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Journal

Laboratory Investigation, 66(5)

ISSN

1530-0307

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Publication Date

1992-05-01

Peer reviewed

Reorganization of Basement Membrane Matrices by Cellular Traction Promotes the Formation of Cellular Networks *In vitro*

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Vascular endothelial cells that are cultured on layers of gelled basement membrane matrix organize rapidly into networks of cords or tubelike structures. Although this phenomenon is a potential model for angiogenesis *in vivo*, we questioned whether basement membrane matrix directs the differentiation of endothelial cells in a specific manner. In this study, we have examined factors that influence the formation of cellular networks *in vitro* in an attempt to define a basic mechanism for this process. We found that endothelial cells, fibroblasts, smooth muscle cells, and cells of the murine Leydig cell line TM3 formed networks on basement membrane matrix in much the same fashion. Light and electron microscopy, combined with time-lapse videomicroscopy, revealed that cells organized on a tesselated network of aligned basement membrane matrix that was generated by tension forces of cellular traction. Cellular elongation and progressive motility across the surface of the gel were restricted to tracks of aligned matrix and did not occur until the tracks appeared. The formation of cellular networks on basement membrane matrix was inhibited by reducing the thickness of the matrix, by including native type I collagen in the matrix, or by disrupting cytoskeletal microfilaments and microtubules. Cell division was not required for network formation. Bovine aortic endothelial cells that formed networks did not simultaneously transcribe mRNA for type I collagen, a protein synthesized by endothelial cells that form tubes spontaneously *in vitro*. Moreover, levels of mRNA for fibronectin and SPARC (Secreted Protein that is Acidic and Rich in Cysteine) in network-forming cells were similar to levels seen in endothelial cells that did not form networks. Endothelial cells and TM3 cells that were plated on highly malleable gels of native type I collagen also formed cords and aligned matrix fibers into linear tracks that resembled those generated on basement membrane matrix, although the structures were not as well-defined. Our observations suggest that the mechanochemical properties of extracellular matrices are able to translate the forces of cellular traction into templates that direct the formation of complex cellular patterns.

Additional key words: Endothelial cells, TM3 cells, Angiogenesis, Extracellular matrix, Matrigel, Type I collagen, SPARC.

Results of studies *in vivo* and *in vitro* suggest that the extracellular matrix (ECM) influences a variety of cell behaviors that potentially contribute to morphogenesis. ECM is thought to activate or direct cell migration (4, 21, 28, 35, 39, 40, 41, 54), alter expression of gene products (2, 5, 6, 9, 33, 38), influence cell division (1, 25, 34, 45), and modify both cell shape and intercellular association (3, 13, 16, 20, 47, 55).

A striking example of the influence of ECM on multicellular organization is seen *in vitro* when vascular endothelial cells are cultured on layers of gelled basement membrane matrix (BMM). On plastic, endothelial cells grow as monolayers and assume flattened, polygonal shapes, whereas on BMM the cells organize within a few hours into two-dimensional networks of cellular cords or tubelike structures that resemble beds of capillaries (14, 15, 31). The mechanism that generates the cellular networks has not been elucidated; however, the phenomenon is thought to be an example of cell-specific differentiation that is induced by components of the ECM. This interpretation is called into question by our current finding that murine Leydig cells grown on BMM organize into cords that closely resemble networks of endothelial cells (50). Moreover, similar networks are formed by bovine retinal pigmented epithelial cells and lens cells that are cultured under similar conditions (29). We have conducted further studies of network formation by cells *in vitro* to define a mechanism that explains this apparent lack of cellular specificity. Our observations indicate that formation of tesselated cords on BMM by several cell

types, including endothelial cells, results from an interaction between cell contractility and matrix malleability. Cellular networks arise when cells associate with narrow, linear tracks of matrix that are generated by forces of cellular traction. These data support the notion that the formation of networks by endothelial cells on layers of BMM is not, by itself, an indicator of specific morphological differentiation. However, traction-induced reorganization of ECM might play an important role in directing certain aspects of cell behavior that contribute to angiogenesis and other morphogenetic processes *in vivo*.

EXPERIMENTAL DESIGN

To compare the ability of different cell types to form networks, bovine aortic endothelial cells (BAEC), cells of the murine Leydig cell line TM3, human fibroblasts, human smooth muscle cells, and murine PYS-2 cells were cultured for up to 48 hours on layers of BMM (Matrigel) that were gelled on a slope to generate a continuous gradient of thickness. After 24 hours, the cultures were assayed for the presence of cellular networks by phase-contrast and dark-field light microscopy. BAEC, TM3 cells, and fibroblasts that were cultured either on plastic or on layers of BMM of uniform thickness were monitored for up to 24 hours by time-lapse, phase-contrast videomicroscopy to follow cell divisions and to observe the movements of cells and/or matrices. The capability of cells to reorganize BMM was studied by culturing BAEC, TM3, or PYS-2 cells for up to 24 hours on layers of highly malleable BMM and viewing the result by light- or scanning electron microscopy. The influence of the cytoskeleton on the formation of cellular networks was examined by culturing cells on BMM in the presence of drugs that disrupt microfilaments (cytochalasin D) or microtubules (colchicine). To assess network formation on BMM of modified composition, BAEC or TM3 cells were cultured on BMM supplemented with fibrillar native type I collagen, heat-denatured type I collagen, or culture medium. The ability of BAEC or TM3 cells to reorganize forms of ECM other than BMM was studied by culturing the cells on gels made of native type I collagen at concentrations of 0.12–2.5 mg/ml. Northern blot analyses were performed to determine whether BAEC altered the synthesis of specific components of ECM during cord formation on BMM. RNA from BAEC that were cultured on plastic, gels of type I collagen, or BMM was resolved by agarose gel electrophoresis, transferred to nitrocellulose, and hybridized with probes specific for the $\alpha 1(I)$ chain of type I collagen, fibronectin, and SPARC.

RESULTS AND DISCUSSION

The ability of BMM to modulate cell shape and association was examined by culturing different cell types on Matrigel with a gradient of thickness. Results are shown in Figure 1. BAEC, TM3 cells, fibroblasts, and smooth muscle cells attached to BMM within 15 minutes. After 16 to 24 hours of culture, cells on thin (10 to 50 μm) layers of BMM had assumed flattened, irregular shapes,

whereas cells on regions of BMM that were thicker (60 to 600 μm) became bipolar and organized into cords that were arranged in tesselated networks of triangles, quadrilaterals, and polygons. As the thickness of BMM increased, cells formed longer cords, which defined the borders of increasingly larger tesserae. For all cell types, this transition occurred more abruptly on layers of BMM prepared with a greater slope (Fig. 1C).

The observation that the thickness of BMM influenced the formation of cellular networks suggested that the mechanical properties of this substrate might be important in determining the shape and spatial arrangement of cells. We explored this concept further by culturing BAEC or TM3 cells on gelled BMM of constant thickness (1.0 mm) that was made more rigid by the addition of varying amounts of gelled native type I collagen. Cells of either type grown on BMM that was supplemented with 0.6 mg/ml collagen organized within 24 hours into clearly demarcated networks (Fig. 2A, D). In contrast, cells cultured on BMM containing collagen at 2 mg/ml were flattened, spread, and unorganized (Fig. 2B, E) and resembled cells cultured on uncoated tissue culture plastic (Fig. 2C, F). Cells grown on BMM containing intermediate concentrations of type I collagen (1.3 mg/ml) developed poorly defined networks intermixed with flattened, unorganized cells (not shown). The ability of type I collagen to suppress cord formation was not due to simple dilution of constituents of BMM by the collagen solution, because networks formed readily when BMM was diluted with a similar volume of Dulbecco's modified Eagle's medium (DMEM) that lacked collagen. BMM that was supplemented with 2 mg/ml of denatured type I collagen formed highly malleable gels that did not suppress network formation by BAEC or TM3 cells. The mild heat-denaturation rendered the collagen unable to gel at physiologic pH, ionic strength, and temperature but did not affect its ability to support the attachment of BAEC and TM3 cells when applied to bacteriologic plastic as an air-dried layer.

