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Isolation and Cultivation of Microvascular Endothelial Cells from Rat Lungs: Effects of Gelatin Substratum and Serum

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Microvascular endothelial cells from rat lungs were cultured in serum-free medium supplemented with an endothelial growth substance, insulin, hydfocortisone and so on. Five to seven days after plating, cultured cells formed a monolayer. They were identified as endothelial cells by morphology and by positive immunohistochemistry for factor VIII-related antigen, a marker for endothelia cells. Differences between gelatin coated culture plates and plastic culture plates in endothelial cell proliferation were evaluated. Cells plated on uncoated plastic plates had a spindle-shaped morphology and did not express factor VIII-related antigen. Two types of medium, serum-free medium containing endothelial growth substance and basal medium supplemented with 20% fetal calf serum, were also compared in primary culture. In contrast with the serum-free medium, cells cultured in the serum-containing medium showed fibroblast-like morphology and did not express factor VIII-related antigen. These results suggest that a gelatin substratum and serum-free medium containing endothelial growth supplement are necessary for in vitro proliferation of microvascular endothelial cells isolated from rat lungs. The culture method and conditions outlined here allow the proliferation of pure microvascular endothelial cells from rat lungs. It may be useful in studying hematogenous metastasis to the lung and the role of microvascular endothelium in other pulmonary disease.

Key Words: Rat lung, microvascular endothelium, serum-free medium, gelatin, factor VIII-related antigen

Since the first reports of *in vitro* culture of vascular endothelium, most successes have been achieved with endothelial cells derived from large vessels (Jaffe *et al.* 1973; Booyse *et al.* 1975). Many authors have investigated chemotaxis, extravasation, and intercellular adherence of neutrophils (Taylor *et al.* 1981; Harlan 1985), lymphocytes (Masuyama *et al.* 1986), monocytes (Wallis *et al.* 1985) and tumor cells (Poste and Fidler 1980; Rice *et al.* 1988) in contact with large vessel endothelial cells. However, it has become clear that there are many fundamental differences between large vessel endothelial cells and microvascular endothelial cells (Zetter 1980; Zetter 1981; Gumkowski *et al.* 1987; Speiser *et al.* 1987). Microvascular endothelial cells actually engaged in many physiologic and pathologic functions *in vitro*. Thus, the inferences that have been made about the mechanisms of angiogenesis, inflammation, and tumor metastasis based on a study of large vessel endothelium may not be valid. *In vitro* culture of microvascular endothelium remains a difficult procedure because it is not easy to isolate the microvascular endothelial cell population and to satisfy their fastidious culture requirements. Nowadays, microvascular endothelial cells from various organs of many species are used for the study of intercellular adhesion, tumor metastasis and various microvascular diseases (Folkman *et al.* 1979; Sherer *et al.* 1981; Alby and Auerbach 1984; Kramer *et al.* 1985; Auerbach *et al.* 1987; Cheng and Kramer 1989). However, few attempts have been made to culture microvascular endothelium from the lung, which plays a more important role to hematogenous metastasis than endothelium from other organs. To develop more representative *in vitro* mod-

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els of hematogenous metastasis, we attempted to culture microvascular endothelial cells isolated from the marginal vessels of rat lungs.

In tis study, we describe the conditions required to isolate and cultivate microvascular endothelial cells from rat lungs. The identification and growth pattern of cultured cells in different conditions are described. We also describe the effects of serum and gelatin in selective proliferation of microvascular endothelial cells isolated from lungs. This report should be useful in the study of hematogenous metastasis from primary tumor site to lung.

MATERIALS AND METHODS

Media

Basal medium consists of a 1 : 1 mixture of Dulbecco's modification of Eagle's medium (DMEM, Hazleton, Lenexa, KS) and Ham's F 12 medium (HyClone, Logan, UT). It also contains penicillin (120 mg/L), streptomycin (270 mg/L), fungizone (2 mg/L), and N-2-hydroxyethyliperazine-N'-2-ethane sulfonic acid (HEPES, Hazleton, Lenexa, KS) 15 mmol/L. Chemically defined serum-free medium was made by adding the following to basal medium; endothelial growth supplement (100 μg/ml), epidermal growth factor (20 ng/ml), triiodothyronine (10^{-10} mon/L), insulin (5 μg/ml), transferrin (5 μg/ml), hydrocortisone (10^{-7} mol/L), cholesterol (1.25 μg/ml), ascorbic acid (40 μmol/L), glutathione (1 μg/ml), sodium selenite (8×10^{-8} mol/L), 2-aminoethanol (10 μmol/L), phosphatidylcholine (12.5 μg/ml), and mercaptoethanol (10 μmol/L). Endothelial grwoth supplement, triiodothyronine, insulin, transferrin, hydrocortisone, cholesterol, sodium selenite, 2-aminoethanol, and phosphatidylcholine were purchased from Sigma chemical Co,. St. Louis, MO. Ascorbic acid, glutathione, and mercaptoethanol were purchased from Merck, Darmstadt, West Germany. Epidermal growth factor was purchased from GIBCO, Grand Island, NY. All reagents were sterilized by filtration (0.22 μm pore size, Millipore) before use.

