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MODULATION OF MICROVASCULAR GROWTH AND MORPHOGENESIS BY RECONSTITUTED BASEMENT MEMBRANE GEL IN THREE-DIMENSIONAL CULTURES OF RAT AORTA: A COMPARATIVE STUDY OF ANGIOGENESIS IN MATRIGEL, COLLAGEN, FIBRIN, AND PLASMA CLOT

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SUMMARY

Rings of rat aorta cultured in Matrigel, a reconstituted gel composed of basement membrane molecules, gave rise to three-dimensional networks composed of solid cellular cords and occasional microvessels with slitlike lumina. Immunohistochemical and ultrastructural studies showed that the solid cords were composed of endothelial sprouts surrounded by nonendothelial mesenchymal cells. The angiogenic response of the aortic rings in Matrigel was compared to that obtained in interstitial collagen, fibrin, or plasma clot. Morphometric analysis demonstrated that the mean luminal area of the microvascular sprouts and channels was significantly smaller in Matrigel than in collagen, fibrin, or plasma clot. The percentage of patent microvessels in Matrigel was also markedly reduced. Autoradiographic studies of ^{3}H-thymidine-labeled cultures showed reduced DNA synthesis by developing microvessels in Matrigel. The overall number of solid endothelial cords and microvessels was lower in Matrigel than in fibrin or plasma clot. A mixed cell population isolated from Matrigel cultures formed a monolayer in collagen or fibrin-coated dishes but rapidly reorganized into a polygonal network when plated on Matrigel. The observation that gels composed of basement membrane molecules modulate the canalization, proliferation, and organization into networks of vasoformative endothelial cells in three-dimensional cultures supports the hypothesis that the basement membrane is a potent regulator of microvascular growth and morphogenesis.

Key words: angiogenesis; basement membrane; extracellular matrix; endothelium.

INTRODUCTION

Angiogenesis, i.e. the formation of new blood vessels, is regulated by a complex process of interaction between soluble factors, endothelial cells, and extracellular matrices (ECM). Recent studies indicate that soluble factors that are angiogenic in vivo such as basic fibroblast growth factor (8,14,44), tumor necrosis factor-alpha (25), and transforming growth factor-beta (40) stimulate angiogenesis in vitro when endothelial cells are cultured on or within three-dimensional gels of interstitial collagen (25,30,34). In the absence of a three-dimensional ECM, angiogenic factors modulate endothelial proliferation or migration or both without inducing formation of microvessels (1,2,43). Endothelial cells cultured on plastic or gelatin-coated surfaces form capillarylike tubes if allowed to age for several weeks or if kept under restrictive conditions of growth (11,26). However, the presence of a three-dimensional matrix accelerates considerably the angiogenic process allowing formation of microvessels in 2 to 3 d rather than in several weeks as observed on plastic surfaces (29,32,35). Moreover, the microvessels that form in three-dimensional matrices develop patent lumina and are lined by a polarized endothelium (32,37), whereas the capillarylike structures formed in the absence of exogenous substrates accumulate an endogenous matrix in their lumen and have been compared by some to inside-out capillaries (10,11,26). These findings suggest that the ECM is an essential regulator of microvascular morphogenesis.

The sprouting endothelium of developing microvessels during angiogenesis migrates through a variety of matrices. Among the major components of the ECM in sites of angiogenesis are the interstitial collagen of the perivascular stroma and fibrin, which frequently accumulates as a provisional matrix during wound healing and tumor angiogenesis (6). In addition, endothelial cells, in response to soluble angiogenic factors, remodel the surrounding ECM through a combination of enzymatic degradation of preexisting substrates (16,22,31,33) and deposition of a newly formed basement membrane (13,21,37,38).

In vitro experiments have demonstrated that interstitial collagen, fibrin, and basement membrane matrices modulate the phenotype of endothelial cells and facilitate the formation

of microvessels (27,29,32,35). However, little is known about the specific role that each of these matrices plays in angiogenesis. Particularly unclear is the function of the microvascular basement membrane during angiogenesis. Data obtained from two-dimensional cultures indicate that components of the basement membrane such as laminin and type IV collagen affect the shape, proliferation, and organization of endothelial cells (13,20). But a gap exists in our understanding of the effect of the basement membrane on the development of microvessels in three-dimensional matrix cultures, i.e. in a setting that more closely simulates the in vivo environment where angiogenesis is a three-dimensional phenomenon.

The present study was designed to investigate the effect of Matrigel, a reconstituted gel composed of basement membrane molecules isolated from the Engelbreth-Holm-Swarm (EHS) mouse sarcoma, on angiogenesis in three-dimensional cultures of rat aorta. We previously observed that aortic rings cultured in plasma clot gave rise to extensive networks of branching microvessels (35,37,38). Here we compare with light microscopic, immunohistochemical, ultrastructural, autoradiographic, and morphometric techniques the angiogenic response of aortic rings cultured in Matrigel, fibrin, interstitial collagen, or plasma clot. Our results indicate that gels composed of basement membrane molecules allow formation of microvascular networks but interfere with the proliferation, maturation, and canalization of the endothelial sprouts. In contrast, fibrin, interstitial collagen, and plasma clot behave as permissive substrates allowing the development of histotypic microvessels. This suggests that the basement membrane, which is an integral component of the microvascular wall, contains potent regulatory signals that can affect the growth and morphogenesis of new microvessels.

