GROWTH AND MORPHOLOGY OF RABBIT MARGINAL VESSEL ENDOTHELIUM IN CELL CULTURE

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ABSTRACT

A procedure for the isolation and cultivation of endothelium from the marginal vessels of the rabbit ear is described. Endothelial cells, isolated by slow perfusion with a trypsin solution, are cultured in minimal essential medium supplemented with 10% fresh rabbit serum for up to 6 mo. In primary culture, marginal vessel endothelial cells grow in an expanding circular pattern with closely apposed cell membranes. Weibel-Palade bodies, subcellular organelles unique to endothelial cells *in situ*, are present in both primary and in serially cultivated cells (12 passages). In intact skin, Weibel-Palade (W-P) bodies are observed in the perinuclear cytoplasm in close proximity to the cell membrane facing the vascular lumen. 8–16 tubules of 200 Å diameter are present in each body. In primary and subcultured cells, W-P bodies of identical size are seen in the vicinity of the Golgi apparatus and in close proximity to the outer cell membrane. At the optimum serum concentration (10%), a cell doubling time of 72–96 h is observed. When growth in normal rabbit serum and in platelet-poor serum is compared, a slower growth rate is observed in the absence of platelets, suggesting that factors released by platelets affect endothelial cell proliferation. However, addition of crude platelet factor does not substitute for complete serum. Fibroblast growth factor is not mitogenic for rabbit marginal vessel endothelium in vitro.

Valuable information on many aspects of endothelial cell function and biochemistry has been obtained using the techniques developed in recent years for the in vitro cultivation of endothelium from large vessels such as the umbilical vein and aorta (8, 13, 31). Attempts have been made to adapt these methods for microvascular endothelium (19, 34), but the long-term cultivation of such cells has not been achieved. In the skin, the role of the dermal vasculature in the maintenance of homeostasis is becoming increasingly apparent. For example, sprouting of new vessels occurs during revascularization after skin injury (23), and extensive vascular networks evolve to support the thickened epidermis seen in several skin disorders (22) and during tumor growth (6). To date, studies on the regulation of the dermal vasculature have been limited to in vivo models. Differences in the ultrastructure of endothelium from the various regions of the vascular network, with respect to the number of Weibel-Palade (W-P) bodies (30), type of endothelial cell junctions (29), or the extent of the cytoplasmic microfilaments (15), have indicated that large vessel endothelium cultivated in vitro may not be a suitable model for study of microvascular function. Classically, the marginal vessels of the rabbit ear have been used as a model system for the study of microvessel proliferation and the

development of a vascular network (26, 32). To simplify investigation of the role of endothelial cells in these processes, we have developed a method to isolate and maintain in vitro endothelial cells from the marginal vessels. A mixed population of endothelial cells from the marginal artery and vein together with microvessel endothelium from arterioles, venules, and capillaries is obtained after perfusion of the vasculature with trypsin solution. The identification of the released cells as endothelial in type by morphologic and ultrastructural criteria, and their growth characteristics and requirements in vitro are described. A preliminary report of this work has appeared (4).

MATERIALS AND METHODS

Isolation of Cells

Adult New Zealand rabbits (2–3.5 kg) were killed by barbiturate overdose and the ears were removed. The pelage was clipped closely with an electric clipper (Oster Corp., Milwaukee, Wis.), and a collecting point was cut at the base of the ear as illustrated in Fig. 1. The cut surface was immersed in sterile Hanks' balanced salt solution containing penicillin (400 U/ml) and streptomycin (200 μg/ml) for 10 min. A small incision was made in the marginal vein, and a polyethylene cannula (polyethylene tubing Intramedic No. 7405, Clay-Adams, Div. Becton, Dickinson & Co., Parsippany, N. J.; i.d. 0.015 inches, o.d. 0.043 inches) with a beveled edge was inserted and pushed ~1 cm into the vein. Blood was flushed from the vascular network of the ear with ~2 ml of an isotonic salt solution (GKN) pH 7.2 (per liter: 1 g glucose, 8 g NaCl, 0.4 g KCl). This was replaced with GKN containing 1% trypsin (ICN Pharmaceuticals, Inc., Life Sciences Group, Cleveland, Ohio), and the cannulated ear was placed in a large petri dish and incubated at 37°C for 50 min. After removal of the ear from the incubator, ~1 ml (15–25 drops) of trypsin solution was perfused gently through the ear vasculature. The perfusate was collected in a centrifuge tube containing 2 ml of minimal essential medium (MEM) (Grand Island Biological Co. [GIBCO] Grand Island, N. Y.) containing 10% calf serum (Irvine Scientific Co., Santa Ana, Calif.) and antibiotics as shown in Fig. 1, and the ear was returned to the incubator for an additional 30-min incubation after which the collection of cells was repeated. The cells released from several ears were pooled and collected by centrifugation, resuspended in MEM containing 10% rabbit serum, penicillin (200 U/ml), and streptomycin (100 μg/ml), and plated onto 35 × 10 mm plastic petri dishes (Lux Scientific Corp., Newbury Park, Calif.) at a cell density of ~1 to 3×10^4 cells/dish.