Preparation of gelatin-coated culture plates

Gelatin (Sigma Chemical Co., St. Louis, MO) was dissovled in cell-culture grade distilled water at a concentration of 1% and sterilized with 0.22-μm filter. The gelatin solution (0.5 ml/well) was poured into the 24-well culture plates (Costar, Cambridge, MA) and incubated at 37°C. After 2 hours, the excess gelatin was aspirated off and the plates were dried.

Animals

Male Sprague-Dawley rats weighing 65 to 85 g were used in this study. All rats were sacrificed by cervical dislocation.

Tissue dissociation and cell separation

Isolation of microvascular endothelial cells was performed according to a modified method of Schumann *et al* (1989). The fresh lungs isolated from the sacrificed rats were washed three times with 50 mL basal medium. The outer edges of the lung lobes, which did not contain large blood vessels, were cut off (by this procedure, the majority of larger vessel types can be excluded) and finely minced in basal medium using iris scissors. The minced lung tissue was suspended in 50 mL of basal medium and centrifuged at $250\times g$ for two minutes. The pellet was incubated in the type IV collagenase (Boehringer-Mannheim, West Germany) solution (2.25 mg/ml collagenase in basal medium) at 37°C in a CO_2 incubator. After ten minutes, 40 mg/ml H_2O dispase (Boehringer-Mannheim, West Germany) were added and incubation at 37°C was continued. Fifteen minutes later, the suspension was centrifuged for two minutes at $250\times g$, and the pellet was washed twice with 50 mL of basal medium. The final pellet was resuspended in 20 mL of basal medium and 0.5 mL of this medium was seeded in gelatin coated or uncoated 24-well culture plates. Two hours later, non-adherent cells and debris were removed and cultures were rinsed and fed serum-free medium, or basal medium containing 10% fetal calf serum. Cultures were incubated at 37°C in humidified air containing 5% CO_2. After 5 days, 0.5 mL of new medium was added per well, and thereafter, the medium was replaced every 3 days.

Identification of the endothelial origin of isolated cells

Enodthelial cells in culture were identified by their characteristic cellular morphology, growth pattern and the presence of factor VIII-related antigen. Factor VIII-related antigen was demonstrated by commercially available kits of Biotin-Streptavidin System (BioGenex laboratories, San Ramon, CA). Briefly explained, cells grown on culture plates were routinely rinsed twice with phosphate buffered saline (PBS) and fixed with methanol at -20°C for 15 minutes. After blocking endogenous peroxi-

dase and non-specific protein binding with 3% hydrogen peroxide and normal rabbit serum, the cells were incubated with rabbit anti-human factor VIII-related antibody for 30 minutes at room temperature. The cells were then incubated with biotinylated goat anti-rabbit immunoglobulin for 30 minutes at room temperature. Subsequently, the peroxidase labeled streptavidin was applied to the cells. The substrate chromogen (3-amino-9-ethylcarbazole) and hydrogen peroxide solution in acetate buffer were added to the cultured cells and a rad to brownish-red color change was observed. Controls for non-specific binding were included.

Subcultivation

The medium was aspirated off, the cells were briefly washed twice with basal medium at 4°C, and 0.05% (wt/vol) trypsin in basal medium was added (0.5 ml per well). The trypsin solution was aspirated off 10 seconds after its addition, leaving only a thin film of fluid over the cells. Then, 1 ml of basal medium containing 5% fetal calf serum was added to each well after 2-3 minutes at room temperatrue, when the cells were rounded up and were beginning to detach from the surface of plates. By gentle agitation with a Pasteur pipette, endothelial cells were released from the culture surface. The suspension was then centrifuged at 250× *g* for 2 minutes, The cell pellet was resuspended in basal medium containing 20% fetal calf serum and the cells were plated at a 1 : 3 ratio into gelatin-coated wells. The medium was replaced every 3 days.

RESULTS

Morphological appearance

When the isolated cells were seeded to gelatin coated culture plates, explants and clumps of 15～30 rounded cells could be seen floating in serum-free medium. Virtually all of these cells from the initial inoculom attached to the gelatin-coated culture plates within 24 hours. Explants and isolated cellular clumps were examined daily by phase contrast microscopy. Proliferation of endothelial cells started at

