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Isolation and Properties of Tumor-derived Endothelial Cells from Rat KMT-17 Fibrosarcoma

Naoki Utoguchi, Adul Dantakean, Hiroo Makimoto, Yukiko Wakai, Yasuo Tsutsumi,[1] Shinsaku Nakagawa and Tadanori Mayumi

Faculty of Pharmaceutical Sciences, Osaka University, 1-6 Yamadaoka, Suita, Osaka 565

Rat KMT-17 fibrosarcoma-derived endothelial cells were isolated by Percoll gradient centrifugation with an attaching-speed separation technique, and their properties in culture were examined. The primary cultured tumor-derived endothelial cells (TEC) showed angiotensin-converting enzyme activity, positivity for Factor VIII-related antigen staining, and typical capillary-like formation on Matrigel. The primary cultured TEC monolayer showed greater permeability than normal tissue-derived endothelial cell (aorta, vena cava and epididymal fat capillary) monolayers on FITC-dextran diffusion (molecular weight 70,000). Leukocyte adhesion to TEC was reduced compared to that to fat-derived capillary endothelial cells. These characteristics resembled those of tumor vascular endothelium, and were observed both in the primary and first-passage cell cultures, but not in the fourth-passage cell cultures. Our findings indicate that primary or subcultured TEC are applicable for studies of the physiological characteristics of tumor endothelial cells.

Key words: Endothelial cell — Primary culture — Tumor — KMT-17

Angiogenesis is required for the growth of solid tumors.[1-3] Many investigators have reported that suppression of tumor angiogenesis leads to the inhibition of solid tumor growth.[4-8] There are several tumor angiogenesis mediators which are secreted by tumor cells and tumor stroma cells, including basic and acidic fibroblast growth factors, tumor necrosis factor-α, transforming growth factor-α and -β, and vascular endothelial growth factor.[9-11] It is thought that the properties of endothelial cells in tumor tissues are affected directly or indirectly by tumor and stroma cells. It is well established that great heterogeneity exists not only between endothelial cells in macro- and microvessels but also among microvessels and endothelial cells from different organ sites. Brain microvessel endothelial cells form a blood-brain barrier through which macromolecules cannot pass,[12, 13] whereas liver and spleen microvessels, known as discontinuous capillaries, possess open cellular junctions which allow the passage of macromolecules.[14, 15] Tumor blood vessels also show important differences from those of normal tissues in terms of structure and function.[16-19] Many investigators have reported that tumor vessels are more permeable than normal tissue vessels.[20-25] The various tissue-derived endothelial cells which have been cultured and examined to date include those from human umbilical vein, human adrenal capillary, rat cerebral microvessel and bovine retinal microvessel.[26-29] However, tumor-derived endothelial cells have not yet been isolated and cultured. In this study, we isolated and cultivated endothelial cells from tumor tissue and examined their properties in culture.

[1] To whom all correspondence should be addressed.

MATERIALS AND METHODS

Rats Inbred WKA/Hok rats, 8–12 weeks old, were obtained from Shimizu Experimental Animal Co., Ltd., Kyoto.

Tumors KMT-17 is a transplantable fibrosarcoma induced by 3-methylcholanthrene in WKA rats and maintained in ascites form.[30-32] KMT-17 tumor cells (3×10^5) were implanted s.c. into the right flank of WKA rats, and the tumors were removed for study 10–14 days later when they had reached a weight of 10–15 g.

Tumor-derived endothelial cell isolation The tumors were aseptically removed and placed in cold Hanks' balanced salt solution (HBSS) containing 140 μg/ml penicillin, 200 μg/ml streptomycin, and 50 μg/ml kanamycin. After removal of the peripheral and necrotic portion of the tissue, the remaining tumor tissue was minced with a razor. The minced tissue was digested with 0.75% collagenase (Wako Pure Chem., Osaka) containing 0.5% bovine serum albumin (BSA) in HBSS for 1 h with constant mixing at 37°C. The resultant cell suspension was passed through a Nylon mesh (300 μm), and washed twice in minimum essential medium (MEM) containing 10% fetal calf serum (FCS) by centrifugation at 4°C for 10 min. The pellet from the final wash was resuspended in MEM containing 10% FCS, and 2 ml of the cell suspension (1.0×10^8 cells) was carefully layered onto the top of a pre-established 45% Percoll (Pharmacia, Sweden) gradient containing Percoll (9 ml), 10× MEM (1 ml), and MEM with 10% FCS (10 ml), and centrifuged at 20,000g in an angled roter for 15 min. The gradient tubes were centrifuged at 1,500g in a swinging rotor for 10 min, and the gradient was continuously

fractionated from the top of the Percoll gradient at 2 ml intervals. Eleven fractions were obtained at this step.