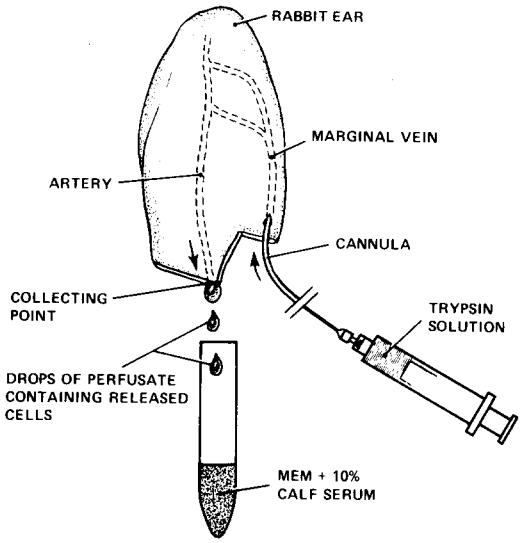


FIGURE 1 Schematic representation of steps involved in the perfusion and collection of endothelial cells from the marginal vessels of the rabbit. The vascular network of the rabbit ear is perfused with a 1% trypsin solution via a polyethylene cannula inserted into the marginal vein. After incubation at 37°C a small volume of trypsin solution (1.0 ml) is perfused through the vasculature. Drops of perfusate containing released endothelial cells are collected (at a point cut at the base of the ear) in a centrifuge tube containing MEM and 10% calf serum.

Subcultivation

Primary cultures which achieved at least 50% confluence within 2 wk were used for subculture. Cells were released from the petri dish surface by incubation with 0.1% trypsin, 0.33% EDTA in phosphate-buffered saline (PBS), pH 7.3 (per liter: 8 g NaCl, 2 g KCl, 1.15 g Na_2HPO_4, 0.2 g KH_2PO_4) for 4 min at 37°C, dispersed by gentle agitation with a Pasteur pipette, and collected by centrifugation at 800 *g* for 1 min. The cells were resuspended in MEM with 10% rabbit serum with antibiotics and plated at a cell density of 5–10 × 10^4 cells/35-mm petri dish. As subcultured cells approached confluence, they were further serially cultivated using the procedure described above.

