

Basal Lamina Formation by Cultured Microvascular Endothelial Cells

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ABSTRACT The production of a basal lamina by microvascular endothelial cells (MEC) cultured on various substrata was examined. MEC were isolated from human dermis and plated on plastic dishes coated with fibronectin, or cell-free extracellular matrices elaborated by fibroblasts, smooth muscle cells, corneal endothelial cells, or PF HR9 endodermal cells. Examination of cultures by electron microscopy at selected intervals after plating revealed that on most substrates the MEC produced an extracellular matrix at the basal surface that was discontinuous, multilayered, and polymorphous. Immunocytochemical studies demonstrated that the MEC synthesize and deposit both type IV collagen and laminin into the subendothelial matrix. When cultured on matrices produced by the PF HR9 endodermal cells MEC deposit a subendothelial matrix that was present as a uniform sheet which usually exhibited lamina rara and lamina densa-like regions. The results indicate that under the appropriate conditions, human MEC elaborate a basal lamina-like matrix that is ultrastructurally similar to basal lamina formed *in vivo*, which suggests that this experimental system may be a useful model for studies of basal lamina formation and metabolism.

Capillary endothelium lie on a basal lamina that has a number of important functions. Not only does it provide the substratum for endothelial cell attachment and tissue organization (52), but it also has a role in blood vessel permeability (15), in the initiation of blood clotting (2), and provides a barrier to cellular migration, the violation of which has importance for angiogenesis (17), leukocyte emigration (55), and tumor metastasis (35, 38). Alterations in vascular basal lamina have been implicated in a number of pathological states, including diabetes mellitus, thrombogenesis, atherosclerosis, and renal failure (3, 15, 28, 53).

The vascular basal lamina is a complex structure. Because of difficulties in isolating sufficient quantities of purified material, biochemical studies of its molecular composition and structure have been limited. It is known, however, that vascular basal lamina is composed of a number of macromolecules including type IV collagen, proteoglycans, and glycoproteins (reviewed in 32, 37, 50). Studies on the synthesis of basal lamina macromolecules and their assembly into mature basal lamina by endothelia has been even more difficult. Recent advances in the isolation and long term culture of microvascular endothelial cells (3, 10, 17, 40) provides the opportunity for the study of these events.

In this report we describe the conditions necessary for the production and deposition of ultrastructurally identifiable basal lamina-like matrices by cultured microvascular endothelial cells isolated from human dermis.

MATERIALS AND METHODS

Preparation of Biosubstrates: To evaluate the influence of the substratum on matrix formation, microvascular endothelial cells (MEC)¹ were cultured on dishes coated with extracellular substrates elaborated by different cell types including skin fibroblasts, aortic smooth muscle cells, corneal endothelial cells, and endodermal cells.

Smooth muscle cells were isolated from rat aortas as described by Ross (43). Bovine corneal endothelial cells (20) were a gift of Dr. Denis Gospodarowicz (University of California, San Francisco, CA). Human foreskin fibroblasts were isolated as described (8). PF HR9 endodermal cells were derived by Dr. A. E. Chung (6) and obtained through the courtesy of Drs. E. Hayman and E. Rouslahti (La Jolla Cancer Research Foundation, La Jolla, CA). All cell lines except the corneal endothelial cells were cultured in Dulbecco's modified Eagle's minimal essential medium (DME) (Gibco Laboratories, Grand Island, NY) and 10% fetal bovine serum (Sterile Systems, Logan, UT); the corneal endothelial cells were cultured in DME plus 10% calf serum (Sterile Systems). The

¹ *Abbreviations used in this paper:* DME, Dulbecco's modified Eagle's minimal essential medium; MEC, microvascular endothelial cells.

various cell types were plated at $5 \times 10^4/\text{cm}^2$ in 35-mm-diam culture dishes precoated with bovine plasma fibronectin derived as previously described (10–12). For coating, dishes were covered with a solution of fibronectin (50–100 $\mu\text{g}/\text{ml}$) in PBS, incubated for 30 min at 22°C, and rinsed twice with PBS. Using similar conditions others have found that $\sim 0.5\text{--}1.0 \mu\text{g}/\text{cm}^2$ of fibronectin is bound to the plastic substratum (22, 26). The cell cultures were maintained at confluence for 7–10 d. Medium was replaced every 3 d and fresh recrystallized ascorbic acid (Calbiochem-Behring Corp., La Jolla, CA) was added to a final concentration of (50 $\mu\text{g}/\text{ml}$) every 24 h. The cells were removed from the extracellular matrix material they had deposited by rinsing twice with hypotonic buffer (5 mM Tris-HCl, 0.5 mg/ml BSA, 0.1 mM CaCl₂, pH 7.5), incubating with the same buffer for 8–10 min at 37°C followed by two brief extractions (1 min each) with 0.5% NP-40 non-ionic detergent in hypotonic buffer at 37°C; the cell-free matrices obtained were washed $5 \times$ with PBS containing 10% calf serum. The extracellular matrix produced by human microvascular endothelial cells cultured on these biosubstrates was compared with that obtained when cells were cultured on fibronectin-coated 35-mm plastic dishes (Falcon Labware, Oxnard, CA).