Cell culture The cells separated by Percoll gradient fractionation were washed twice with MEM to remove Percoll solution. The cells were then resuspended in Dulbecco's modified Eagle's medium (DMEM) containing 10% FCS and 25 μg/ml endothelial cell growth supplement (Sigma, Missouri) and plated on culture dishes. After 24 h of culture, the cells were washed with phosphate-buffered saline (PBS) 3 times to remove non-adhering cells.

Angiotensin-converting enzyme activity Each cell suspension fraction was washed with HBSS twice to remove cell debris and medium. The pellets were then resuspended in borate buffer (pH 8.5) and sonicated. The angiotensin-converting enzyme (ACE) activity in each fraction was determined according to Friedland and Silverstein,[33] and expressed per μg of cell protein. The amount of protein was assayed using a Bio-Rad protein assay kit (Bio-Rad, California) with BSA as a standard.

Tube formation assay Tube formation assay was examined with two types of cells; Percoll gradient-separated cells and cells cultured on tissue culture dishes. The tube formation assay was performed by the method of Kubota *et al.* as described previously.[34] Matrigel (Collaborative, Massachusetts), an extract containing basement membrane components, was applied to 24- or 48-well culture plates and incubated at 37°C, which induced gelling. The gradient-separated cell suspensions (1.0×10^6/ml) in the medium were pipetted onto the gels. After 24 h of cultivation, the gels were washed gently with the medium. Primary cultured cells were detached from culture dishes by treatment with 0.025% trypsin and suspended in medium at 1.0×10^5/ml before being plated onto the gels. After 4 h, tube formation was examined.

Factor VIII staining Factor VIII-related antigen staining was performed using a modification of the method described by Cole *et al.*[35] Rabbit anti-human Factor VIII antibody and the second antibody, fluorescein isothiocyanate (FITC)-conjugated goat anti-rabbit IgG, were obtained from DAKO (Denmark). To examine the validity of Factor VIII staining, we also examined the staining using rabbit normal serum as the primary antibody. The fluorescence of normal serum-stained cells was weak or negligible.

Isolation of normal tissue-derived endothelial cells Rat epididymal fat pad-derived capillary endothelial cells (RCEC) were isolated according to Madri and Williams.[36] Aortic endothelial cells were isolated by the explant method of McGuire and Orkin.[37] Vena cava endothelial cells were isolated by the same explant technique. The RCEC, aortic and vena cava-derived endothelial cells were cultured in the same medium as the tumor-derived endothelial cells (TEC). Cells were identified as endothelial cells on the basis of the presence of Factor VIII-related antigen and tube formation assay.

Permeability assay In the assay of the permeability of endothelial monolayers, Intercell (Kurabo, Osaka) was used as a permeation chamber, and FITC-dextran (molecular weight 70,000) was used to evaluate macromolecular diffusion. FITC-dextran is transported through the intercellular spaces of endothelial cells (junctional transport).[38] The Intercell was coated by the following method: coating solution (50 μl), composed of 1 volume of collagen type I (Nitta-gelatin, Osaka) and 3 volumes of 60% ethanol, was applied onto the Teflon Intercell membrane, and the collagen-coated membrane was then dried at room temperature, and endothelial cells were cultured on the Intercell. The permeability assay was performed when the cells were confluent. The FITC-dextran permeability assay was performed as described.[39] The permeability coefficient of the cell layer alone was calculated from the following relationship[40]:

$$1/P_{EC}=1/P_{total}-1/P_{mem}$$

where, P_{EC}, P_{total}, and P_{mem} are the permeability coefficients of the cell layer alone, the Teflon membrane and cell layer combined, and the Teflon membrane alone, respectively.