Cell Identification

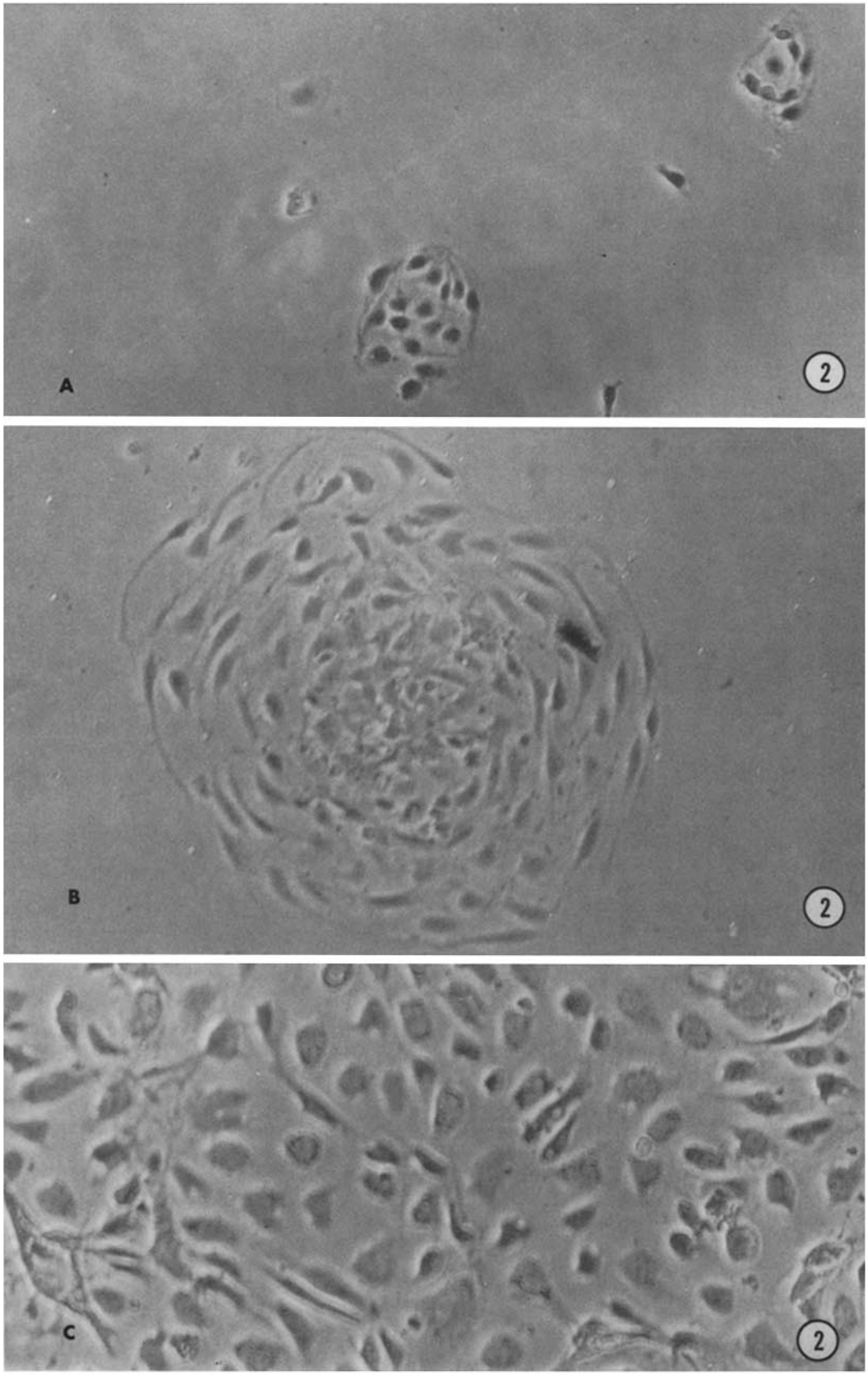
Other Cell Types

For morphologic comparison, rabbit epidermal keratinocytes were isolated and grown in vitro as described by Liu and Karasek (16). Fibroblasts were isolated from cell outgrowths of rabbit skin organ cultures (3).

Preparation of Tissue Cells for Electron Microscopy

Cells were released from the culture surface by incubation with 0.1% trypsin, 0.33% EDTA in PBS as described above for subcultivation, resuspended in MEM with 10% rabbit serum (2.0

FIGURE 2 Attachment and growth of marginal vessel endothelium in primary culture. (*A*) 24 h after plating; (*B*) day 3 in culture; (*C*) day 11 in culture. × 3,926.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ml/dish), then fixed by the addition of 3.0 ml of 2.0% buffered glutaraldehyde solution, pH 6.8 (9, 25). After centrifugation (1 min at 800 *g*), the cell pellet was covered with a layer of fresh fixative. After rinsing in isotonic wash solution, the cell pellet was postfixed in 1% osmium tetroxide for 1 h, dehydrated, and embedded in Maraglas (Marblette Co., Div. of Allied Products Corp., Long Island City, N. Y.) (5, 18). 1-μm-thick sections of the plastic-embedded preparation were stained with metachromatic dyes for examination by light microscopy (21). Ultrathin sections stained with uranyl acetate and lead citrate were examined in a Siemens Elmiskop 101 (33).

