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SPONTANEOUS TRANSFORMATION AND IMMORTALIZATION OF HUMAN ENDOTHELIAL CELLS

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SUMMARY

A new cell line from the human umbilical vein has been established and maintained for more than 5 yr (180 generations; 900 population doublings). This strain, designated ECV304, is characterized by a cobblestone monolayer growth pattern, high proliferative potential without any specific growth factor requirement, and anchorage dependency with contact inhibition. Karyotype analysis of this cell line reveals it to be of human chromosomal constitution with a high trisomic karyotype (mode 80). Ultrastructurally, endothelium-specific Weibel-Palade bodies were identified. Although one of the endothelial cell markers, Factor VIII-related antigen (VIIIR:Ag) was negative in this cell line, immunocytochemical staining for the lectin Ulex europaeus I (UEA-I), and PHM5 (anti-human endothelium as well as glomerular epithelium monoclonal antibody) was positive, and angiotensin-converting enzyme (ACE) activity was also demonstrated. In addition, ECV304 displayed negativity for alkaline and acid phosphatase and for the epithelial marker keratin. All of these findings suggest that ECV304 cells originated from umbilical vein endothelial cells by spontaneous transformation. Ultrastructurally, no viruslike particles have been detected intracellularly. Nude mouse tumorigenicity and rabbit cornea tests were both positive. This is a report on a novel case of phenotypic alteration of normal venous endothelial cells of human origin in vitro, and generation of a transformant with indefinite life spans. This line may be useful in studies of some physiologically active factors available for medical use.

Key words: endothelial cells; spontaneous transformation; Weibel-Palade body; immortalization.

INTRODUCTION

Endothelial cells have been used extensively for studies of physiologic fibrinolysis (10,17,31,33), blood vessel permeability (14), response of the blood vessel to various physiologic and pathologic stimuli (3,4,6,7,29) and tumor angiogenesis (9,20). Several workers have reported on efforts to establish a permanent human endothelial cell line for such studies, using SV-40 or sarcoma virus (8,13).

We have been engaged for several years in the cultivation of endothelial cells from over 600 umbilical cords. Meanwhile, a certain endothelial cell population from one umbilical vein (donor number 304) was found to migrate and proliferate very actively. When subcultivated, these cells readily grew to confluence without any specific growth factor requirements, exhibiting a typical epithelial growth pattern. These cells, designated ECV304, have been serially subcultivated and maintained for 5 yr.

Rodent cells cultured in vitro have often been known to transform spontaneously into cell lines with permanent life spans (37). Cells from bovine origin have also been reported to undergo transformation spontaneously (15) or after treatment with certain defined agents, such as virus (26) or chemical carcinogens (16). In human cell cultures, however, spontaneous transformation followed by immortalization rarely, if ever, occurs (23).

We report here that an immortalized endothelial cell line from human umbilical vein origin has been established. In addition, inasmuch as ECV304 cells are supposed to have been immortally transformed, their angiogenic and tumorigenic capacities were studied by rabbit cornea and mouse implantation assays, respectively.

MATERIALS AND METHODS

Cells

Human endothelial cells were obtained from the umbilical vein by the method of Maruyama (25) and Jaffe et al. (22), with a slight modification. The umbilical vein, obtained from a healthy donor, was perfused with Ca^{++}-free and Mg^{++}-free Dulbecco's balanced salt solution [phosphate buffered saline (PBS)] by way of an 8 Fr. catheter (Toray Medical Inc., Tokyo, Japan) inserted into one end of the vessel. The other end was clamped, and dispase II (500 U/ml; Godo Shusei, Tokyo, Japan) solution was infused into the vessel with slight pressure. Twenty minutes after incubation at 37° C, the enzyme solution containing detached cells was flushed from the vessel. The cells were pelleted,

suspended in M199 medium (GIBCO Laboratories, Grand Island, NY) supplemented with 20% fetal bovine serum (FBS; Flow Laboratories, North Ryde, Australia, or Filtron, Australia), 100 U/ml penicillin (GIBCO), 100 mg/ml streptomycin (GIBCO), and 2.5 μg/ml Fungizone (GIBCO) and cultivated in Petri dishes (60-mm diameter; Miles Laboratories, Naperville, IL) at 37° C under an atmosphere containing 5% CO_2. After confluency, Cytodex III (Pharmacia Fine Chemicals, Uppsala, Sweden) suspended in the fresh medium was added to the culture. The cells migrated over the beads and grew to confluence in 5 d. Beads were then detached from the culture dish by gentle pipetting, collected in the centrifuge tube, and the cells on the beads were peeled off with 0.05% trypsin:0.02% EDTA (Whittaker M. A. Bioproducts, Walkersville, MD, and Wako Pure Chemical, Tokyo, Japan), pelleted, suspended in the culture medium and plated onto two Petri dishes (35 mm; Miles Laboratories). After 23 d, one culture actively grew to confluence, exhibiting a cobblestone appearance. These cells, ECV304, were passaged every week in the ratio of 1:20. ECV304 cells at Passage 20 to 168 [population doublings (PD) 100–840] and human umbilical vein endothelial cells (HUVEC) at primary or second passage were used in these studies. The HUVEC cells used as controls were obtained from healthy normal umbilical veins on an as-needed basis.

Growth Rate

The doubling time of ECV304 cells was measured at Passages 47 (PD 235), 82 (PD 410), 153 (PD 765), and 163 (PD 815) in media containing 2 and 10% FBS, respectively. In Passage 2 HUVEC, doubling time was measured in the medium containing 20% FBS. The cells were plated onto 12-well tissue culture plates (Coster, Cambridge, MA) at 10^4 cells/well and cultivated. After 17 h the culture was washed with PBS, placed into fresh medium, and cell counting was performed every 48 h by a cell counter (CC-108, Toa Sysmex, Kobe, Japan). The doubling time of the cells was calculated from the exponential part of the growth curve.

Immunocytochemical Examination

To examine whether ECV304 cells were derived from the endothelium or from any other cells (such as monocytes), immunocytochemical studies were done.

