In Vitro Cell. Dev. Biol. 29A:91-93, February 1993

0883-8364/93 $01.50+0.00

Letter to the Editor

MORPHOLOGIC STUDY OF ANGIOGENESIS IN VITRO

Dear Editor:

The mechanism of angiogenesis has not been defined precisely, but it is likely that the process in vivo includes endothelial cell migration, proliferation, and lumen formation (1). The successful formation of capillary-like tubes has been described in cultures of endothelial cells from various types of blood vessels, including bovine adrenal capillaries (3,10,11,12), bovine aorta (2), human umbilical vein (5–7), human dermal microvessels (5), and capillaries obtained from rat epididymal fat pads (8,9). However, the precise nature of angiogenesis in vitro has not been elucidated. In the present study we used light and electron microscopy to study the angiogenic processes of cultured endothelial cells that had been isolated from bovine retina.

Bovine retinal capillary endothelial cells (BRCECs) were isolated by the methods described by Folkman et al. (4). Ten bovine eyes were dissected 5 mm posterior to the limbus and retinas were removed. The homogenized retinas were centrifuged at 400 *g* for 5 min and the resultant pellet was resuspended in 20 ml of cold Dulbecco's modified Eagle's medium (DMEM, Nissui Pharmaceutical, Tokyo, Japan). Microvessels were trapped on an 83 μm nylon mesh, transferred to a petri dish containing 15 ml of 0.5% collagenase (Sigma Chemical Co., St. Louis, MO), and incubated on a gyratory shaker for 30 min. The resultant vessel fragments were trapped on a 53 μm nylon mesh, washed with cold DMEM and centrifuged at 400 *g* for 5 min. The pellets were finally resuspended in 20 ml DMEM with 10% fetal calf serum (FCS) supplemented by 1 ng/ml of basic fibroblast growth factor (bFGF). After about 3 days, colonies of BRCECs began to appear. BRCECs (2×10^5) were seeded on Type I collagen gels (Kokencellgen, Koken Co., Tokyo, Japan), derived from bovine dermis, in 12-well plates (Corning Glass Works, Corning, NY) and cultured in DMEM containing 10% FCS and 1 ng/ml bFGF. The type I collagen gels were prepared as described by Yasunaga et al. (12). Specimens were prepared for light and electron microscopy at Days 1, 3, 5, and 7 after being placed into culture. Endothelial cells plated on collagen gels were fixed in situ with 4% glutaraldehyde in 0.1 *M* cacodylate buffer solution for 24 h. The gels were detached from the dishes and cut into small pieces. After washing in 0.1 *M* cacodylate buffer and postfixation for 90 min in 1% cacodylate-buffered osmium tetroxide, the specimens were dehydrated in a series of graded alcohols and embedded in an epoxy resin. Semithin sections measuring 1 μm were cut for light microscopy and stained with azur II; thin sections were cut on an ultramicrotome (Porter-Blum MT II), stained with uranyl acetate and lead citrate, and examined with an electron microscope (JEM-100CX). Some specimens were also serially thin-sectioned.

The isolated BRCECs were characterized as endothelial cells by immunohistochemical staining for von Willebrand's factor. BRCECs grown to confluency on the surface of collagen gels formed a monolayer of closely apposed cells at 1 day after plating. Many of these endothelial cells were connected by intercellular junctions. At this same time the initial stage of sprouting was observed in the cytoplasm of some of the endothelial cells in the monolayer. The sprouting portion of the cytoplasm had microfilament bundles. At 3 days of culture, BRCECs sprouted into the collagen gels. The sprouting portion of the endothelial cytoplasm had many microfilament bundles. Mitotic figures were found occasionally in the BRCECs of the monolayer. Some BRCECs formed capillary-like tubes that were usually located just beneath the surface monolayer (Fig. 1). This tube formation had increased in both number and length by 5 and 7 days of culture. Four distinct processes of capillary-like tube formation were observed in this experiment.

