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INTRACELLULAR MECHANISMS INVOLVED IN BASEMENT MEMBRANE INDUCED BLOOD VESSEL DIFFERENTIATION IN VITRO

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SUMMARY

The extracellular matrix, particularly basement membranes, plays an important role in angiogenesis (blood vessel formation). Previous work has demonstrated that a basement membranelike substrate (Matrigel) induces human umbilical vein endothelial cells to rapidly form vessel-like tubes (Kubota, et al., 1988; Grant et al., 1989b); however, the precise mechanism of tube formation is unclear. Using this in vitro model, we have investigated morphologic changes occurring during tube formation and the cytoskeletal and protein synthesis requirements of this process. Electron microscopy showed that endothelial cells attach to the Matrigel surface, align, and form cylindrical structures that contain a lumen and polarized cytoplasmic organelles. The cytoskeleton is reorganized into bundles of actin filaments oriented along the axis of the tubes and is located at the periphery of the cells. The addition of colchicine or cytochalasin D blocked tube formation, indicating that both microfilaments and microtubules are involved in this process. Cycloheximide blocked tube formation by 100%, indicating that the process also required protein synthesis. In particular, collagen synthesis seems to be required for tube formation because cis-hydroxyproline inhibited tube formation, whereas either the presence of ascorbic acid or the addition of exogenous collagen IV to the Matrigel increased tube formation. Our results indicate that endothelial cell attachment to Matrigel induces the reorganization of the cytoskeleton and elicits the synthesis of specific proteins required for the differentiated phenotype of the cells.

Key words: endothelium; basement membrane; laminin; differentiation; blood vessel; mechanisms.

INTRODUCTION

Endothelial cells line the lumen of blood vessels and form a continuous nonthrombogenic surface. Together with their underlying basement membrane, these cells provide a barrier between the blood and the extravascular connective tissue. These cells are polarized epitheloid cells with distinct apical and basal cytoplasmic domains (Kramer, 1985; Muller and Gimbrone, 1986). These cells are usually in a "quiescent" state, with complex cell-to-cell junctions, and are lined by a basement membrane (Grant et al., 1989b; Risau and Lemmon, 1988). They may become "activated", i.e. highly proliferative and migratory, especially during embryonic development, in wounds, and in inflamed tissues (Folkman, 1985; Folkman and Klagsbrun, 1987; Madri and Pratt, 1987; Risau and Lemmon, 1988). Angiogenesis, the formation of blood vessels, involves four stages: a) the cells sprout from the parent vessel, b) degrade and penetrate through the adjacent basement membrane, c) proliferate and migrate in the underlying stroma, and d) then realign and differentiate into new vessels.

Many cell- and tissue-specific factors including lymphokines (Bussolino et al., 1988; Ingber and Folkman, 1989b), heparin-binding growth factors (Folkman, 1985; Folkman and Klagsbrun, 1987; Maciag, 1989), and cytokines (deGroot et al., 1987; Maciag, 1989; Montesano et al., 1983; Montesano et al., 1987) have been shown to stimulate cell migration and proliferation in vitro (Folkman and Haudenschild, 1980; Folkman and Klagsbrun, 1987; Gosposdarowicz et al., 1978; Ingber and Folkman, 1989a; Lioté et al., 1987; Maciag, 1989; Madri et al., 1988; Sato and Rifkin, 1988). How these as well as other extracellular factors induce the resulting cascade of events which results in endothelial cell differentiation has not been elucidated due to the difficulties involved in examining blood vessel formation in vivo. Several in vitro systems have been developed to study angiogenesis. When endothelial cells are maintained at a superconfluent state for several days, approximately 30% of the cells form capillarylike structures spontaneously, with the remainder maintained in a monolayer (Folkman and Haudenschild, 1980; Maciag et al., 1982; Madri and Pratt, 1986). This is also seen if cells are first cultured and then maintained in the absence of the growth factors. Capillarylike structures will also form when the cells are cultured on substrata such as collagen I, fibronectin, and gelatin or on a fragment of aorta in a fibrin clot (Madri and Pratt, 1987; Montesano, 1986; Montesano et al., 1983; Mori et al., 1988; Nicosia et al., 1984). Depending on the culture system, endothelial differentiation usually requires from 2 days to 8 wk and the vessels may be reversed, i.e. secretion of basement membrane substances may be directed towards the lumen (Maciag et al., 1982).

The extracellular matrix plays an important role in angiogenesis. Endothelial cells utilize matrix molecules during their migration

(Risau et al., 1988). At late stages in angiogenesis the cells produce various components of basement membrane including laminin, collagen IV, entactin, and heparan sulfate proteoglycan. These matrix components may allow the cells to align themselves and form the capillarylike structures (David and LaCorbiere, 1982; Fukuda et al., 1988; Furcht, 1986; Giltay and Mourik, 1988; Grabel and Watts, 1987; Grant et al., 1989b; Ingber et al., 1989a,b; Kramer and Fuh, 1985; Kubota et al., 1988; Madri et al., 1983; Montesano, 1986; Risau and Lemmon, 1988). An in vitro system was developed that mimics end-stage angiogenesis, employing a reconstituted matrix (Matrigel) which had similar properties to normal basement membranes (Kleinman et al., 1982; Kleinman et al., 1985; Kubota et al., 1988) as a culture substratum for the endothelial cells. When large vessel or microvascular endothelial cells were incubated on this substratum, the cells attached, aligned themselves, and within 18 h formed a network of tubes on the Matrigel surface that resembled a capillary network (Grant et al., 1989b; Kubota, et al. 1988; Lawley and Kubota, 1989). This system has been used to examine the role of laminin in endothelial cell differentiation (Grant et al., 1989b; Kubota et al., 1988). The differentiated phenotype could be perturbed by the addition of antibodies to basement membrane components or by the addition of biological active laminin-derived synthetic peptides. Endothelial cells attach to Matrigel via an RGD site on the laminin molecule, then a second site containing the pentapeptide sequence, YIGSR, induces the cells to align and form the capillarylike network (Grant et al., 1989b). Here we have used this in vitro model system to investigate the mechanism of this tube formation by examining the intracellular events occurring during endothelial differentiation on Matrigel. First, we examined the morphologic changes and cytoskeletal requirements during tube formation. Using inhibitors of protein synthesis, we demonstrate that protein synthesis and, in particular, collagen IV are required for vessel formation (Maragoudakis et al., 1988; McAuslan, et al., 1988). These studies help to define the events involved in the morphologic and biosynthetic process occurring during differentiation of endothelial cells.