Materials and Methods

Living cultures. The aorta of 2- to 3-mo.-old Fischer 344 male rats was removed and dissected as previously described (35,37). One-millimeter long aortic rings were embedded in three-dimensional gels of type I collagen, fibrin, Matrigel, or plasma clot. The gels were prepared in specially designed culture chambers which we have termed sponge-slide culture wells (SSCW). The SSCW is a cylindrical chamber of standard volume (155 mm³) which is obtained by gluing with nontoxic silicone rubber a cylindrical ring of cellulose sponge to a histology glass slide (36,37). After gelation, the slide holding the culture chamber with the aortic ring was placed in a large Leighton tube (Bellco, Vineland, NJ) containing Eagle's minimal essential medium (MEM) supplemented with 20% fetal bovine serum, 1 m*M* L-glutamine, 10 m*M* HEPES

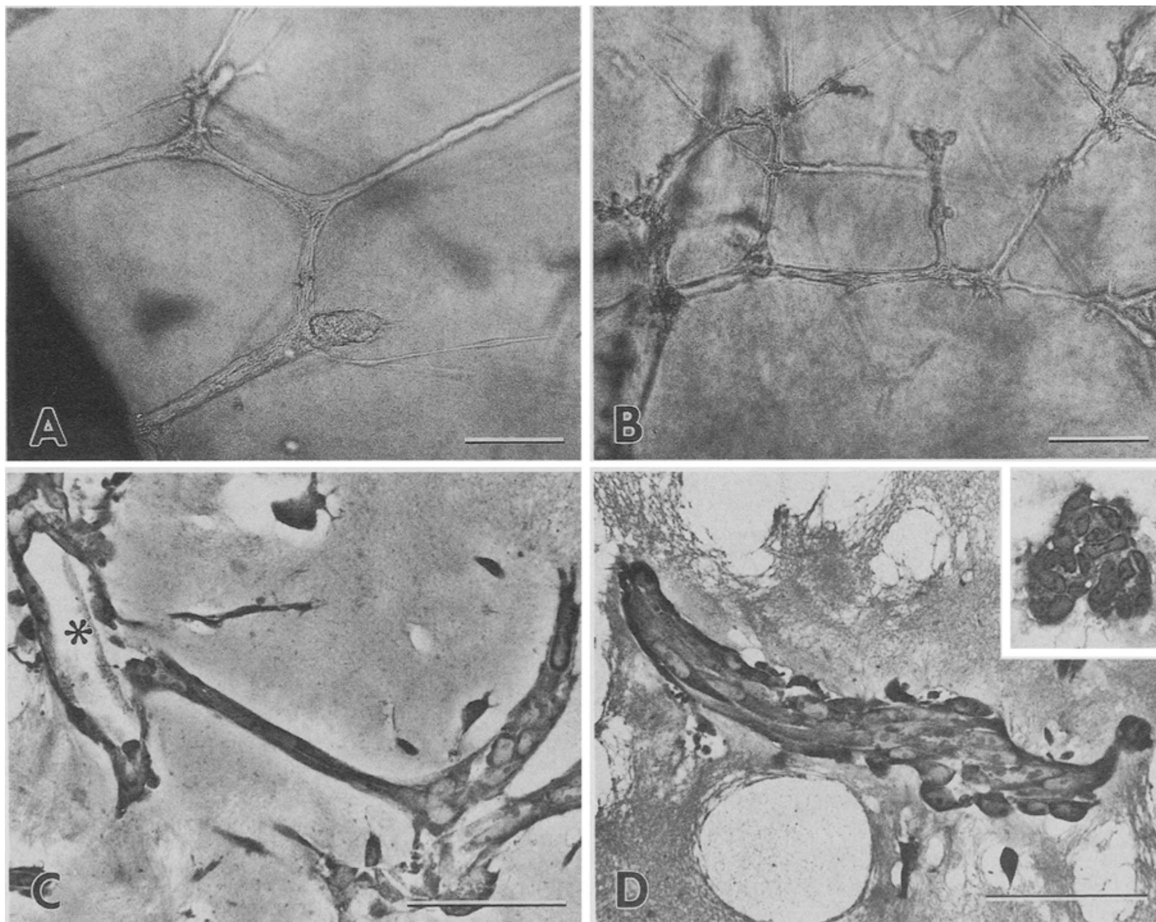

FIG. 1. Angiogenesis in Matrigel. Microvascular outgrowths forming loops (*A*) and polygonal networks (*B*) in 10-d-old living cultures of rat aorta. Histologic preparations demonstrate longitudinal (*C,D*) and cross (*D, inset*) sections of solid sprouts and occasional patent microvessels (* in *C*). *Bars: A* and *B*, 100 μm; *C* and *D* (including inset) 50 μm.

buffer, and 50 μg/ml gentamicin and kept at 35.5° C. All cultures were observed daily with an Olympus IM inverted microscope equipped with a 35mm camera. The SSCW has unique characteristics when compared to a plastic well. a) Three-dimensional growth occurs in a setting that facilitates diffusion of nutrients and gasses not only from the top surface but also from the lateral surfaces of the gel through the pores of the sponge. b) The Leighton tube in which the SSCW is placed holds 14 ml of medium. The high ratio between amount of medium and gel volume guarantees an optimal supply of nutrients even at later stages of growth when the outgrowth has become very dense. c) The SSCW can be easily detached from the slide at the end of the experiment and the gel can be removed from the sponge to be processed for morphologic studies. d) Microvessels grow in radial fashion from an explant of standard size in a cylindrical chamber of standard volume. This allows us to quantitate angiogenesis using whole mount histologic sections and digitizing morphometry.

Gels of ECM

Collagen. Gels of interstitial collagen were prepared according to Montesano et al. (32) by quickly mixing at 4° C 7 vol of rat-tail collagen solution in 0.1× MEM with 1 vol 10× MEM and 2 vol of 11.7 mg/ml sodium bicarbonate. Gelation was allowed to take place by incubating the culture at 35.5° C for 30 min. Type I collagen was obtained from rat-tail tendon as described by Elsdale and Bard (7).