Isolation and Culture of Human Cutaneous Microvascular Endothelial Cells: Microvascular endothelial cells (MEC) were isolated from the cutaneous vessels of human newborn foreskin and cultured on a fibronectin substrate in Eagles' minimal essential medium or Leibowitz L-15 medium supplemented with 50% pooled heat-inactivated human serum and 3.3×10^{-4} M 3-isobutyl-1-methylxanthine and 5×10^{-4} M dibutyryl cyclic AMP as previously described (3, 10–12).

Established MEC from passages 1–7 were plated at 1×10^5 cells/cm² onto fibronectin-coated plastic dishes or dishes coated with cell-free extracellular matrices produced by the various cell lines and cultured in the medium described above with daily addition of ascorbic acid to give a final concentration of 25 $\mu\text{g}/\text{ml}$. MEC even at passage seven exhibit typical endothelial cell morphology, stain with antiserum to factor VIII associated antigen, contain Weibel-Palade bodies, and form tube-like structures (3, 10, 12, 30).

Electron Microscopy: Monolayers of MEC in culture dishes were processed for transmission electron microscopy as previously described (10–12). Briefly the cultures were fixed by overlaying the cell layer with a 1:1 mixture of culture medium with 3% phosphate-buffered glutaraldehyde solution at room temperature. This fixative was replaced 15 min later with a mixture of 2% phosphate-buffered glutaraldehyde solution. The cultures were rinsed with phosphate buffer prior to a 1-h treatment with buffered 1% OsO₄. After dehydration in ethanol, the cells were embedded *in situ* in epoxy plastic. Ultrathin sections cut perpendicular and parallel to the plastic culture dish were stained with uranyl and lead salts and examined in an Elmiskop 101 electron microscope.

Indirect Immunofluorescent Microscopy: For immunofluorescent studies, MEC were grown on Lux plastic or glass coverslips precoated with fibronectin as described above. Microwell culture chambers (5-mm diam) were formed using a silicone gasket apparatus (Bellco Glass Co., Vineland, NJ). MEC were seeded at confluence and after 10 d of culture, the subendothelial matrix was isolated as described above for the other extracellular matrices. The matrices were treated at 4°C for 10 min in fresh 0.5% formaldehyde in PBS, washed extensively in PBS, and reacted for 1 h with either affinity-purified rabbit anti-type IV collagen or rabbit antilaminin antibodies, at a concentration of 10 and 26 $\mu\text{g}/\text{ml}$, respectively. The antibodies were diluted in Tris-saline buffer (50 mM Tris-HCl, 150 mM NaCl, pH 7.5) containing 1% normal goat serum. For controls, matrices were incubated with normal, nonimmune rabbit IgG (25 $\mu\text{g}/\text{ml}$) (Boehringer Mannheim Biochemicals, Indianapolis, IN). The matrices were subsequently washed extensively with PBS and treated 1 h with affinity-purified goat anti-rabbit IgG conjugated with rodamine (Boehringer Mannheim Biochemicals) at 9 $\mu\text{g}/\text{ml}$ in Tris-saline buffer. Following five washes in buffer, the coverslips were mounted in glycerol-PBS solution (1:1) and photographed with Kodak Tri-X film under epi-illumination. The antibodies to laminin and type IV collagen were a gift from Drs. Hynda K. Kleinman and George R. Martin (National Institute of Dental Research, National Institutes of Health, Bethesda, Maryland). The antibodies were prepared against antigens isolated from the EHS tumor and their specificities have been verified in previous studies (37, 49). In addition, antiserum specific to type IV collagen (1) and laminin (48) obtained from other sources (gifts from Dr. Hans Peter Bächinger at the University of North Carolina, and Drs. Eva Engvall and Erkki Ruoslahti, La Jolla Cancer Research Foundation, respectively) gave identical results.

RESULTS

Localization of Type IV Collagen and Laminin in the Subendothelial Matrix

Subendothelial cell matrices were isolated from confluent

cultures of MEC grown on fibronectin-coated substrata. When these matrices were observed by indirect immunofluorescence following staining with affinity-purified type IV collagen antibodies, the antigen was found localized in a fibrillar pattern of fairly uniform nature (Fig. 1).