Leukocyte-endothelial cell adhesion assay Preparation and fluorescence labeling of leukocytes: Rat leukocytes were isolated from heparinized blood; the blood was layered over Mono-poly resolving medium (ICN Biomedicals Inc., Ohio), and the leukocytes were separated

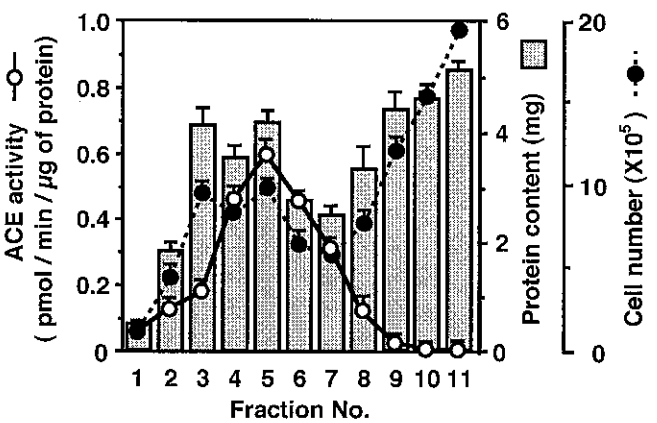

Fig. 1. Angiotensin-converting enzyme (ACE) activity and protein content of each fraction. Tumor tissue was minced and digested by collagenase, and cells were separated by centrifugation through a Percoll gradient. ACE activity, protein content and number of cells of each fraction were determined. Values represent the mean±SD of four separate assays.

by density gradient centrifugation at 350*g* for 30 min at room temperature. Erythrocytes contaminating the leukocyte pellet were removed by treatment with 0.2% NaCl solution followed by 1.6% NaCl solution. The leukocytes were labeled with fluorescent dye using a modification of the method described by Luscinskas *et al.*[41] Briefly, the leukocytes (1.0×10^6 cells/ml HBSS) were incubated with the dye, 2′,7′-bis(2-carboxyethyl)-5-(and-6)-carboxyfluorescein acetoxymethyl ester (BCECF-AM; Molecular Probes, Inc., Oregon), at a concentration of 1.7 μg/ml for 30 min at 37°C. The leukocytes were then washed twice with HBSS by centrifugation to remove free BCECF-AM, and labeled leukocytes were resuspended in HBSS at 1.0×10^6 cells/ml. The cell viability, measured as trypan blue exclusion, was greater than 98%.

Adhesion assay: Endothelial cells were plated on 96-well tissue culture plates. When the cells reached confluence, fluorescence-labeled leukocytes (1.0×10^5/100 μl) were added to the wells. After 30 min of incubation at 37°C, adhesive and nonadhesive leukocytes were separated by a modification of the method described by Pauli and Lee,[42] i.e., the wells were filled with HBSS and the plates were sealed with rubber sheets. The inverted plates were centrifuged at 50*g* for 5 min. The plate sealer was removed with the plate still in the inverted position, and the remaining buffer was drained by tapping the plate on a filter pad. The cells were lysed by addition of 200 μl of 2% Triton X-100. Aliquots (150 μl) were taken from each well and mixed with 750 μl of Tris-HCl buffer (pH 8.5), and the fluorescence intensity was measured using a

