In Vitro Rapid Organization of Endothelial Cells into Capillary-like Networks Is Promoted by Collagen Matrices

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ABSTRACT   We have studied the behavior of cloned capillary endothelial cells grown inside a three-dimensional collagen matrix. Cell monolayers established on the surface of collagen gels were covered with a second layer of collagen. This induced the monolayers of endothelial cells to reorganize into a network of branching and anastomosing capillary-like tubes. As seen by electron microscopy, the tubes were formed by at least two cells (in transverse sections) delimiting a narrow lumen. In addition, distinct basal lamina material was present between the abluminal face of the endothelial cells and the collagen matrix. These results showed that capillary endothelial cells have the capacity to form vessel-like structures with well-oriented cell polarity in vitro. They also suggest that an appropriate topological relationship of endothelial cells with collagen matrices, similar to that occurring in vivo, has an inducive role on the expression of this potential. This culture system provides a simple in vitro model for studying the factors involved in the formation of new blood vessels (angiogenesis).

The endothelial lining of blood capillaries consists of a single layer of flattened cells whose basal surface interacts with the extracellular matrix, while the apical surface delimits the lumen of the vessel. The important role played by microvascular endothelium in a wide range of normal and pathological processes has stimulated the development of culture methods for the growth of capillary endothelial cells in vitro (1–3, and references therein). These studies have been so far carried out on two-dimensional substrata, on which capillary endothelial cells usually grow as typical monolayers, although a tendency to form tubular structures has been reported in some conditions (4).

Since blood capillaries in vivo are entirely surrounded by extracellular matrix (containing collagen as a major component), we have studied the behavior of capillary endothelial cells grown inside collagen gels (5, 6) in vitro. Collagen gels provide a more physiological, isotropic environment that has been shown to promote the organization of different cell types into three-dimensional, tissue-like structures (7–16). Here we show that capillary endothelial cells embedded in collagen gels organize rapidly into a network of anastomosing capillary-like tubes.

MATERIALS AND METHODS

Preparation of Collagen Gels: Type I collagen was solubilized by stirring adult rat tail tendons for 48 h at 4°C in a sterile 1:1,000 (vol/vol) acetic acid solution (300 ml for 1 g of collagen) (17). The resulting solution was filtered through a sterile triple gauze and centrifuged at 16,000 g for 1 h at 4°C. The supernatant was then extensively dialyzed against $\frac{1}{10}$ Eagle's minimal essential medium (Gibco Laboratories, Grand Island, NY) and stored at 4°C.

Gels of reconstituted collagen fibers were prepared by simultaneously raising the pH and ionic strength of the collagen solution according to a modification (18) of the method originally described by Elsdale and Bard (5). This was achieved by quickly mixing 7 vol of cold collagen solution with 1 vol of 10 × minimum essential medium and 2 vol of sodium bicarbonate (11.76 mg/ml) in a sterile flask kept on ice to prevent immediate gelation. The cold mixture was then dispensed into 35-mm plastic culture dishes (Falcon Plastics, Div. of Bioquest, Oxnard, CA) (~0.8 ml per dish) and allowed to gel for 10 min at 37°C.

Endothelial Cell Culture: Cloned capillary endothelial cells from bovine adrenal cortex (4) were obtained from Dr. B. Zetter (Children's Hospital, Boston, MA) and serially passaged in gelatin-coated (4) tissue culture flasks (Falcon Plastics). The culture medium was a 1:1 mixture of Dulbecco's modified Eagle's medium and conditioned medium (prepared as described in reference 4) from mouse sarcoma cells, which were also obtained from Dr. B. Zetter. The mixture was supplemented with 10% calf serum, 4 mM glutamine, 500 U/ml penicillin, and 100 μg/ml streptomycin.

For collagen gel cultures, the endothelial cells were seeded into 35-mm collagen-coated dishes and allowed to grow on the surface of the gels for 2–3 d to obtain a subconfluent monolayer. The culture medium was then removed and ~0.8 ml of the cold collagen mixture described above were poured on the top of the first gel and allowed to polymerize for 10 min at 37°C. Fresh medium was added after the collagen had gelled and was renewed at 48-h intervals. The reorganization of the endothelial cell monolayer was monitored and photographed with a Zeiss inverted phase contrast photomicroscope.