Fibrin. Fibrin gels were obtained by quickly mixing 1 vol of a 3 mg/ml bovine fibrinogen solution (Calbiochem, San Diego, CA) in MEM with 0.02 vol of a 50 U/ml solution of human thrombin (Sigma Chemical Co., St. Louis, MO) in MEM. Fibrin gels formed rapidly at room temperature within 1 min.

Matrigel. Matrigel is a laminin-rich extract of the EHS mouse sarcoma which contains also type IV collagen, heparan sulphate, and entactin (3,23). Matrigel was obtained from Hynda Kleinman (National Institute of Health, Bethesda, MD) or from Collaborative Research (Bedford, MA). Gelation occurred in 30 to 45 min at 35.5°C.

Plasma clot. Plasma clots were obtained by mixing 2 vol of reconstituted chicken plasma (Difco Laboratories, Detroit, MI) with 1 vol of chick embryo extract as previously described (35).

Histologic Studies

After 14 d of culture the gels were fixed in 10% buffered formalin and removed intact from the SSCW using a razor blade under a dissecting microscope. They were then washed in phosphate buffered saline (PBS), pH 7.4, dehydrated through graded ethanols and processed for glycol methacrylate embedding (JB4, Polysciences, Warrington, PA). Two-micrometer-thick histology sections were cut with Ralph knives mounted on an LKB Historange microtome and stained with 0.1% toluidine blue in 0.1% borax. Sections were collected at intervals of 10 μm and their depth into the culture was recorded on each slide.

Morphometric Studies

For morphometric studies we analyzed histology sections using a Leitz Laborlux K microscope equipped with an MTI high resolution videocamera, a Hipad digitizer tablet, an Audiotronics black and white monitor, and an IBM AT computer with Bioquant IV image analysis software. The sections were taken from the center of the culture at the level of the aortic ring where the maximum number of microvessels was usually observed. In contrast to paraffin, glycol methacrylate embedding did not cause artifactual distortion of the gel and gave us a high resolution of microvascular details in whole mount sections of each culture. Six sections from three to six cultures per experimental group were examined. All the microvessels and solid endothelial sprouts present in each section were counted and measured by scanning the slides under oil immersion (1000×). Large vessels were measured at 100× or 400×. Approximately 400 to 800 microvessels per experimental group were analyzed. The following parameters were measured: a) number of microvessels per section; b) vascular area (VA); c) luminal area (LA); d) vascular wall area (VWA = VA-LA); e) luminal index (LI = LA/VA × 100); f) patency index. Parameters b, c, d, and e were analyzed by measuring individual vessels. The luminal index measured the percentage of the vascular area occupied by the lumen. The patency index was arbitrarily defined as the percentage of microvessels with luminal index greater than 10%. Analysis of variance was used to determine if there were significant differences among the experimental groups. Thereafter, the Student-Newman-Keuls test was used to identify the specific groups that were significantly different from each other.

Autoradiography

For the study of DNA synthesis all cultures were labeled for 24 h with 5 μCi/ml ^{3}H-thymidine (TdR), fixed in buffered formalin, embedded in glycol methacrylate, and sectioned.

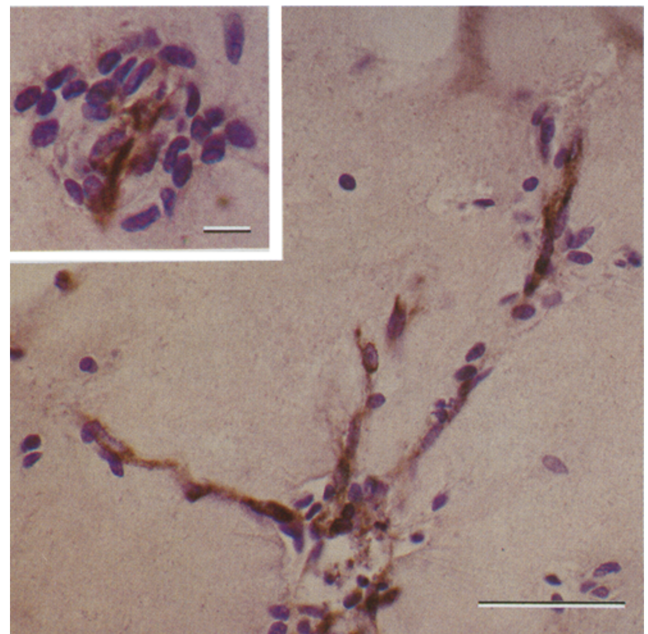

FIG. 2. Angiogenesis in Matrigel. Endothelial sprouts immunostained for FVIII-RAg. *Inset* shows multilayering of nonendothelial cells around a FVIII-RAg-positive endothelial core. *Bars:* 50 μm; *inset* 10 μm.

Sections were dipped in Ilford KD5 photographic emulsion, exposed for 1 wk, developed in Kodak D19 developer, fixed, and stained with toluidine blue. The percentage of labeled nuclei (labeling index) in each culture was obtained by counting the nuclei of both endothelial and periendothelial cells of all the microvessels present in each section at the level of the aortic ring. Sections were selected as described above. Analysis of variance followed by Student-Newman-Keuls test was used for statistical analysis.

Ultrastructural Studies

For electron microscopy the cultures were fixed in 4% formaldehyde, 1% glutaraldehyde in 0.1 *M* phosphate buffer, trimmed to 1-mm^3 pieces, postfixed in OsO_4, dehydrated in graded ethanols, and embedded in Epon. Thin sections were cut with an LKBIII ultramicrotome, stained with methanolic uranyl acetate and lead citrate and examined with a Zeiss 10A transmission electron microscope.