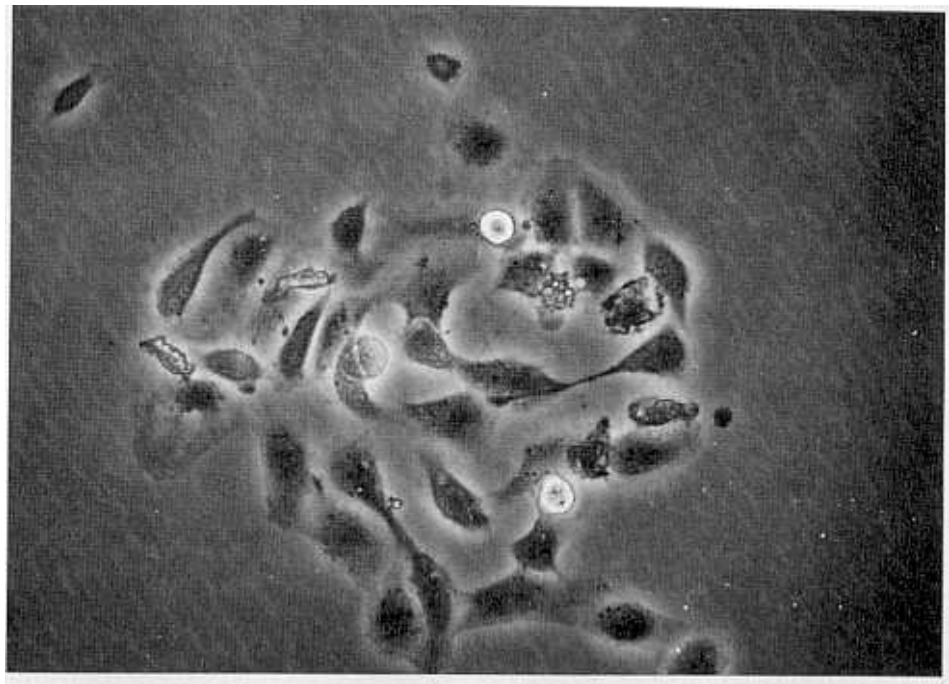

Fig. 1. A. Aggregates of microvascular endothelial cells. Endothelial aggregates composed of several microvascular endothelial cells. Some of these cells show cytoplasmic extension. Twenty for hours after plating. One percent gelatin coated culture plate and serum free medium containing endothelial cell growth supplement were used. × 320

the periphery of explants and isolated clumps of cells (Fig. 1, A and B). The growth of each colony occurred rapidly and was directed radially away from its center. The cells were homogenous, closely opposed, flat and polygonal with round or oval nuclei containing several prominent nucleoli. Cytoplasmic extensions were observed in some cells. This unique growth pattern was maintained throughout the culture, and the colonies increased in size as the cells proliferated. After 7 days in primary culture, the cells approached confluence and the number of cytoplasmic extensions decreased. They formed a monolayer which demonstrated contact inhibition and the uniform cobblestoned appearance typical of endothelial cells (Fig. 2, A and B). Primary isolations with the culture conditions of gelatin-coated surfaces and serum-free medium have been repeatedly performed with no fibroblast or smooth muscle cell contamination noted by phase-contrast microscopy and immunohistochemical study. when the cells were allowed to remain confluent for longer than 3 weeks in primary culture, they became large and many vacuoles were formed in cytoplasms (Fig. 2C). The cells also had loss of surface glittering.

Subcultivation

According to the method described above, the cells have been subcultured up to 3 passages over a period of 1 month. The split ratio was 1 : 3. Basal medium containing 20% fetal calf serum was used and no exogenous growth factors were added. The cells maintained their uniform appearance through 3 passages suggesting that they were essentially pure cultures (Fig. 6). When immunohistochemical staining was performed on secondary cultures, the cells were found to express factor VIII-related antigen.

Immunohistochemisty

The cultured cell monolayer grown on gelatin-coated culture plates was stained for factor VIII-related antigen. Virtually 100% of the cells stained positively for factor VIII-related antigen, the endothelial cell-specific product. Namely, they demonstrated a pattern of dot staining localized in cytoplasmic granules, especially the perinuclear region (Fig. 3A). Non-endothelial L929 cells were negative

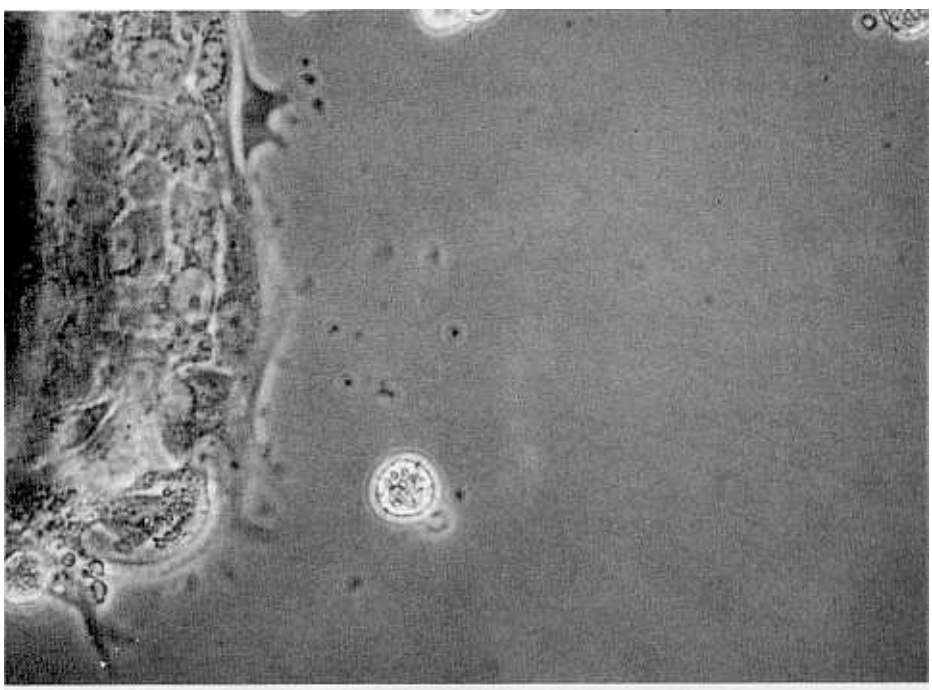

Fig. 1. B. Microvasular endothelial cells growing from a tissue explant. Twenty four hours after plating. One percent gelatin coated culture plate and serum free medium containing endothelial cell growth supplement were used. × 320