Cell materials were cultured on four-chamber Lab-Tek slides (Miles Laboratories). After subconfluency, the cultures were washed in PBS, fixed and stained by the avidin-biotin peroxidase complex (ABC) method as described below. For the immunocytochemical identification of Factor VIII-related antigen (VIIIR:Ag) and keratin, the cell materials were fixed in 0° C 100% methanol for 15 min, rinsed, and incubated with mouse monoclonal antihuman VIIIR:Ag (Cooper Diagnostics, Cochranville, PA) and mouse antihuman keratin monoclonal antibody (molecular weight 50–67 Kd; Immunotech, Marseille), respectively, then maintained at room temperature. In the case of PHM5 (Australian Monoclonal Development, Artarmon, Australia) (monoclonal antibody to human glomerular epithelium) (18), the cells were fixed in 0° C 100% acetone for 15 min; PHM5 was then added and the preparations were maintained at room temperature. After 30 min all the preparations were washed, biotinylated rabbit antimouse IgG (Vector Laboratories,

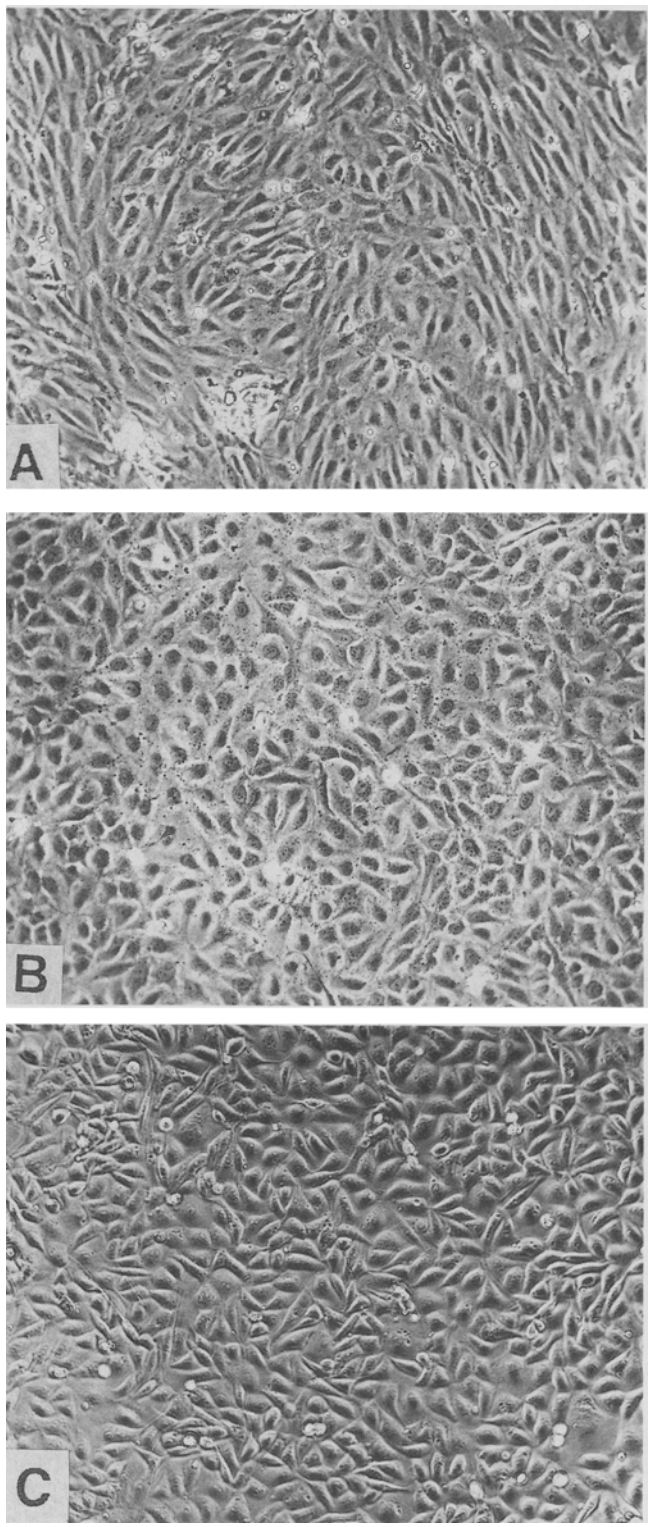


FIG. 1. Microphotographs of HUVEC and ECV304. *A*, HUVEC (primary culture); *B*, ECV304 (Passage 17; PD 85); *C*, ECV304 (Passage 184; PD 920). All three confluent cultures exhibit cobblestone monolayer morphology.

Burlingame, CA) was added, and they were maintained for 30 min at room temperature. After washing, the cells were treated with avidin-biotin complex (ABC, Vector Laboratories) for 60 min at room temperature and washed. The peroxidase reaction was developed with 0.3 mg/ml 3-,3'-diaminobenzidine (Wako) in the buffer containing 0.05% hydrogen peroxide (Wako) for 5 to 10 min at room temperature. These preparations were then counterstained with hematoxylin, mounted, and photographed. Control staining was performed by omission of primary antibodies. For staining with UEA-I, the cell materials were fixed with absolute methanol for 15 min and incubated with biotinylated UEA-I (E.Y Laboratories, San Mateo, CA) for 30 min at room temperature. Then the cells were incubated with ABC and treated in the same manner as described above.

Alkaline and acid phosphatase. The cell materials were fixed with 3% paraformaldehyde (TAAB Laboratories, Reading, England) in 0.1 *M* cacodylate buffered to pH 7.4.

Alkaline phosphatase: To the cell preparation we added 0.4 mg/ml naphthol AS-BI phosphate (Wako pure chemical), 6 μl/ml dimethylformamide (Wako), and 1 mg/ml Fast red TR (Sigma Chemical, St. Louis, MO) in 0.05 *M* propanediol buffer (pH 9.45).

Acid phosphatase: To the cell preparation we added 0.4 mg/ml naphthol AS-BI phosphate, 6 μl/ml dimethylformamide, and 0.6 mg/ml Fast red ITR (Sigma) in 0.1 *M* acetate buffer (pH 5.4) containing 3 drops of 10% manganese chloride (Wako).

Both samples were incubated for 5 to 10 min at room temperature, rinsed and counterstained with hematoxylin, and examined microscopically.

Angiotensin-Converting Enzyme (ACE)

ECV304 and HUVEC cells were seeded into 12-well culture plates and cultivated. For measurement of activity in the culture supernatant, confluent cultures were rinsed with PBS, 1 ml of substrate (7.48 mg hippuryl-glycyl-glycine/ml in Hanks' solution; Boehringer, Indianapolis, IN) was added to each well, and the cells were incubated for 30 min at 37° C. To measure the cell-associated ACE activity, confluent cultures were washed, extracted with 0.5 ml/well of 0.5 % Triton X-100 (Wako) before addition of substrate, and the sample was incubated for 30 min at 37° C. These reaction mixtures were then centrifuged for 15 min at 3300 rpm. We mixed 375 μl of each supernatant with 0.5 ml of 100 m*M* sodium borate buffer (pH 9.6) and 25 μl of 2,4,6-trinitrobenzenesulfonic acid (Boehringer, 20.4 mg/ml), and the mixture was maintained for 15 min at 37° C; released glycyl-glycine was colorimetrically determined at the wave length of 405 nm. One unit of ACE is defined as the amount of enzyme required to release 1 μmol of hippuric acid per minute at 37° C.