Type I: degeneration of cytoplasm within a single endothelial cell. The tube began with the appearance of an area of degeneration or cytolysis in a single endothelial cell (Fig. 2 *a,b,c*). Serial thin sections revealed the initial stage of tube formation to consist of the formation of an osmiophilic membranous structure in the area of degeneration (Fig. 2 *a*). The area of degeneration gradually became larger, and resembled a vacuole (Fig. 2 *b,c*). The luminal side of the vacuole had many pinocytotic vesicles, while portions of the abluminal side were covered by a basal lamina-like material.

Type II: folding over of a single endothelial cell to form a junction with itself. Two processes emanating from a single endothelial cell became connected by a junctional complex, thereby forming a small tube (Fig. 3).

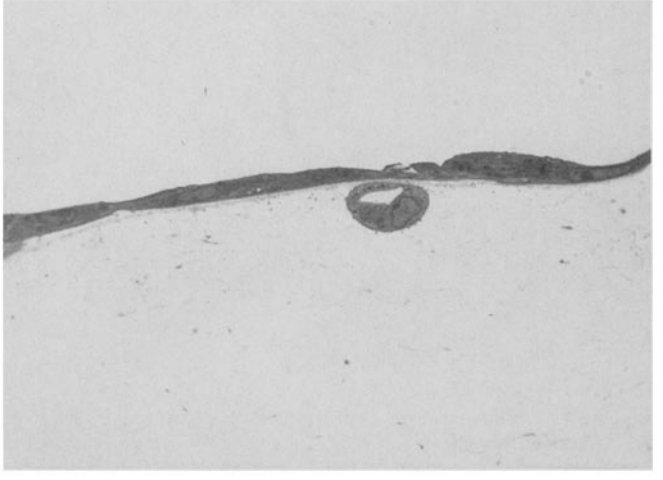

FIG. 1. Light micrograph of cultured BRCECs at 7 days. BRCECs form a capillary-like tube that is located just beneath the surface monolayer. (×670).