Immunohistochemical Studies

The endothelial nature of the microvascular outgrowths was confirmed by staining selected cultures for factor VIII-related antigen (FVIII-RAg). The cultures were fixed in buffered formalin and processed for paraffin embedding. Sections were mounted on poly-D-lysine-coated histology slides, partially digested with 0.1% trypsin in 0.1% $CaCl_2$, tris buffer, pH 7.8, and processed for immunostaining using rabbit antihuman FVIII-RAg Ig diluted 1/200 (Dako, Santa Barbara, CA) as primary antibody, biotynilated goat antirabbit antibody diluted 1/200 (Vector, Burlingame, CA) as linking reagent, and the avidin-biotin complex (Vector) as detection system. The sections were reacted with diaminobenzidine and counterstained with iron hematoxylin. The percentage of solid outgrowths containing endothelial cells was determined by counting the number of both FVIII-RAg positive and negative cords or cohesive aggregates that were composed of three or more cells.

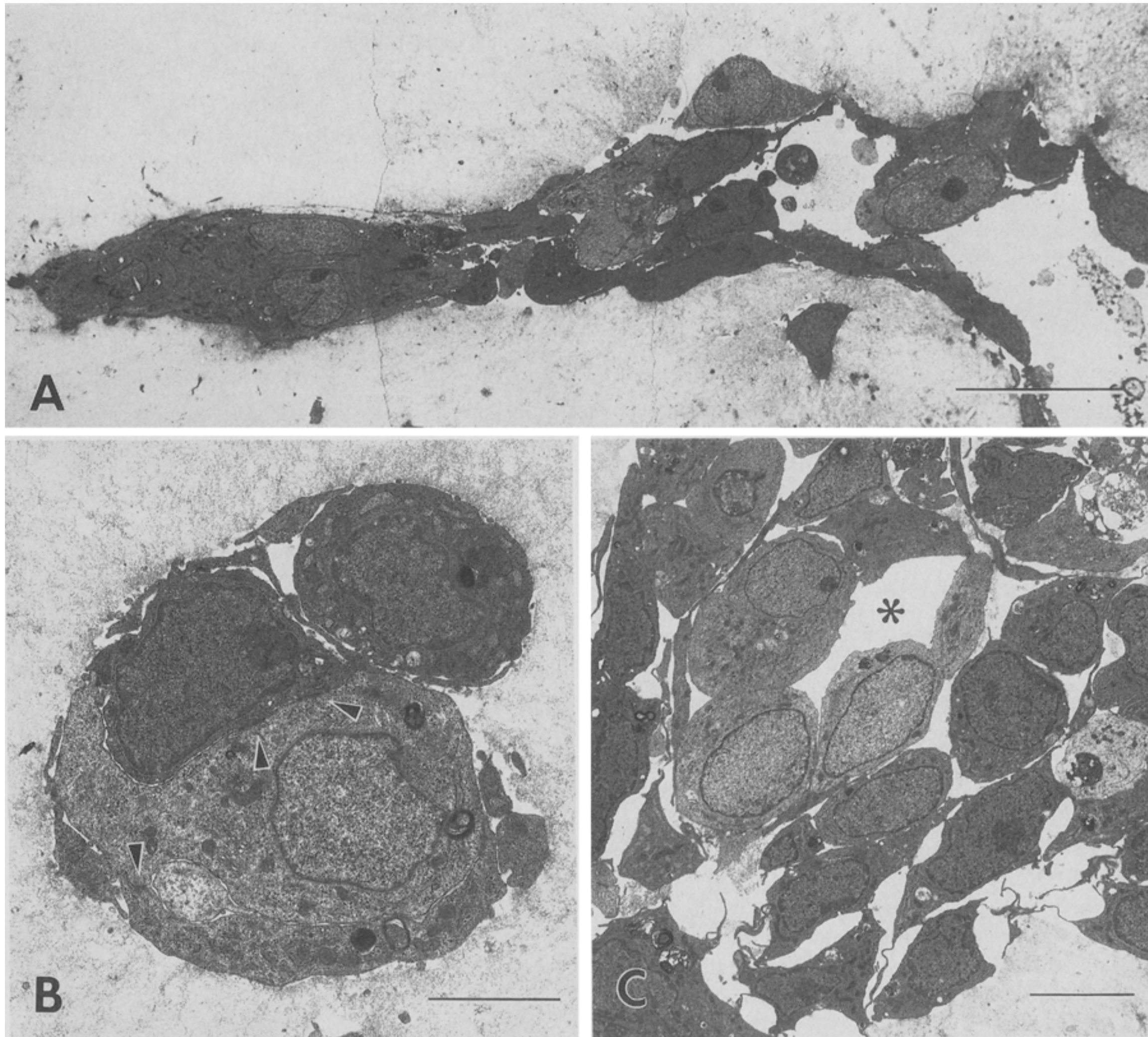

FIG. 3. Angiogenesis in Matrigel. Electron micrographs of longitudinal (*A*) and cross (*B*) sections of solid microvascular sprouts arising from the endothelium lining the surface of the gel. *Arrowheads* indicate junctions between endothelial cells (*B*). Cross section (*C*) of endothelial channel with small lumen (*) demonstrates perivascular accumulation of undifferentiated mesenchymal cells. *Bars: A* and *C*, 5 μm; *B*, 2 μm.

RESULTS

Angiogenesis in Matrigel. The outgrowth in Matrigel cultures was composed of solid cords and spindle cells. The solid cords were seen to arise from the aortic intima as early as 3 to 4 d after the beginning of the experiment. Spindle cells migrated primarily from the adventitia. The solid outgrowths, which were composed of cohesive cells with ill-defined boundaries, branched and anastomosed with each other giving rise to loops (Fig. 1 *A*) and polygonal networks (Fig. 1 *B*). The hubs of the network were at times composed of solid multicellular aggregates. Microvessels with slitlike lumina were occasionally observed, particularly toward the end of Week 2 of culture. However, the network at this stage was primarily composed of an admixture of solid cords and spindle-shaped cells which had proliferated into the aortic lumen and the surrounding gel. Partial lysis of the gel was observed around the explant.