Electron Microscopy

The cells, grown to confluence on four-chamber Lab-Tek culture chamber slides, were rinsed thoroughly with PBS and prefixed with 2.5% glutaraldehyde (Nakarai Chemicals, Kyoto, Japan) for 4 h at room temperature. After washing, postfixation was done with 1% osmium tetroxide solution (TAAB Laboratories) at room temperature. After 2 h, the cell preparations were washed again, dehydrated through graded ethanols, and embedded in Epon 812 (Oken, Tokyo, Japan) by the inverted gelatin capsule method.

Cornea Test

This test was performed according to the method of Gimbrone et al. (11), using three male New Zealand white rabbits (body weight 3.2 to 3.3 kg). Under a general anesthetic, a 1-mm wide opening was made in the central part of the cornea and tiny pockets were cut into the avascular corneal stroma of each eye, to a depth of approximately 1-mm and reaching to 1-mm from the limbal vessel. By way of a microtube inserted into the pocket, a 5-μl cell suspension containing 1.4×10^6 cells of ECV304 was injected into one pocket, and HUVEC into the other. The HUVEC cells served

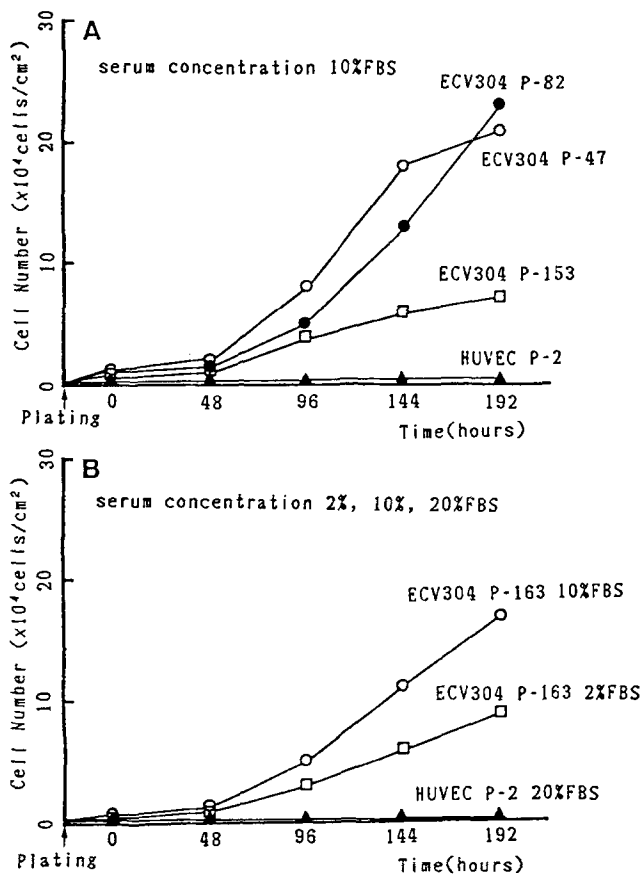


FIG. 2. Comparison of the growth curve of ECV304 with that of HUVEC. *A*, growth curve of HUVEC Passage 2 (*solid triangles*), ECV304 Passage 47; PD 235 (*open circles*), Passage 82; PD 410 (*solid circles*) and Passage 153; PD 765 (*open squares*). Cells were cultured in M199 containing 10% FBS; the cells were plated in 12-well culture plates at a density of 10^4 cells/well. After 17 h, the medium was replaced with fresh medium and cell counting was performed every 48 h. ECV304 grew fast as compared to HUVEC. The fast growth of ECV304 cells is especially notable at early passages. *B*, growth curves of ECV304 Passage 163 (PD 815), cultivated with M199 containing 10% (*open circles*) or 2% FBS (*open squares*). Cultivation and cell counting were performed in the same manner as before.

as controls, and the use of right or left eye was counter balanced. The eyes were examined with a slit-lamp microscope at Days 3, 7, 10, and 14 after implantation and photographed at 14 d.

Tumorigenicity Assay

A suspension of ECV304 (5.8 × 10^7 cells/0.2 ml PBS) was injected subcutaneously into the armpit of 5-wk-old BALB/c nu/nu female mice irradiated with 450 rad x-ray 4 d before injection. Tumors appeared at the inoculation surfaces approximately 1 mo. after inoculation, and the tumor was surgically removed and subjected to histopathologic examination.

RESULTS

Microcarriers had been added to the confluent primary culture of HUVEC cells obtained from donor number 304 5 yr ago. When the cells that grew on the beads were subcultured, they proliferated actively as a confluent monolayer which assumed a cobblestone appearance. The confluent monolayer culture profile at Passages 17 (PD 85) and 184 (PD 920) are shown in Fig. 1. These cells were morphologically quite different from smooth muscle cells or fibroblasts.

Cell Growth

Growth rates of ECV304 at various passage levels were compared with those of HUVEC. At any passage examined, ECV304 cultivated in M199 containing 10% FBS for 8 days exhibited a growth rate 37 to 110 times faster than did HUVEC at Passage 2 (Fig. 2 *A*). ECV304 proliferated especially actively at early passages. Population doubling time was 32.9 h for ECV304 grown in the medium with 10% FBS and 35.3 h in 2% FBS (Fig. 2 *B*). This contrasted with the prolonged population doubling time of HUVEC, 89.3 h when cultivated in 20% FBS medium. Addition of growth factors, such as basic fibroblast growth factor, endothelial cell growth supplement, or epidermal growth factor, did not have any stimulatory effect on the growth of ECV304, and ECV304 grew significantly in serum-