Isolated MEC matrices were also examined for the presence of laminin, a basement membrane-specific glycoprotein (49, 50). Antibodies specific for laminin stained the subendothelial matrix in a pattern similar to that outlined by antibodies specific to type IV collagen (Fig. 1). In contrast to the positive staining obtain with the above antibodies, control nonimmune rabbit IgG failed to stain the matrix (not shown). At least at the level of the light microscope, laminin and type IV collagen appear to be co-distributed within the extracellular matrix. Similar studies with the matrix produced by the MEC when cultured on the PF HR9 substrates were not possible due to the presence of the antigens in the PF HR9 matrix itself (1, 16, 47, 48).

Ultrastructure of the Subendothelial Matrix Deposited by Cells Cultured on a Fibronectin-coated Substrate

Confluent MEC monolayers maintained on fibronectin-coated dishes were examined at selected time intervals after plating by transmission electron microscopy. An extracellular matrix was deposited only at the basal surface of the cell layer. With increasing time in culture a discontinuous, multilayered filamentous matrix was produced (Fig. 2A). The thickness of the matrix varied substantially from one area to another. The endothelial cells were in close contact with the uppermost portion of the matrix and long segments of matrix microfibrils were commonly present juxtaposed to the cell's basal surface.

Ultrastructure of a Subendothelial Matrix Deposited by Cells Cultured on Biosubstrates

FIBROBLAST-DERIVED SUBSTRATUM: The fibroblast-derived substratum consisted of an abundance of fine collagen fibrils interspersed with amorphous, moderately electron dense material (Fig. 2B). Between the substratum and the overlying MEC were scant deposits of relatively coarse matrix material that appear to have been elaborated by the MEC. This matrix was usually in close apposition to the endothelial cell's basal surface.

BOVINE CORNEAL ENDOTHELIAL CELL-DERIVED SUBSTRATUM: In contrast to the fibroblast-derived substratum, that produced by corneal endothelial cells was thicker and consisted of electron dense aggregates of collagen (Fig. 2C). The MEC grew in close apposition to this substratum and a thin, discontinuous layer of amorphous and finely fibrillar material was observed.

AORTIC SMOOTH MUSCLE CELL-DERIVED SUBSTRATUM: The substratum produced by smooth muscle cells consisted of a loose layer of coarse collagen and elastin fibrils interspersed with faintly electron opaque amorphous material (Fig. 2D). In close apposition to the overlying MEC was a relatively well formed, discontinuous matrix of varying thickness. This matrix, as well as that present beneath the MEC cultures on corneal endothelial cell-derived substrates, resembled a lamina densa in some areas. Clusters of cytoplasmic vesicles were present along the basal surface of the MEC with some of them open to the extracellular environment.