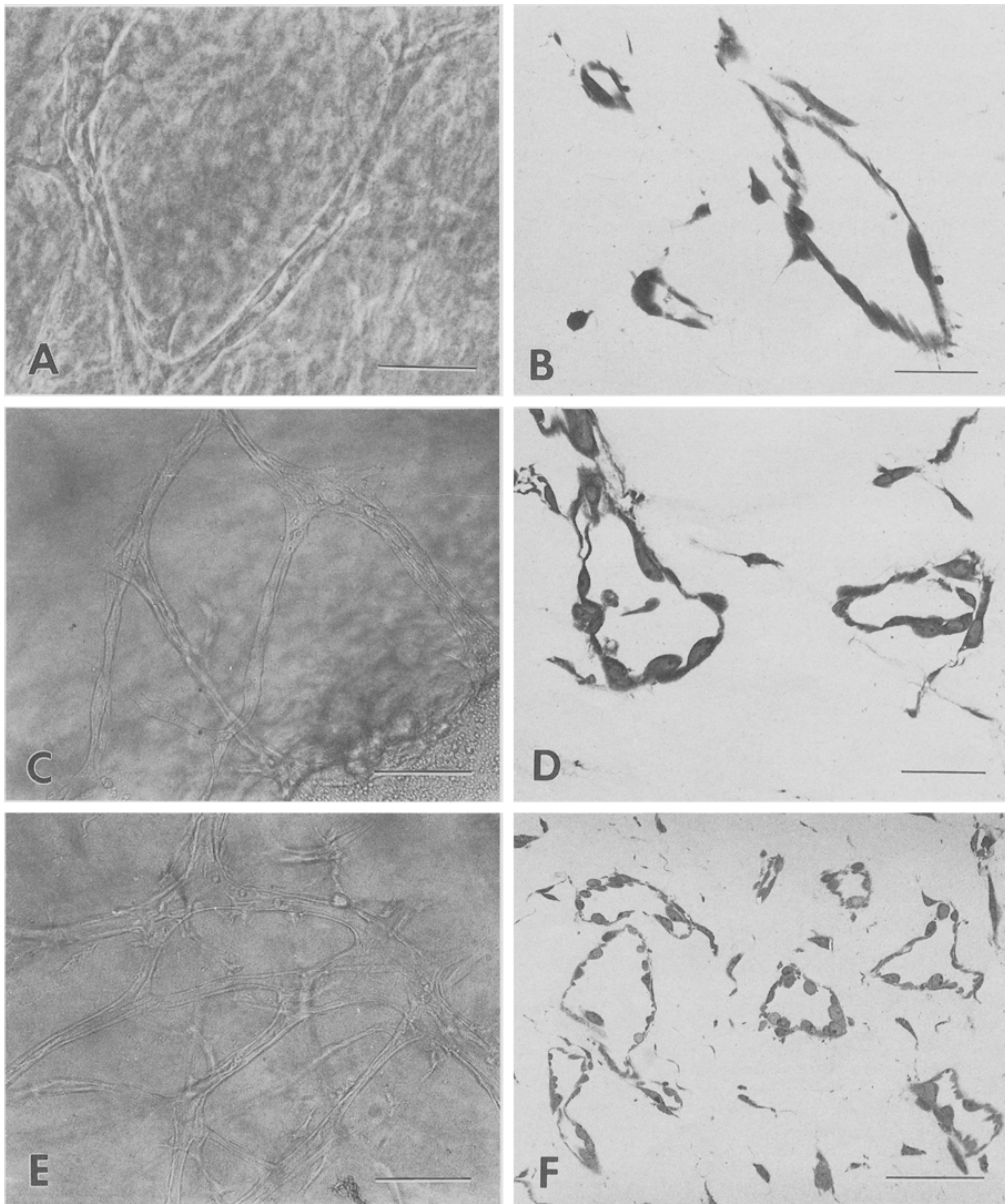

FIG. 4. Angiogenesis in collagen, fibrin and plasma clot. Living cultures (*A,C,E*) and histologic sections (*B,D,F*) showing widely patent microvessels in collagen (*A,B*), fibrin (*C,D*), and plasma clot (*E,F*) cultures of rat aorta during Week 2 of growth. *Bars: A,C,E* and *F*, 50 μm; *B* and *D*, 30 μm.

Histologic studies revealed that the intimal endothelium had migrated as a sheet from the explant onto both the adventitia and the adjacent gel forming the lining of a chamber. In addition, solid cords composed of cohesive cells had sprouted from the surface endothelium into the surrounding gel. Some outgrowths near the explant had patent lumina and were easily recognizable as newly formed microvessels (Fig. 1 *C*). However, the majority of the cohesive outgrowths consisted of solid cords or aggregates of cells without recognizable lumina (Fig. 1 *D*). Interspersed among the patent microvessels, the solid cords and the aggregates were also spindle-shaped nonendothelial mesenchymal cells.

Immunohistochemical stain for FVIII-RAg demonstrated that 40% of the nonluminal cohesive outgrowths which were composed of three or more cells displayed a core of endothelial cells surrounded by one to several layers of satellite nonendothelial mesenchymal cells (Fig. 2). The remaining 60% of the nonluminal cohesive outgrowths were FVIII-RAg negative and were therefore considered of nonendothelial nature. All patent microvessels were FVIII-RAg positive.

Electron microscopy studies confirmed that the microvascular sprouts originated from the surface endothelium lining the gel (Fig. 3 *A*) and were composed of closely apposed endothelial cells connected by junctions (Fig. 3 *B*). Newly formed patent microvessels were lined by endothelial cells showing pinocytotic activity. Some microvessels were surrounded by up to four layers of nonpolar undifferentiated mesenchymal cells (Fig. 3 *C*). Periendothelial cells were observed around solid endothelial sprouts but they were best recognizable by electron microscopy in the mature microvessels after the establishment of endothelial polarity and the formation of the lumen. There was no evidence of Weibel-Palade bodies in the endothelial outgrowth or in the endothelium of the aortic intima.