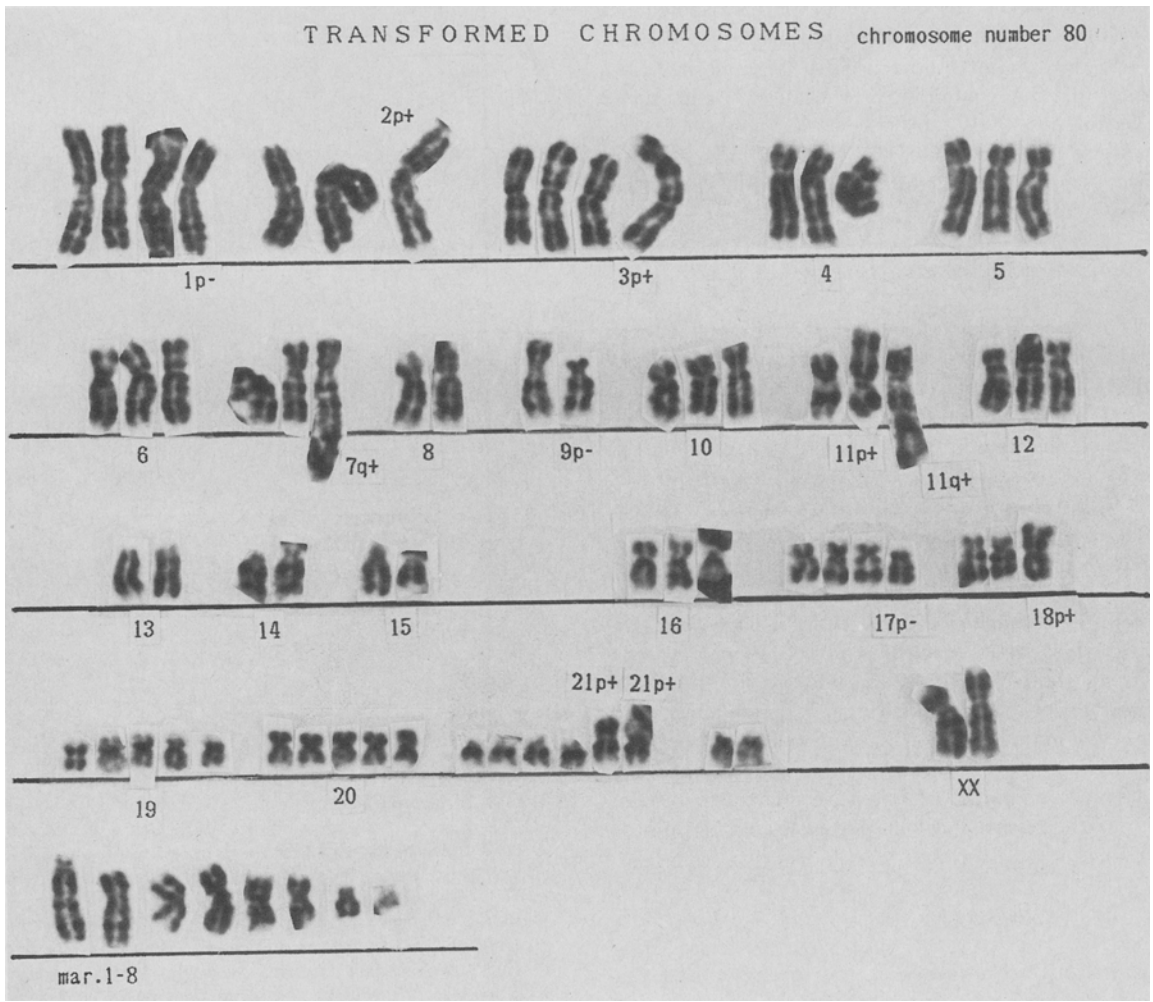


FIG. 3. A representative karyotype of ECV304 at Passage 20. Eighty chromosomes in ECV304 at Passage 20 were classified by the trypsin G-banding method.

TABLE 1

DISTRIBUTION OF CHROMOSOME NUMBERS IN 50 ECV304 CELLS, PASSAGE 20

Number of Chromosomes	Cells
73	1
74	2
75	0
76	0
77	3
78	8
79	5
80	21
81	6
82	2
83	1
84	1

free medium (data not shown). From these observations, the original HUVEC cells appeared to have been spontaneously transformed.

Chromosome Analysis

Cytogenetic analysis of ECV304 was performed at several passages. The karyotype at Passage 20 (PD 100) and its karyotype distribution in 50 cells are illustrated in Fig. 3 and Table 1, respectively. The karyotypic profile was generally trisomic; i.e. nos. 2, 3, 4, 5, 6, 7, 11, 12, and 16 chromosomes were trisomic but nos. 1, 17, and 18 were tetrasomic, nos. 19 and 20 were pentasomic, no. 21 was hexasomic, and the rest were diploid. Similar modal chromosome numbers of the cell sample from Passages 20 (PD 100), 28 (PD 140), 49 (PD 245), 84 (PD 420), and 170 (PD 850) were noted (80, 79, 77, 75, and 74, respectively). This cell line was therefore confirmed to be of human origin.

Immunocytochemical Examination of ECV304

Table 2 summarizes the results of immunostaining reactions of ECV304 and HUVEC cells with respect to various endothelial markers. With the exception of VIIIR:Ag, the immunostaining reactions are identified. VIIIR:Ag, a prominent endothelial marker (27), is immunocytochemically negative in ECV304 (Fig. 4 *B*) at any passage and is quantitatively undetectable in the culture supernatant by enzyme immunoassay (Boehringer kit; data not shown). However, ECV304 did have distinct binding activity for UEA-I lectin (Fig. 4 *D*), a sensitive marker for endothelium of human origin (21). Moreover, ECV304 stained with PHM5 (Fig. 4 *F*), which is positive for not only human glomerular epithelium (18) but also human endothelium (unpublished). Elimination of the primary antibody in each immunocytochemical reaction resulted in negative staining. These findings suggest that except for VIIIR:Ag, the main immunocytochemical characteristics of endothelium origin are still retained in ECV304 even after serial subcultivations.

Angiotensin-Converting Enzyme Activity

The ACE activity associated with the cells was compared with that secreted into the culture medium (Fig. 5). The cell-associated enzyme activities of ECV304 cells were essentially at the same levels as HUVEC cells, and the difference in enzyme activities in the medium conditioned with ECV304 were much lower than HUVEC. This finding further supports the idea that ECV304 cells were derived from endothelial cells (19).

Electron Microscopy

In HUVEC cells (Fig. 6 *A*) abundant microfilaments were observed but intermediate filaments were sparse. Numerous pynocytotic vesicles were located in the vicinity of the cell membrane, and Weibel-Palade bodies were found in the cytoplasm.

ECV304 cells (Fig. 6 *B*, *C*, and *D*) exhibited abundant cell organelles, vesicles, microtubules, and thick filaments adjacent to the cell membrane. In addition, distinct nuclear alterations with an increase in nucleoli and heterochromatins were recognized (Fig. 6 *C*), indicating that these cells are functionally active. In particular, presence of Weibel-Palade bodies located near the Golgi complex (Fig. 6 *D*) suggests that ECV304 were derived from the endothelium (34). Moreover, no viruslike particles were found.

Rabbit Cornea Test

Vascular response to injection of ECV304 was not observed until 1 wk after the injection, when slight capillary development was recognized with a slit lamp microscope. Two weeks later, a distinct neovascularization was detected in all three corneas tested (Fig. 7 *B*). In contrast, the HUVEC injection did not elicit any vascular response (Fig. 7 *A*).