PF HR-9 ENDODERMAL CELL-DERIVED SUBSTRATUM

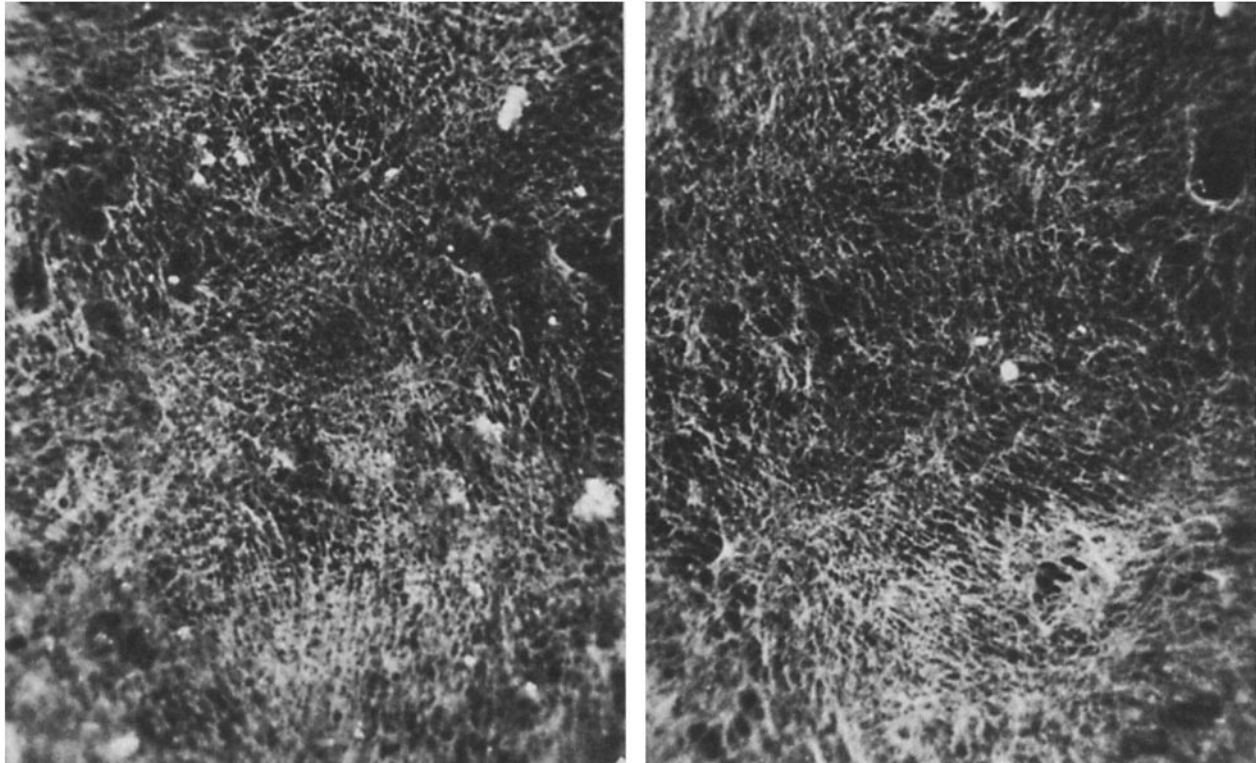


FIGURE 1 Localization of type IV collagen and laminin in the MEC matrix. MEC were cultured at confluence for 10 d on fibronectin-coated substrata. The matrices were isolated and processed for indirect immunofluorescence for type IV collagen (left) or laminin (right) as described in Materials and Methods section. Note that both type IV collagen and laminin are distributed in a fibrillar pattern. $\times 150$.