Angiogenesis in collagen, fibrin, and plasma clot. Angiogenesis in these gels was characterized by an orderly sequence of morphogenetic events. Solid endothelial sprouts developed from the explant within 3 to 4 d. The sprouts anastomosed with each other and matured into a network of branching microvessels with patent lumina toward the end of Week 1 and throughout Week 2 of culture. The establishment of patency was a characteristic of these cultures which allowed us to easily recognize the microvessels and to distinguish the endothelial outgrowths from the surrounding nonendothelial spindle-shaped cells (Fig. 4). The microvessels with the largest lumina were observed in plasma clot culture.

Immunohistochemical stain for FVIII-RAg of collagen, fibrin, and plasma clot cultures demonstrated that 100% of the cohesive outgrowths including solid cords and patent microvessels were composed of endothelial cells. Periendothelial cells and scattered spindle-shaped mesenchymal cells were negative for FVIII-RAg.

Ultrastructurally the microvessels were lined by a polarized endothelium resting on a discontinuous basal lamina. Endothelial cells were connected by junctions and displayed pinocytotic activity. A single layer of periendothelial cells

TABLE 1

QUANTITATIVE ANALYSIS OF ANGIOGENESIS IN THREE-DIMENSIONAL MATRIX CULTURE[a]

	Collagen, $n = 6$	Fibrin, $n = 6$	Matrigel, $n = 6$	Plasma Clot, $n = 6$
Number of microvessels	64.17 (7.12)	85.00[b] (9.43)	39.83[c] (3.91)	176.33[d] (14.63)
Vascular area	512.81[c] (89.01)	890.12[e] (126.35)	425.72[c] (39.51)	1049.81[e] (169.59)
Luminal area	200.62[b] (49.11)	258.81[b] (60.64)	44.27[d] (20.7)	415.39[d] (67.27)
Vascular wall area	311.60[c] (42.54)	622.67[e] (70.09)	378.92[c] (21.39)	624.72[e] (101.86)
Luminal index, %	21.91[b] (2.55)	17.94[b] (1.39)	5.36[d] (1.48)	27.86[d] (1.83)
Patency index, %	67.65 (5.01)	59.07[b] (3.01)	11.43[d] (4.54)	76.79[f] (2.85)
Labeling index, %	22.93[d] (0.89)	31.48[d] (2.29)	10.24[d] (3.12)	47.98[d] (1.27)

[a]Data expressed as means. Values in parenthesis indicate standard error of the mean. Areas measured in μm^2. n = number of histologic sections studied. Data evaluated by analysis of variance-Student-Newman-Keuls test. Matrigel values include data from 100% of patent microvessels and 40% of nonluminal cohesive outgrowths which on paraffin sections contained endothelial cells by FVIII-RAg stain. Data from the remaining FVIII-RAg negative 60% of nonluminal cohesive outgrowths were not included. Data for the other gels were obtained from 100% of both patent microvessels and solid outgrowths which on paraffin sections were all positive for FVIII-RAg.

[b]Significantly different from Matrigel and plasma clot ($P < 0.05$).

[c]Significantly different from fibrin and plasma clot ($P < 0.05$).

[d]Significantly different from the other matrices ($P < 0.05$).

[e]Significantly different from collagen and Matrigel ($P < 0.05$).

[f]Significantly different from fibrin and Matrigel ($P < 0.05$).

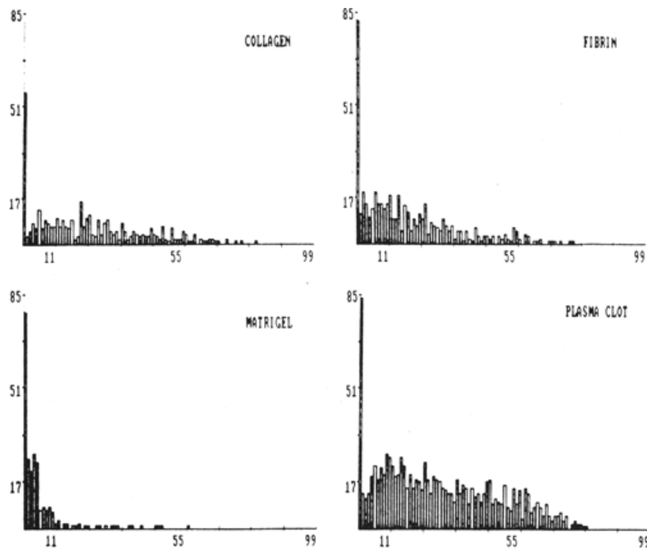

FIG. 5. Computer-generated distribution of luminal index values (percentage of vascular area occupied by lumen) of newly formed microvessels in 14-d-old cultures of rat aorta. Graph illustrates a marked reduction in number of microvessels with higher luminal index values in Matrigel cultures. X axis: luminal index. Y axis: number of microvessels.

similar to the perivascular mesenchymal cells of Matrigel cultures was observed in mature microvessels as previously described (34).

Quantitative Analysis of Angiogenesis

Morphometric studies. The data obtained from analysis of the microvessels with digitizing morphometry are summarized in Table 1. Microvascular outgrowths in Matrigel had a significantly smaller luminal area and luminal index than the microvessels formed in collagen, fibrin, or plasma clot. Calculation of the patency index and distribution analysis of all luminal index values demonstrated a marked reduction in Matrigel cultures of microvessels with luminal index greater than 10% (Table 1 and Fig. 5). In contrast, no significant reduction was noted in the solid sprouts with luminal index values of 0 to 1% (Fig. 5). This indicated that Matrigel influenced the canalization of the microvessels and interfered with the maturation of the endothelial sprouts into patent channels. The vascular wall area, vascular area, and number of microvessels were smaller in Matrigel than in fibrin or plasma clot cultures. The highest number of microvessels and the largest luminal area, luminal index, and patency index were observed in plasma clot cultures. There were no significant differences between fibrin and collagen gel cultures for all the parameters examined except for the vascular wall and vascular areas which were larger in fibrin clot cultures.