Processing for Light and Electron Microscopy: The collagen gel cultures were fixed in situ with either 2.5% glutaraldehyde, 1% tannic acid (Mallinckrodt Inc., Science Products Div., St. Louis, MO), in 0.1 M sodium cacodylate buffer, or 2.5% glutaraldehyde, 0.2% tannic acid, 0.05% saponin in 0.1 M phosphate buffer (19). After extensive washing in buffer, the collagen
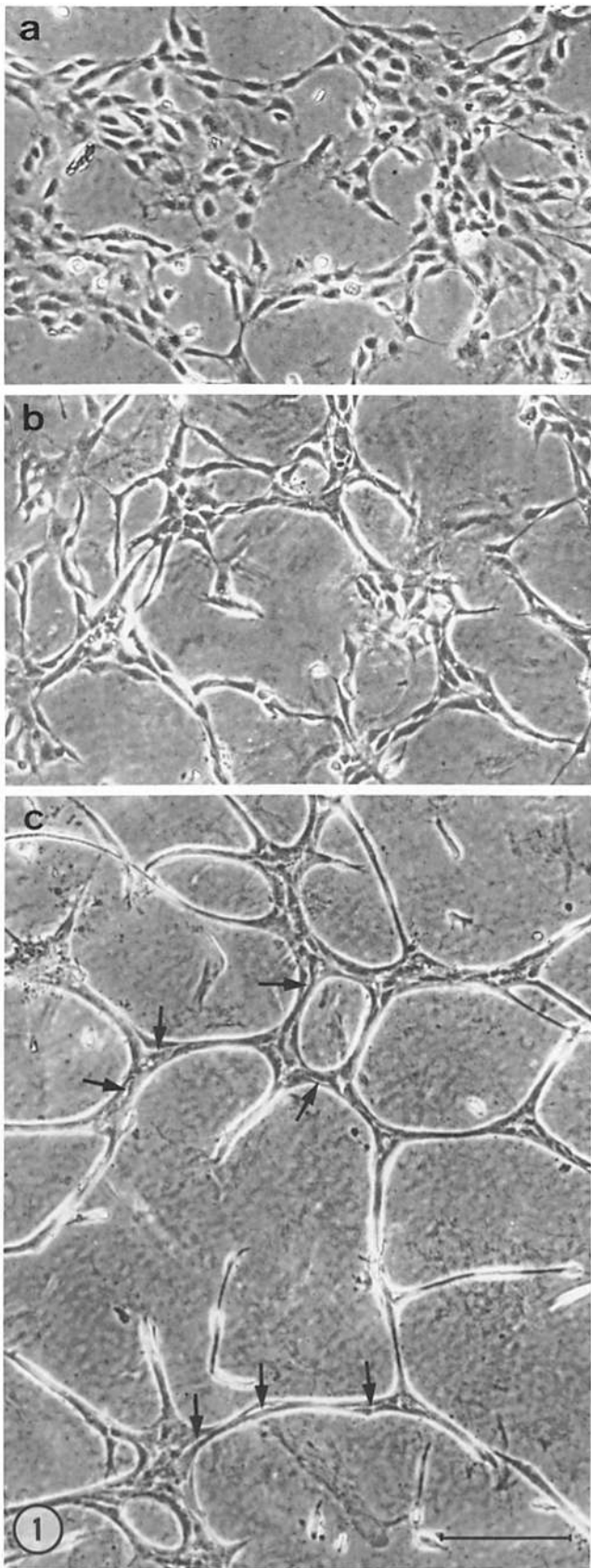
RESULTS

Capillary endothelial cells plated either in plastic culture dishes or on the top of collagen gels formed typical monolayers. In contrast, preformed monolayers established on the surface of collagen gels underwent a dramatic reorganization when covered with a second collagen layer. The sequential changes of subconfluent monolayers of capillary endothelial cells are illustrated in Fig. 1. Collagen overlay was followed by a progressive retraction of the monolayer (Fig. 1 b), which resulted, within ~2 d, in the appearance of a network of branching and anastomosing cords of cells (Fig. 1 c). By phase contrast microscopy, most of these cords showed a central, translucent cleft along their axis, which suggested lumen formation. Confluent monolayers responded to collagen overlay with a less complete retraction, and for reasons not clearly understood, areas of cell necrosis were observed at the intersection points of the network. Therefore, most experiments were performed with subconfluent monolayers. Formation of anastomosing networks of capillary endothelial cells was obtained not only by the reorganization of preformed monolayers, but also by (a) resuspending freshly trypsinized cells in gelling collagen solutions or (b) allowing endothelial cells to attach to the surface of a collagen gel and covering them with a second collagen layer before the occurrence of cell spreading (results not shown). Examination of semithin sections cut perpendicularly to the culture plane clearly demonstrated that virtually all the endothelial cells delimited a narrow lumen, so as to form complete tubular structures resembling blood capillaries (Fig. 2, a and b). Thin sections showed that the endothelial tubes consisted of at least two contiguous cells (Fig. 2, c-e) whose plasma membranes had focal points of fusion (not shown), suggesting tight junction formation. They also revealed the presence of abundant cell debris in the lumen, as well as the occurrence of basal lamina-like material at the interface between endothelial cell plasma membrane and collagen matrix (Fig. 2 e).