To examine the process of network formation in greater detail, BAEC or TM3 cells were plated at 25% confluency on 1.0 mm thick layers of undiluted BMM and monitored by time-lapse videomicroscopy. The behaviors of both cell types were similar. Two to 4 hours after attaching to the BMM, adjacent cells moved slowly toward one another and cohered to form small, randomly distributed aggregates. Cells approached one another as a consequence of local distortions of the BMM. Cells were not progressively motile. They remained spherical, exhibited no lamellipodia, and did not move past fixed reference points (*i.e.*, small granular inclusions) in the gel. As cells aggregated, some cells elongated, acquired a bipolar shape, and formed bridges between adjacent aggregates. With time, networks of multicellular cords became clearly defined. Networks were remodeled continuously: some polygonal tesserae closed in a sphincterlike manner as cellular cords that defined their walls began to shorten, whereas other polygons opened and linearized to form the sides of larger tesserae. Reorganization of cellular cords was accompanied by distortion of underlying BMM. BAEC did not divide on BMM,
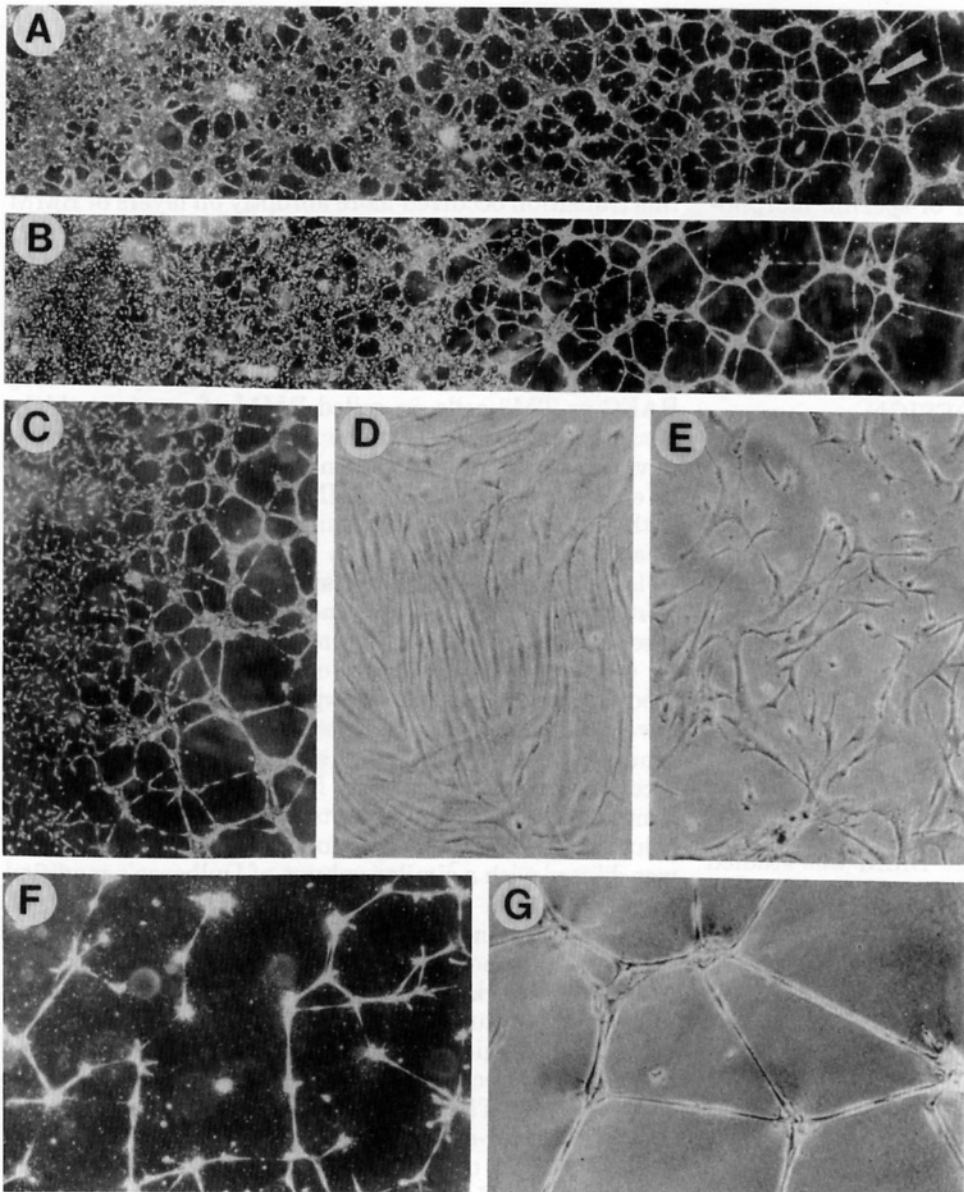
FIG. 1. The formation of networks by different cell types is influenced by the thickness of the underlying ECM. *A,* BAEC cultured 19 hours on BMM with a gradient of thickness that increases from 10 μm (*far left*) to 500 μm (*far right*). On thin BMM, cells are flattened and arranged randomly. As the matrix increases in thickness, cells elongate and organize into tesselated networks of progressively longer, narrower cords (*arrow*). In this photomontage, distance from far left to far right is 17 mm. *B,* TM3 cells cultured on a substrate similar to *A*. The relationship between matrix thickness and network formation is nearly identical between TM3 cells and BAEC. *C,* TM3 cells cultured 19 hours on BMM with a steep gradient of thickness increasing from 10 μm (*far left*) to 400 μm (*far right*) over a distance of 4 mm. Transition from flattened, stellate cells (*far left*) to networks (*right*) occurs more abruptly than that seen in *A* or *B*. Twenty hours after plating, SK5 fibroblasts (*D, F*) and human smooth muscle cells (*E, G*) assume flattened shapes on thin (20 μm) layers of BMM (*D, E*) but form tesselated networks on thick (300 μm) layers of this matrix (*F, G*). *A, B, C, F* are darkfield images; *D, E, G* are viewed by phase-contrast. Figure 1*A, B,* and *F,* ×8; *C,* ×13; *D* and *E,* ×130; *G,* ×39.

whereas occasional divisions of TM3 cells were observed after cords were fully formed. Occasionally, the sides of tesserae were composed of straight, narrow, acellular lines (Fig. 3*A* and *B*) rather than of cellular cords. Time-lapse monitoring revealed that the lines, which measured from 25 μm to over 500 μm in length, were formed between cells by "condensing" from the surrounding BMM. Matrix lines were never curved, but the lines elongated, grew shorter, or changed their angle with respect to other matrix lines or cords of cells as the BMM was remodeled. All single bipolar cells rested upon a single matrix line which passed directly beneath the long axis of the cell (Fig. 3*B*). Moreover, multicellular cords which were incomplete revealed an underlying line of BMM. BAEC and TM3 cells rapidly assumed a bipolar morphology upon contact with matrix lines. After contacting the lines, cells remained permanently associated with them and in many cases began to move progressively back and forth along them. Isolated cells which did not make contact with matrix lines neither altered their spherical shape nor moved progressively over the matrix,

even after 48 hours of culture. Matrix lines were also observed in association with networks of smooth muscle cells (Fig. 3*C*) and fibroblasts (not shown).

Our time-lapse studies suggested that matrix lines (which from now on will be referred to as "tracks" because progressive cell movement is restricted to them) served as templates for the formation of cellular cords. The manner in which tracks were formed was investigated in greater detail by plating either BAEC or TM3 cells on a highly malleable gel prepared from freeze-thawed Matrigel. Time-lapse analyses revealed that both cell types dramatically remodeled the substrate into a network of matrix cables that resembled the pattern of tracks within untreated BMM. After 24 hours of culture, aggregated cells had pulled nearly all of the BMM into islands upon which the cells rested. Cellular aggregates remained connected, however, by narrow, straight cables of matrix that formed a geometric network (Fig. 4*A* to *D*). The cables were connected to cellular aggregates at their ends but were elevated above the tissue culture plastic throughout most of their length. Although many