Materials and Methods

Cells used in the assay. Human umbilical vein endothelial cells (HUVECs) were isolated from freshly obtained cords by digesting with 0.1% collagenase (Worthington Biochemical Co., Freehold, NJ) (Jaffe et al., 1973). The cells were grown in a Medium 199 containing 20% fetal bovine serum (Hyclone Lab. Inc., Logan, UT), 200 μg/ml of ECGS (Collaborative Res., Inc., Waltham, MA), heparin (Sigma Inc., St. Louis, MO), 100 U/ml penicillin-streptomycin, 50 μg/ml gentamicin, and 2 m*M* L-glutamine. The nasal mucosal endothelial cells (NMECs) were obtained from surgically removed human nasal mucosa (Fukuda et al., 1989). The tissue was washed with Dulbecco's phosphate buffer saline (DPBS) and then the epithelial and periosteal areas were separated. The submucosal tissues were cut into 4-mm^2 sections and incubated in 0.05% collagenase (type II) plus 1% EDTA for 1 h at 37° C. The submucosal tissue was washed with Dulbecco's minimum essential medium (DMEM) and then the tissue sections were pressed to release the endothelial cells. The endothelial cells were resuspended in DMEM supplemented with 20% fetal bovine serum and antibiotics. The microvascular endothelial cells from human adipose tissue (HMVEC) were isolated by 0.4% collagenase digestion (Williams, 1987) and centrifugation through a 5% bovine serum albumin gradient. The cells were grown in a defined, low-serum-containing endothelial cell growth medium (Clonetics Co., San Diego, CA). All three cultures were characterized as endothelial cells by positive staining for angiotensin converting enzyme and by the uptake of acetylated dil-LDL (Knedler and Ham, 1987).

Matrigel substratum and assay. The tube formation assay on Matrigel was carried out as previously described (Grant et al., 1989b; Kubota, et al., 1988). Matrigel, prepared from the EHS tumor (Kleinman et al., 1986), was used to coat 16-mm Costar wells (0.25 ml/well). After polymerization of the Matrigel at 37° C, endothelial cells (40 000 cells/0.5 ml) were added to each well. Type IV collagen was prepared as previously described (Kleinman et al., 1982). The cytoskeletal inhibitors, colchicine and cytochalasin D, the protein synthesis inhibitor, cycloheximide (Sigma), the collagen inhibitor, cis-hydroxyproline (Sigma), and the collagen synthesis stimulator, ascorbic acid, were individually added directly to the medium with the cells. In addition, cycloheximide was also added 1 h before plating the cells on Matrigel and in some assays at 1, 3, and 24 h after plating the cells. After 18 h at 37° C, the medium was removed, the tissue was fixed with "DifQuick," and the tubes were stained blue for quantitation using the Optomax digital analyzer connected to an IBM PC (Grant et al., 1989b). This analyzer measured the darkly stained tubes and converted the total tube area into pixels, which was representative of the tube area in each well. In each experiment, this tube area in each test well is compared to control wells. Thus, the data are represented as a relative number and expressed as the percent tube area/control. Each compound was examined at several different concentrations (in duplicate) and each experiment was repeated at least 3 times.

The effect of cycloheximide and cis-hydroxyproline on endothelial cell protein synthesis was also examined by plating endothelial cells in plastic 24-well plates (50 000 cell/well) in a methionine-free medium, including the same drug concentrations as outlined above, along with 10 μCi/ml of [^{35}S]methionine per well. The cells were incubated with the isotope at 37° C for 4 h. Each well was then washed and the cells were lysed with 100 μl of lysis buffer [0.1 *M* Tris-HCl-NaCl, 1% Triton X-100, 1% deoxycholate, 0.1% sodium dodecyl sulfate (SDS), 1.0% aprotinin (Sigma), 0.02% sodium azide and 2 m*M* phenylmethylsulfonyl fluoride]. Then 10 μl from each well was used to obtain trichloroacetic acid (TCA) precipitable counts.

Electron microscopy. After tube formation on Matrigel, the medium was aspirated and the well was rinsed with phosphate buffered saline (PBS). After fixation for 2 h at room temperature in 2.5% glutaraldehyde in PBS, the preparation was washed in PBS and then immersed in 1% buffered OsO_4 for 2 h at room temperature. For transmission electron microscopy, the preparation was washed with water, dehydrated in ethanol-propylene oxide, and routinely embedded in the Epon substitute, Ladd LX-112 Resin Kit (Ladd Res. Ind. Inc., Burlington, VT). Thin sections were cut perpendicular to the surface of the Matrigel to obtain cross sections of the tubes, and were viewed in a Zeiss electron microscope. Pictures from 20 different areas on different sections were taken in the electron microscope, and enlarged micrographs were examined carefully. For scanning electron microscopy, the preparation was rinsed in phosphate buffer and then incubated in a saturated aqueous solution (1% wt/vol filtered) of thiocarbohydrazide for 10 min. The preparation was then rinsed with distilled water 5 times and reincubated