Nude Mouse Tumorigenicity Assay

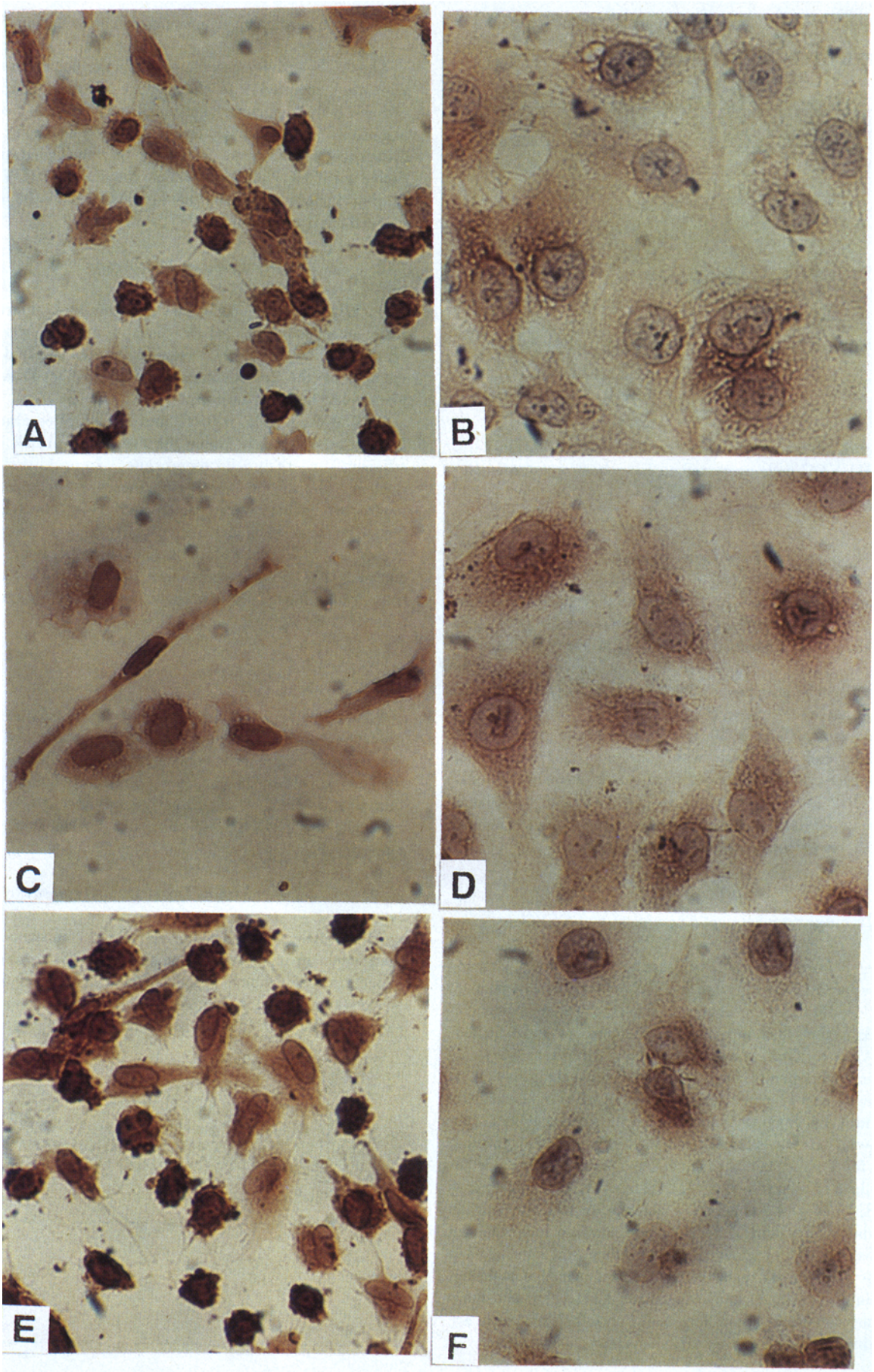
The mice formed distinct solid tumors (10 mm diameter) within 1 mo., but the animals still survived.

Sections of tumor tissue excised from the nude mice were stained with hematoxylin-eosin and showed that the tumor mass was filled with actively growing tumor cells

TABLE 2

A COMPARTION OF ECV304 AND HUVEC CELLS WITH RESPECT TO THE IMMUNOSTAINING REACTIONS FOR VARIOUS ENDOTHELIAL MARKERS

Markers	HUVEC	ECV304
VIIIR:Ag	+	−
UEA-I	+	+
Keratin	−	−
PHM5	+	+
Alkaline phosphatase	−	−
Acid phosphatase	−	−


A
B
C
D
E
F

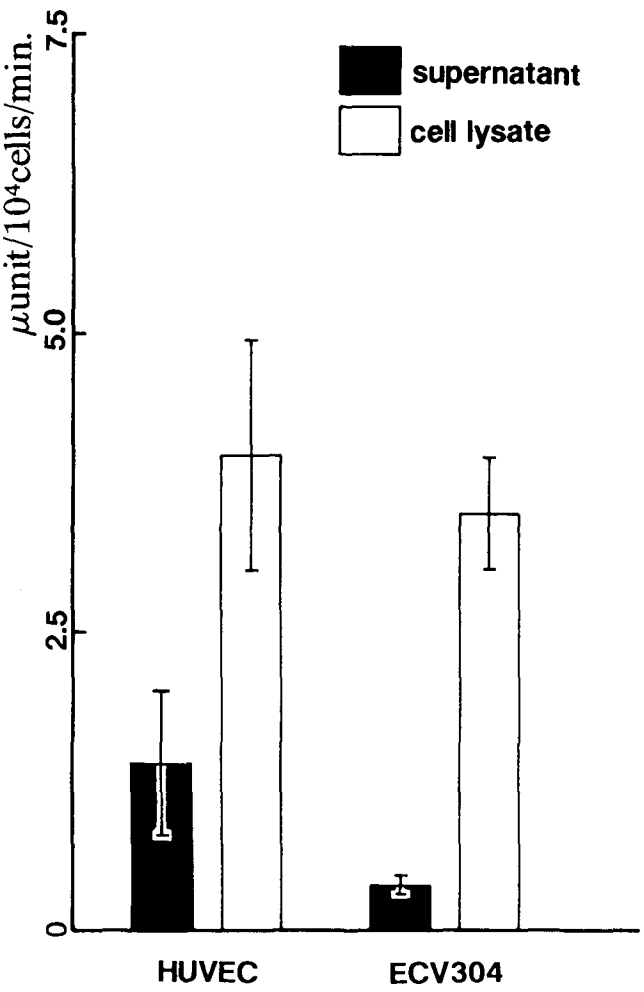


FIG. 5. ACE activity associated with cells and that released from cultured cells. ACE activity in the culture supernatant (*solid squares*) and in the cells lysed with 0.5% Triton X-100 (*open squares*) were measured by incubation with a synthetic substrate hippuryl-glycyl-glycine for 30 min at 37° C.