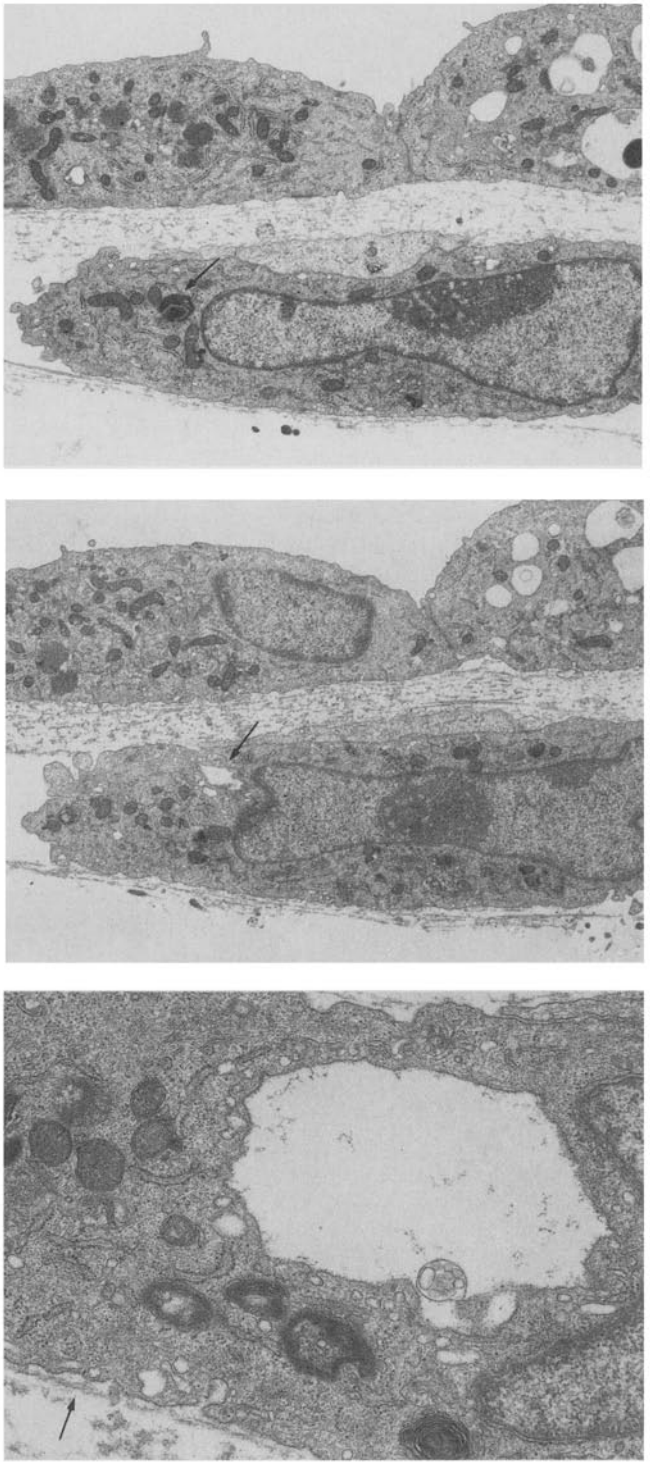

FIG. 2. Electron micrographs of serial sections of a capillary-like tube. *a*. An endothelial cell with an area of degeneration (*arrow*). The area of degeneration has an osmiophilic membranous structure. (×5100). *b*. The electron lucent areas join together and become larger (*arrow*). (×4900). *c*. The area of degeneration has a tube-like appearance. The luminal side of the tube has pinocytotic vesicles and the abluminal side has areas with basal lamina-like material (*arrow*). Other osmiophilic membranous structures are found around the lumen. (×18 800).

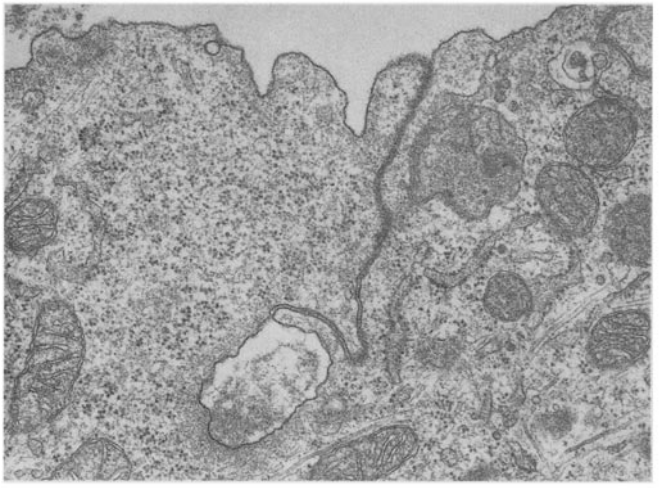

FIG. 3. Electron micrograph of a capillary-like tube. The small tube consists a single endothelial cell folded over onto itself. A junctional complex is present between the cytoplasmic extensions of the endothelial cell. (×18 300).