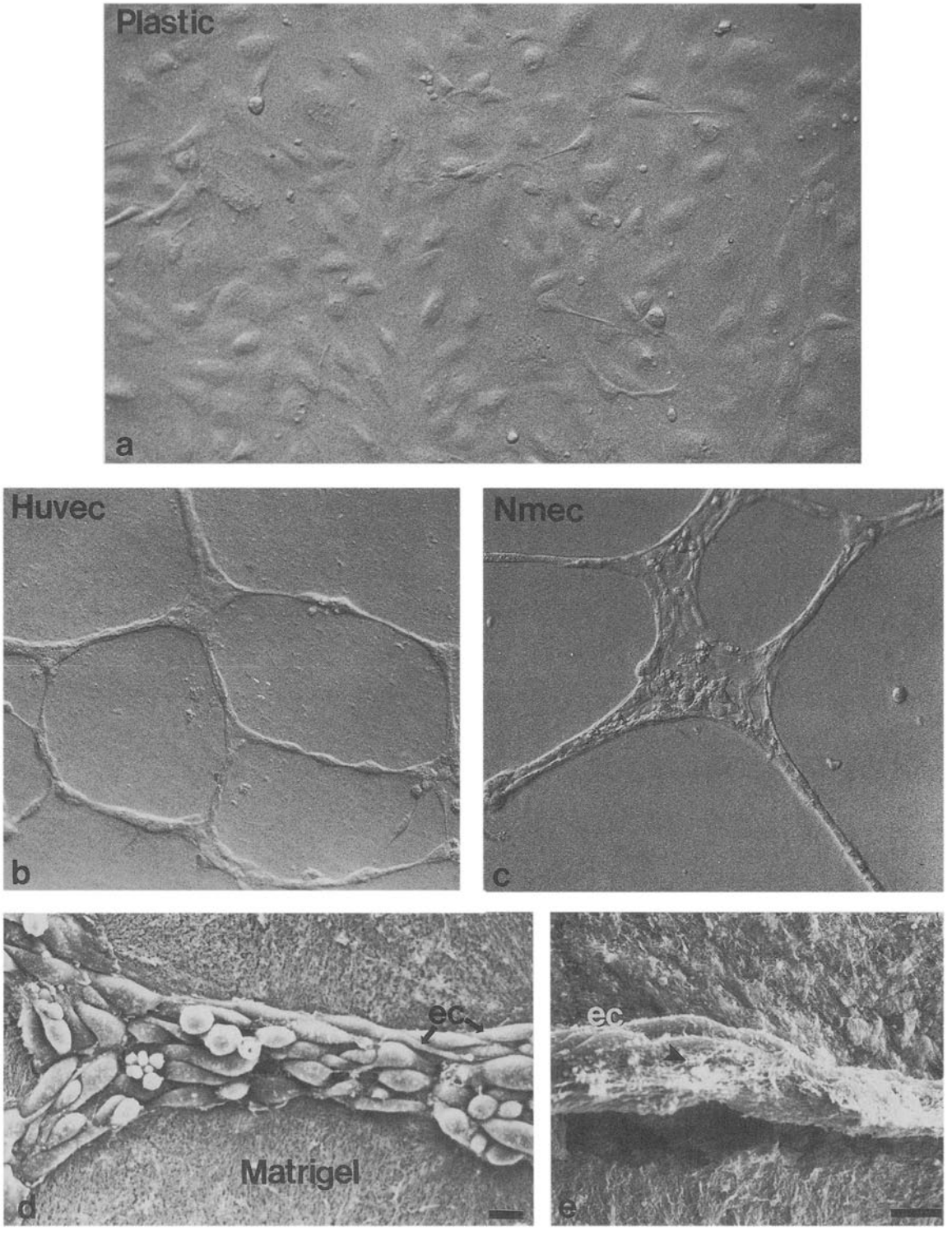

FIG. 1. Light and electron microscopic morphology of endothelial cells on plastic and Matrigel. Human umbilical vein endothelial cells (*a* and *b*) and human nasal mucosal endothelial cells (*c*) are shown on plastic (*a*) and on Matrigel (*b* and *c*) (×400); *d* and *e* show scanning electron microscopy of a HUVEC tube at 6 h (*d*) and 24 h (*e*) on Matrigel. *Note* the elongated individual cell (*ec*) making up the tube at 6 h. and the particles of fuzzy material on the tube surface after 24 h. *Bars* = 10 μm.