FIG. 2. Addition of native type I collagen to BMM inhibits the formation of cellular networks. TM3 cells (*A, B, C*) or BAEC (*D, E, F*) were cultured 19 hours. Cells organize into networks on BMM supplemented with 0.6 mg/ml of gelled native type I collagen (*A, D*). Cells grown on BMM supplemented with 2.0 mg/ml of native type I collagen (*B, E*) are flattened, spread, and unorganized like cells on plastic (*C, F*). *A, D* are darkfield images; *B, C, E, F* are viewed by phase-contrast. Figure 2*A* and *D*, ×26; *B, C, E,* and *F*, ×128.
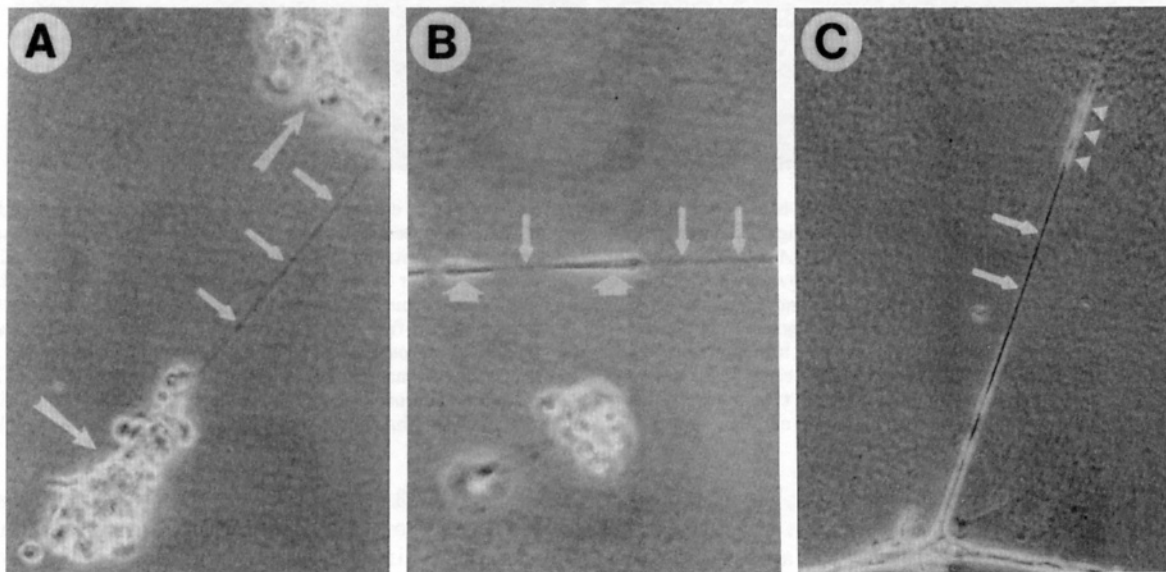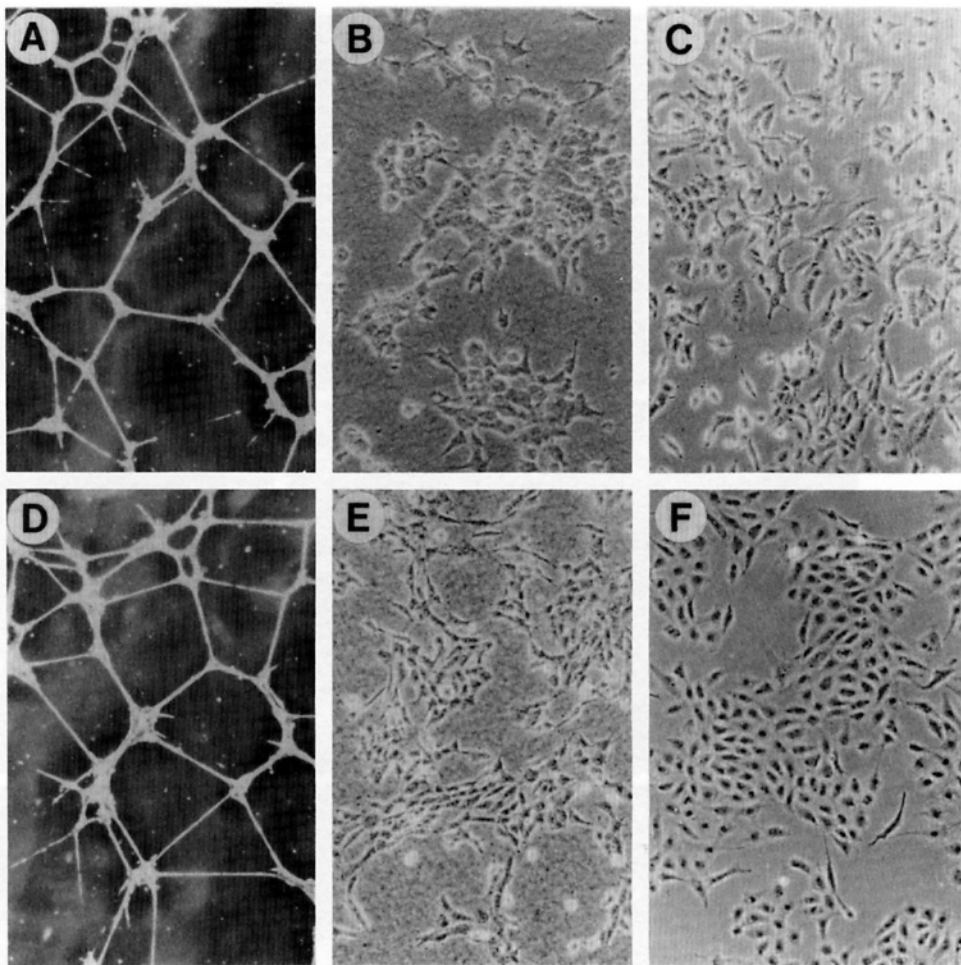

FIG. 3. Cells generate lines in BMM. *A*, BAEC cultured 24 hours on a 300-μm thick gel of BMM. A thin, straight line of reorganized matrix (*small arrows*) has appeared between adjacent cell aggregates (*large arrows*). Similar lines are often covered by overlying cellular cords. *B*, Line in BMM (*narrow arrows*) generated by aggregated TM3 cells (beyond photo) bears two cells (*broad arrows*) that are moving toward one another. *C*, Line in BMM (*arrows*) formed by human smooth muscle cells bears several elongated, refractile cells. Three are indicated by *arrowheads. A–C* are viewed by phase-contrast. Figure 3*A* and *B*, ×200; *C*, ×120.

cables were free of cells, most bore cellular cords or smaller numbers of motile, bipolar cells (Fig. 4*E* and *F*). Cellular cords formed on highly malleable BMM and on more rigid, untreated BMM in a similar manner, *i.e.*, by