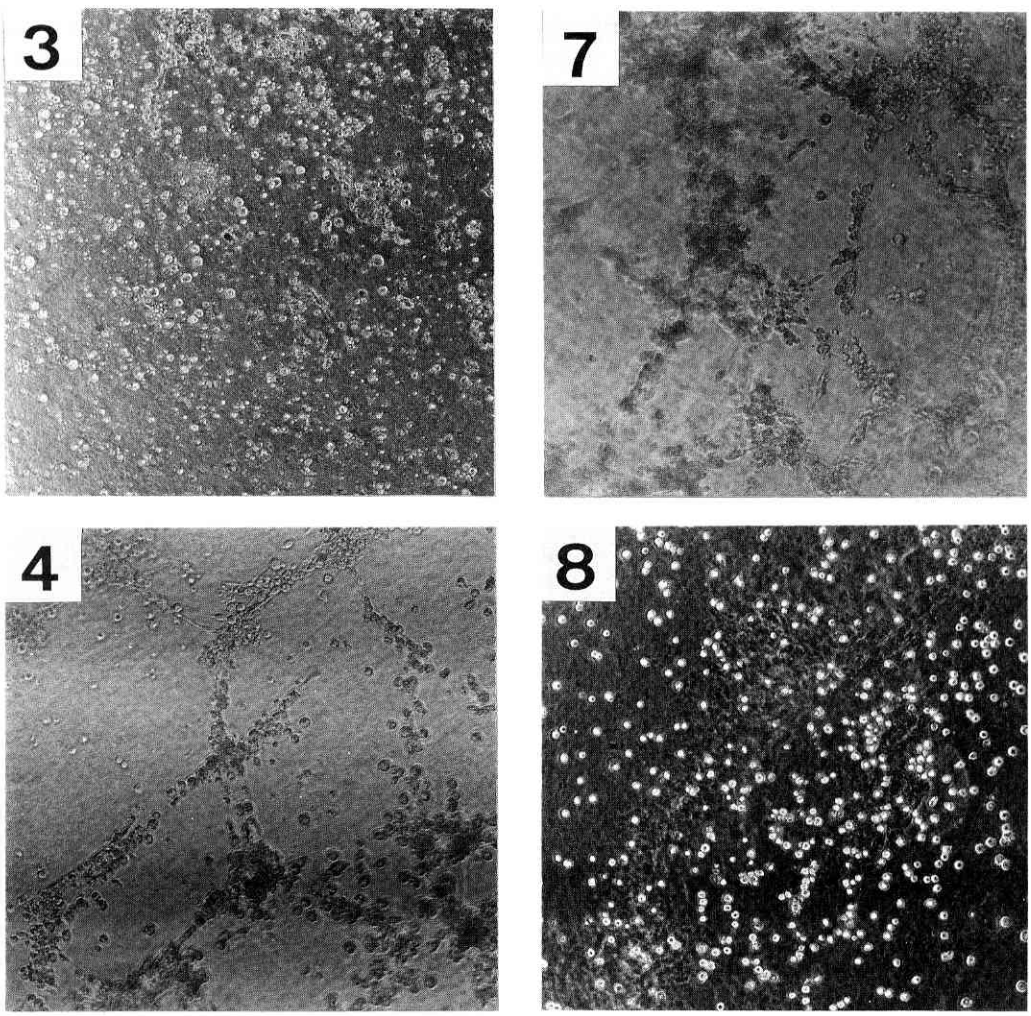

Fig. 2. Phase-contrast micrographs of tube formation on Matrigel. Percoll gradient-separated cells were plated onto Matrigel. After 24 h, non-adhering cells were removed by washing-out, and tube formation was observed. The number on each of the photographs represents the fraction number.

spectrofluorophotometer (excitation 508 nm, emission 531 nm).

RESULTS

ACE activity and tube formation The cell suspension, which was prepared from rat KMT-17 fibrosarcoma by collagenase digestion, was separated on a Percoll gradient, which was continuously fractionated from the top of the gradient. Cells of each fraction were washed with HBSS twice. The ACE activity, protein content and number of cells of each fraction were assayed (Fig. 1). The ACE activity per μg of cell protein was the highest in Fraction 5, indicating that the endothelial cells were the most abundant in this fraction. Although there were many cells in Fractions 8 to 11, the ACE activities in these fractions were very low or negligible.

We examined what fractions contain endothelial cells using the tube formation assay described in "Materials and Methods" (Fig. 2). By 24 h after plating, most of the cells did not adhere to the Matrigel. After washing-out of non-adhering cells, vessel-like tubes were observed in Fractions 4 to 7, but not in any other fractions. These findings with regard to ACE activity and tube formation assays suggested that endothelial cells could be separated from the mixture containing many cell types by using Percoll gradient centrifugation.

Primary culture of tumor-derived endothelial cells The cells from Fractions 3 to 8 were cultured on tissue culture dishes (2.2×10^5 cells/cm^2). By 7–10 days after plating, the cells reached confluence. At this time, the cultured cells were observed to be heterogeneous by phase contrast microscopy, and 10–50% of them were positively stained for Factor VIII-related antigen (data not shown). The endothelial cells adhere more rapidly to the culture dishes as compared with smooth muscle cells. Therefore, the attaching-speed separation technique is useful for culturing endothelial cells without contamination by other cell types.[43,44] After 24 h of cultivation, non-adhering cells were removed by washing-out with PBS, and only the adhering cells were cultured. The plating density of the cells was 2.2×10^5 cells/cm^2, and the number of cells adhering 24 h after plating was 0.27×10^4/cm^2. After 7 days of cultivation, the cells were confluent, and the cell number was 4.9×10^4/cm^2. Fig. 3 shows a phase-contrast photomicrograph of cultured cells at confluence. Tumor-derived cultured cells were morphologically homogeneous under observation by phase contrast microscopy (Fig. 3A). The cultured cells formed a monolayer on the dish. The cultured adhering cells from Fractions 4 to 7 were all positive for Factor VIII staining (Fig. 4). The cells from Fractions 3 and 8 showed low positivity (less than 10%) or were negative. The specificity of Factor VIII staining was confirmed with rat aortic, venous, and epididymal fat-derived endothelial cells, KMT-17 and fibroblasts. All types of endothelial cells were positive for the staining, and both KMT-17 and fibroblasts were negative (data not shown).