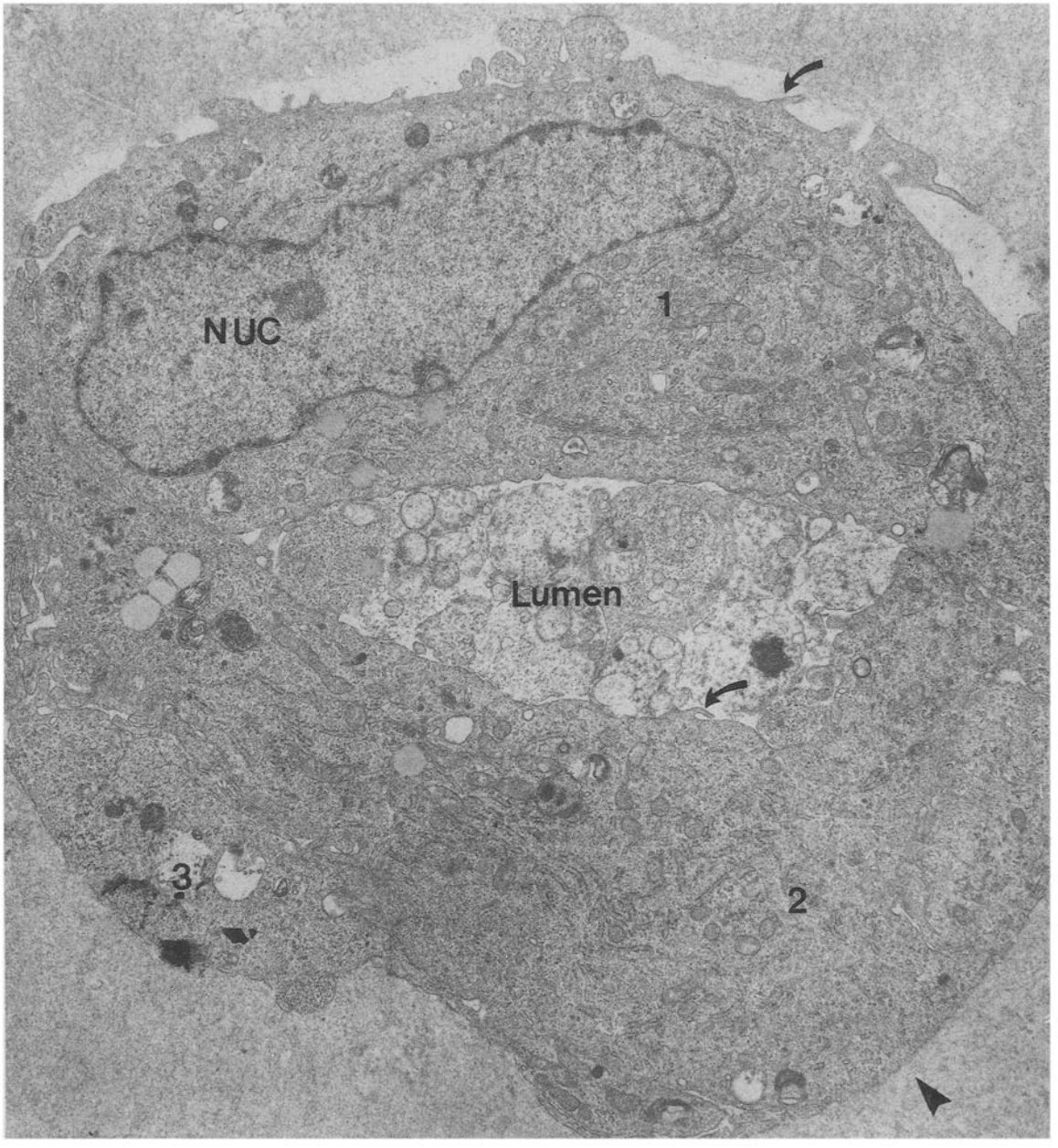

FIG. 2. A transmission electron micrograph of a cross section through one of the HUVEC tubes on Matrigel. There are three cells (*1,2,3*) which make up this cross section. They surround a central lumen which contains cellular debris. The cell surface in contact with the Matrigel is smooth (*arrowhead*), in contrast to the adlumenal processes seen at the top of the tube and the membrane lining the lumen (*arrows*). ×18 000.

with 1% OsO_4 for 30 min. After rinsing with distilled water, the specimen was dehydrated in acetone, critical point dryed, and mounted. The surface of the Matrigel containing the tube network was carbon coated and observed in a JOEL scanning electron microscope.

Staining for intracellular F-actin filaments. Endothelial cells (HUVECs and HMVECs) plated at 10^4 and 10^5 cells/well were incubated overnight in two chamber glass-slides with and without a 0.5-mm thick Matrigel coat. After 12 and 18 h, the wells were washed with warm PBS and then immediately fixed with 3.7% formaldehyde in PBS for 10 min. Wells were washed 3 times with PBS and then the preparation was incubated in 0.1% digitonin (at 37° C) for 15 min. The wells were washed with PBS 3 times, then the cells were incubated with 10 μl/ml of rhodamine phalloidin solution (Molecular Probes Inc., Eugene, OR) in PBS for 1 h at 37° C. Wells were washed twice with PBS and mounted in 90% glycerol in PBS. The cells were viewed on a Nikon Fx microscope equipped with fluorescence optics. Images were taken using a 40× objective.

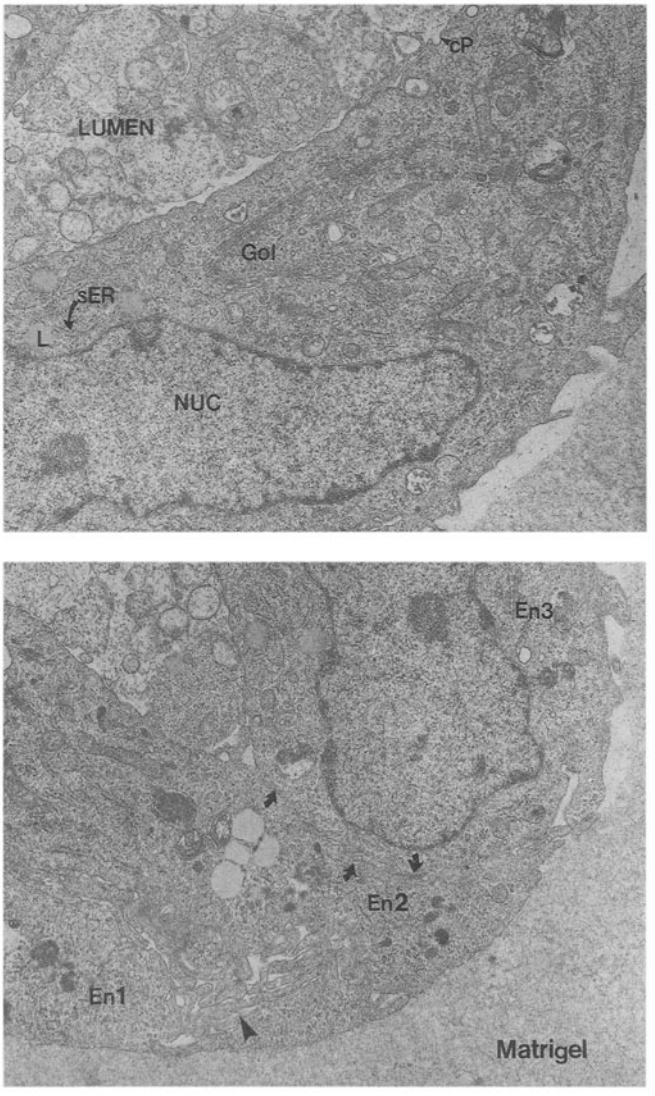

FIG. 3. Cross section of a tube on Matrigel. *Top*, a portion of a endothelial cell with the lumen in the *upper left* and Matrigel in *lower right*. The cell's cytoplasmic organelles are polarized exhibiting a large Golgi apparatus (*Gol*), smooth endoplasmic reticulum (*sER*), coated pits (*cP*), lipid granules (*L*), and numerous granules in the apical cytoplasm above the nucleus (*NUC*). ×28 750. *Bottom*, the junction between three cells. There are numerous interdigitating processes between cells 1 and 2 (*arrowhead*). Cells 2 and 3 show numerous areas in which junctional specializations are present (*arrows*). *Note* that the cell membrane in contact with the Matrigel is smooth. ×27 500.

The cells on Matrigel were processed on a Tracor Nothern 8502 image analyzer to reduce background fluorescence.