elongation and cohesion of groups of cells that were situated over developing linear tracks of matrix, or by migation of cells from aggregates onto cell-free tracks.
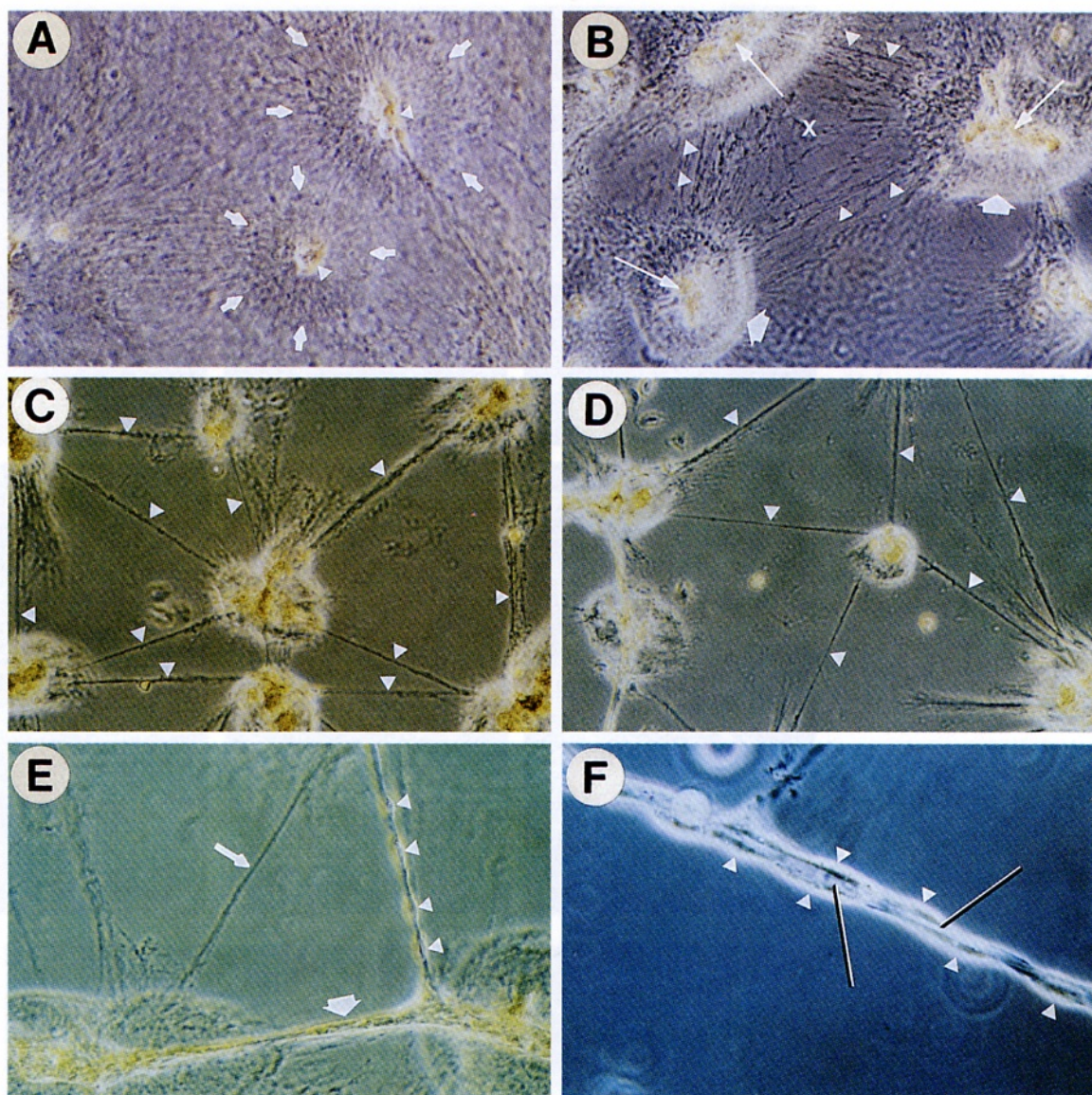
Scanning electron microscopy showed that the BMM

FIG. 4. Cells reorganize highly malleable BMM into networks. *A* to *C*, TM3 cells were grown on a 200-μm layer of Matrigel that was freeze-thawed before gelation. After 6 hours of culture (*A*), fibers of BMM are pulled toward spherical cellular aggregates (*arrowheads*) to form radiating patterns, the perimeters of which are indicated by *arrows*. After 14 hours of culture (*B*) three cellular aggregates (*thin arrows*) rest on circular islands of BMM (2 islands are indicated by *broad arrows*). BMM is being pulled from the geometric center (*X*) between the aggregates and aligned into poorly defined tracts (*arrowheads* indicate linear stretches of matrix) that link the cellular aggregates.

After 24 hours of culture (*C*), 7 cellular aggregates have pulled nearly all of the BMM beneath them but remain connected by narrow, cell-free cables of matrix (*arrowheads*). BAEC (*D*) form similar cables (*arrowheads*) after 24 hours of culture on malleable BMM. In *E*, three consequences of matrix reorganization by BAEC are seen. One cable of matrix is cell-free (*narrow arrow*), whereas another cable bears isolated cells (*arrowheads*). Cells are forming a cord (*broad arrow*) on a third cable of matrix. In *F*, elongated, highly refractile TM3 cells (*arrowheads*) surround a narrow cable of BMM (*two-tone lines*). *A* to *F* are viewed by phase-contrast. Figure 4*A*–*E*, ×120; *F*, ×300.

within the cables was aligned into long, closely-associated parallel strands (Fig. 5*A* and *B*). A correlation between the formation of cellular cords and reorganization of BMM was further suggested by the negative response of PYS-2 cells to these matrices. PYS-2 cells attached firmly and spread on highly malleable BMM but did not form cellular networks or reorganize the matrix into networks of cables (Fig. 5*C*).

Reorganization of BMM required forces generated by living cells because linear tracks did not appear within BMM that was incubated for 72 hours in culture medium lacking cells, in medium containing concentrated lysate from frozen-thawed BAEC cells, or in the presence of

BAEC or TM3 cells that were killed with formaldehyde either before or immediately after their attachment to the matrix. Since the cytoskeleton is involved in the generation of cellular forces, we examined whether network formation by BAEC was affected by interfering with the formation of cellular microfilaments or microtubules. We observed that the microfilament-disrupting drug cytochalasin D inhibited network formation in a dose-dependent manner (Fig. 6*A* to *F*). The effects of cytochalasin were reversible. Cultures on BMM that were inhibited by exposure to cytochalasin D for 19 hours formed matrix tracks and cellular cords within 24 hours after the drug was washed from the wells (Fig. 6*G*).
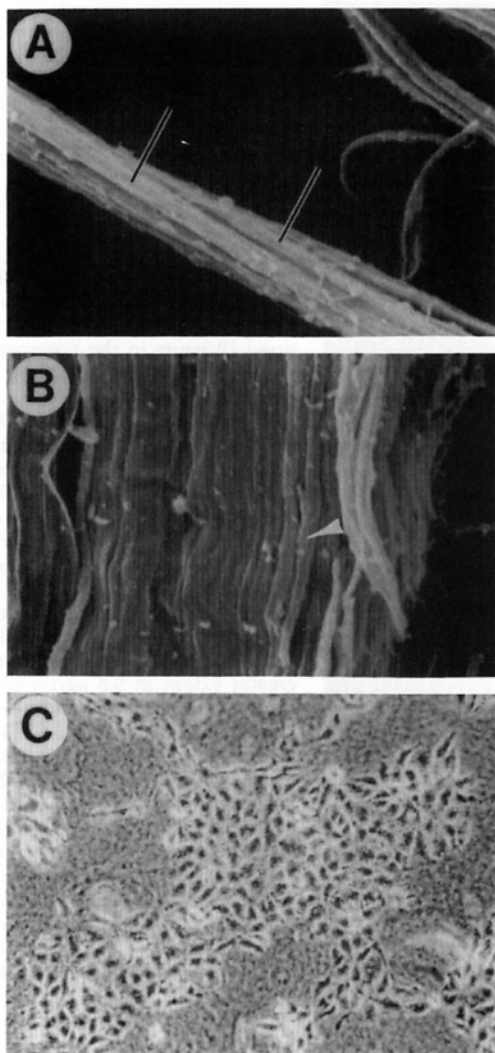
FIG. 5. Small (*A*) and large (*B*) cables generated by cellular reorganization of highly malleable BMM are comprised of closely-associated parallel strands of matrix. *A,* Two strands are indicated by two-tone lines. *B,* One strand is indicated by the *arrowhead.* PYS-2 cells (*C*) cultured 48 hours on similar layers of BMM acquire a flattened shape, do not form cellular networks, and do not reorganize matrix into cables. *A, B* are viewed by scanning electron microscopy. *C* is viewed by phase-contrast. Figure 5*A,* ×8,600; *B,* ×6,600; *C,* ×150.

Network formation by BAEC was also inhibited by colchicine, a microtubule-disrupting drug (Fig. 6*H*). The inhibitory effects of colchicine, like those of cytochalasin, were overcome by removal of the drug from the cultures.

At this juncture, we questioned whether the reorganization of BMM by cellular contractile processes and the associated formation of cellular networks were unique to this substrate or could occur on other forms of ECM. Therefore, we studied the behavior of BAEC on gels of native type I collagen, since this protein is not a component of Matrigel. Cells cultured 19 hours on 1 mm thick hydrated gels of 2.5, 2.0, or 1.0 mg/ml collagen resembled cells grown on plastic. They were unorganized and assumed flattened, spread shapes (Fig. 7*A*). Cells did not align collagen fibers within the gels. In gels formed from 0.25–0.4 mg/ml collagen, limited alignment of fibers and cells over short distances (2–4 cell lengths) contributed to poorly-defined networks (Fig. 7*B*). Cells reorganized fibers in highly malleable gels of 0.12–0.16 mg/ml collagen into linear arrays up to 300 μm in length. Like the tracks in BMM, the aligned collagen fibers bridged adjacent cells or cell aggregates (Fig. 7*C*). Elongated cells were arranged along the aligned collagen in linear chains and occasionally formed thicker cords (Fig. 7*D*). Organization of cells into polygonal networks was not as extensive as on BMM. TM3 cells aligned fibers within type I collagen gels in a manner similar to that of BAEC (not shown).