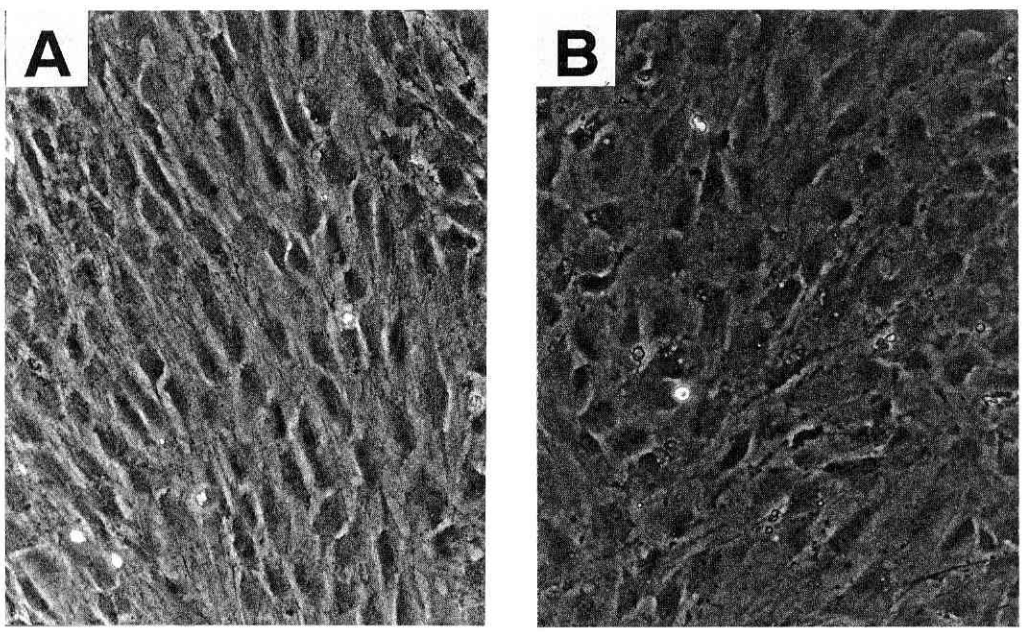

Fig. 3. Phase-contrast micrographs of rat endothelial cells. A: Tumor-derived endothelial cells. B: Epididymal fat capillary endothelial cells.