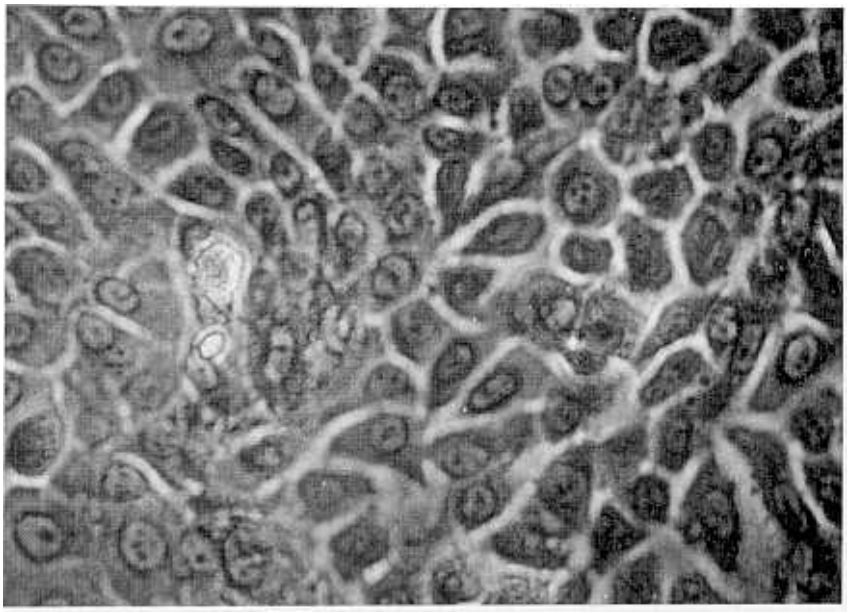

*Fig. 2. **A.** A monolayer of microvascular endothelial cells grown from aggregates. Cultured cells formed a monolayer which demonstrated contact inhibition and the uniform cobble-stoned appearance typical of endothelial cells. Note their flat and polygonal morphology, round or oval nuclei, and prominent nucleoli. Seven days after plating. One percent gelatin coated culture plate and serum free medium containing endothelial cell growth supplement were used. × 320*

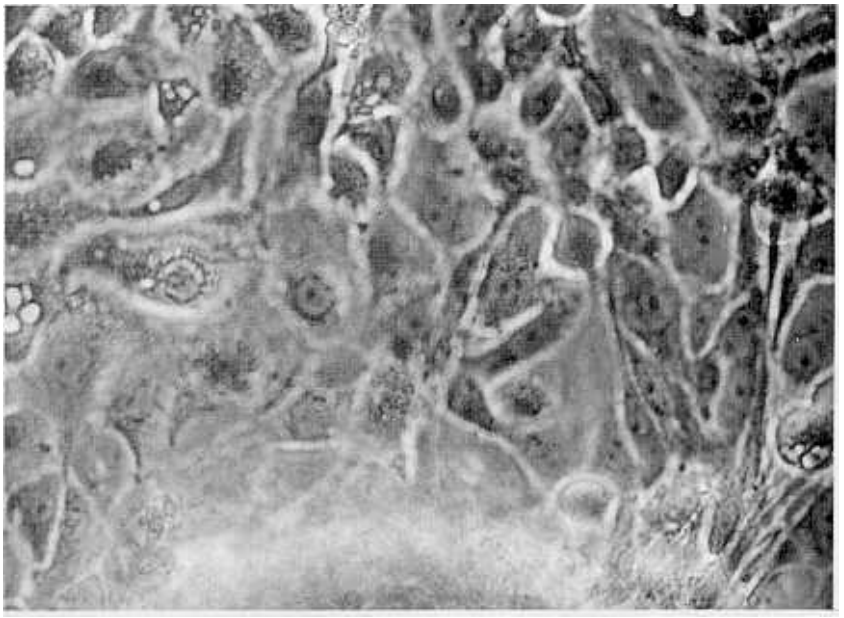

*Fig. 2. **B.** A monolayer of microvascular endothelial cells grown from a tissue explant. Cultured cells spread out from a tussue explant and formed a typical endothelial monolayer. Seven days after plating. One percent gelatin coated culture plate and serum free medium containing endothelial cell growth supplement were used. × 320*