Our observations suggested that cells exerted tension to align fibers and to generate networks of tracks within ECM. We further examined the relationship between tensile and compressive forces exerted by BAEC and TM3 cells by culturing the cells on thin films of silicone rubber (18, 19, 48) and observing the distortion patterns that arose in this substrate as a consequence of cell contractility. BAEC and TM3 cells adhered to films of rubber within 24 to 48 hours of plating. The rubber was not cytotoxic to either cell type, since the rate of cell division was not affected after 7 days of culture. Distortion of the rubber was most apparent around and beneath cellular aggregates. Aggregates were surrounded by a radiating field of linear tension wrinkles. Adjacent aggregates were frequently linked by one or more tension wrinkles that were deeper and much longer than wrinkles projecting from other regions of the aggregates (Fig. 8*A*). Moreover, smaller cellular aggregates and isolated cells were often linked by a network of tension wrinkles that resembled the network of tracks generated by cells on BMM (Fig. 8*B*). The projecting tension wrinkles arose as the result of compression of the rubber substrate beneath cells. Compression was particularly notable beneath cellular aggregates, where the rubber film was tightly packed to form folds that were oriented circumferentially (Fig. 8*C*) or creases that were arranged in a dense, disorganized mass. Distortion of the rubber film could be completely reversed by a partial dislodgment of the aggregates from the substrate with a microneedle. Reduction in cell contact with the rubber caused all tension and compression wrinkles to disappear within a few seconds as the rubber expanded from beneath the aggregates to assume its original smooth contour.

Endothelial cells isolated from capillaries and selected clones of BAEC are capable of forming networks of cords or tubelike structures after several days or weeks of culture in the absence of exogenously supplied layers of ECM (11, 12, 23, 26). Cells that form these structures are associated with fibronectin (11) and exhibit elevated levels of protein and messenger RNA (mRNA) that correspond to type I collagen and SPARC (26) (a protein which has been shown to be associated with morphogenesis and changes in cellular shape) (44). As components of the ECM, these proteins might be important in promoting spontaneous tube formation by endothelial cells *in vitro* and angiogenesis *in vivo*. We were interested, therefore, in determining whether the BAEC used in our studies (which do not form cords in the absence of exogenous ECM) expressed elevated levels of mRNAs for these three proteins during their formation of cords on BMM. Relative amounts of mRNA, normalized to levels of 28S ribosomal RNA (rRNA), are shown in the bar graph in Figure 9. BAEC that formed networks on
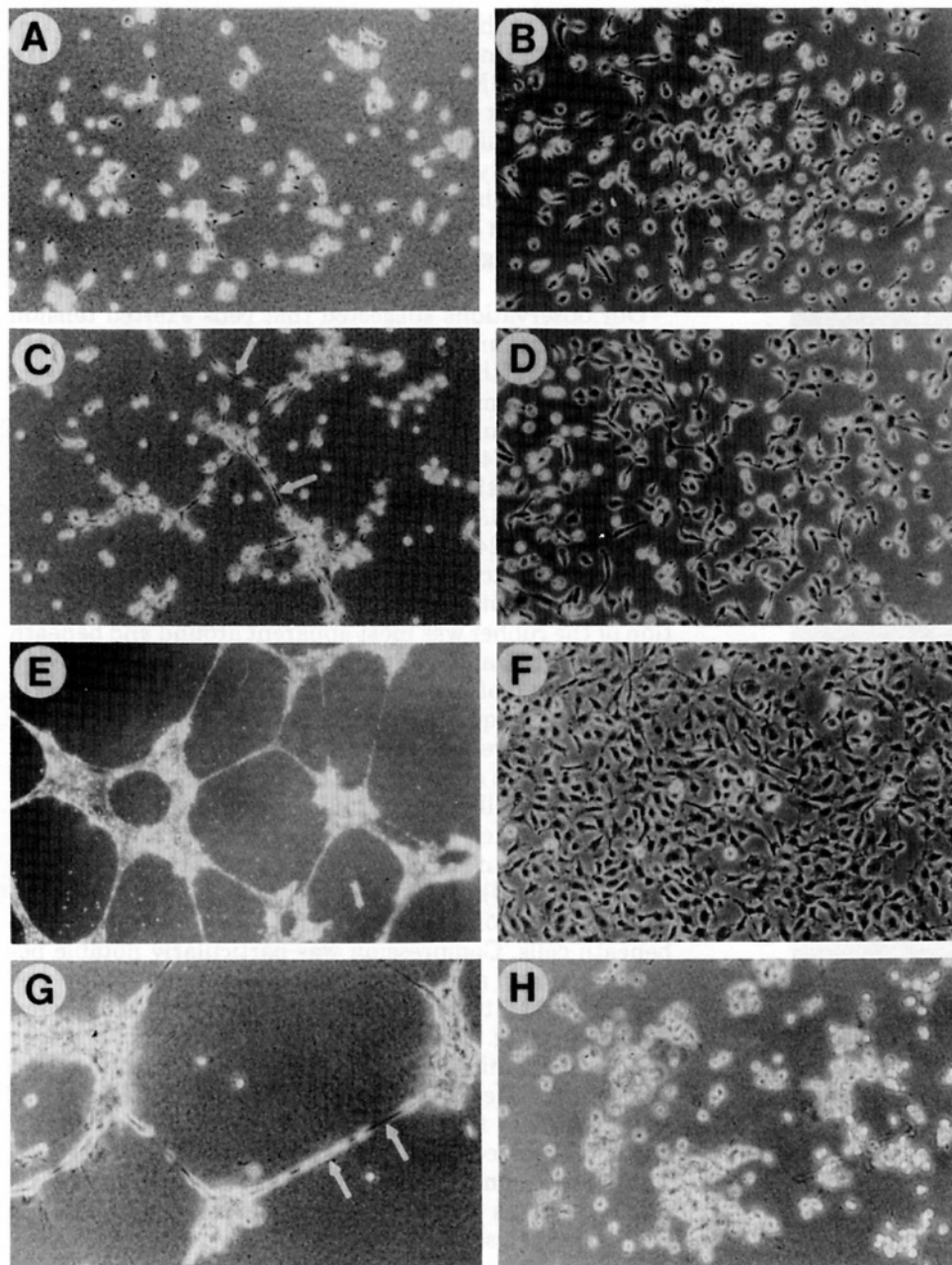
FIG. 6. Disruption of microfilaments or microtubules suppresses formation of cellular cords on BMM. BAEC were cultured 19 hours in the presence of cytochalasin D or colchicine on uncoated plastic or on gels of BMM. In 1.5 μg/ml cytochalasin (*A, B*), cells on BMM (*A*) are spherical and do not form networks. Cells on plastic (*B*) are not spread. Attachment of cells to BMM or plastic was firm and was not affected by cytochalasin. In 0.75 μg/ml cytochalasin (*C, D*), some cells on BMM (*C*) form short linear chains (*arrows*), whereas approximately 50% of cells on plastic (*D*) exhibit some degree of spreading. Networks on BMM were developed to a greater extent in 0.5 μg/ml of the drug (not shown). In control cultures exposed to 0.2% DMSO, cells on BMM (*E*) organize into fully developed networks, and cells on plastic (*F*) are fully spread. When cytochalasin is removed from the cell culture depicted in *A*, networks (*G*) of matrix tracks (*right arrow*) and associated cells (*left arrow*) begin to form within 24 hours. Network formation on BMM is also blocked by 2.5 μM colchicine (*H*). *A* to *H* are viewed by phase-contrast. Figure 6*A* to *D* and *F* to *H*, ×105; *E*, ×27.

BMM and BAEC that assumed flattened shapes on plastic or gelled type I collagen expressed similar levels of mRNA for fibronectin. Cells grown on BMM expressed levels of SPARC mRNA that were the same as those expressed by cells grown on type I collagen, but which were slightly lower than those found in cells cultured on plastic. mRNA for type I collagen was not detected in BAEC grown on plastic, collagen, or BMM. In contrast, cells comprising cords that formed spontaneously in culture (26) expressed high levels of mRNA for type I collagen and contained levels of mRNA for SPARC and fibronectin that were, respectively, 2.1- and 3.8-fold higher than levels expressed by BAEC that formed cords on BMM.

