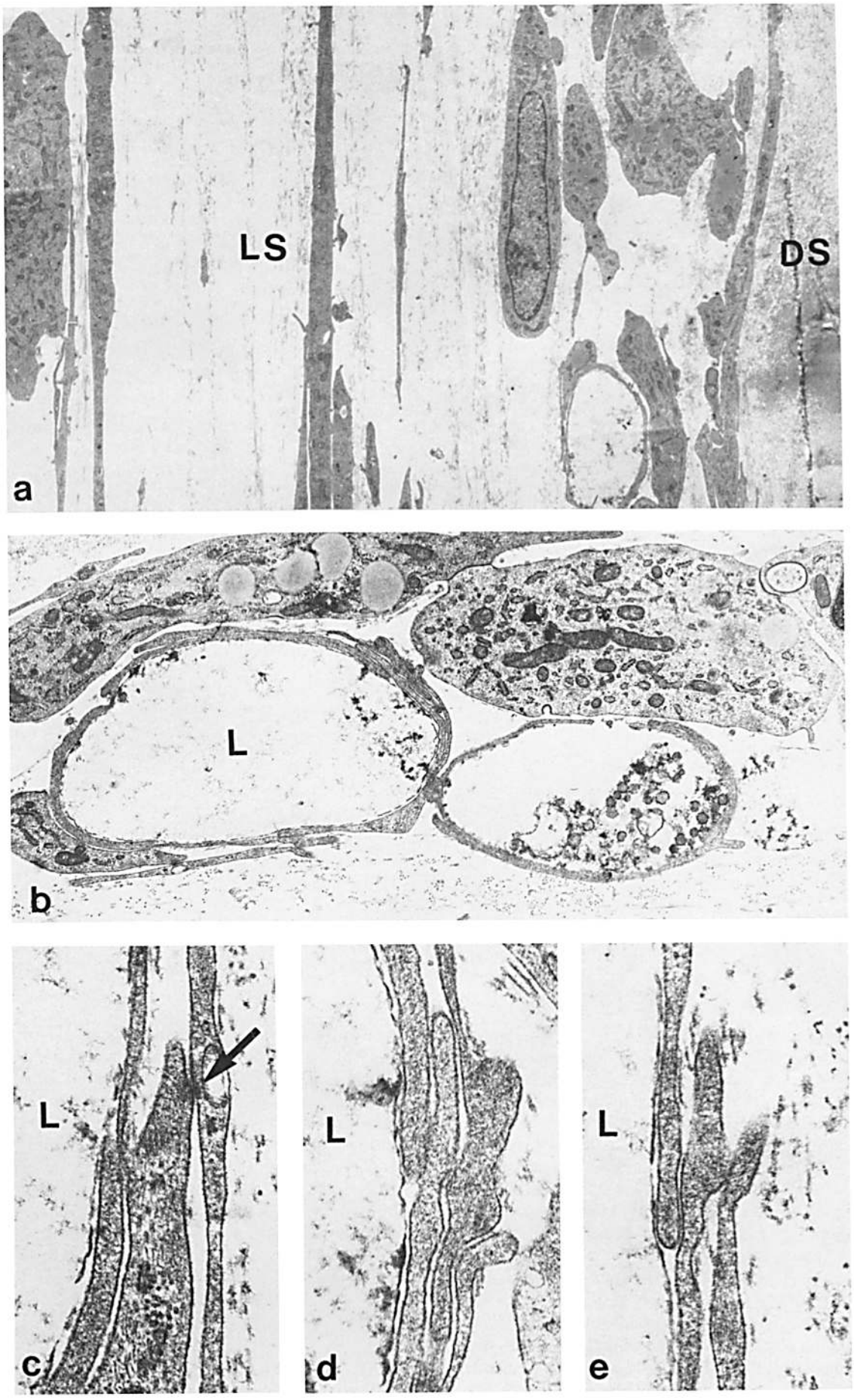
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FIGURE 9 (*a*) Long-term culture of cells grown on stromal aspect of the amnion. Cells are noted to be migrating into the stroma and by day 21 of culture are observed to form tubelike structures with lumina. × 1,700. (*b*) High-power micrograph of cell processes forming a tubelike structure. × 6,500. (*c*, *d*, *e*) Higher power micrographs of the cell processes forming a tubelike structure illustrated in (*b*) revealing interdigitating cell processes in close opposition with occasional junctional complexes (arrow). × 24,200. *L*, lumen; *LS*, loose stromal area of amnion; *DS*, dense stromal area of amnion.