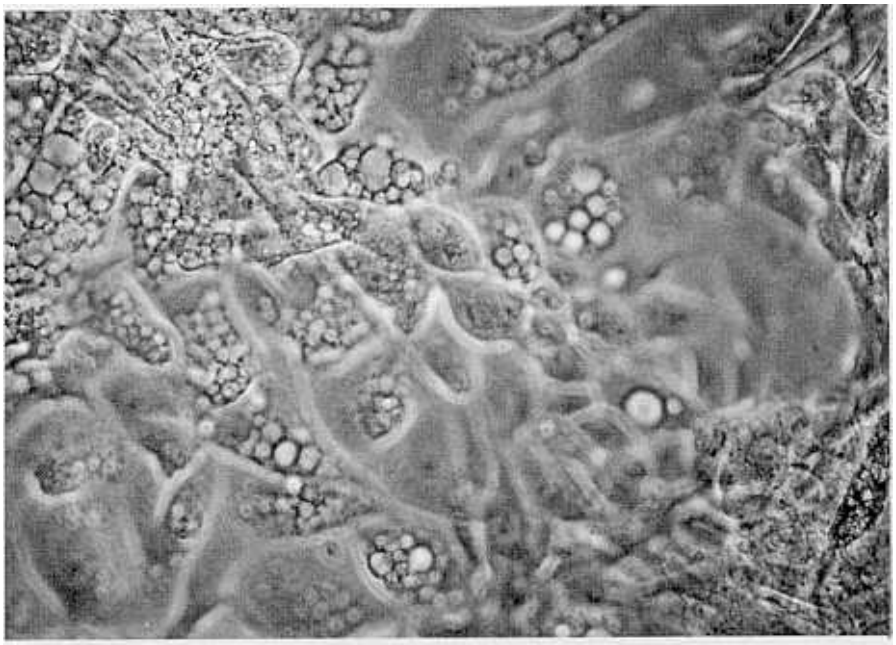

Fig. 2. C. Microvascular endothelial cells consisted of enlarged cells with numerous vacuoles. At the end of lifespan, microvascular endothelial cells showed increased cell size and accumulated numerous vaculoes. Twenty one days after plating. × 320

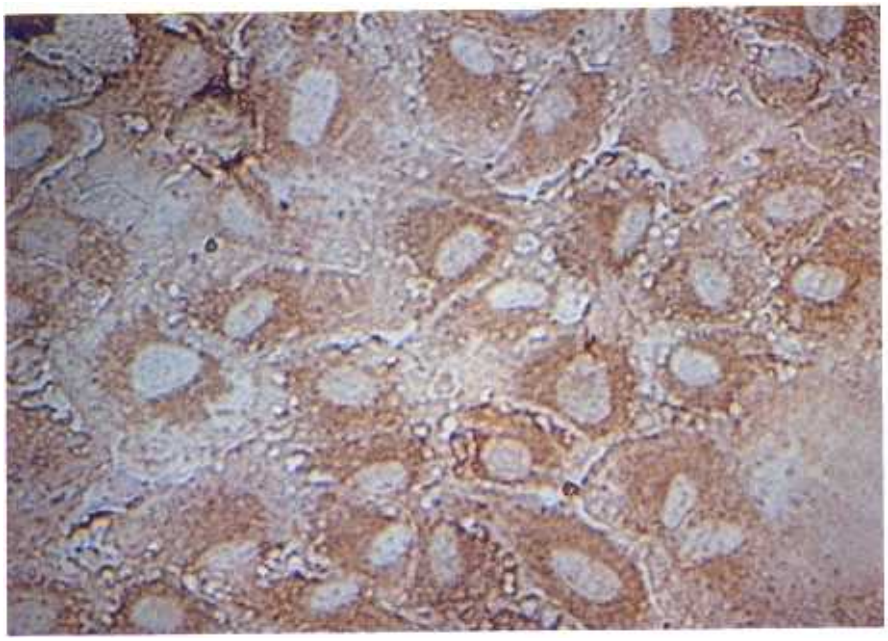

Fig. 3. A. Immunohistochemical test for factor VIII-related antigen in microvascular endothelial cells. Cultured cells expressed factor VIII-related antigen in virtually 100% of cells. Note the cytoplasmic color change. × 320

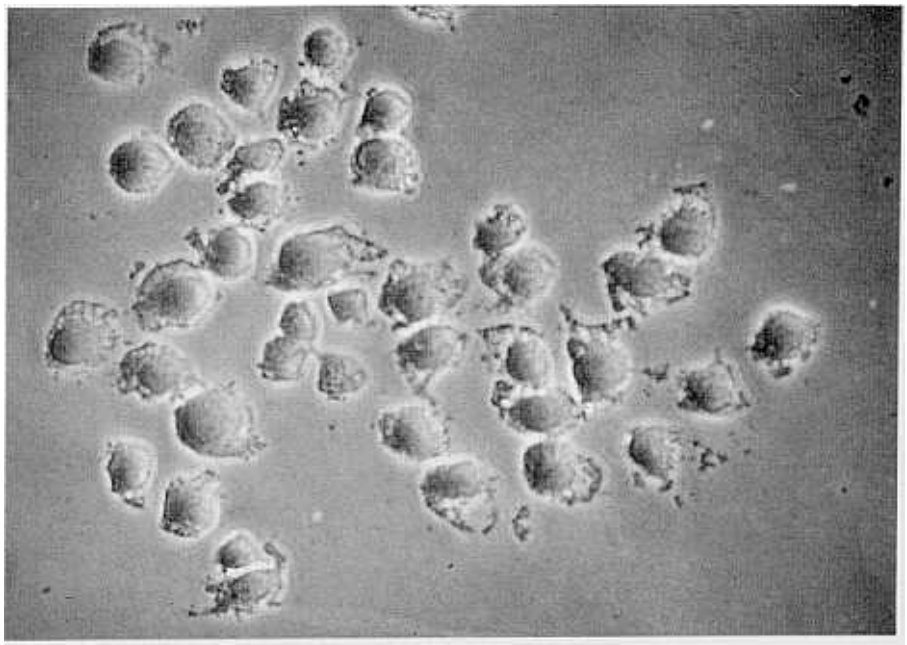

Fig. 3. B. Immunohistochemical test for factor VIII-related antigen in L929 cells. L929 cells did not express factor VIII-related antigen. × 320