The organization of cells into networks of cords or tubes on layers of BMM has been interpreted in previous studies as a morphological expression of cellular differentiation *in vitro* (14, 15, 31, 53). Our studies indicate, however, that different cell types form networks by a physical/mechanical process that is not necessarily a consequence of specific cellular differentiation. Cells pull BMM toward them, which draws neighboring cells together, promotes cellular aggregation, and aligns BMM in narrow tracks that connect adjacent cells or cell aggregates. The tracks, which promote cellular elongation and migration, act as templates for the formation of cellular cords.

CELLULAR TRACTION INDUCES CO-ALIGNMENT OF CELLS AND BMM

Our results indicate that cellular force-generating processes involving microfilaments and microtubules provide the energy for tension-based reorganization of BMM and subsequent formation of cellular cords. The formation of linear tracks in BMM as a consequence of
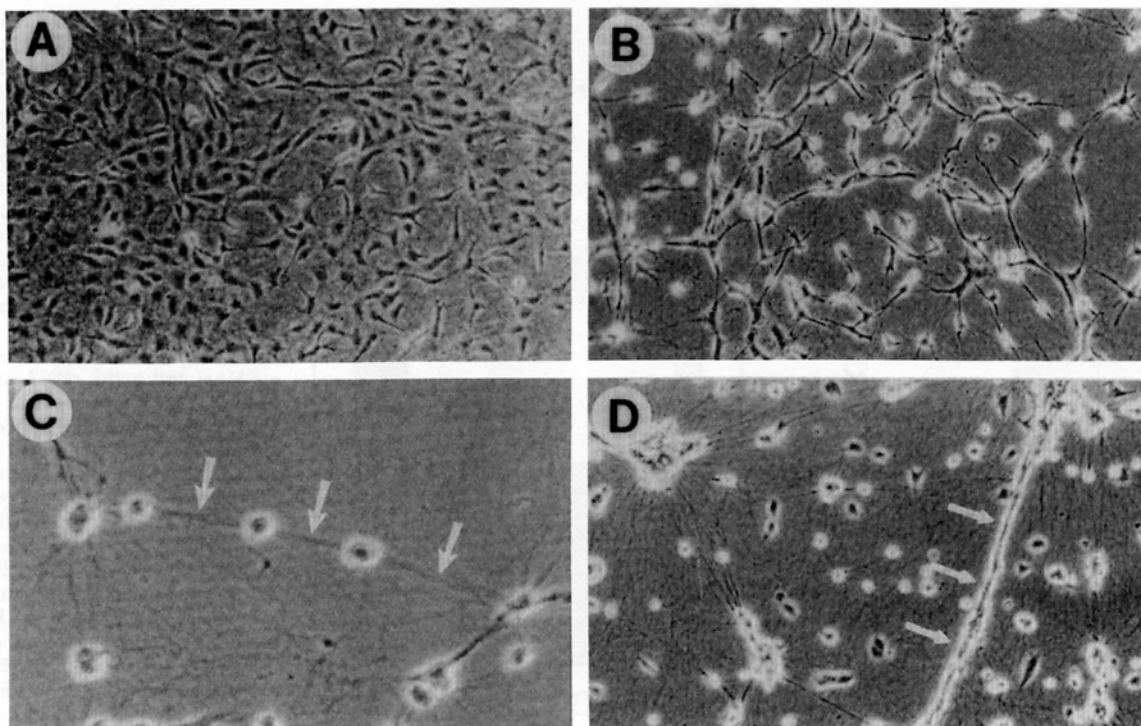
FIG. 7. BAEC cultured 19 hours on a gel of native type I collagen at concentrations of 2 mg/ml (*A*), 0.32 mg/ml (*B*), or 0.16 mg/ml (*C*, *D*), exhibit different morphologies. *A*, Cells are flattened, spread, and unorganized. *B*, Cells have elongated along bundles of collagen fibers to form a poorly organized network. *C*, Reorganized collagen fibers form linear arrays (*arrows*) that bridge adjacent spherical cells. Occasionally (*D*), cells form a cord (*arrows*). *A* to *D* are viewed by phase-contrast. Figure 7*A*, *B*, and *D*, ×120; *C*, ×250.

FIG. 8. BAEC and TM3 cells distort films of silicone rubber. *A*, In this photomontage, a narrow array of parallel tension wrinkles, which appear as alternating light and dark lines by phase-contrast (*large arrow*), bridges adjacent aggregates (*agg*) of TM3 cells. Tension wrinkles that radiate from other regions of the aggregates (one wrinkle is indicated by the *small arrow*) are much shorter. *B*, Small aggregates of BAEC (*arrows*) are connected by narrow tension wrinkles (*arrowheads*). *C*, Rubber has been compressed beneath an aggregate of TM3 cells to form many closely spaced wrinkles (one wrinkle is indicated by the *small, black arrow*). Tension wrinkles (*arrowheads*) project from the curving edge of the aggregate (*broad arrow*) at right angles to the compression wrinkles. Figure 8*A* and *C*, ×97; *B*, ×117.
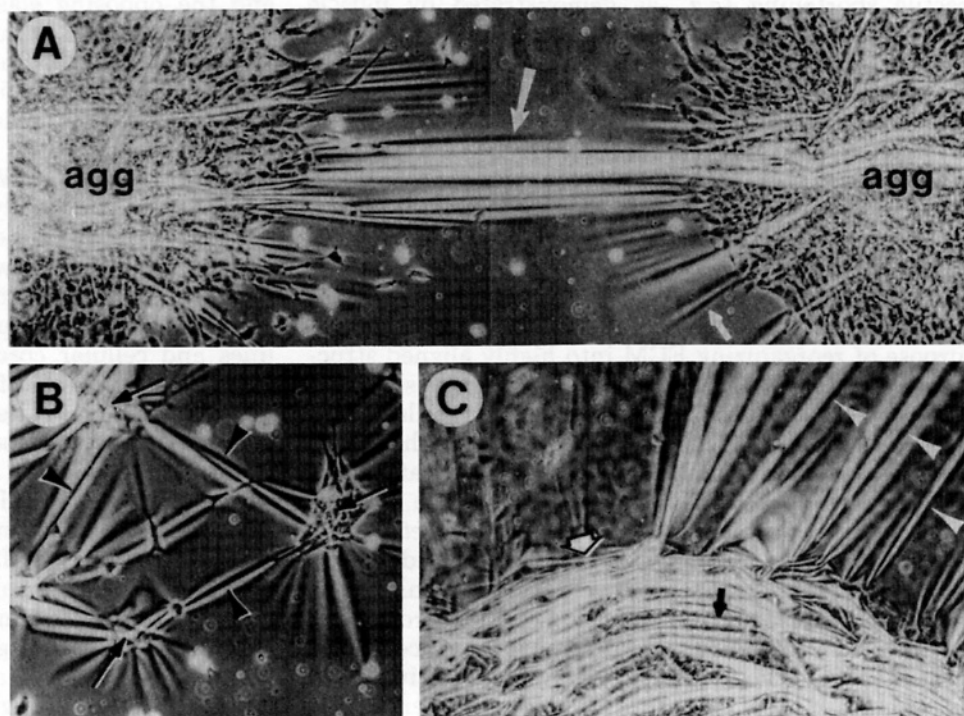
forces applied by cells is a phenomenon that is not unique to this form of ECM. Tensile forces exerted by dispersed fibroblasts or fragments of explanted tissue cause fibers within type I collagen gels to align into an interlocking network of triangles, quadrilaterals, and polygons (17) that closely resembles cell-generated patterns within BMM. The distance over which type I collagen fibers can be aligned is comparable to BMM and can exceed 1 cm (18, 48). Cell-generated patterns within BMM resemble the linear tension wrinkles that develop between aggregates of fibroblasts (18, 19), TM3 cells, or BAEC as they deform films of silicone rubber. Tension is generated as cells compress the rubber into a series of folds that form directly beneath each cell. This process has been
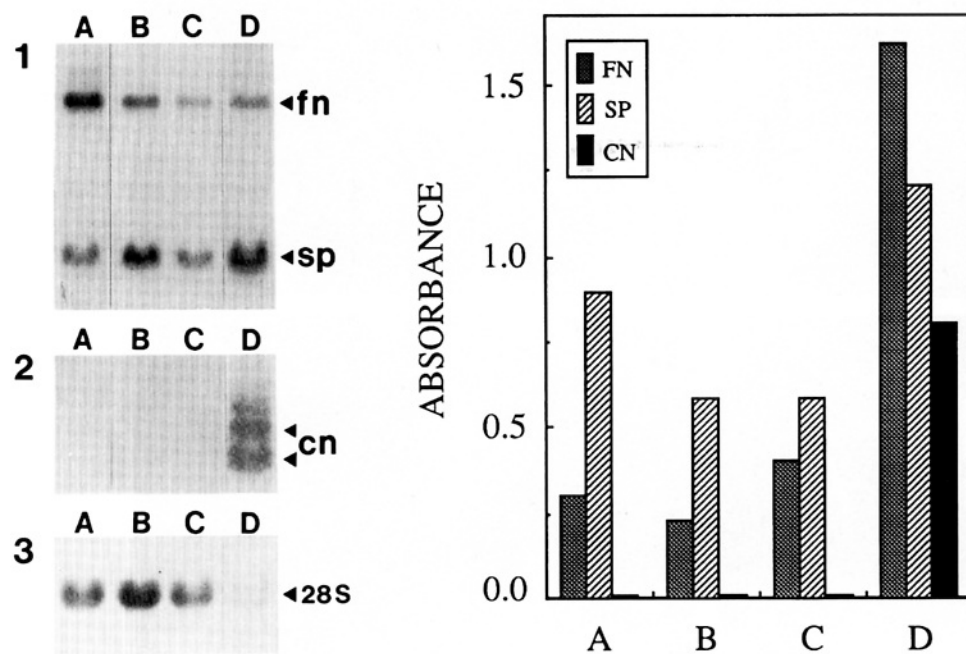
FIG. 9. Expression of mRNAs for ECM components by BAEC cultured on different substrates. Total RNA from BAEC that were cultured 24 hours on uncoated plastic (*A*), 2.0 mg/ml type I collagen gel (*B*), or gelled BMM (*C*), and total RNA from cells that comprise networks of cords/tubes that form spontaneously on plastic (*D*) were subjected to Northern blot analysis. Image *1* is a Northern blot hybridized with fibronectin (*fn*) and SPARC (*sp*) cDNA probes. Images *2* and *3* show the same blot reprobed with an $\alpha 1(I)$ collagen (*cn*) cDNA (*arrowheads* denote 4.8 kb and 5.8 kb mRNAs) and a 28S rRNA probe (*28S*), respectively. The *bar graph* shows the relative quantities of mRNAs for fn, sp, and cn that were measured from autoradiographs *1* and *2* by densitometry and corrected for RNA sample quantity according to the levels of 28S rRNA shown in *3*. mRNA for type I collagen is expressed at high levels in BAEC undergoing spontaneous cord formation, but is not detectable in flattened BAEC cultured on plastic or collagen, or in cord-forming cells on BMM. BAEC undergoing spontaneous cord formation expressed levels of SPARC and fibronectin that were, respectively, 2.1- and 3.8-fold higher than levels expressed by cord-forming BAEC on BMM.