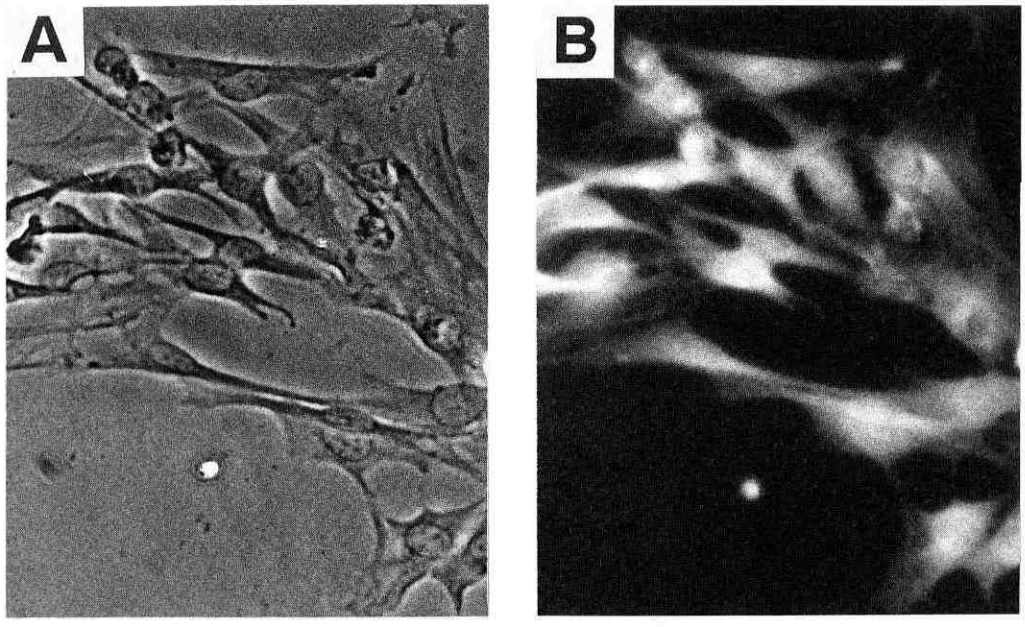

Fig. 4. Phase-contrast and fluorescence micrographs of KMT-17 fibrosarcoma-derived endothelial cells from Fraction 5. A: Phase-contrast micrograph. B: Fluorescence micrograph of Factor VIII staining by indirect immunofluorescence labeling.

The ACE activity of the primary cultured cells from Fractions 4 to 7 was 20.5 pmol/min/mg of protein. All primary cultures from Fractions 4 to 7 formed tube-like structures on Matrigel, but those from Fractions 3 and 8 did not (Fig. 5). We confirmed that KMT-17 did not form any tube-like structure on Matrigel (data not shown). All the cultured cells from Fractions 4 to 7 were positive for endothelial marker, such as Factor VIII-related antigen and tube formation on Matrigel. These findings suggested that the cultured cells which were isolated by a combination of the two separation methods were endothelial cells; these cells were successfully cultured without contamination by other cell types.

Permeability of endothelial monolayers We examined whether the isolated cells presented *in vivo* tumor-derived properties under culture conditions. Tumor vessels are more permeable than normal tissue vessels *in vivo*.[20–25] The TEC monolayers showed greater permeability than the aortic, venous and epididymal fat-derived endothelial monolayers on FITC-dextran (molecular weight 70,000) diffusion (Table I). The confluence of each cell type and its contact with other cells was observed under phase-contrast microscopy. The differences between TEC and normal tissue-derived endothelial cells in the shared area of the cells on the permeation chamber did not account for the differences in their characteristics.

Leukocyte adhesion Leukocyte adhesion to tumor vessel endothelium is reduced compared to normal tissue vessel endothelium.[45] We examined this feature with our cultured cells, and found that leukocyte adhesion to TEC was reduced compared to that to FCEC (Table II). Adhesion was expressed as number of adhering leukocytes per cm^2 and per endothelial cell number. Therefore, this low adhesion did not depend on the number of endothelial cells at confluence.

Passage and TEC characteristics The effects of culture passage on the hyperpermeability and low leukocyte adhesion of TEC were examined (Fig. 6). The cells were passaged at confluence at a split ratio of 1:4. In the first passage, the TEC monolayer was still more permeable than that of FCEC, but after the 4th passage, the permeability of TEC was not different from that of FCEC. Similarly, while low adhesion of leukocytes to TEC was observed in first-passage cultures, after the 4th passage, there was no significant difference compared to that of FCEC.

DISCUSSION

In the present study, tumor-derived endothelial cells were cultured by a two-step separation technique and their properties in culture were observed. The isolated and cultured cells were pure endothelial cells. The cultured cells from Fractions 4 to 7 were homogeneous, and all the cell was positive for Factor VIII-related antigen staining and formed tube-like structures on Matrigel.