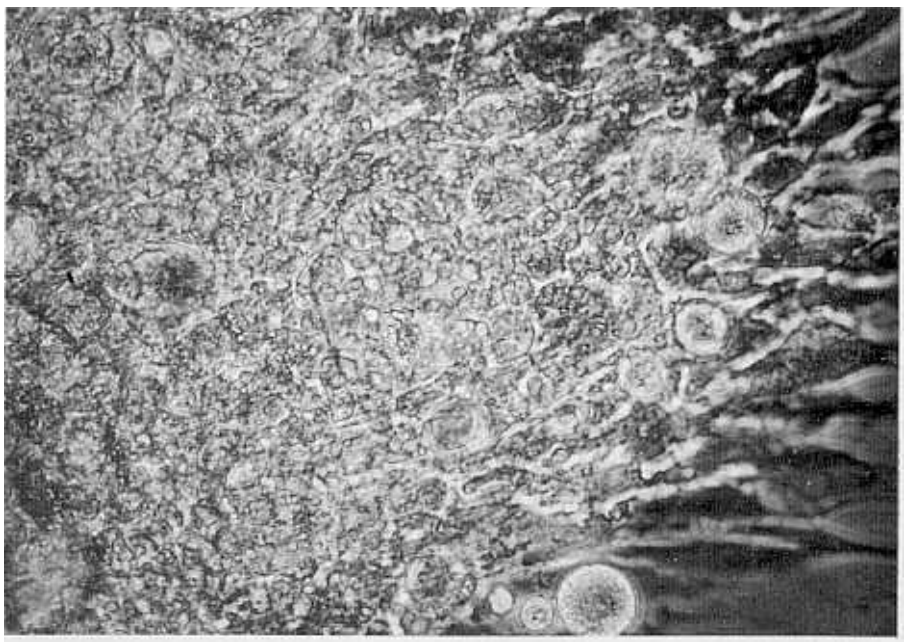

Fig. 4. Cells cultured in uncoated plastic culture plaste. Note their spindle-shaped morphology. Seven days after plating. × 320

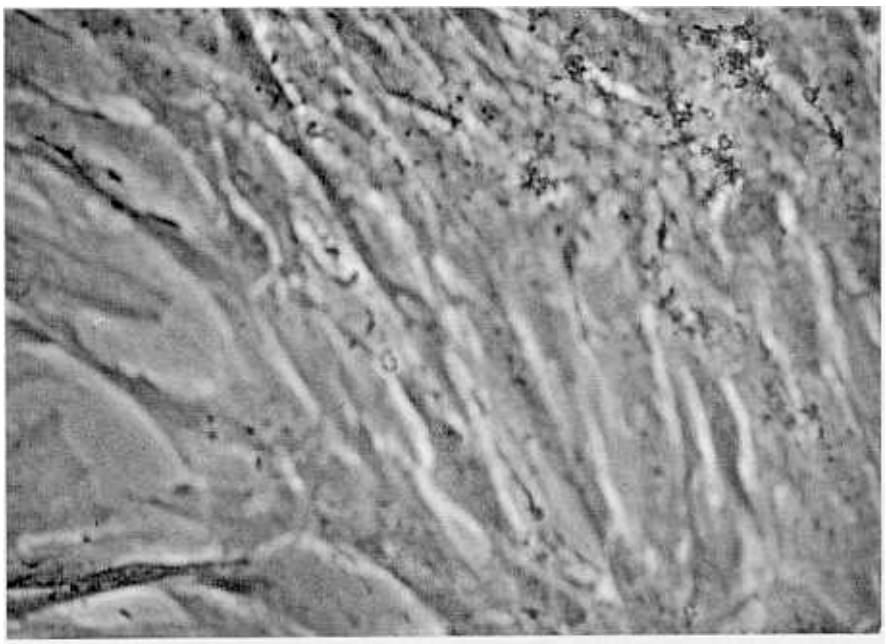

*Fig. 5. **A.** Cells cultured in RPMI 1640 medium supplemented with 10% fetal calf serum. They had a spindle-shaped, fibroblast-like morphology. Five days after plating. × 320*