Radioautographic studies. The synthesis of DNA (labeling index) by newly formed microvessels was significantly different for each of the matrices studied (Table 1). The lowest labeling index values were found in Matrigel. This finding correlated with the reduced number of newly formed microvessels, indicating a decreased angiogenic response of the aortic rings in Matrigel. The highest labeling index and number of microvessels were found in plasma clot cultures. This was probably due to the combined angiogenic effect of fibrin (6) and the growth factors of the chick embryo extract.

Behavior of Mixed Endothelial-Nonendothelial Cell Cultures on the Surface of Matrigel, Fibrin, or Type I Collagen

In one of the Matrigel cultures a mixed outgrowth of both endothelial and nonendothelial mesenchymal cells migrated to the bottom of the culture well forming cords with slitlike

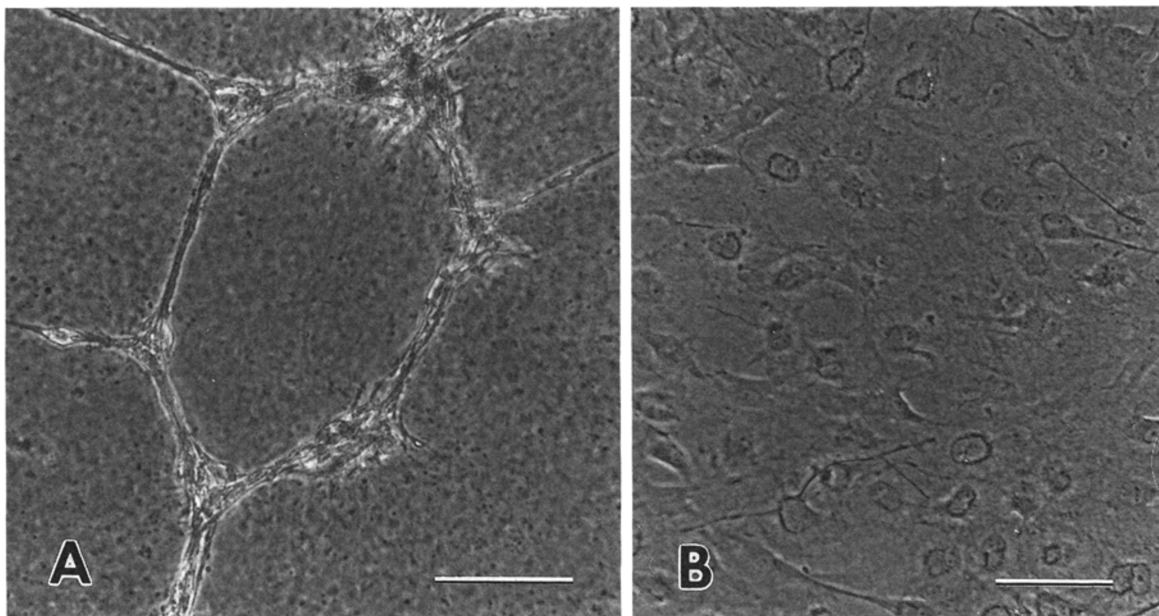

FIG. 6. A mixed population of endothelial and nonendothelial mesenchymal cells derived from a Matrigel culture of rat aorta rapidly (24 h) reorganized into a polygonal network when plated on a Matrigel-coated dish (*A*). These cells formed a monolayer on type I collagen (*B*) or fibrin (not shown). *Bars: A* and *B*, 100 μm.

lumina on the glass surface beneath the gel. To better understand the process of cord formation, the gel holding the aortic explant was detached with a razor blade and the slide was returned to the incubator. The following day, the cells were seen to migrate out of the cords. Within 2 to 3 d the cords had disappeared and the cells were actively proliferating as a monolayer. To investigate if the formation of cords was dependent on the type of ECM, the cells were trypsinized and plated on 35-mm dishes coated with Matrigel, fibrin, or type I collagen gel. The cells on Matrigel rapidly reorganized into branching cords forming a polygonal network in 1 d (Fig. 6 *A*). In contrast, the same cells on fibrin or type I collagen formed a monolayer (Fig. 6 *B*). The mixed composition of cell populations isolated from three-dimensional cultures of rat aorta was demonstrated by FVIII-RAg stain which showed the presence of both endothelial and nonendothelial cells (data not shown).

Discussion

Our studies indicate that Matrigel, a reconstituted basement membrane gel composed of laminin, type IV collagen, heparan sulphate, and entactin, is a potent modulator of angiogenesis in three-dimensional cultures of rat aorta. A comparative analysis of the vasoformative response of aortic rings in different biomatrices demonstrated the following effects of Matrigel on angiogenesis: a) incomplete maturation of solid microvascular sprouts into patent microvessels; b) reduction in size of microvascular lumina of patent microvessels; c) decreased vascular proliferation. In addition, Matrigel induced rapid formation of cords and networks by mixed populations of endothelial and nonendothelial mesenchymal cells in two-dimensional cultures. In contrast, matrices such as fibrin, plasma clot, and type I collagen, which do not contain basement membrane components, behaved as permissive substrates allowing the formation of histotypic patent microvessels in three-dimensional cultures without inducing rapid formation of networks in two-dimensional cultures.