(Fig. 8 *A*, *B*). Capillary vessels from the host were found to be distributed widely within the tumor tissue. Consequently, the tumor cells were located as small nests dispersed between the mesenchymal structure, which mainly consisted of the host vasculature. Indeed, some lumenlike spaces, surrounded by the injected cells, looked like the initial stage of vasculogenesis, but no blood corpuscles were found in these cavities. However, these luminal cavities also seemed to be an artifact originating from the necrotic detachment of the growing cells located in the central part of tumor.

In ECV304-induced tumor tissue, actively proliferating ECV304 cells were negative for VIIIR:Ag (Fig. 8 *C*) but positive for UEA-I (Fig. 8 *D*).

DISCUSSION

From the karyological, immunocytochemical, and ultrastructural observations described above, ECV304 were shown to be an immortal endothelial cell line of human origin.

Inasmuch as ECV304 underwent transformation within 3 to 4 wk after preparation, some histiocytes may have been contaminated and transformed during cultivation. Alkaline and acid phosphatase are both known to be representative

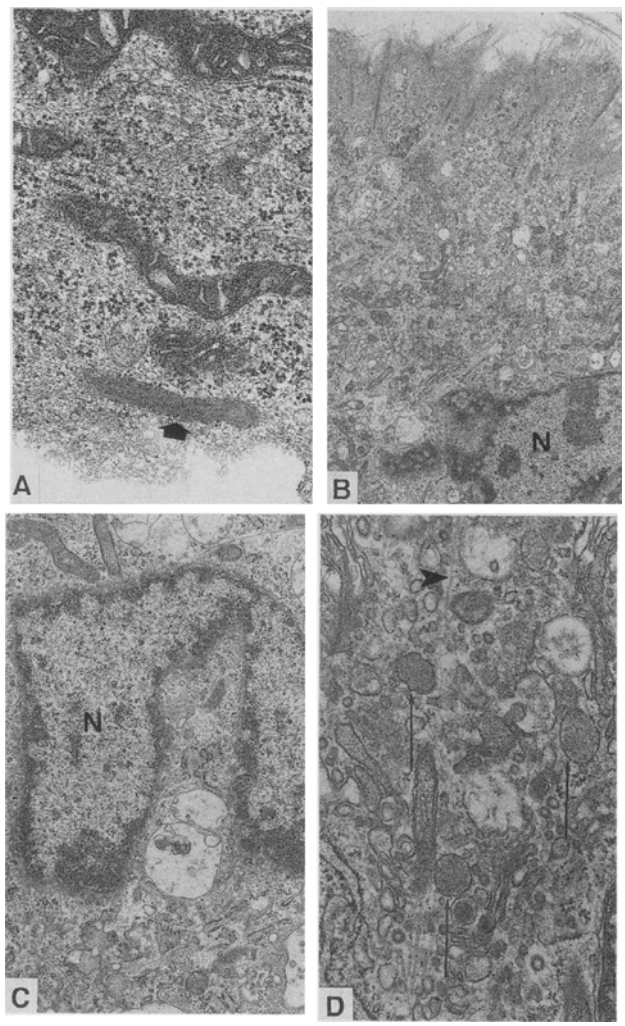


FIG. 6. Electron micrograph of cultured HUVEC (Passage 2; *A*) and ECV304 (Passage 20, PD 100; *B*, *C*, *D*). Weibel-Palade body is observed in the cytoplasm (*arrow* in *A*) and many vesicles are located in the vicinity of HUVEC cell membranes (*A*; ×30 333). ECV304 have abundant organelles, vesicles in cytoplasma and thick filaments beneath the cell membrane (*B*; ×4667). At higher magnifications, distinct nuclear (*N*) alterations with an increase in nucleoli and heterochromatins are observed (*C*; ×11 667). Near the Golgi complex, microtubules (*arrowhead* in *D*; ×23 333) and Weibel-Palade bodies (*arrow* in *D*) are observed.

FIG. 4. Immunocytochemical staining of VIIIR:Ag, UEA-I, and PHM5. ×336. HUVEC at Passage 2 (*A*, *C*, *E*) and ECV304 at Passage 166; PD 830 (*B*, *D*, *F*) were fixed and stained for VIIIR:Ag (*A*, *B*), UEA-I (*C*, *D*), and PHM5 (*E*, *F*) by the ABC method.