Pieces of skin measuring 1–2 mm^3 were removed from the rabbit ear, immediately fixed in 2% phosphate-buffered glutaraldehyde solution (pH 6.8), and subsequently prepared for electron microscope examination as described above for the isolated cells.

Cell Proliferation Studies

Growth Requirements

SERUM TYPE AND CONCENTRATION: Freshly isolated or first-passage serially cultivated rabbit marginal vessel endothelial cells were suspended in MEM containing 10% heat-inactivated rabbit serum prepared in the laboratory (see below) and plated onto 35-mm plastic petri dishes at a plating density of 1 to 5 × 10^4 cells/dish. Cells were maintained in a humidified atmosphere of 94% air, 6% CO_2 at 37°C. The medium was removed 24 h later and the cells were refed with either the same medium or MEM containing 10% calf or horse serum (Irvine Scientific Co.), pooled human serum (see below), or rabbit serum (GIBCO). All sera were heat inactivated at 56°C for 30 min. For other experiments, cells were grown in MEM supplemented with 0.5, 2.0, or 10% rabbit serum. In all cases cells were fed twice weekly for 14 d. Growth achieved during this period was determined by counting the cell number or by $^{32}PO_4^{3-}$ incorporation as described below.

PLATELET-POOR SERUM: Platelet-poor serum (PPS) was prepared from fresh rabbit blood carefully collected via heart puncture in a chilled plastic syringe. The blood was immediately cooled to 4°C and spun in a refrigerated centrifuge at 1,000 *g* for 10 min to sediment the blood elements. The plasma was removed and further centrifuged at 4°C at 10,000 *g* for 10 min. The plasma was decanted and clotting was initiated by the introduction of a glass stirring rod. PPS was squeezed from the fibrin clot and filtered through a Millipore filter (Millipore Corp., Bedford, Mass.) before heat inactivation at 56°C. Fresh rabbit blood allowed to clot before removal of the blood elements was used as the control rabbit serum in these experiments. The absence of platelet factors in PPS was demonstrated by the failure of 3T3 cells to proliferate in the presence of PPS alone.

PLATELET EXTRACT: A platelet growth factor was prepared from human platelets obtained from outdated platelet-rich plasma no longer suitable for clinical use, by the method of Antoniades and Scher (1). The protein content of this boiled platelet extract was determined by the method of Lowry et al. (14) and its activity quantitated by use of a 3T3 cell assay (20). Addition of the platelet extract (1.5–3 μg protein) to cultures of 3T3 cells maintained in PPS was found to support the growth of 3T3 cells which failed to survive in PPS alone.

MEDIA: The growth of rabbit marginal vessel endothelium in MEM supplemented with either serum or PPS was compared to that achieved in Dulbecco's modified essential medium (DMEM).

FIBROBLAST GROWTH FACTOR: Fibroblast growth factor (FGF) of bovine pituitary origin was kindly supplied by Dr. Dennis Gospodarowicz (Cancer Research Institute, University of California, San Francisco). The lyophilized FGF was dissolved in MEM and diluted such that final concentrations of between 50 and 500 ng/ml culture medium were achieved.

Determination of Cell Proliferation

CELL NUMBER: Endothelial cells were released from the culture surface by brief trypsinization as described above for subcultivation. The number of cells in an aliquot of the resulting cell suspension was counted with a haemocytometer and a Nikon inverted phase-contrast microscope.

INCORPORATION OF $^{32}PO_4^{3-}$: Primary and serially cultured endothelial cells were incubated in complete medium containing 2 μCi/ml $^{32}PO_4^{3-}$ (carrier free, Schwartz/Mann Div., Becton, Dickinson & Co., Orangeburg, N. Y.). $^{32}PO_4^{3-}$ incorporation into TCA-precipitable material was determined by scintillation counting as described previously (16).