RESULTS

In culture, endothelial cells (HUVECs) appear as a flat cobblestone monolayer (Fig. 1 *a*). The nasal mucosal endothelial cells (NMECs) are similar to the HUVECs, except that the NMECs tend to be more elongated and spindle shaped. Within 18 h on Matrigel, both cell types attach and organize into an irregular network of tubes on the surface of the Matrigel (Fig. 1 *b,c*). As observed by scanning electron microscopy, these tubes were comprised of many cells (*ec*) which overlapped and adhered to one another (Fig. 1 *d*). In some assays, differentiated cells (after being on Matrigel for 24 h) demonstrated an accumulation of a filamentous network similar to the Matrigel itself. Transmission electron microscopy of cross sections through the tube revealed two to three cells that surround a central lumen (Fig. 2). Areas of the endothelial cell membrane which directly contacted the Matrigel were fairly smooth (*arrowhead*), whereas the lumenal cell membrane and upper surface of the tube not in contact with Matrigel was studded with micro villus-like projections (Fig. 2, *arrows*). Often the lumen of these tubes contained cellular debris (Fig. 2). There was some polarization of the cellular organelles with the nucleus in a basolateral location, whereas the Golgi apparatus, numerous vesicles, and granules were proximal to the lumen (Fig. 3, *top*). In addition, coated pits were present on the lumenal plasmalemma. The cytoplasmic membranes of adjacent cells were closely associated, and the junctional specializations between the cell membranes were present (Fig. 3, *bottom*). Recently, experiments performed by Fernando Varges (FDA), using lucifer yellow injections, demonstrated that these cells are physically and electrically coupled (unpublished results). Often, numerous interdigitating processes were seen between the cells (Fig. 3, *bottom*).

Examination of actin filaments in HMVECs stained by phalloidin-rhodamine, demonstrated that the cytoskeleton of HMVEC cells grown on plastic either at low or high density was arranged into starlike clusters with some criss-crossing actin filaments (Fig. 4). The actin distribution resembled the stress fibers observed with other cell types. Endothelial cells forming tubes on Matrigel had a quite different distribution of actin. The filaments ran parallel to one another along the axis of the tubes from cell to cell and were concentrated along the edges of many tubes. In addition, the actin network seemed to run continuously from one cell to the next.

We studied the role of the cytoskeleton in tube formation using the microtubule and microfilament inhibitors colchicine and cytochalasin D. Addition of increasing amounts of colchicine (0 to 20 μg/ml) at the time of plating endothelial cells on Matrigel inhibited tube formation, with maximal inhibition of tube formation at 60 to 70% of that normally observed (Fig. 5). In contrast, cytochalasin D inhibited 100% of tube formation at a concentration of about 10 μg/ml. It should be noted that the cells remained attached to the Matrigel but did not align. We conclude that both microtubules and microfilaments are required for tube formation.

To examine the involvement of protein synthesis in tube formation, cycloheximide was added (1 to 5 μg/ml) at the start of the assay. Cycloheximide caused a dose-dependent inhibition of tube formation with almost complete inhibition at 3 μg/ml (Fig. 6). At a dose of 2 μg/ml, less than 10% of the added cells were found floating in the media containing cycloheximide, indicating the cells had not detached (data not shown). The TCA precipitable counts of incorporated [^{35}S]Methionine also demonstrated that almost 100% of protein synthesis was inhibited in these cells by the addition of 3 μg/ml of cycloheximide. In addition, cycloheximide (2 μg/ml) was added at various times preceeding or following the plating of endothelial cells (Fig. 7). At early time points (−1, 0, and 1 h), tube formation was significantly inhibited (60 to 70%), whereas after 3 and 24 h tube formation was reduced by only 30%, suggesting that