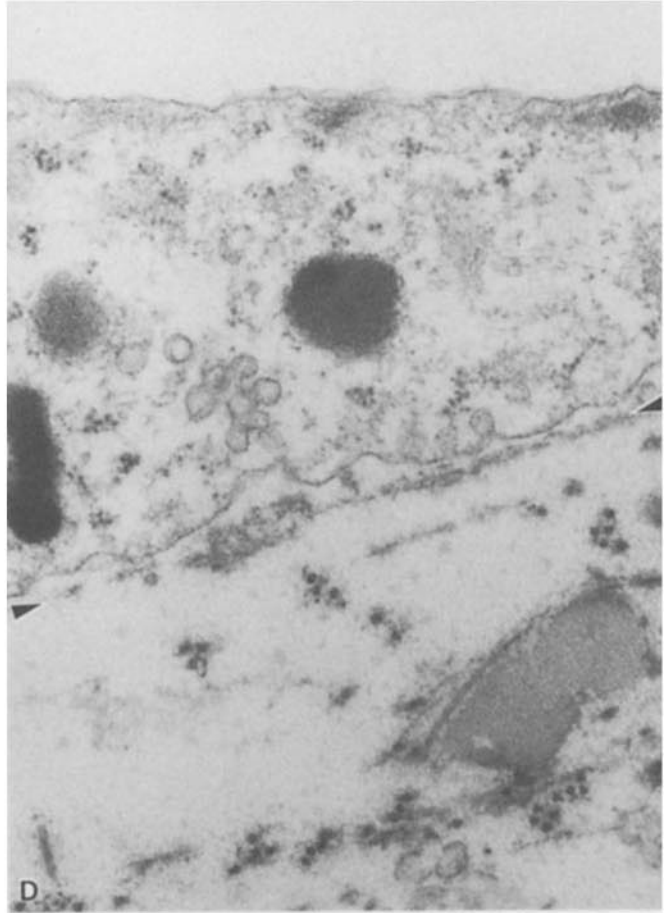
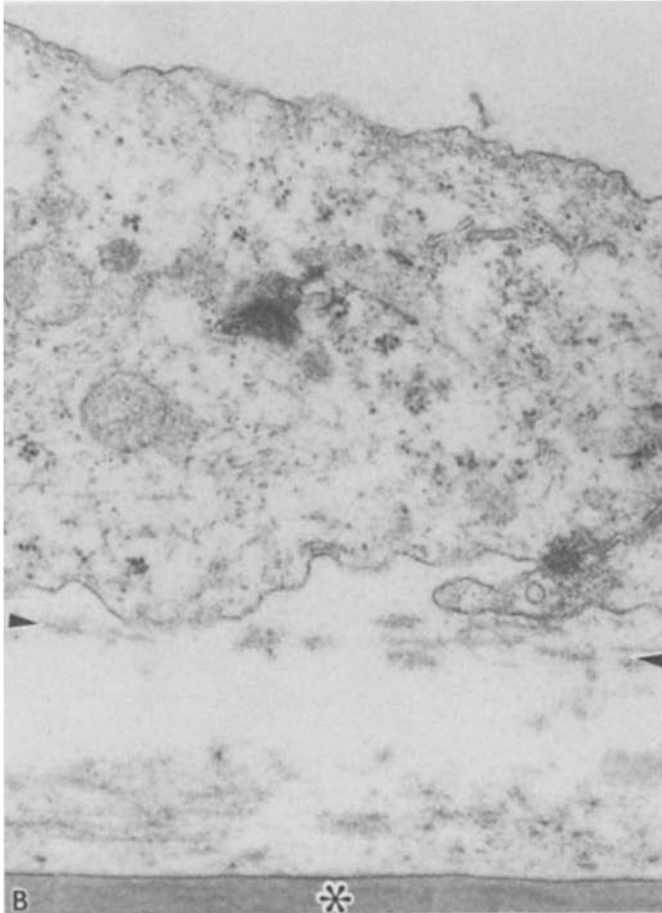
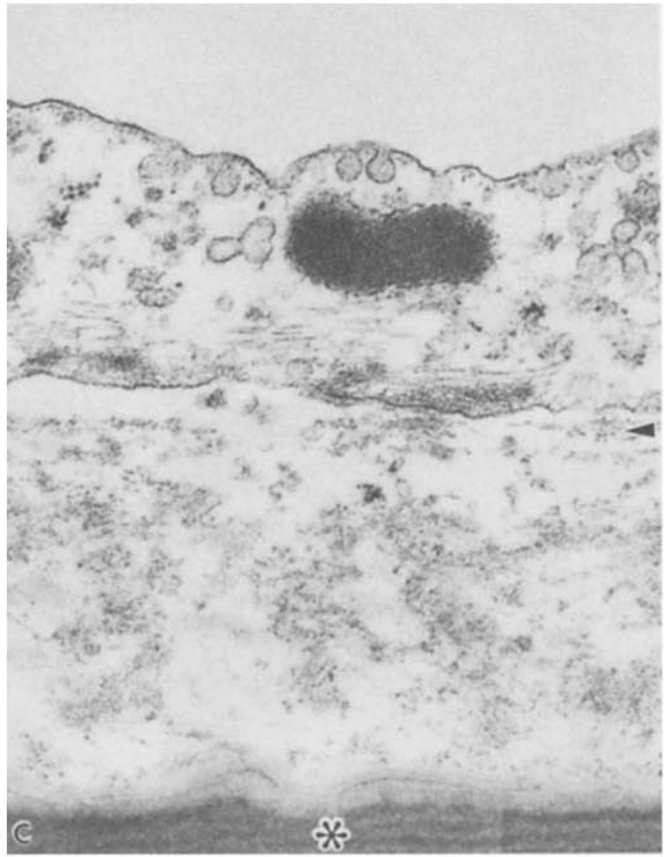
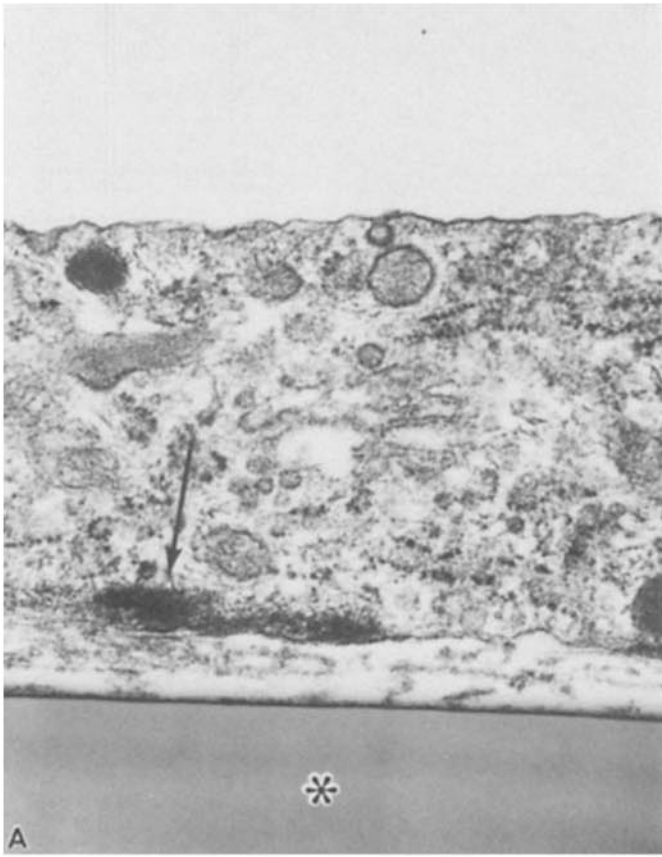
TUM: The PF HR9 endodermal cells elaborate a substratum that is composed of a meshwork of fine filaments interspersed with small granular structures (34) (Fig. 3A). Although this substratum is a continuous sheet, its thickness is uneven and the upper surface has a scalloped appearance (34). MEC cultured on the PF HR9 substrate produced an extracellular matrix that usually consisted of a continuous thin sheet of electron dense material corresponding to basal lamina found in vivo. The formation of basal lamina-like structures by MEC cultured on PF HR9-derived substrata was studied at selected time periods after seeding. At day one, the MEC had attached and spread, forming a continuous monolayer; no significant deposition of matrix material was evident at this time (Fig. 3A). By day three, discontinuous deposits with a high electron density were present along the entire length of the basal surface (Fig. 3B). From days 9–14, the formation of long segments of linear deposits was common and appeared