RESULTS

Isolation of Cells

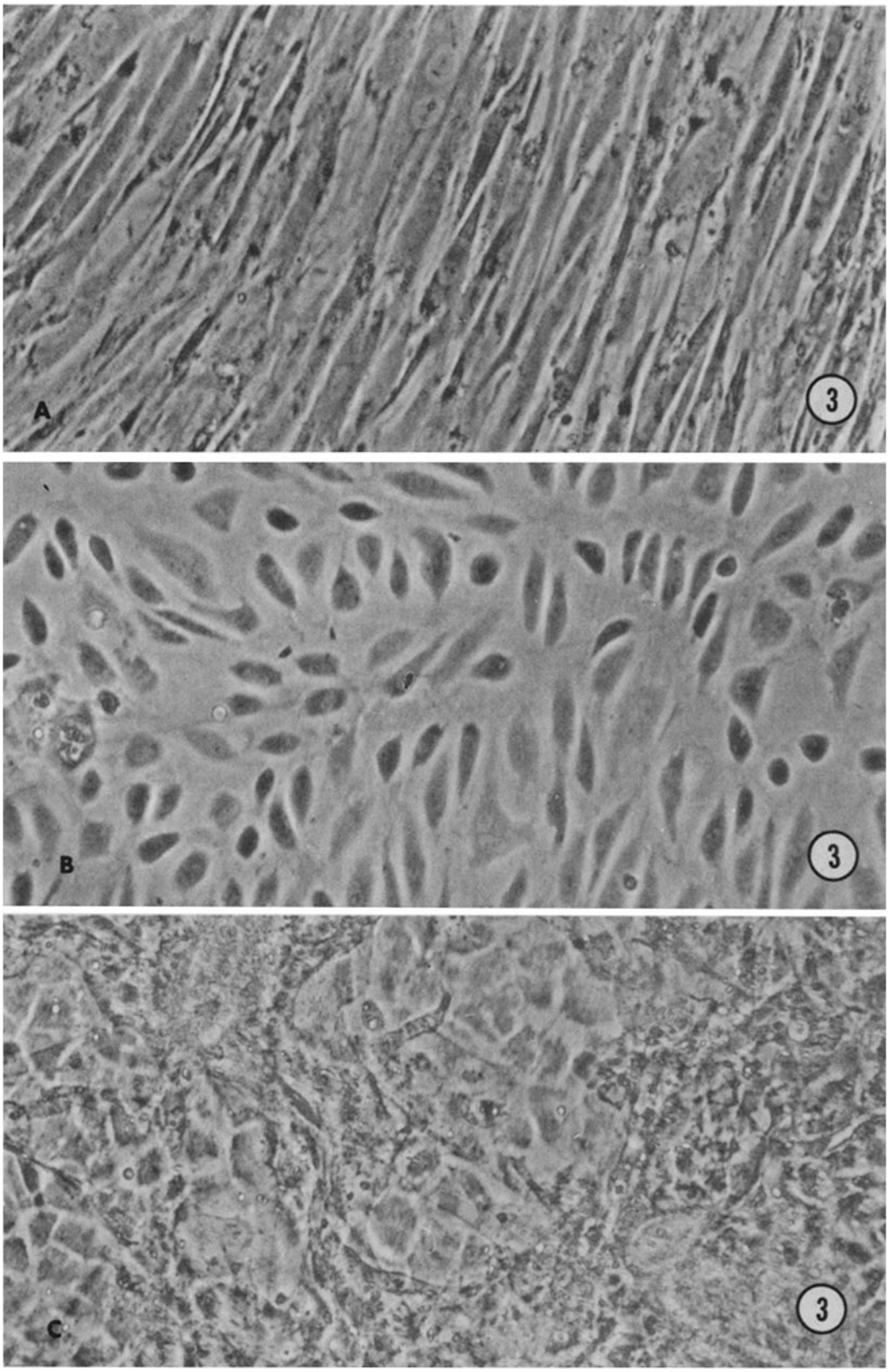
At trypsin concentrations below 1%, few endothelial cells were released even after extended (2-h) incubation at 37°C. Trypsin infiltration of the surrounding tissues was a common occurrence, resulting in the release of various other nonendothelial cell types. The maximum yield of endothelial cells essentially free of other cell types was obtained after a 50-min incubation with higher concentrations of trypsin solution (between 1 and 2% in GKN) followed by a further 30-min incubation and a second endothelial cell harvest. The percentage of nonendothelial cell types increased markedly if the incubation time was extended.