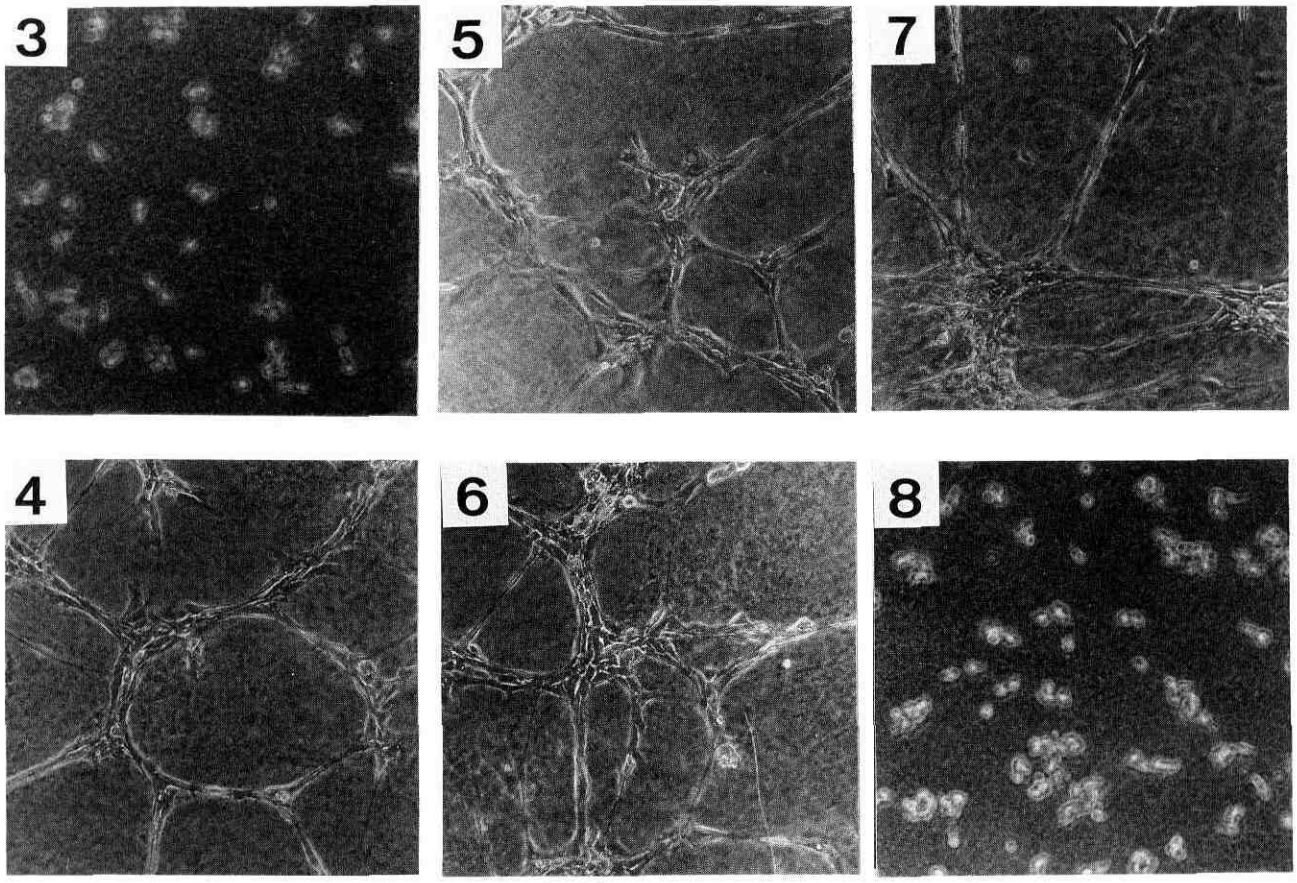

Fig. 5. Phase-contrast micrographs of tube formation on Matrigel. Cultured cells derived from each fraction were plated onto Matrigel, and after 4 h, tube formation was observed. The number on each of the photographs represents the fraction number.

Table I. Permeability of Rat Endothelial Monolayers to FITC-dextran

Source	Permeability coefficient ($\times 10^{-3}$ cm/h)
Aorta	17.23±2.52
Vena cava	8.43±1.96
Fat capillary	19.49±3.57
Tumor capillary	41.10±3.99

Rat aortic, vena cava, epididymal fat capillary and tumor-derived endothelial cells were cultured on the permeation chamber. When the cells were confluent, the permeability of FITC-dextran (molecular weight 70,000) was determined. Mean±SD. N=4.

Table II. Numbers of Leukocytes Adhering to Endothelial Cells

	$\times 10^5$ cells/cm^2	$\times 10^5$ cells/10^5 cells
FCEC	1.58±0.12	2.83±0.22
TEC	0.86±0.14[a)]	1.75±0.28[a)]

Rat epididymal fat-derived endothelial cells (FCEC) and tumor-derived endothelial cells (TEC) were cultured on a 96-well tissue culture plates. When the cells were confluent, rat leukocyte adhesion to endothelial cells was assayed.
a) $P<0.001$ compared with FCEC by Students' t test. Mean±SD. N=4.