similar to mature basal lamina with regions analogous to the lamina rara interna (Fig. 3C). Higher magnification of the subendothelial space reveals an electron-lucent lamina rara-like region that separates the plasmalemma from the lamina dense-like electron-dense zone of amorphous matrix (Fig. 3D).

DISCUSSION

With the recent progress made in the development of methods to culture endothelial cells from large and small vessels, studies on the synthesis and deposition of basement membrane components by these cells are now feasible. Although endothelial cells obtained from large blood vessels such as the bovine aorta have been shown to produce subendothelial matrices in vitro (19, 35, 54) their ultrastructure does not accurately resemble basal lamina found in vivo. Moreover,

FIGURE 2 Comparison of the pattern of deposition of extracellular matrix material by human microvascular endothelial cells (MEC) cultured for 14 d on various substrata. (An asterisk marks the plastic culture dish.) (A) On fibronectin-coated plastic, a discontinuous and multilayered filamentous matrix is deposited between the basal surface of the endothelial cell and the substratum. Intracytoplasmic subplasmalemmal condensations, consisting of bundles of microfilaments (arrow), are seen at sites where extracellular matrix filaments are in particularly close apposition with the cell membrane. (B) Human fibroblast-derived substratum containing ill-defined fibrils can be seen overlying the plastic culture dish. A scant, irregular, coarsely fibrillar matrix is observed subjacent to the basal surface of the MEC (linear structure between arrowheads). (C) Bovine corneal endothelial cell-derived substratum consists of a thick layer of collagen filaments embedded in amorphous material covering the plastic culture dish. On this substratum, a very thin but discontinuous layer of matrix material is observed (see arrowhead). Numerous pinocytotic vesicles are present near the luminal surface of the MEC. (D) Rat aortic smooth muscle cell-derived substratum is composed of a thick layer of coarse collagen and elastic fibers. On this substratum, MEC deposit a distinct linear subcellular matrix (arrowheads), which is of varying thickness and discontinuous. Pinocytotic vesicles are prominent in the MEC cytoplasm near the basal surface. $\times 40,000$.