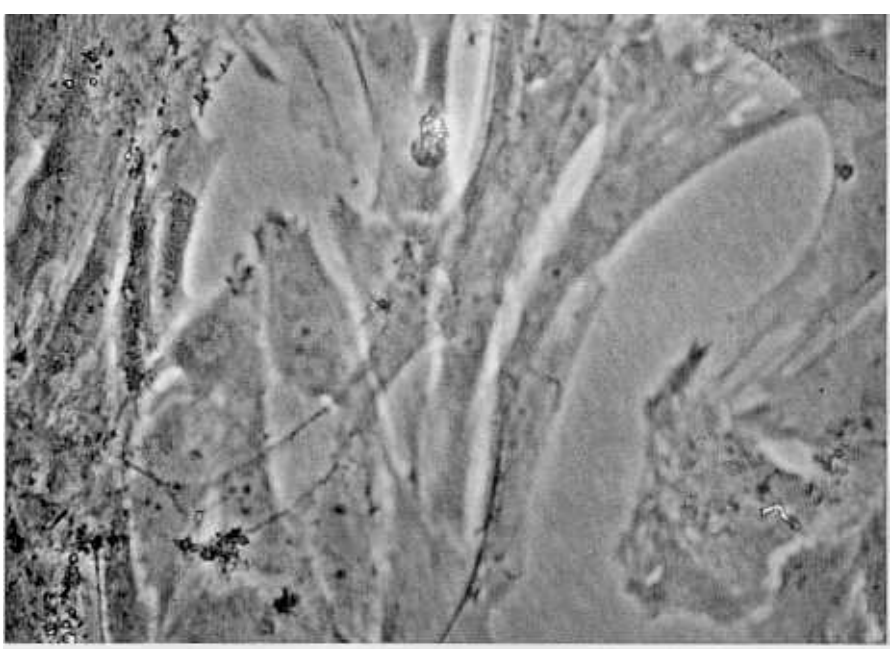

*Fig. 5. **B.** Immunohistochemical test for tactor VIII-related antigen in cells cultured in RPMI 1640 medium containing 10% fetal calf serum. These cells did not express factor VIII-related antigen. × 320*

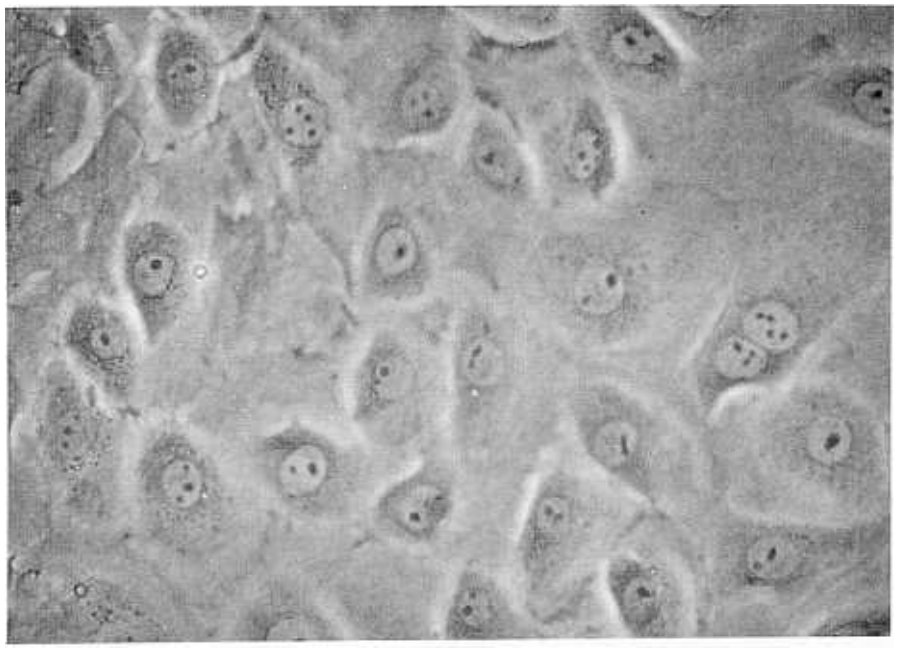

Fig. 6. Secondary passage of microvascular endothelial cells. The characteristic morphology of endothelial cells was shown. Seven days after plating. One percent gelatin coated culture plate and basal medium containing 20% fetal calf serum were used. × 320

for factor VIII-related antigen (Fig. 3B).

Effect of an extracellular matrix

The effect of gelatin in culturing microvascular endothelial cells was evaluated. Morphological and immunohistochemical studies showed that use of an estracellular matrix, gelatin, is essential to microvascular endothelial culture. In the absence of gelatin, proliferation of microvascular endothelial cells was not observed in the phase contrast microscope. Instead, the growth of spindle-shaped cells was observed (Fig. 4). This morphology and growth pattern were distinct from those seen in microvascular endothelium cultured on gelatin coated culture plates. Morevoer, they did not express factor VIII-related antigen. These spindle-shaped cells are thought to be fibroblasts.

Effect of serum in primary culture

In 20% serum-containing basal medium, extensive outgrowth of cells morphologically similar to fibroblasts was observed. These cells were long, slender, and spindle-shaped. They grew close to one another in parallel arrays with whorling and multiple overlapping layers (Fig. 5A). They did not express factor VIII-related antigen (Fig. 5B). It was not possible to select the cell populations of endothelial morphology in such medium.