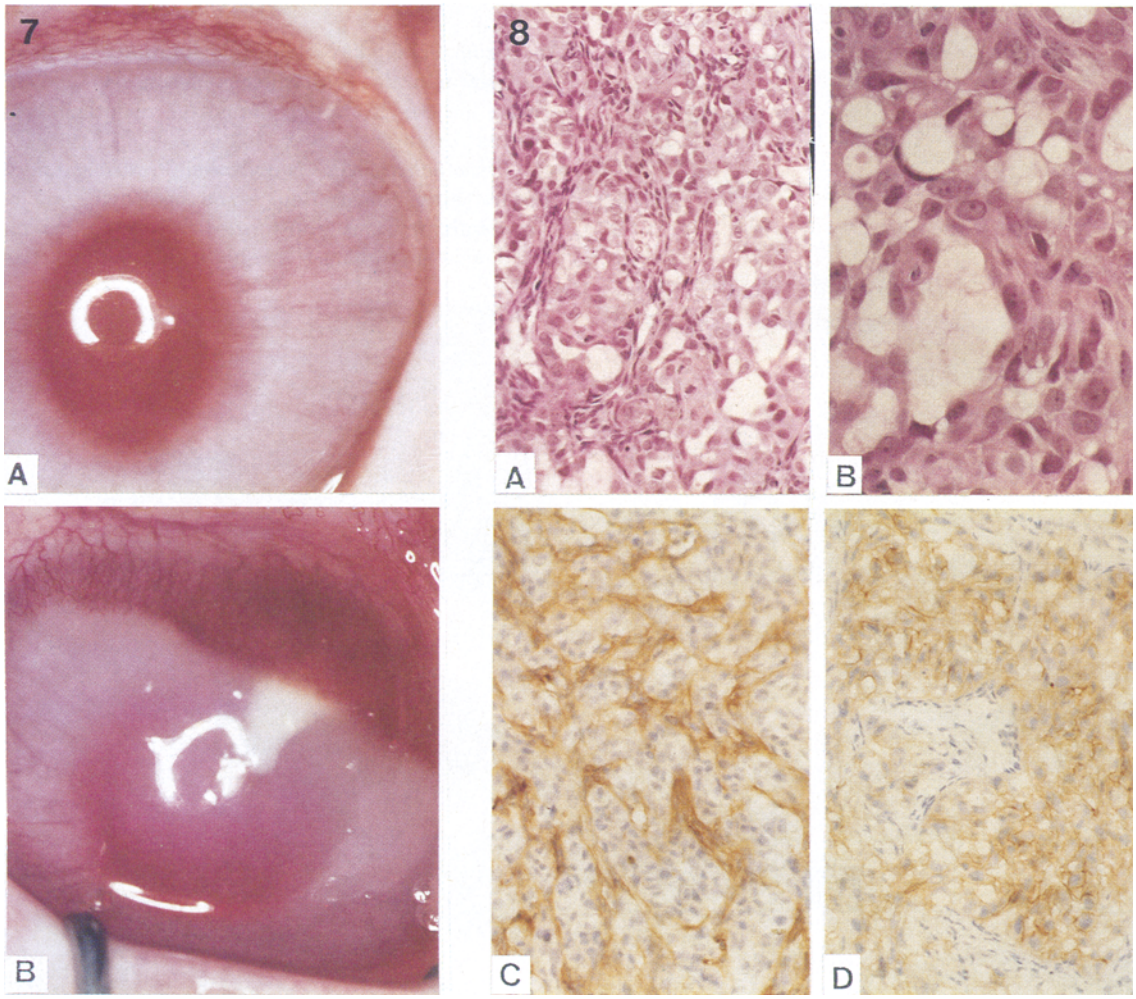


FIG. 7. Cornea test. Difference of vasoproliferative activities between HUVEC (*A*) and ECV304 (*B*) was compared 14 d after injection. Neovascularization was clearly observed only in the ECV304-implanted cornea.

FIG. 8. Histology of tumor tissue induced by implantation of ECV304 (Passage 94) into a nude mouse. Sections were stained with hematoxylin-eosin (*A*: ×520; *B*: ×1040). ECV304 grew in mass, forming nests subdivided by host blood vessels. Several lumenlike spaces are observed in which blood cells are not detectable. Only mouse-derived endothelium is VIIIR:Ag positive (*C*: ×520). The implanted ECV304 alone reacted with UEA-I (*D*: ×520).

markers for histiocytes and macrophages (30). Although VIIIR:Ag was not detectable in ECV304, the cells have ACE activity, Weibel-Palade bodies, and UEA-I and PHM5 reactivities, and are negative staining for human keratin (molecular weight 50 to 67 Kd) (5), alkaline and acid phosphatase, as well as for several monoclonal antihuman monocyte antibodies (data not shown). These observations suggest that ECV304 originated from HUVEC by spontaneous transformation but from neither histiocytes nor from mesothelial cells. Moreover, because ECV304 are continuing to passage after 5 yr (180 generations; PD 900) and to grow actively, they are considered to have been immortally transformed.

In HUVEC, high levels of polyploidy and karyotypic alterations have been found by Nichols et al. (28). Their results seem to support the spontaneous transformation observed in this study. Attempts to transform and immortalize by infection with SV40 resulted in the loss of endothelial cell-specific markers (13). Recently, however, HUVEC immortalized by infection with certain sarcoma viruses have been reported (8) to be morphologically unchanged and to produce prominent endothelial cell markers such as VIIIR:Ag and Weibel-Palade bodies. Our ECV304, with the exception of VIIIR:Ag deficiency, still retain the same immunocytochemical, biochemical, and morphological characteristics as HUVEC.

As described above, no apparent viruslike particles were ultrastructurally noticeable in the cell preparation. Addition of ECV-304-conditioned medium to the primary HUVEC culture, followed by subsequent prolonged cultivation, resulted in neither morphologic nor proliferative alteration, suggesting that this cell line has not been produced by virus infection. The donor of ECV304, a 5-yr-old boy, has been clinically well, and family studies have not

revealed any genetic abnormalities. The cultured cells were also confirmed not to be contaminated by mycoplasma by the method of Chen (1).

Immortalization of cells has been proposed to be a pathophysiologically important step leading to neoplastic transformation. However, solid tumors produced by ECV304 implantation failed to kill the host animals, which suggests that ECV304 cells have not yet undergone malignant neoplastic transformation. The finding that ECV304 cells injected into nude mice were VIIIR:Ag negative but UEA-I positive, indicates that ECV304 cells still retain their original antigenic characteristic during tumor formation. In contrast, nude mouse-originated vascular endothelium was positive for VIIIR:Ag, but negative with UEA-I, which had also been reported (21). From these observations, it is unlikely that ECV304 proliferation would to form a vasculature in nude mice.

Although endothelial cells from various tissues need some growth factors (24,32), addition of growth factors, such as basic fibroblast growth factor, endothelial cell growth supplement, or epidermal growth factor, did not have any stimulatory effect on the growth of ECV304. In this respect, ECV304 can be favorably available for biomedical research.

In addition, whether administration of certain dedifferentiation-promoting agents, such as dimethylsulfoxide, dibutyryl cyclic AMP, phorbol myristate acetate, and vitamin A and D, would enable ECV304 to redifferentiate into its original endothelium was studied using colorimetric assay of VIIIR:Ag. However, the results with these test substances proved to be negative for both the induction of morphologic alteration and the production of VIIIR:Ag. This suggests that these cells were not capable of being redifferentiated by these agents under our experimental conditions (data not shown).

Pre- and neoplastic transformation of certain tissues, such as mammary gland and bladder, have been reported to exhibit angiogenic activity (2,12). Inasmuch as ECV304 cells have been transformed and immortalized spontaneously, we attempted to examine whether they acquired angiogenic and tumorigenic activity. From the observation that ECV304 cells induced neovascularization, ECV304 were thought to have acquired angiogenic ability by transformation, although the results could have been due simply to the greater ability of the ECV304 cells to replicate.

We also have confimed the presence of plasminogen activator (33) and its inhibitor (31) in ECV304 culture medium without FBS (unpublished). Endothelin, a vasoconstrictor that was first isolated and characterized from porcine aortic endothelium (36), has recently been reported to be also detectable in ECV304 (35).