DISCUSSION

We have shown that capillary endothelial cells grown inside a three-dimensional collagen matrix organize into a network of branching and anastomosing tubes resembling capillary beds in vivo. This demonstrates that endothelial cells, even after repeated passage in culture, maintain the capacity to form vessel-like structures in vitro, if they are provided with an adequate environment. Although previous studies have indicated that this potential can also be expressed to a certain extent on conventional substrata (4, 21), in our experimental conditions the organization process was rapid, uniform throughout the entire culture, and easily reproducible. Endo-

FIGURE 1 Time course of the reorganization of capillary endothelial cells grown initially on top of a collagen gel, then covered with a second layer of collagen (phase contrast microscopy). (a) Just before collagen overlay, the endothelial cells form a subconfluent monolayer. (b) 8 h after being overlaid, the monolayer has retracted to form interconnecting cords of cells. (c) After 2 d, the culture appears as a network of branching and anastomosing cords of endothelial cells showing translucent clefts along their axis (arrows). Bar, 200 µm. × 90.

thelial cells from bovine aorta have been previously cultured within collagen gels: the cells assumed a mycelial pattern without any obvious organization and there was no evidence of lumen formation (6). Whether this reflects differential properties (discussed in reference 2) of large vessel versus microvascular endothelium remains to be established.

Endothelial cells in vivo are highly polarized, since they have a nonthrombogenic apical surface delimiting the vascu-
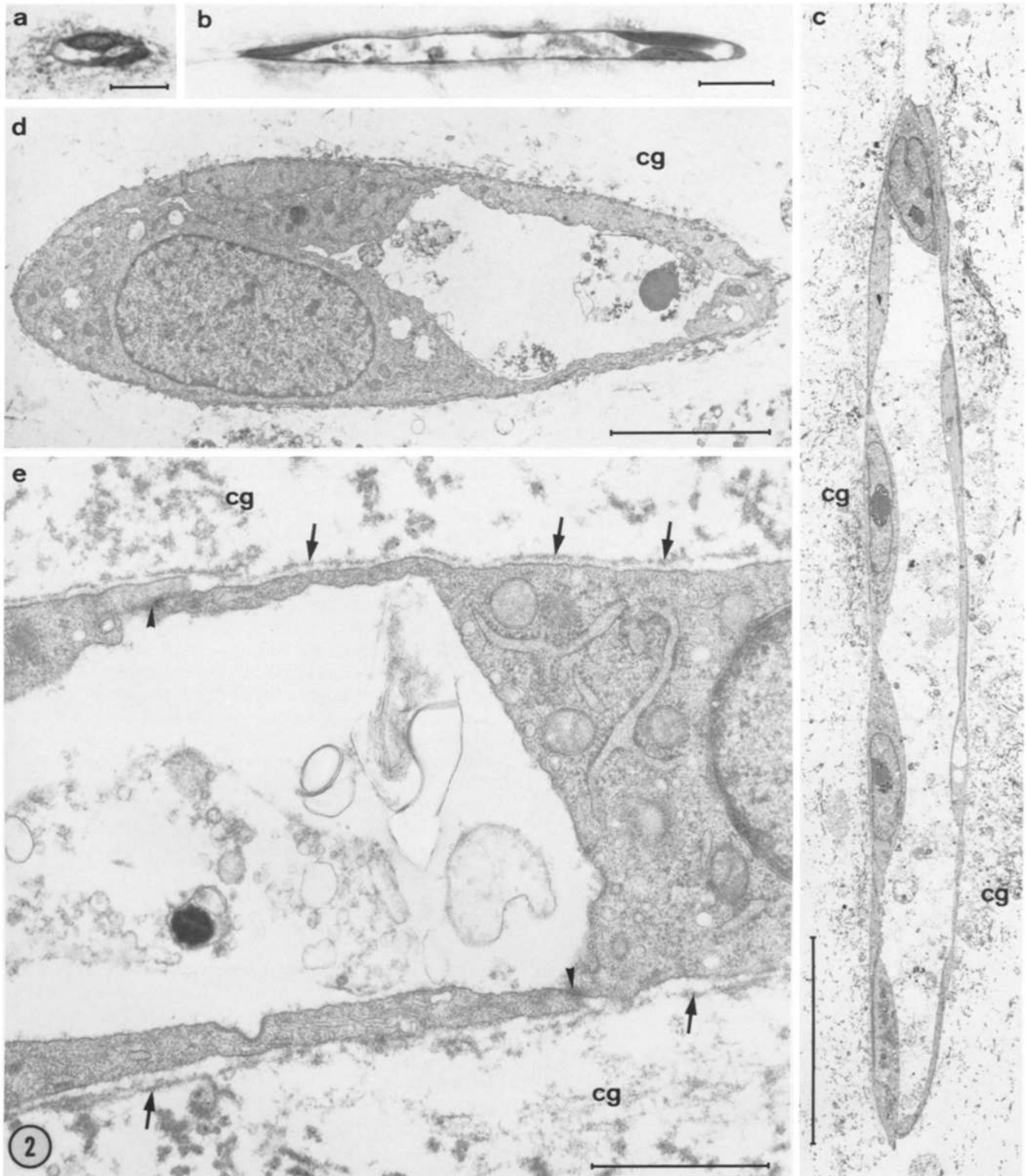