DISCUSSION

Microvascular endothelium differs from large vessel endothelium in several ways (Zetter 1980; Zetter 1981; Gumkowki *et al.* 1987; Speiser *et al.* 1987). Microvascular endothelial cells are involved in angiogenesis (Form *et al.* 1986), inflammation (Harlan 1985), hematogenous metastasis (Alby and Auerbach 1984; Auerbach *et al.* 1987) and many pathologic states (Williams *et al.* 1981; Marks *et al.* 1985). They also act as a permeability barrier to plasma, allowing the selective, active transfer and metabolism of many substances (Scholefl 1963). Therfore, it is inappropriate to use endothelial cells cultured from major blood vessels in these kinds of studies.

The study of vascular endothelial cell biology, in general, has increased since the routine culture and

identification of these cells isolated from umiblical veins were first demonstrated (Jaffe 1973). However, the cultivation of microvascular endothelial cells is more difficult than that of large vessel endothelial cells. Early attemps to culture microvascular endothelial cells were unsuccessful because of an inability to grow them in homogeneous populations. The main problems are difficulties in isolation of microvascular endothelial cells and fastidious culture conditions. In this study, the cells isolated from rat lungs were cultured using gelatin coated surface and serum-free culture medium supplemented with endothelial growth supplement. After 7 days in culture, the cultured cells displayed a monolayer with the typical cobble stoned appearance (Fig. 2, A and B). The cells are characterized as endothelial cells by the presence of factor VIII-related antigen, the most commonly employed marker for vascular endothelial cells (Fig. 3 A). Factor VIII-related antigen is only found elsewhere in platelets and megakaryocytes, so it can provide a mean of identification and a measure of non-endothelial contamination (Jaffe 1977). In this study, nearly all cultured cells were positive for factor VIII-related antigen.

Primary endothelial cells began to display degenerative changes and lost their proliferative capacity after 3 weeks in culture. They became large and contained many cytoplasmic vacuoles (Fig. 2 C). Possible mechanisms of the cessation of proliferation are thought to be terminal differentiation and inadequate culture conditions (Mueller *et al.* 1980; Rosen *et al.* 1981)

The use of extracellular matrix such as gelatin, collagen, fibronectin, and laminin is an important factor for the active proliferation of microvascular endothelial cells (Madri and Williams 1983; Kleinman *et al.* 1987; Carley *et al.* 1988; Nicosia and Ottinetti 1990). We compared the different pattern of proliferation and morphology of cultured cells between gelatin coated surface and uncoated plastic surface. The cells grown on gelatin coated surface showed active proliferation. Their morphology and immunohistochemical study confirmed that they were microvascular endothelial cells (Fig. 2, A and B; Fig. 3A). In contrast, the cells, cultured on plastic surface had spindle shape and did not express factor VIII-related antigen (Fig. 4). They did not achieve confluence and showed degenerative changes after 2 weeks in culture. These findings may be explained by the higher attachment rate of freshly isolated endothelial cells onto the gelatin treated culture plate than the bare plastic culture plate (Ingber and Folkman 1989). It is also possible that gelatin activated the substance which stimulates the proliferation of microvascular endothelial cells (Schor *et al.* 1979). In other words, there is the possibility that the endothelial cells plated into uncoated plastic surface could not express the binding sites for growth factors or that the binding sites could be masked: Additional work is required to define these possible mechanisms. Furthermore, the role of other extravascular matrix, such as collagen, laminin and fibronectin in proliferation of microvascular endothelial cells should be also studied.

In primary culture of microvascular endothelial cells, the effects of serum were also evaluated. The cells cultured in serum-free medium showed typical morphology of endothelial cells and stained positively for factor VIII-related antigen (Fig. 2, A and B; Fig. 3 A). On the other hand, the cells cultured in serum-containing medium were spindle-shaped with several processes. These cells were microscopically quite unlike the cultured endothelial cells. They revealed features very much like fibroblasts, i. e., long, slender cells that show non-random orientation with the long axis parallel to each other (Fig. 5A). They grow as multilayered arrays of spindle-shaped cells. In addition, they did not express factor VIII-related antigen (Fig. 5B). This is especially true that serum-containing medium favors the overgrowth by fibroblasts, although the endothelial cells isolated from skin are rountinly cultured in serum-containing medium (Davison *et al.* 1980; Davison *et al.* 1983; Marks *et al,* 1985). The cells cultured in serum-containing medium in this study are thought to be fibroblasts. However, the serum-free medium used in this study did not allow continuous passages of microvascular endothelial cell culture. In serial subcultures, basal medium containing 20% fetal calf serum was more effective. After elimination of non-endothelial cells by using serum-free medium, serum-containing culture medium is thought to be necessary for effective serial cultivations.

We have shown that chemically defined serum-free medium and gelatin-coated culture plates can prevent overgrowth of fibroblasts or other undesired cells. In culturing microvascular endothelial cells from rat lungs, these two culture conditions are essential for selective proliferation, especially during the early stage of culture. Further investigation for the exact mechanisms of cell-matrix interaction is thought to be required. In addition, the degenerative changes of microvascular endothelial cells could be delayed by optimizing the culture conditions such as cell number in plating, split ratio

in subculture and the interval of medium change.

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