REFERENCES

1. Chen, T. R. In situ detection of mycoplasma contamination in cell cultures by fluorescent Hoechst 33258 stain. Exp. Cell Res. 104:255-262; 1977.
2. Chodak, G. W.; Haudenschild, C.; Gittes, R. F., et al. Angiogenic activity as a marker of neoplastic and preneoplastic lesions of the human bladder. Ann. Surg. 192:762-771; 1980.
3. Clemmons, D. R.; Isley, W. L.; Brown, M. T. Dialyzable factor in human serum of platelet origin stimulates endothelial cell replication and growth. Proc. Natl. Acad. Sci. USA 80:1641-1645; 1983.
4. Cohen, S.; Johnson, A. R.; Hurd, E. Cytotoxicity of sera from patients with scleroderma. Arthritis Rheum. 26:170-178; 1983.
5. Connell, N. D.; Rheinwald, J. G. Regulation of the cytoskeleton in mesothelial cells: reversible loss of keratin and increase in vimentin during rapid growth in culture. Cell 34:245-253; 1983.
6. Eldor, A.; Fridman, R.; Vlodavsky, I., et al. Interferon enhances prostacyclin production by cultured vascular endothelial cells. J. Clin. Invest. 73:251-257; 1984.
7. Emeis, J. J.; Kooistra, T. Interleukin 1 and lipopolysaccharide induce an inhibitor of tissue-type plasminogen activator in vivo and in cultured endothelial cells. J. Exp. Med. 163:1260-1266; 1986.
8. Faller, D. V.; Kourembanas, S.; Ginsberg, D., et al. Immortalization of human endothelial cells by murine sarcoma viruses, without morphologic transformation. J. Cell. Physiol. 134:47-56; 1988.
9. Folkman, J.; Cotran, R. Relation of vascular proliferation to tumor growth. Int. Rev. Exp. Pathol. 16:207-248; 1976.
10. Gelehrter, T. D.; Sznycer-Laszuk, R. Thrombin induction of plasminogen activator-inhibitor in cultured human endothelial cells. J. Clin. Invest. 77:165-169; 1986.
11. Gimbrone, M. A.; Cotran, R. S.; Leapman, S. B., et al. Tumor growth and neovascularization: an experimental model using the rabbit cornea. JNCI 52:413-427; 1974.
12. Gimbrone, M. A.; Gullino, P. M. Neovascularization induced by intraocular xenografts of normal, preneoplastic, and neoplastic mouse mammary tissues. JNCI 56:305-318; 1976.
13. Gimbrone, M. A.; Fareed, G. C. Transformation of cultured human vascular endothelium by SV40 DNA. Cell 9:685-693; 1976.
14. Gore, I.; Takada, M.; Austin, J. Ultrastructural basis of experimental thrombocytopenic purpura. Arch. Pathol. 90:197-205; 1970.
15. Gorman, S. D.; Hoffman, E.; Nichols, W. W., et al. Spontaneous transformation of a cloned cell line of normal diploid bovine vascular endothelial cells. In Vitro 20:339-345; 1984.
16. Grinspan, J. B.; Mueller, S. N.; Levine, E. M. Bovine endothelial cells transformed in vitro by benzo(a)pyrene. J. Cell Physiol. 114:328-338; 1983.
17. Hajjar, K. A.; Harpel, P. C.; Jaffe, E. A., et al. Binding of plasminogen to cultured human endothelial cells. J. Biol. Chem. 261:11656-11662; 1986.
18. Hancock, W. W.; Atkins, R. C. Monoclonal antibodies to human glomerular cells: a marker for glomerular epithelial cells. Nephron 33:83-90; 1983.
19. Hayes, L. W.; Goguen, C. A.; Ching, S. F., et al. Angiotensin-converting enzyme: accumulation in medium from cultured endothelial cells. Biochem. Biophys. Res. Commun. 82: 1147-1153; 1978.
20. Hobson, B.; Denekamp, J. Endothelial proliferation in tumours and normal tissues: continuous labelling studies. Br. J. Cancer 49:405-413; 1984.
21. Holthöfer, H.; Virtanen, I.; Kariniemi, A. L., et al. Ulex europaeus I lectin as a marker for vascular endothelium in human tissues. Lab. Invest. 47:60-66; 1982.
22. Jaffe, E. A.; Nachman, R. L.; Becker, C. G., et al. Culture of human endothelial cells derived from umbilical veins. J. Clin. Invest. 52:2745-2756; 1973.
23. Kakunaga, T. Neoplastic transformation of human diploid fibroblast cells by chemical carcinogens. Proc. Natl. Acad. Sci. USA 75:1334-1338; 1978.
24. Maciag, T.; Kadish, J.; Wilkins, L., et al. Organizational behavior of human umbilical vein endothelial cells. J. Cell Biol. 94:511-520; 1982.
25. Maruyama. Y. The human endothelial cell tissue culture. Z. Zellforsch. Microsk. Anat. 60:69; 1963.
26. Meischke, H. R. C. In vitro transformation by bovine papilloma virus. J. Gen. Virol. 43:473-487; 1979.
27. Mukai, K.; Rosai, J.; Burgdorf, W. H. C. Localization of factor VIII-related antigen in vascular endothelial cells using an immunoperoxidase method. Am. J. Surg. Pathol. 4:273-276; 1980.

28. Nichols, W. W.; Buynak. E. B.; Bradt, C., et al. Cytogenetic evaluation of human endothelial cell cultures. J. Cell Physiol. 132:453-462; 1987.
29. Olander, J. V.; Bremer, M. E.; Marasa, J. C., et al. Fibrin-enhanced endothelial cell organization. J. Cell. Physiol. 125:1-9; 1985.
30. Rabinovitch, M.; Andreucci, D. A histochemical study of "acid" and "alkaline" phosphatase distribution in normal human bone marrow smears. Blood 4:580-594; 1949.
31. Sprengers, E. D.; Verheijen, J. H.; Van Hinsbergh, V. W. M., et al. Evidence for the presence of two different fibrinolytic inhibitors in human endothelial cell conditioned medium. Biochem. Biophys. Acta 801:163-170; 1984.
32. Thornton, S. C.; Mueller, S. N.; Levine, E. M. Human endothelial cells: use of heparin in cloning and long-term serial cultivation. Science 222:623-625; 1983.
33. Van Hinsbergh, V. W. M.; Binnema, D.; Scheffer, M. A., et al. Production of plasminogen activators and inhibitor by serially propagated endothelial cells from adult human blood vessels. Arteriosclerosis 7:389-400; 1987.
34. Weibel, E. R.; Palade, G. E. New cytoplasmic components in arterial endothelia. J. Cell Biol. 23:101-112; 1964.
35. Yamashita, Y.; Kobayashi, M.; Hasegawa, S., et al. Establishment of serum-free culture of human vascular endothelial cells. Tissue Culture Research Communication 7:139(abstract); 1988 (in Japanese).
36. Yanagisawa, M.; Kurihara, H.; Kimura, S., et al. A novel potent vasoconstrictor peptide produced by vascular endothelial cells. Nature 332:411-415; 1988.
37. Yuspa, S. H.; Hawley-Nelson, P.; Koehler, B., et al. A survey of transformation markers in differentiating epidermal cell lines in culture. Cancer Res. 40:4694-4703; 1980.

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