referred to as "traction" (48) to distinguish it from contractile shortening (*e.g.*, as demonstrated by muscle cells). A cell exerting traction does not shorten along its length but in fact elongates in the direction of primary lines of tension. Forces of cellular traction are transmitted to the substrate through focal contacts (52) and are generated by the same mechanism that causes fibroblasts and other cell types to move slowly over rigid surfaces such as glass or plastic. It has been estimated, however, that the traction force exerted by fibroblasts *in vitro* is 100 to 1000 times greater than that which is sufficient to accomplish locomotion (18). It has been proposed that fibroblasts *in vivo* exert forces of this magnitude for the purpose of reorganizing ECM into highly aligned structures (*e.g.*, tendons, ligaments, organ capsules) rather than for cell movement. Indeed, we find that cells cannot move progressively over gelled BMM until they have generated paths of aligned matrix. Evidence that cells *in vivo* align fibers of ECM and subsequently use them as paths for migration was obtained by Markwald *et al.* (35), who described the alignment of the disorganized, acellular ECM of the cardiac atrioventricular cushion into tracks that projected from the ends of endocardial cells toward the myocardium. Migrating endothelial cells were associated with these tracks and formed putative adhesive connections with them. ECM of atrioventricular cushions did not become aligned after cellular microfilaments were disrupted by cytochalasin.

MECHANICAL PROPERTIES OF BMM MODULATE CELLULAR ORGANIZATION

The shape that cells assume when they are attached to a substrate *in vitro* is dependent, at least in part, on the equilibrium between the forces of cell contractility and the opposing elastic forces generated within the substrate as it is deformed (22, 24). Cells flatten and spread on rigid substrates such as plastic, thin layers of collagen, or aldehyde-fixed matrices of either collagen or BMM. Conversely, cells acquire rounded forms on malleable supports such as floating collagen gels or unfixed ECM (6, 10, 38, 47). We find that the mechanical properties of BMM not only modulate the shapes of individual cells but also influence the two-dimensional organization of cells into networks. Decreasing the malleability of BMM by either reducing its thickness over a rigid support, or adding type I collagen, suppresses tension lines and cellular cords, and enhances cell spreading. Type I collagen might facilitate cell spreading by providing cell attachment sites that are highly adhesive and/or present in large numbers. We find, however, that cord formation by murine Leydig cells, which attach very poorly to gels of type I collagen, is inhibited *in vitro* by the same concentrations of type I collagen that suppress cord formation by TM3 cells and BAEC, both of which bind firmly to type I collagen (50). It appears more likely that the long, stiff, randomly oriented bundles of fibrils that form during the gelation of native type I collagen (8) suppress cord formation by decreasing the malleability of BMM. Accordingly, cord formation is not inhibited by supplementing BMM with heat-denatured type I collagen, which will not form long fibers under physiological conditions.

ECM AND ANGIOGENESIS *IN VITRO*

Vascular endothelial cells form interconnecting, tesselated cords or tubelike arrays when cultured on layers

of BMM (31). Since these structures resemble microvasculature in vivo and do not form on tissue culture plastic, it has been proposed that they arise by a type of cell-specific morphological differentiation (14, 15, 31), triggered by the binding of specific molecules of BMM to complementary receptors on the endothelial cell surface. The YIGSR sequence within the B1 chain of laminin is reported to induce individual endothelial cells to assume a ringlike appearance in vitro that has been interpreted as the basic unit of capillary structure (15). However, it is apparent that these highly flattened, spread cells with lumens that seem to be large vacuoles bear little resemblance to cells of the capillary wall. Moreover, a role for laminin as a primary signal for differentiation is questionable because endothelial cells rapidly form tubelike structures when they are exposed to other forms of ECM that include substrate-bound fibronectin (23) or fibrils of type I collagen introduced into culture medium (27).

The best evidence that the organization of endothelial cells into networks on BMM is not by itself an indicator of vascular differentiation is that the phenomenon occurs among nonvascular cell types, including rat osteoblasts (53), murine Leydig cells (50), the murine Leydig cell line TM3, human fibroblasts, and human smooth muscle cells. Moreover, Kennedy et al. (29) observed that bovine retinal microvascular endothelium, retinal pigmented epithelial cells, and lens cells formed branching tubes on BMM that were very similar in appearance. Formation of networks can also be uncoupled from certain aspects of cellular differentiation. For example, murine Leydig cells grown on BMM form either tesselated cords or spherical aggregates that lack cords, depending on the thickness of the matrix layer. Cells of either morphology, however, appear equally "differentiated," in that they express high levels of 3β-hydroxysteroid dehydrogenase and do not divide. In contrast, flattened, "undifferentiated" Leydig cells grown on plastic proliferate rapidly and cease production of this steroidogenic enzyme (50).