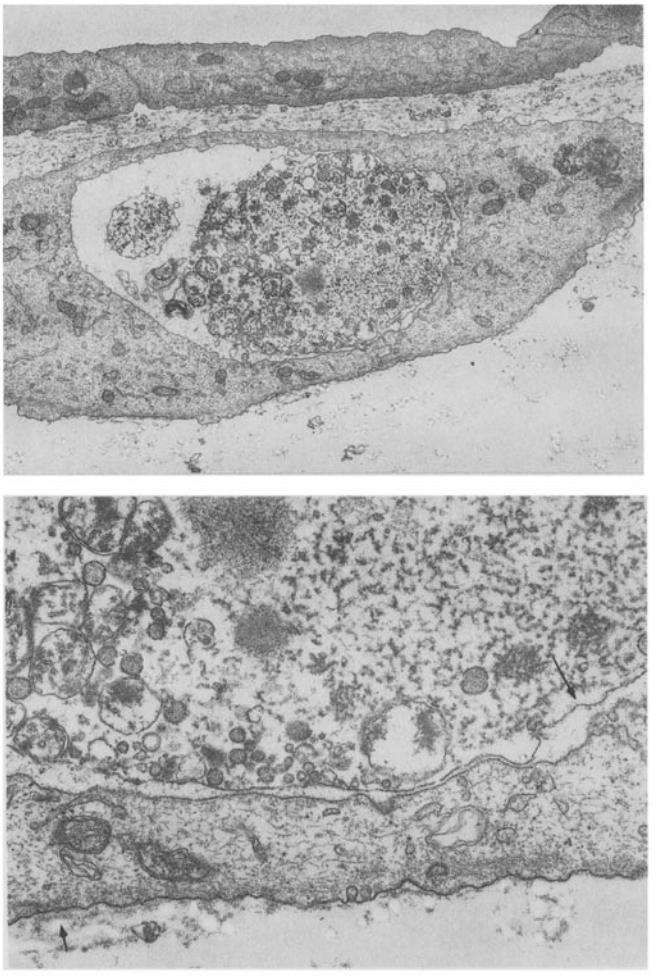

FIG. 4. Electron micrographs of serially sections of a capillary-like tube. *a*. A tube consists of a single endothelial cell and contains cell debris that consists of vesicular, membranous and amorphous structures. (×4800). *b*. Higher magnification of Figure 4*a*. The membrane, which probably represents the plasma membrane of a degenerative endothelial cell, is present within the tube (*long arrow*). Basal lamina-like material is present in some areas (*short arrow*). (×18 300).

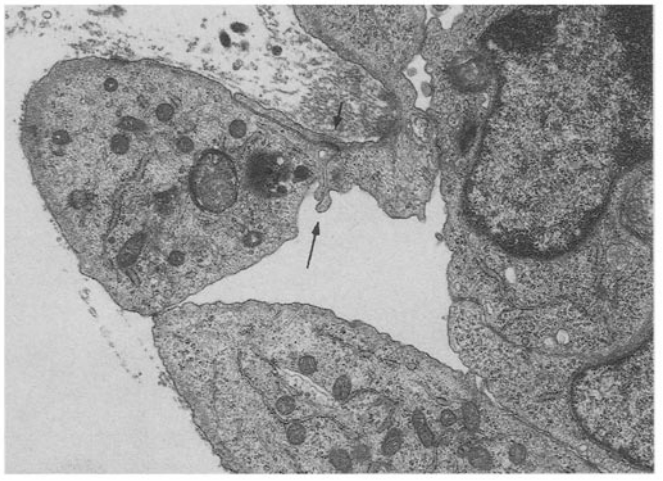

FIG. 5. Electron micrograph of a capillary-like tube. A tube is composed of five endothelial cells that are connected by intercellular junctions. Basal lamina-like material is present along portions of the albuminal side of the tube. No cellular debris is seen within this tube. There are marginal folds (*long arrow*) and an intercellular junction (*short arrow*) are present between two endothelial cells. (×9500).

Type III: degeneration of a centrally located endothelial cell. In some cultures a centrally placed cell within a cluster of endothelial cells underwent degeneration, thereby creating a tube (Fig. 4 *a,b*). The degenerative cell was surrounded by either a single intact endothelial cell (Fig. 4 *a,b*) or by several endothelial cells that were connected to each other by junctional complexes. These intact endothelial cells had areas with basal lamina-like material (Fig. 4 *b*). This type of tube contained cell debris, which represented the remnants of the cell that had degenerated.