Various tissue-derived endothelial cells have been cultured, and it is very important to determine whether the isolated cells retain their tissue-derived properties in culture. Endothelial cells present not only tissue-derived characteristics, but also the properties of the region of the vessels from which they were derived (aorta, vena cava and capillaries). Histamine induces plasma protein leakage in the venous or capillary vessels *in vivo*. It also increases venous or capillary-derived endothelial permeability in culture systems.[46,47] However, histamine decreases bovine aortic endothelial cell permeability.[48] These findings suggest that, in the study of tissue-specific

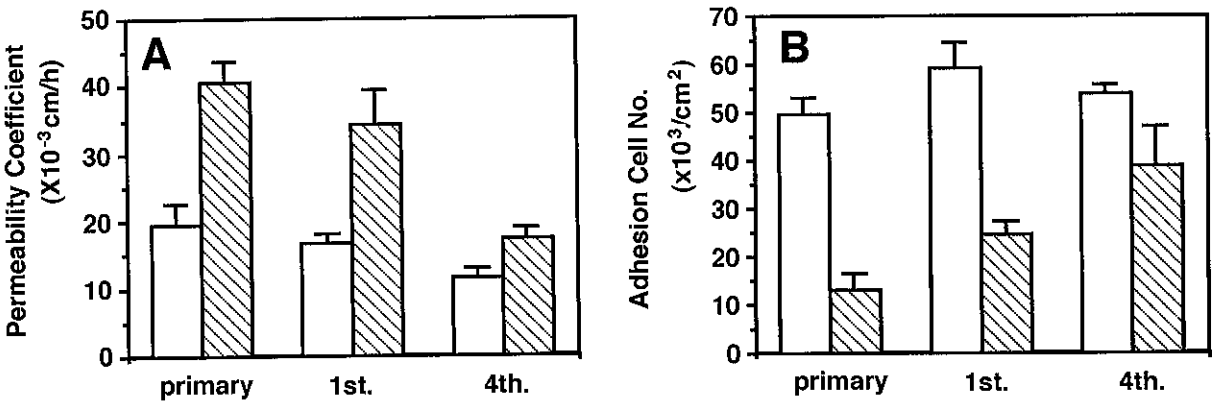

Fig. 6. Diminution of tumor-derived endothelial properties by successive passaging. TEC and FCEC were passaged at a split ratio of 1:4. FCEC, open columns; TEC, slashed columns. Columns and bars show the mean±SD of four separate assays. A: Passaged cells were cultured on the permeation chamber. When the cells reached confluence, the permeability of endothelial monolayers was determined. B: Passaged cells were cultured in a 96-well microplate. When the cells were at confluence, the leukocyte adhesion was determined.

endothelial cell properties *in vitro*, it is necessary to study the endothelial cells derived from the specific tissue. Primary cultured TEC showed hyperpermeability to macromolecule diffusion and low adhesion of leukocytes, properties similar to those of tumor vascular endothelium *in vivo*.

In culture, the characteristic activities of brain endothelial cells, γ-glutamyltranspeptidase and alkaline phosphatase activity, decrease with increasing passage number.[49] The tumor vessel properties of the TEC were observed not only in primary culture but also in the early subcultures. This observation suggests that to a certain extent continual interactions between endothelial and tumor cells were not necessary for the continued expression of the tumor-derived endothelial properties, at least in the early passages. In the 4th-passage subcultures, these tumor-derived endothelial properties were no longer observed. In the absence of interactions between the tumor and the endothelial cells, for example via soluble factors, direct attachment, or the extracellular matrix, the features of TEC appear to revert to those of normal tissue endothelium.

It is unclear why tumor vessels have more permeability than normal tissue vessels. Vascular permeability factor (VPF), also known as vascular endothelial growth factor, which is secreted by tumor cells, increases vascular permeability *in vivo* and is thought to induce tumor vessel permeability.[50–52] We do not know whether the tumor-derived endothelial cells we isolated and cultured would be affected by VPF *in vivo*, and the cells were cultured *in vitro* in medium containing no VPF. Despite the lack of VPF in the culture medium, the TEC showed hyperpermeability during early subculture. Maeda *et al.* reported that nitric oxide mediated the hyperpermeability of solid tumor vessels.[53] Bradykinin also has an important role in the hyperpermeability of tumor vessels.[54, 55] However, the details of the mechanism of regulation of endothelial permeability by these substances remain unclear. The TEC may be of use in the study of the mechanism of tumor vessel hyperpermeability, and may serve as a model in which to study the physiological characteristics of tumor endothelial cells.

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