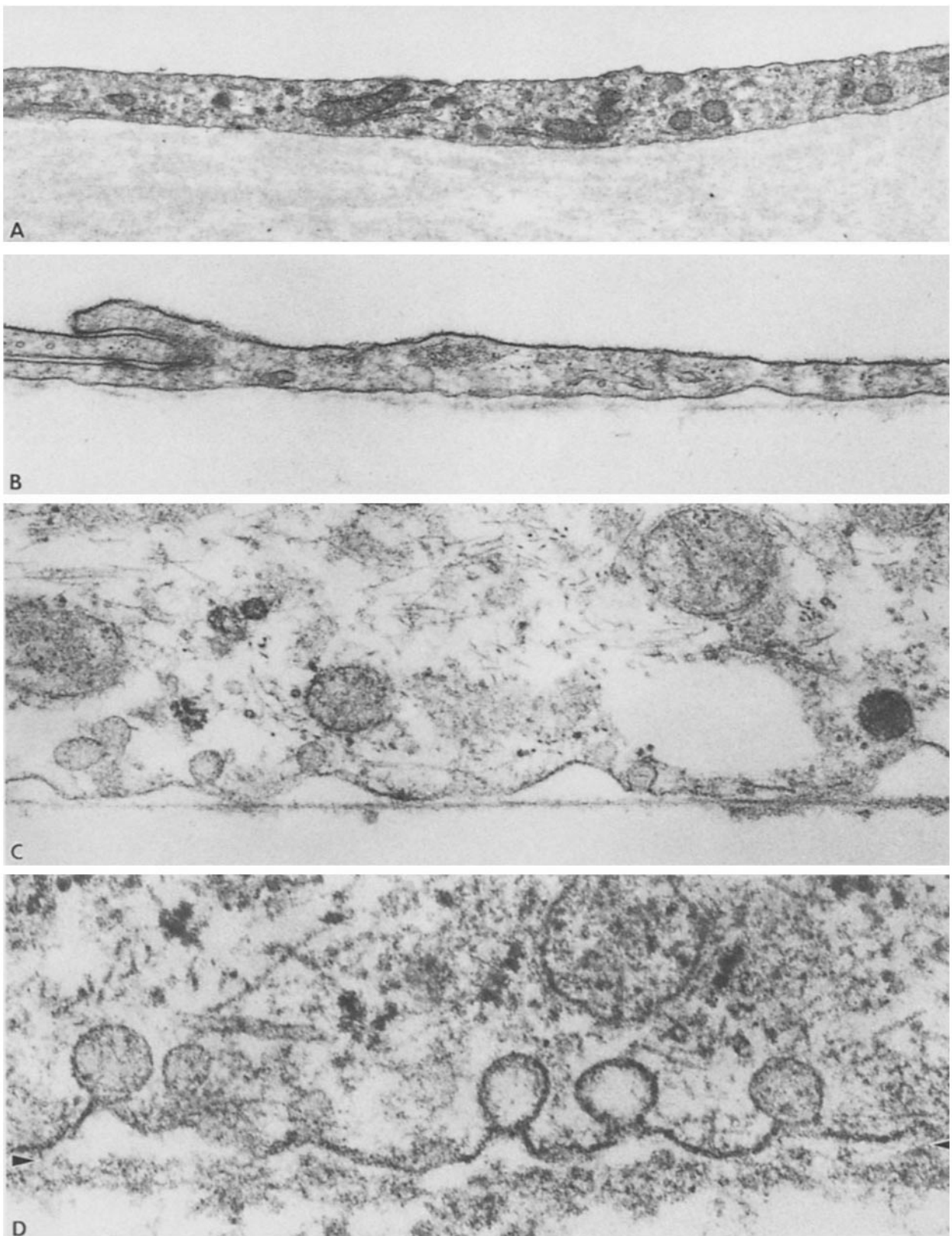


FIGURE 3 Deposition of a basal lamina-like extracellular matrix by human MEC cultured on PF HR9 endodermal cell-derived substrata. (A) MEC cultured for 1 d. The endothelial cell is closely adherent to the surface of the PF HR9 cell substratum which consists of a meshwork of fine filaments interspersed with small granular structures. At this time deposition of subendothelial matrix is not observed. (B) After 3 d of incubation, a thin linear layer of matrix material of high electron density is visible in close apposition to the basal surface of the cell. A cell-cell junction is present. In this micrograph, the MEC and its associated basal lamina-like matrix is separated from the underlying PF HR9 cell-derived substratum which is not visible. (C) At 14 d, a continuous linear basal lamina-like matrix of uniform thickness is deposited beneath the basal surface of the MEC. As in Fig. 3B, the MEC and its associated matrix has separated from the underlying PF HR9 cell-derived substratum (not visible). (D) High magnification of the MEC basal lamina-like matrix after 14 d of culture shows an electron-lucent zone (arrowheads) between the plasmalemma and the lamina densa-like region. Note the extensive pinocytotic activity. $\times 19,800$ (A); $\times 60,000$ (B); $\times 66,000$ (C); $\times 136,000$ (D).

biochemical analysis reveals that these matrices usually contain interstitial collagens (types I and III) with only small amounts of basement membrane collagen (type IV) (45, 46, 51). An exception to these findings is the exclusive production of type IV collagen by human endothelial cells from the umbilical vein (44).

Studies of cultured bovine adrenal capillary cells by Sage et al. (46) failed to detect the secretion of type IV collagen, an important constituent of the basal lamina. As with aortic endothelial cells, these capillary cells secrete large amounts of interstitial collagen types I and III. Since capillary endothelium is known to deposit type IV collagen *in vivo* (36, 37), it appears that in the case of the bovine capillary cells the expected secretory phenotype may have been lost during the process of isolation or passage *in vitro*. However, Madri and Williams (40) have demonstrated that capillary endothelial cells, from the rat epididymal fat pad, secrete mostly type IV and type V collagen when cultured on substrata of basement membrane collagens. Furthermore, our studies suggest that cultured human dermal MEC synthesize and deposit into the subendothelial matrix mainly type IV collagen (33).