Angiogenesis, under optimal conditions of growth in vivo as well as in vitro, is characterized by an orderly sequence of morphogenetic events. The migrating endothelium forms solid sprouts that branch and anastomose, giving rise to loops and networks. As the solid sprouts mature into microvessels they become canalized and acquire a patent lumen. This process is accompanied by the remodeling of the surrounding ECM. The old basement membrane of the preexisting microvessels is digested by the sprouting endothelium and a new basement membrane gradually accumulates around the developing microvessels (5,35,42).

The finding that a gel composed of basement membrane molecules in vitro causes incomplete maturation of microvascular sprouts and interferes with angiogenesis seems paradoxical inasmuch as patent microvessels in vivo are enveloped by basement membranes, and components of the basement membrane have been shown to induce formation of capillary tubes in vitro (29). One possible explanation for this phenomenon is that the premature exposure of developing sprouts in a three-dimensional matrix to unphysiologic concentrations of basement membrane molecules may disrupt the normal sequence of maturation of microvessels. This hypothesis implies that basement membrane components have the ability to directly influence the growth and morphogenesis of microvessels.

Support for this idea comes from previous studies indicating that the microvascular basement membrane undergoes complex maturational changes during angiogenesis. Ultrastructurally, the basement membrane of immature sprouts is either absent or it is represented by a discontinuous basal lamina (35,42). The immature tips of microvessels sprouting in the murine cornea in response to injury are surrounded by laminin but lack type IV collagen (13). Similar studies during embryogenesis demonstrated that early embryonic capillaries expressed fibronectin but little if any laminin (4,39,41,45). We observed that the provisional microvascular matrix during angiogenesis in plasma clot cultures of rat aorta consists of a fibrillary network of fibronectin and type V collagen and of patchy deposits of laminin and type IV collagen (37,38).

The functional significance of the differences between the provisional and the mature microvascular matrix is unknown, but it is possible that developmental changes in the basement membrane are necessary for an orderly growth and morphogenesis of microvessels. The provisional matrix may facilitate sprouting and proliferation, whereas the mature matrix may induce endothelial polarity and the formation of lumina. Because of its static molecular composition, Matrigel may not meet the dynamic compositional requirements necessary in a developing basement membrane for an orderly growth of microvessels. Conversely, angiogenesis in fibrin or type I collagen would proceed undisturbed, because in these gels basement membrane molecules accumulate perivascularly according to a well-defined developmental program of synthesis and secretion by the endothelial cells.

The cause of the reduced proliferation of microvessels in Matrigel is unclear. Both the overall number of microvessels and the synthesis of DNA were reduced in Matrigel as compared to fibrin, type I collagen, or plasma clot cultures. Laminin and type IV collagen are known to affect the proliferation and migration of isolated endothelial cells in two-dimensional cultures. Their effects, however, change with the type of endothelial cells, the experimental model, and the culture conditions (13,18,20,47).

The effect of Matrigel on angiogenesis may be due to other components of the basement membrane such as heparan sulphate or entactin. Heparin, a highly sulphated form of heparan sulphate, binds to growth factors and has been shown to modulate the effect of angiogenic factors and inhibitors in vivo (12,19). Heparin also modulates endothelial migration (46) and proliferation in response to growth factors in vitro. However, its effect on endothelial proliferation can be stimulatory or inhibitory depending on the source of endothelial cells and the type of growth factor (15). Unknown is the function of entactin and its effect on endothelial cells remains to be determined.

A unique characteristic of Matrigel was its ability to induce formation of cords and networks by endothelial and nonendothelial cells in both two-dimensional and three-dimensional cultures. Formation of cords and networks in response to Matrigel has been observed also in cultures of Sertoli cells, hepatocytes, and vascular endothelial cells (3,17,24). This

phenomenon may be due to the presence in Matrigel of laminin and type IV collagen that are believed to play a role in modulating the organization of microvascular endothelial cells (19,24,28,29). Based on these findings, it is intriguing to speculate that the retiform organization of developing microvessels in matrices such as fibrin or type I collagen, which do not contain basement membrane molecules, may be mediated by endogenous production of basement membrane by endothelial cells. Support for this hypothesis comes also from the observation that angiogenesis can be inhibited both in vivo and in vitro through inhibition of collagen synthesis (21 and personal observation) or dissolution of the basement membrane (19).

Nonendothelial mesenchymal cells in Matrigel cultures participated with the endothelial cells in the formation of three-dimensional networks and were frequently seen forming multilayered sheaths around the endothelium. These cells were FVIII-RAg negative and had an undifferentiated phenotype by electron microscopy. The observation that retiform patterns of growth in or on basement membrane gels are not unique to endothelial cells is consistent with the findings by Emonard et al. (9) that calf skin fibroblasts form networks on Matrigel and suggests that the periendothelial accumulation of cells such as pericytes or smooth muscle cells during embryonal development or in pathologic processes may be mediated by components of the basement membrane. It is possible that the nonendothelial mesenchymal cells influenced the formation of microvessels in Matrigel cultures. However, these cells were found also in collagen, fibrin, and plasma clot cultures where microvessels developed and matured in a histotypic fashion. Further studies with isolated cells will be necessary to better elucidate the respective role of endothelial and periendothelial cells in the morphogenesis of microvessels in different biological matrices.

In summary, this study demonstrates that gels composed of basement membrane molecules contain a complex set of signals capable of modulating the growth, maturation, and organization of microvessels in three-dimensional cultures. The observation that a gel composed of basement membrane molecules can influence essential processes such as microvascular proliferation, canalization, and network formation suggests that production of basement membrane by developing sprouts has to be carefully regulated to allow an orderly growth and maturation of microvessels. To better understand the mechanisms through which the basement membrane modulates angiogenesis, future studies will be aimed at elucidating the effect of individual matrix molecules on microvascular growth in chemically defined three-dimensional gels.

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