Type IV: two or more contiguous endothelial cells connected by junctions. This type of tube was composed of two or more endothelial cells that became joined by intercellular junctions (Fig. 5). Pinocytotic vesicles and marginal folds were seen on the luminal side of the tube, and a basal lamina-like material was distributed unevenly along the abluminal side. These tubes contained no cellular debris.

Using an in vivo model, Ausprunk and Folkman (1) determined that new vessel growth took place as a series of sequential steps that were similar regardless of the type of angiogenic stimulus. In brief, they found that endothelial cells within a venule begin to degrade the basal lamina and to protrude through the wall of the vessel; the migration of endothelial cells is associated with their linear alignment as they form a capillary sprout; a lumen is formed within the sprout; mitosis is confined to endothelial cells distal to the leading edge of the sprout. The changes seen in the BRCECs seen in the present study are quite similar to those described above. The initial stage of growth of the BRCECs was the sprouting of endothelial cell cytoplasm into the collagen gels. Mitotic figures were not found in the sprouting endothelial cells, but apparently were present in the unaltered endothelial cells of the monolayer. Four types of tube formation were observed.

REFERENCES

1. Ausprunk, D. H.; Folkman, J. Migration and proliferation of endothelial cells in preformed and newly formed blood vessels during tumor angiogenesis. Microvasc. Res. 14:53–65; 1977.
2. Feder, J.; Marasa, J. C.; Olander, J. V. The formation of capillary-like tubes by calf aortic endothelial cells grown in vitro. J. Cell Physiol. 116:1–6; 1983.
3. Folkman, J.; Haudenschild, C. Angiogenesis in vitro. Nature 288:551–556; 1980.
4. Folkman, J.; Haudenschild, C. C.; Zetter, B. R. *Long-term culture of* capillary endothelial cells. Proc. Natl. Acad. Sci. USA 76:5217–5221; 1979.
5. Jackson, C. J.; Jenkins, K. L. Type I collagen fibrils promote rapid vascular tube formation upon contact with the apical side of cultured endothelium. Exp. Cell Res. 192:319–323; 1991.
6. Kubota, Y.; Kleinman, H. K.; Martin, G. R., et al. Role of laminin and basement membrane in the morphological differentiation of human endothelial cells into capillary-like structures. J. Cell Biol. 107:1589–1598; 1988.
7. Maciag, T.; Kadish, J.; Wilkins, L., et al. Organizational behavior of human umbilical vein endothelial cells. J. Cell Biol. 94:511–520; 1982.
8. Madri, J. A.; Williams, S. K. Capillary endothelial cell cultures: phenotypic modulation by matrix components. J. Cell Biol. 97:153–165; 1983.
9. Merwin, J. R.; Anderson, J. M.; Kocher, O., et al. Transforming growth factor beta-1, modulates extracellular matrix organization and cell-cell junctional complex formation during in vitro angiogenesis. J. Cell. Physiol. 142:117–128; 1990.
10. Montesano, R.; Orci, L.; Vassalli, P. In vitro rapid organization of endothelial cells into capillary-like networks is promoted by collagen matrices. J. Cell Biol. 97:1648–1652; 1983.
11. Montesano, R.; Vassalli, J. D.; Baird, A., et al. Basic fibroblast growth factor induces angiogenesis in vitro. Proc. Natl. Acad. Sci. USA 83:7297–7301; 1986.
12. Yasunaga, C.; Nakashima, Y.; Sueishi, K. A role of fibrinolytic activity in angiogenesis: quantitative assay using in vitro method. Lab. Invest. 61:698–704; 1989.

Tatsuro Ishibashi[1] Toshinori Murata
Katsuo Sueishi Hajime Inomata

Department of Ophthalmology and
First Department of Pathology (K. S., T. M.)
Faculty of Medicine
Kyushu University
Fukuoka 812
Japan

(Received 1 May 1992)

[1] To whom correspondence should be addressed at Department of Ophthalmology, Faculty of Medicine, Kyushu University 60, 3-1-1 Maidashi, Higashi-ku, Fukuoka 812, Japan.