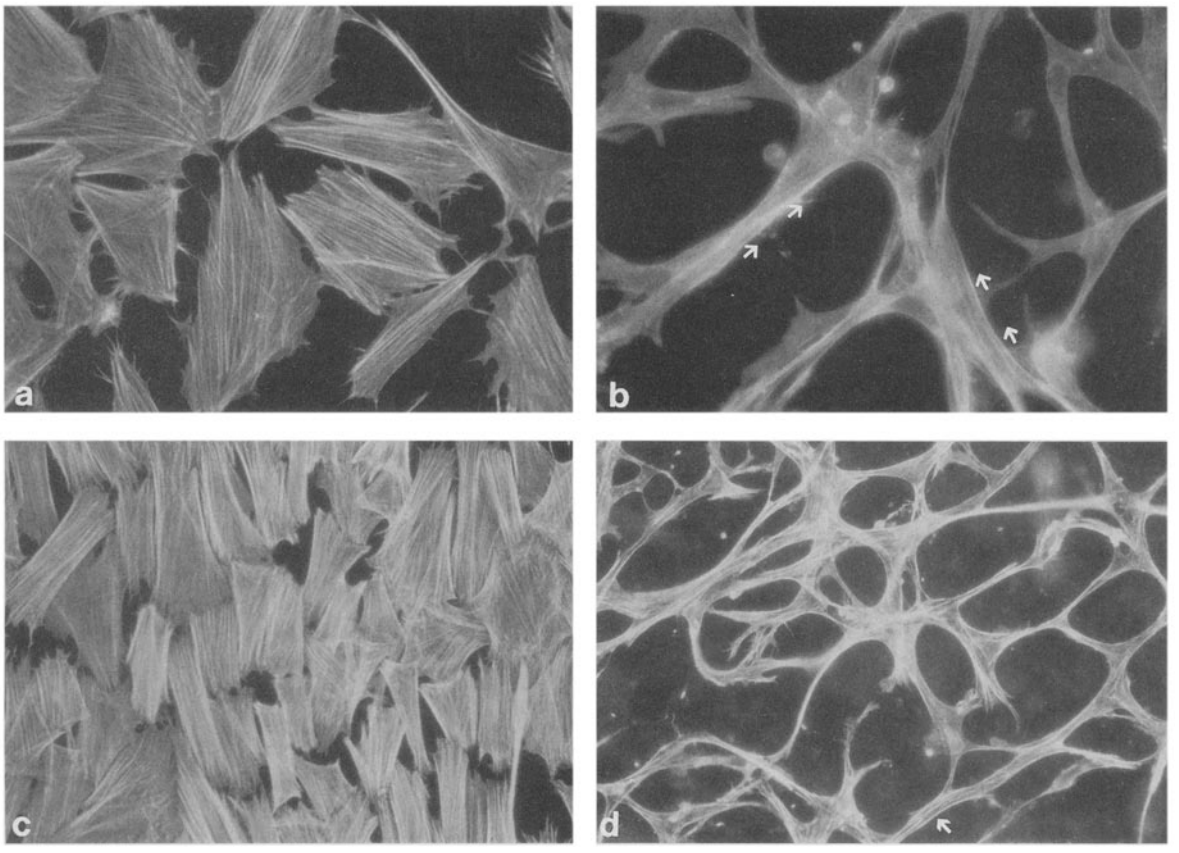

FIG. 4. Florescence microscopy of HUVECs stained with phalloidin-rhodamine. *Upper panel* show that endothelial cells at 10^4 per well and *lower panel*, 10^5 per/well. On plastic the HMVECs (*a* and *c*) contain regular actin filaments in the form of stress fibers; *b* and *d* show the cells plated on Matrigel. Actin filaments in the tubes are organized and run parallel to the tube-axis (*arrows*). ×1000.

protein synthesis is required for early tube formation and only partially needed in later stages or for maintenance of the tubes.

Others have indicated that collagen is required for tube formation by endothelial cells (Kubota et al., 1988; Madri et al., 1980; Madri et al., 1983). Therefore, we examined the role of collagen in tube formation using both HUVEC and NMEC cells. Cishydroxyproline, a specific inhibitor of procollagen secretion, reduced tube formation in a dose-dependent manner (Fig. 8) with 50% inhibition at 100 to 200 μg/ml. This amount of cis-hydroxyproline was not toxic to the cells over an 18-h period as judged by their ability to remain attached to the substrate. Ascorbic acid, a promoter of collagen synthesis, was found to increase tube formation in a dose-dependent manner with 50% more tubes at 50 μg/ml (Fig. 9; Table 1). At higher concentrations (100 μg/ml), ascorbic acid was toxic (data not shown). Matrigel contains less than 10% collagen IV, and addition of collagen IV to the Matrigel promoted tube formation (Fig. 9; Table 1). Cycloheximide and collagen IV were added to the Matrigel (at 25 μg/ml of Matrigel) to determine whether collagen IV could reverse the inhibitory effects of cycloheximide (Fig. 9; Table 1). Collagen IV increased tube formation in the presence of cycloheximide (2 μg/ml), but not to control levels (Table 1). These data demonstrate that collagen IV is important for tube formation. Inasmuch as collagen could not totally reverse the effect of cycloheximide, it is likely that other proteins are also required for tube formation.

DISCUSSION

Endothelial cells are able to align and organize in vitro into capillarylike tubes in the absence of other cell types (Folkman, 1985; Folkman and Haudenschild, 1980). However, the mechanisms involved have not been well defined except that the removal of growth factors and the presence of extracellular matrix promote the process. More recently it has been observed that both microvascular and large vessel endothelial cells will cease proliferation, align, and form capillarylike structures when plated on Matrigel, a reconstituted mixture of basement membrane components (Kubota et al., 1988). The advantage of this system is that tube formation is rapid, occurring in 18 h, and a majority of the cells participate in this process. Laminin, one of the principal components of Matrigel, is able to induce the alignment and organization of the endothelial cells into immature tubes by itself (Grant et al., 1989b). In addition, it has been suggested that basement membrane components may not only directly regulate the cells but may also play a direct mechanical role in changing cell shape (Ingber, et al., 1989a; Li, et al., 1987) and in stimulating differentiation. The rapid differentiation of the cells suggests that the endothelial cells, when plated on Matrigel, overcome the mechanical tension (placed on them on plastic), migrate, and change cell shape and lift away from the substratum, thus freeing the cells and permitting increased cell-to-cell contacts which promote three-dimensional assembly.

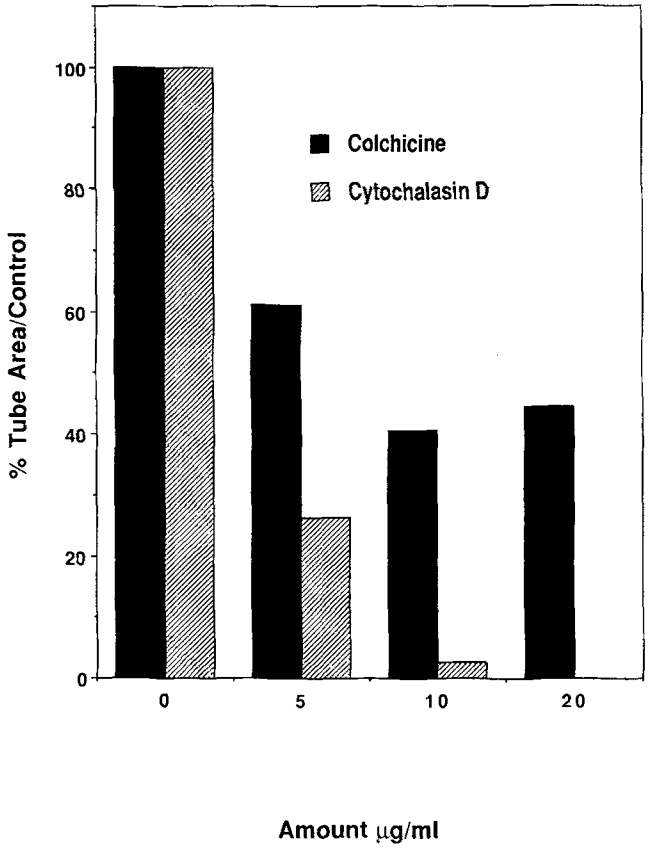

FIG. 5. The effect of colchicine and cytochalasin D on tube formation. The amount of HUVEC tube formation was examined in the presence 0 to 20 μg/ml of each drug. Measurements of the total tube area per well were performed using the Optomax digital analyzer and expressed as the percent tube area formed relative to the control. Data averaged from duplicate experiments. Cholchicine maximally inhibits to 60% whereas cytochalasin D inhibits 100% of forming tubes at 10 to 15 μg/ml.