A physical/mechanical mechanism for network formation in vitro could explain why the process lacks cell- and matrix-specificity. In such a model, cellular networks form when (a) cells exert traction, (b) traction forces are transmitted to the ECM by adhesive couplings between cell and matrix, and (c) the ECM is sufficiently malleable to be aligned by traction forces. Tubelike structures could form when elongated cells ensheathed linear cords of ECM (12). Indeed, tubes made by cultured endothelial cells within 2 to 4 hours after exposure to a solution of fibrillar type I collagen contain bundles of collagen in their lumens (27). Amorphous and fibrillar material is also found within the lumens of endothelial tubes that arise on substrates of BMM (31) and fibronectin (23). Moreover, ECM has been found in lumens of endothelial cell tubes that develop spontaneously in the absence of exogenous ECM (11, 12, M. L. Iruela-Arispe and E. H. Sage, unpublished observations). Ingber and Folkman (23) report that cultures of capillary endothelial cells undergoing spontaneous tube formation accumulate elastic, branching, filamentous webs of ECM upon which cells align and elongate. These webs and their associated endothelial cells, which resemble the structures formed by BAEC and TM3 cells as they reorganize layers of

highly malleable BMM (e.g., Fig. 4F), are likely to arise through tractional restructuring of endogenously synthesized ECM. Although different cell types can utilize ECM from exogenous sources to form networks in vitro, there are no reports that cell types other than vascular endothelial cells can organize endogenously synthesized ECM into a template for network formation in vitro. Capillary formation in vivo might therefore be a consequence of the specific ability of endothelial cells to synthesize gene products that generate a unique combination of ECM malleability, cytoskeletal tension, and cell-ECM adhesion. The elevated synthesis of mRNA for type I collagen and fibronectin by endothelial cells undergoing spontaneous angiogenesis in vitro suggests that these proteins play an important (and most likely structural) role in promoting the formation of tubes. Additional controls over angiogenesis might be mediated by proteins such as SPARC and thrombospondin. These proteins bind to specific components of ECM and reduce adhesion of endothelial cells to surfaces in vitro (42, 44). SPARC and thrombospondin might therefore limit the rate or extent of traction-driven alignment of ECM by interfering with cellular adhesive contacts that couple the force-generating elements of the cytoskeleton to the ECM. Limitations imposed on traction forces during angiogenesis might enhance the stability of newly-formed capillary tubes. The validity of our hypotheses can be determined by further studies of the relationships between cellular traction, matrix synthesis and organization, and cellular morphology.

METHODS

CELL CULTURE

Endothelial cells from adult bovine aorta were isolated, cloned, and passaged as previously described (43). Experiments were performed on cells between passages 4 and 8. The murine Leydig cell line TM3 (37) was purchased from the American Type Culture Collection (Rockville, Maryland). Cultures of smooth muscle cells from newborn human aorta (passages 5–7) and adult human dermal fibroblasts of the line SK5 were gifts from Elaine Raines (Department of Pathology, University of Washington). Cells of the murine line PYS-2, which have characteristics of parietal yolk sac endoderm (32), are cultured routinely in our laboratory. TM3 cells were grown in a 1:1 mixture of DMEM and Ham's F12 (GIBCO BRL, Gaithersburg, Maryland) that contained 5% normal horse serum (Flow Laboratories, McLean, Virginia), 2.5% fetal calf serum (Hy-Clone Laboratories, Logan, Utah), 100 units/ml of penicillin, and 100 μg/ml of streptomycin. All other cell types were cultured in DMEM (GIBCO) containing 10% fetal calf serum and penicillin/streptomycin. Stock cultures of cells were maintained in 75 cm² plastic tissue culture flasks (Corning Glass Works, Corning, New York) in 5% CO_2/95% air at 37° C and 100% humidity. In most experiments, cells were plated at densities of 1,200 to 1,800/mm².

PREPARATION OF CELL CULTURE SUBSTRATES

Substrates of BMM components were formed from Matrigel, an extract of material secreted by the Englebreth-Holm-Swarm murine sarcoma (Collaborative Research, Inc., Bedford, Massachusetts). Hydrated gels of type I collagen were prepared by combining 6 volumes of Vitrogen-100™ (a solution of 0.012 N HCl containing 3 mg/ml type I collagen from bovine skin -

Collagen Corporation, Palo Alto, California) with 1 volume of 7-fold concentrated DMEM containing $NaHCO_3$ at saturation (50). The concentration of collagen was varied by diluting the mixture of Vitrogen/7-strength DMEM with DMEM. Hybrid gels were made by combining Vitrogen/7-strength DMEM with liquid Matrigel in varying volume ratios. In some experiments, the native type I collagen in the hybrid gels was replaced with Vitrogen that was heat-denatured under mild conditions (55° C for 15 minutes). Routinely, 300 μl of the solutions of Matrigel, type I collagen, or collagen/Matrigel were dispensed into plastic tissue culture wells of 16 mm diameter (24 well cluster plate, Corning) and gelled by warming to 37° C for 1 hour. For some experiments, the malleability of BMM was increased by subjecting liquid Matrigel to one freeze-thaw cycle prior to gelling at 37° C.

Substrates of BMM with a gradient of thickness (30) were made by combining 50 μl of DMEM with 250 μl of Matrigel and spreading the ice-cold mixture in an even layer on a 22 mm × 50 mm flame-sterilized coverslip. The Matrigel was applied 10 mm from one narrow edge to provide an uncoated surface for handling. The long axis of the coverslip was maintained at an angle of 8° as the Matrigel was heated at 37° C for 1 hour. In this manner, a gel was formed of a thickness that varied along its 40 mm length from less than 10 μm to approximately 600 μm. The sloped BMM gels were placed in 100 mm diameter tissue culture dishes (Corning) and covered with culture medium to a depth of 6 mm. Cells were seeded at a density of approximately 1,600/mm². After the cells had settled, the coverslips were examined to ensure that the cells were dispersed evenly. To eliminate gravitational effects during culture, dishes were maintained at an angle of 8° and the coverslips were oriented such that the slope of the dish opposed the slope of the gel to form a level culture surface.

To form films of silicone rubber, thin layers of dimethylpolylysiloxane (60,000 centistokes; Sigma Chemical Company, St. Louis, Missouri) were applied to 22 mm glass coverslips and polymerized by heating as described in Stopak and Harris (48). The coated coverslips were placed in 35 mm tissue culture dishes and washed in three changes of cell culture medium before cells were plated.

Microscopy

Cultures were examined by dark-field or phase-contrast illumination with a Zeiss inverted microscope. Photographs were taken with Kodak ASA 160 color slide film. In some studies, the behavior of cells on layers of BMM was monitored continuously by time-lapse videomicroscopy as described previously (50). Examination of cord-forming cultures by scanning electron microscopy was performed by Thomas N. Wight (Department of Pathology, University of Washington). Cultures were fixed 45 minutes in 3% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.3) that contained 6% sucrose and 2 mM $CaCl_2$, postfixed 30 minutes with 1% OsO_4 in cacodylate/sucrose/Ca buffer, dehydrated in ethanols, critical point dried, and coated with gold/palladium. Samples were viewed with a JEOL JSM 35C at 15 kV.

Drug Treatments

BAEC and TM3 cells were treated with cytochalasin D (Sigma) at concentrations that ranged from 0.5–1.5 μg/ml, or with colchicine (Sigma) at 2.5 μM. A stock solution of 1 mg/ml cytochalasin was prepared with dimethylsulfoxide (49). A stock solution of 2.5 mM colchicine was prepared with DMEM + 10% fetal calf serum. Stock solutions were stored at −20° C. Cells were plated on BMM or plastic and allowed to attach for 2 hours before the drugs were added.

Analysis of RNA

BAEC were grown 24 hours in 6-well cluster plates (Corning) on gels formed from BMM, 2.0 mg/ml Vitrogen, or uncoated plastic. Total RNA was extracted from 10^6 cells (from each substrate), purified, resolved by agarose gel electrophoresis, and subjected to Northern blot analysis as described previously (26). [^{32}P]-labeled cDNA probes included a 1.1 kb sequence of α1(I) chain of human type I collagen (7, 26); a 557 bp sequence of murine SPARC (26, 36, 51), and a 2.2 kb sequence of human fibronectin (46). Ten micrograms of total cellular RNA was loaded per lane. Verification of equal loadings was accomplished by re-hybridizing the blots with a [^{32}P]-labeled cDNA derived from 28S rRNA (26). Relative amounts of mRNAs were determined by quantitative densitometry of autoradiographs and were normalized to levels of 28S rRNA. Northern blot analyses were also performed on RNA from cord-forming cells of a clone of BAEC that generates cords and tubes in the absence of exogenous ECM (26).

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