Identification of Cells

Phase Microscopy

We could detect no morphological difference between the cells harvested from the first and second collections. In both cases, the majority of the cells that settle down and attach within 24 h

FIGURE 3 A comparison of the typical in vitro morphology of confluent cultures of various cell types from rabbit skin: (*A*) fibroblasts; (*B*) endothelial cells; (*C*) epidermal keratinocytes. × 4,640.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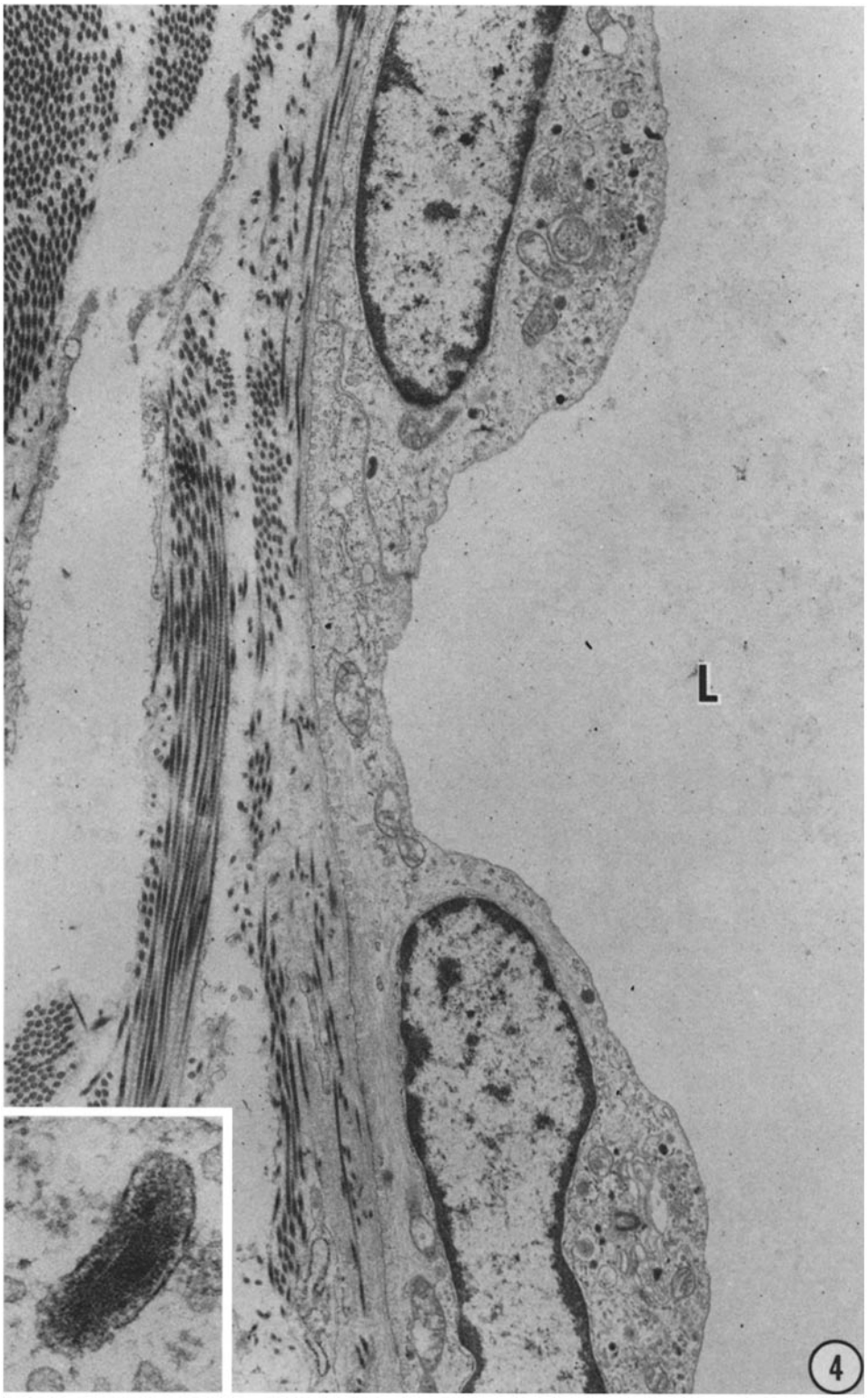
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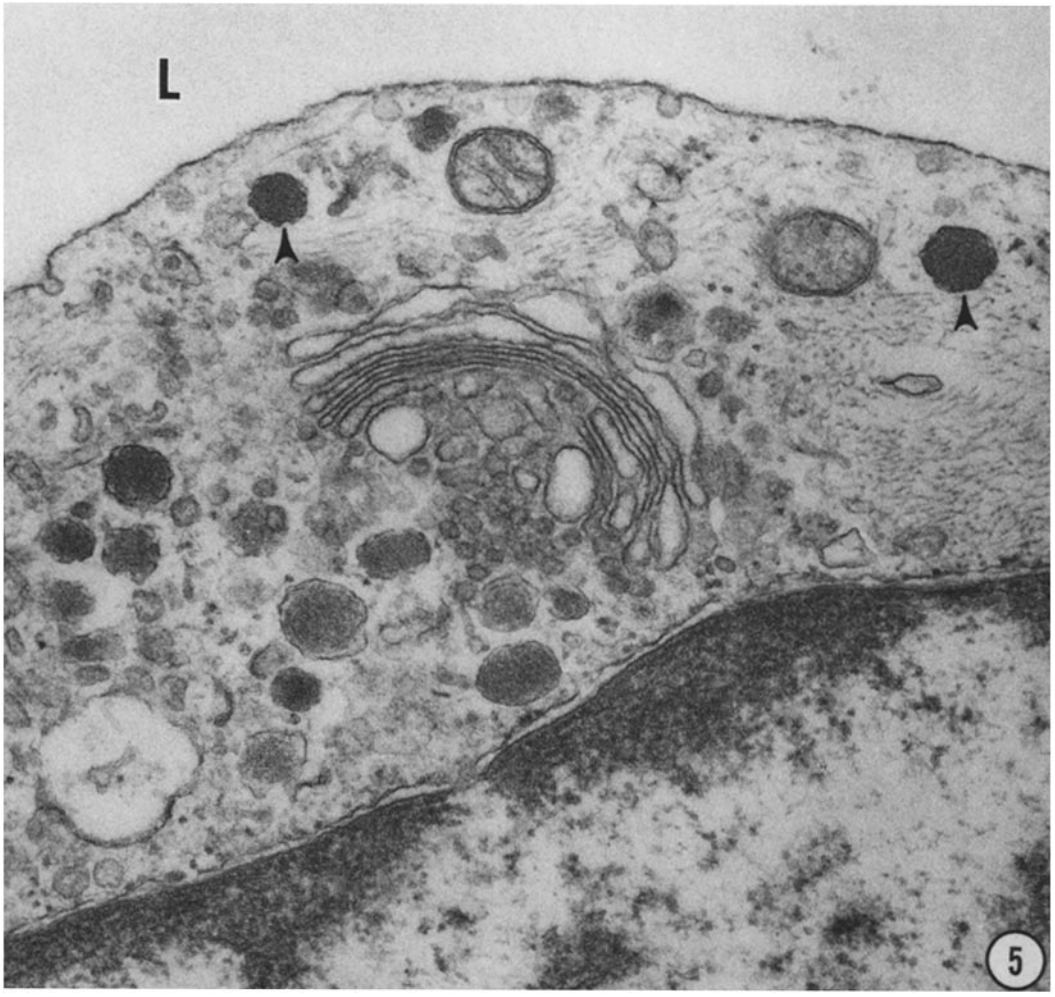