In this study we have examined the role of the cytoskeleton on tube formation using human large vessel and microvessel endothelial cells. Tube formation was accompanied by a rearrangement of actin filaments from the appearance of stress fibers observed when the cells were grown on plastic to a parallel actin network which was often concentrated at the tube surface. Furthermore, because both colchicine and cytochalasin D inhibited tube formation, the interaction of the endothelial cells with Matrigel probably leads to the redistribution of microfilaments observed and allows tube formation to occur. In addition early events such as cell attachment and migration would be effected by the inhibition of the cells' cytoskeletal assembly. The cytoskeletal arrangement may also assist in the redistribution of cytoplasmic organelles as the cells progress from a flattened or rounded morphology to the polarized phenotype seen with tube formation. Endothelial cells are known to be highly polarized. For instance, they have been shown to secrete basement membrane protein basally, whereas serum proteins are secreted apically into the medium (Unemori et al., 1990). In addition, these cells have distinct apical and cytoplasmic protein compositions (Muller and Gimbrone, 1986).

We obtained additional evidence that protein factors were involved in tube formation. Low levels of cycloheximide and cis-hydroxyproline inhibited tube formation. Cis-hydroxyproline, an analogue of proline, is incorporated into the nascent polypeptide chains of collagen-blocking helix formation and secretion of collagen molecules (Kleinman et al., 1981). These observations suggested that collagen might be one of the proteins whose synthesis was required for tube formation. Tube formation can also be inhibited when antibodies to type IV collagen are added to the endothelial cell-Matrigel cultures (Grant et al., 1989a). Indeed ascorbic acid, a stimulator of collagen synthesis, and exogenously added collagen IV increased tube formation. This is not unexpected because capillaries are in contact with a collagen-rich basement membrane. Previously it was shown that laminin is important in promoting the differentiated phenotype of endothelial cells on Matrigel (Grant et al., 1989b). The

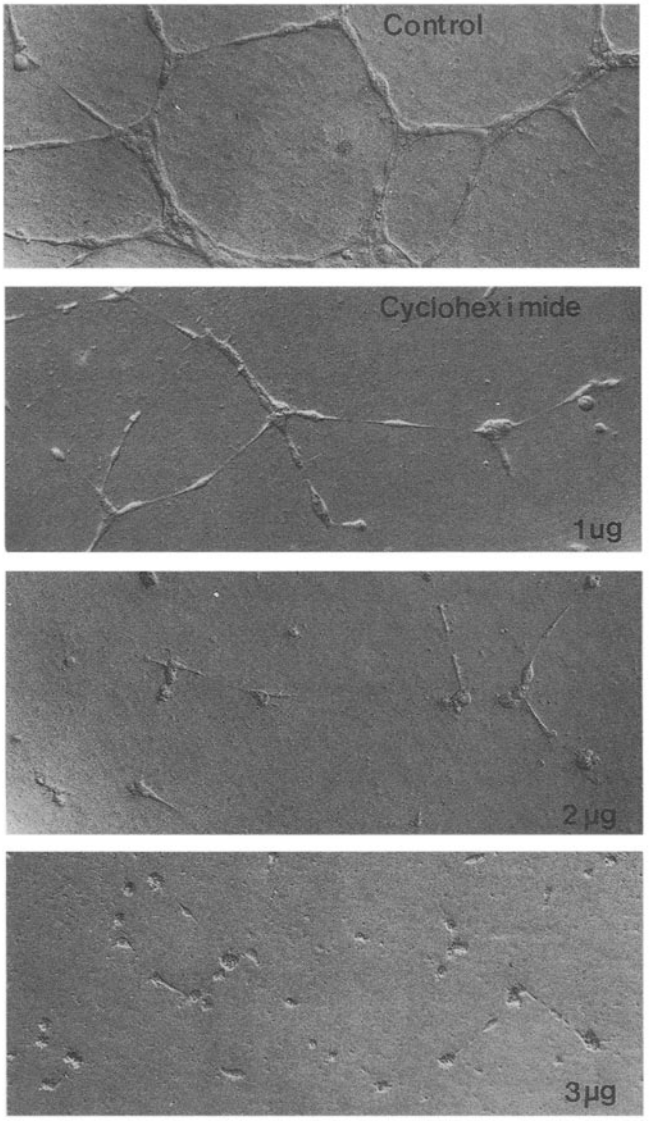

FIG. 6. Morphology of endothelial cells on Matrigel after exposure to cycloheximide at dosages of 1 to 3 μg/ml. Tube formation is inhibited with increasing amount of the drug.

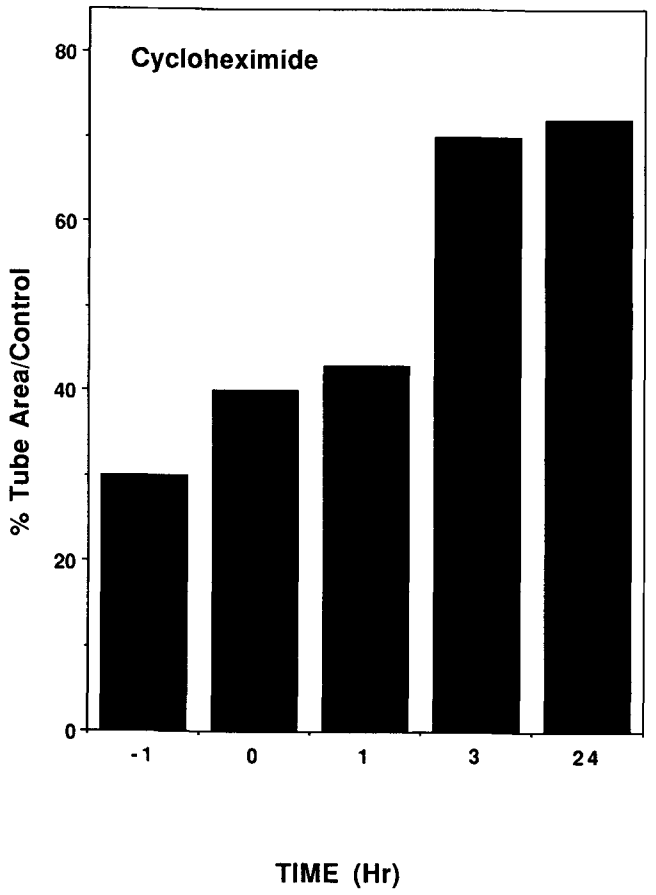

FIG. 7. Quantitation of the effects of cycloheximide on tube formation. The percent tube area/control was measured as in Fig. 5, after addition of 2 μg/ml of cycloheximide at different time points during tube formation. Data obtained from the average of three experiments.