The macromolecular composition of the various matrices tested as growth substrates for MEC has been extensively documented. The matrices produced by the skin fibroblasts (23), aortic smooth muscle cells (13), and corneal endothelial cells (46) have been shown to contain mostly interstitial collagen types I and III. In addition, the corneal endothelial cells contain lesser amounts of collagen types IV and V (46). The PF HR9 matrix is comprised exclusively of type IV collagen (1, 14, 16). Fibronectin glycoprotein is abundant in all the matrices except that elaborated by the PF HR9 cells (23, 29, 46, 48). Thrombospondin, a glycoprotein associated with platelets has been recently identified in the matrices deposited by corneal endothelial cells (46). Laminin and entactin are basement membrane-specific glycoproteins found in the PF HR9 matrix (1, 25, 47, 48); lesser amounts of laminin are also present in the corneal endothelial cell matrix (19). The smooth muscle matrix is unusual in its abundance of elastin fibers (29) (see Fig. 2D). Matrix-associated glycosaminoglycans have been characterized for all cell types except for the smooth muscle cells. Heparan sulfate and chondroitin sulfates are the predominant glycosaminoglycans found in the matrices produced by the fibroblasts (23), the PF HR9 cells (34), and the corneal endothelial cells (42). Although the cell lines listed above appear to exhibit a relatively stable pattern of secretory phenotypes, it is possible that there may be some variability in the matrix composition depending on the cell passage number, age of cultures, serum lots, and other parameters.

The apparent specificity of the PF HR9 matrix in promoting the formation of a matrix resembling basal lamina by the MEC may be related to the high concentration of basement membrane specific macromolecules in the PF HR9 matrix. The other matrices tested appear to be more related to the interstitial variety as defined by the major collagen types present and the predominance of fibronectin rather than laminin glycoprotein. The most significant finding is that conditions have been defined that promote the deposition by cultured microvascular endothelial cells of an ultrastructurally identifiable basal lamina containing lamina densa- and lamina rara-like structures. The immunocytochemical studies presented here demonstrate that the MEC synthesize and deposit type IV collagen and laminin into their subendothelial matrix.

Furthermore, initial biochemical studies indicate that the matrices produced by the MEC are similar whether the cells are cultured on fibronectin- or PF HR9 matrix-coated substrates (33).

The influence of the endodermal matrix-substrate on the production of a basement membrane-like structure by the MEC may be a consequence of a direct interaction between the endothelial cell and the matrix or solubilized matrix component(s). How the endodermal matrix-substrate modifies the subendothelial matrix organization and/or metabolism is unclear. Since PF HR9 endodermal cells produce typical basement membrane macromolecules, it is possible that the endoderm basement membrane-like matrix acts as a template for the assembly of endothelial basement membrane components. Alternatively, specific macromolecules gradually solubilized from the endoderm matrix could co-assemble with subendothelial matrix components resulting in the formation of a stable basement membrane structure.

Previous reports have demonstrated that extracellular matrix materials can modulate basal lamina production. For example, culturing epithelial cells on collagen gels was found to promote the deposition of basal lamina-like structures (9, 24, 41). In addition, Garbi and Wollman (18) have shown that the addition of soluble laminin to explants of thyroid epithelium induces basal lamina formation, which otherwise fails to develop. A number of important studies on the effect of extracellular matrix on cultured vascular endothelial cells have indicated that exogenous matrices or matrix components can influence collagen synthesis. Tseng et al. (51) reported that bovine corneal endothelial cells when grown on plastic synthesize greater amounts of collagen than when cultured on preformed matrices. Madri and Williams (40) found that rat capillary endothelial cells exhibit preferential growth on interstitial collagens and proliferate slowly on basement membrane collagens. Furthermore, when these capillary cells were cultured on basement membrane collagen-coated substrata, a larger proportion of the secreted collagens were represented by basement membrane collagens, while cells cultured on interstitial collagens produced mainly interstitial collagens.

While the actual mechanism by which exogenous matrix modulates basement membrane formation by microvascular endothelial cells remains to be established, it is noteworthy that a unique specificity exists for the type of matrix that induces these effects. The failure of matrices derived from fibroblasts or corneal endothelial cells muscle cells to promote proper basement membrane formation by the MEC could be due to the lack of appropriate levels of basement membrane constituents in these matrices. The culture system described here may provide a useful model for the study of microvascular basement membrane metabolism and the role of bio-substrates in these processes.

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