FIGURE 2   Semithin (a and b) and thin (c–e) sections perpendicular to a layer of capillary endothelial cells grown on a collagen gel and subsequently overlaid with a second collagen layer. (a–d) Endothelial cells are organized around narrow lumina so as to form capillary-like tubular structures. (e) Basal lamina-like material (arrows) is present between the abluminal face of the endothelial cells and the collagen matrix. The arrowheads indicate regions of contact between contiguous endothelial cells. Cg, collagen gel.
(a) Bar, 10 μm; × 870. (b) Bar, 20 μm; × 590. (c) Bar, 20 μm; × 1,700. (d) Bar, 5 μm; × 5,100. (e) Bar, 1 μm; × 29,000.

lar lumen, and a thrombogenic basal cell surface in contact with extracellular matrix components. In collagen gel cultures, the interaction of collagen with the entire surface of endothelial cells probably induces the formation of new apical faces segregated from the collagen matrix and encircling newly formed lumina. A similar mechanism has been proposed to explain the development of tissue-like cavitary structures by different types of epithelial cells grown inside collagen gels (7–14). Collagen gels have also been reported to promote cell migration (5, 6, 18, 22–27) and this could contribute to the establishment of tubular networks of endothelial cells.

That the endothelial channels formed in our experimental condition have the correct polarization (i.e., corresponding to that of vascular endothelium in vivo) is suggested by the presence of basal lamina-like material between the abluminal face of the endothelial cells and the collagen matrix. This is particularly relevant in relation to previous reports of tubular structure formation by endothelial cells grown on conventional substrata. In the latter studies, basal lamina-like material was observed inside the lumen rather than on the outer surface of the endothelial cells (4, 21), which suggests that the structures described were in fact inverted ("inside-out") endothelial tubes.

The origin and significance of cell debris frequently observed within the lumen of the capillary-like tubes formed in the collagen gel cultures is unclear. This debris sometimes contains remnants of pycnotic nuclei and cytoplasmic organelles. It might, therefore, result from the death of cells that have been completely surrounded by other endothelial cells during the organization process and thus excluded from the interaction with the collagen matrix. Whether the debris found in the lumen of endothelial tubes in the studies cited above (4, 21) has a similar or different origin is not known.

The present and previous (7–16) results suggest that an essential factor in some phases of organogenesis is the appropriate topological interaction between growing cells and extracellular matrix components. Such an interaction may be a key event in determining the cell polarization required for organogenesis. In the present case, interaction with the extracellular matrix might be sufficient to induce the correct organization of endothelial cells into capillary-like tubes, while specific angiogenesis factors could be necessary for the growth and perhaps specialization of the endothelial cells.

Formation of new capillary blood vessels (angiogenesis) occurs in a wide range of important biological processes including embryonic development, osteogenesis, luteinization, and wound healing. It is also a component in many pathological processes, such as chronic inflammation and certain immune reactions, and plays a crucial role in tumor growth (28–31). Numerous animal (28–31) and in vitro (4, 21, 32) models have been proposed for the study of angiogenesis. Because of its simplicity, the culture system we have described represents an attractive alternative model to investigate the mechanisms of angiogenesis in vitro and its regulation by diffusible factors (28–31, 33–36).

After this manuscript was submitted for publication, three papers appeared that are relevant to the topic of the present study. Feder et al. (37) reported the formation of "inverted" tubes by calf aortic endothelial cells cultured under standard conditions. Shor et al. (38) described the establishment of three-dimensional networks of bovine aortic endothelial cells grown on or within collagen gels. Although they mentioned that the cells often enclosed lumen-like spaces, the occurrence

of capillary-like tubes was not documented. Madri et al. (39) reported the occasional formation of tubelike structures by capillary endothelial cells grown on various collagen substrata or migrating into the stromal aspect of amniotic membranes. Finally, we have recently obtained preliminary evidence that human umbilical vein endothelial cells also organize into tubular networks when embedded in collagen gels (unpublished observations).

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