present study shows that another basement membrane component, type IV collagen, is also required for tube formation. Endothelial cells have been shown to synthesize collagen IV in vitro (Howard et al., 1976; Madri et al., 1983; Kramer and Fuh, 1985; Ingber et al., 1989b). Some in vivo studies demonstrated that inhibition of collagen IV biosynthesis prevents angiogenesis in the chick chorioallantoic membrane (Maragoudakis et al., 1988). However, collagen IV is not likely to be the only protein necessary, because supplements of collagen IV only partially reversed the inhibition caused by cycloheximide. Thus, our findings that endogenous production of collagen IV stimulated further tube formation suggest that the amount in Matrigel is insufficient for complete differentiation.

Other components in Matrigel may also be necessary for tube formation because neither laminin or type IV collagen alone, in the form of a thin coating, induces tube formation. In fact, both of these components can also promote cell proliferation (Ingber et al., 1987; Madri et al., 1983). Entactin and the heparan sulfate proteoglycan, which are both present in Matrigel, are biologically active when tested alone with cells and are likely to be active in the Matrigel as well (Clement et al., 1989; Mann et al., 1989). Antibodies to entactin, a major glycoprotein in Matrigel, block tube formation (Grant et

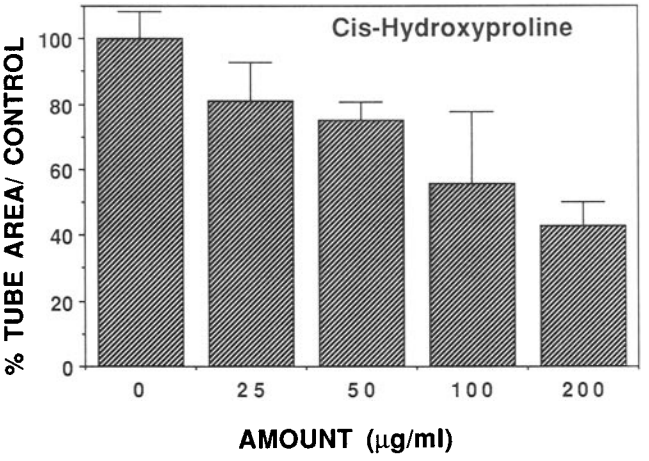

FIG. 8. Effect of cis-hydroxyproline on tube formation. Measurements were performed as in Fig. 5 and the data are shown as percent tube area/control at different dosages.

al., 1989a). Matrigel is also highly enriched in growth factors (Taub et al., 1990). When kidney tubule cells are grown on matrigel from which the growth factors have been removed, differentiation does not occur but can be restored with exogenous epidermal growth factor. Subsequently normal tubules are observed suggesting that the matrix has sufficient information to promote differentiation.

The data to date suggest that the cells must recognize several components in the Matrigel. Cells could interact with these components through multiple receptors, such as integrins (Ruoslahti and Pierschbacher, 1987), laminin binding proteins (Grant et al., 1989b), or the heparan sulfate binding protein (Clement et al., 1989), and interruption of any one of these interactions could abort the entire differentiation process. Others have shown that endothelial cells grown on a collagen I gel can also invade and form vessel-like structures when the cells are stimulated by PMA (Montesano et al., 1983). Therefore, the cells possess a complex receptor repertoire and have the potential to differentiate on different matrices.

In conclusion, Matrigel induces endothelial cells to differentiate as evidenced by both the morphologic changes and a reduction in

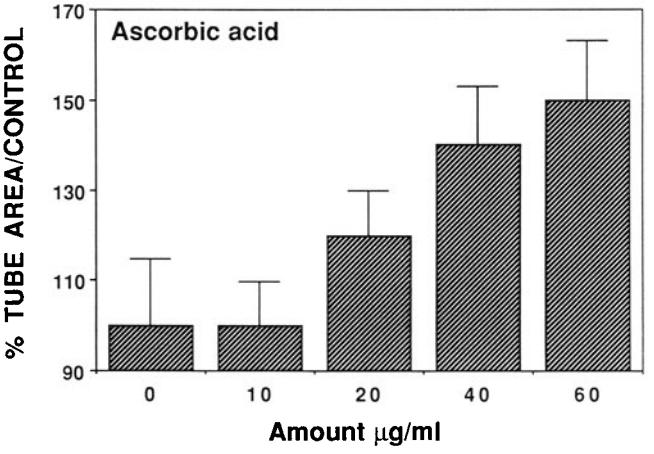

FIG. 9. Dose effect of ascorbic acid on tube formation. Data were obtained from the average of seven experiments.

TABLE 1

EFFECT OF VARIOUS FACTORS ON TUBE FORMATION

Treatment	% Tube Formation
Matrigel	100
Matrigel + Cis-Hydroxyproline (50 μg/ml)	61
Matrigel + Ascorbic acid (50 μg/ml)	145
Matrigel + Collagen IV (25 μg/ml of matrigel)	140
Matrigel + Cycloheximide (2 μg/ml)	50
Matrigel + Cycloheximide (2 μg/ml) & Collagen IV (25 μg)	70

proliferation. This differentiation is not terminal because the cells will proliferate again when separated from the tubes by trypsin. The angiogenic process involves numerous steps including receptor binding, cytoplasmic rearrangement, and protein synthesis. In addition, inasmuch as the endothelial cells become well differentiated on Matrigel, they are likely to be synthesizing many other specific proteins distinct from those synthesized on plastic. Analysis of the early gene products synthesized by cells on Matrigel will yield important additional information on the factors regulating angiogenesis.

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