FIGURE 5 Higher magnification of the Golgi complex showing W-P bodies in various stages of assembly. The mature bodies tend to align near the cell membrane facing the vascular lumen (arrowheads). *L*, vascular lumen. × 56,000.

was released as small cell sheets (Fig. 2*A*) which grew into circular colonies of closely apposed polygonal cells with well-defined nuclei and indistinct cell borders. This circular configuration which was maintained throughout primary culture (Fig. 2 *B* and *C*) differed markedly from the morphology of rabbit dermal fibroblasts or epidermal keratinocytes, Fig. 3). The long, spindle-shaped fibroblasts (Fig. 3 *A*) grew in parallel arrays, contrasting with the cobblestone appearance of monolayer cultures of endothelial cells (Fig. 3 *B*). Epidermal keratinocytes (Fig. 3 *C*) grew in multilayered sheets and were smaller and more granular in appearance than the larger endothelial cells.

FIGURE 4 Part of a venule lined by two endothelial cells. The numerous electron-opaque round and elliptical bodies in the cytoplasm (between the nucleus and vascular lumen in these cells) usually occupied by the Golgi complex are W-P bodies. These bodies are present in much smaller concentrations in the attenuated parts of the endothelial cells (between the nuclei). *L*, vascular lumen. × 12,000. *Inset:* Higher magnification showing a single, tangentially sectioned W-P body located in the attenuated cytoplasm of an endothelial cell. × 82,000.

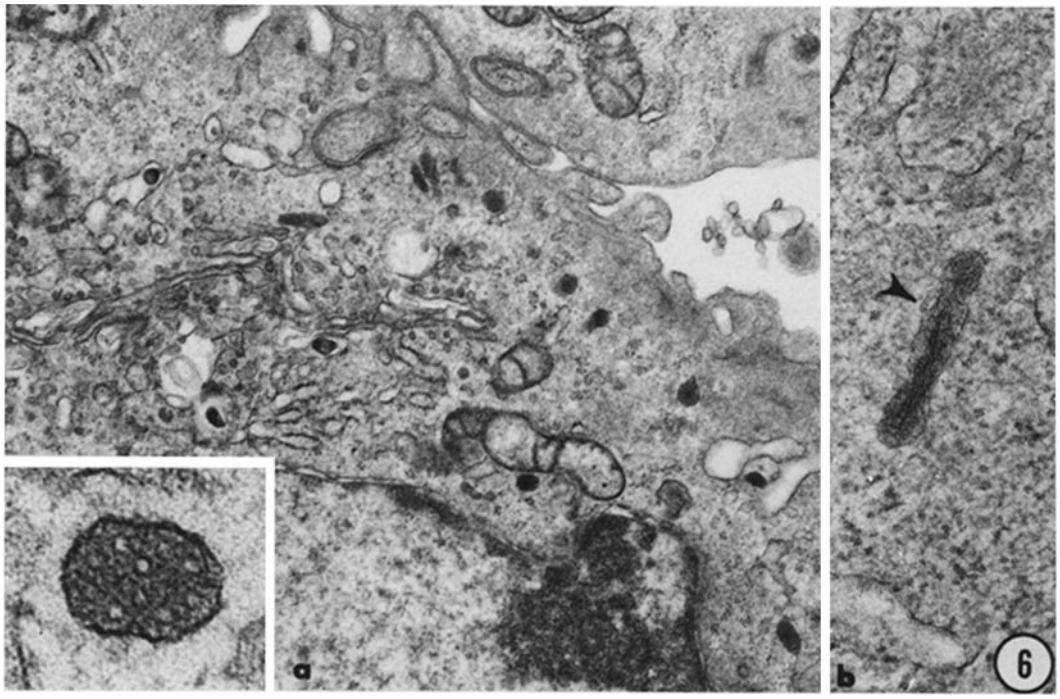

FIGURE 6 (*a*) A primary culture (10 d in vitro) shows both W-P bodies in the Golgi zone and their alignment near the cell surface. × 22,000. *Inset:* A section cut perfectly perpendicular to the long axis of a W-P body shows the body substructure which includes parallel tubules in the electron-opaque matrix of the core. × 190,000. (*b*) W-P body (arrowhead) in a cell of a subculture after 10 passages (5 mo in vitro). × 68,000.

ULTRASTRUCTURE

IN TISSUE SECTION: The endothelial cells lining the blood vessels of the rabbit ear, particularly the venules, contained moderate numbers of regular, rod-shaped structures, usually referred to as Weibel-Palade (W-P) bodies. These organelles were present singly in the attenuated parts of these cells (Fig. 4, *inset*). However, most of these bodies, often in clusters, were encountered in the perinuclear cytoplasm, usually in the vicinity of the Golgi apparatus, which in these cells was present in a bulging mass of cytoplasm between the nucleus and the vessel lumen (Fig. 4). Another notable feature was the propensity of W-P bodies to be located in close proximity to the cell membrane facing the vascular lumen, as demonstrated in Fig. 5. Although it was possible to determine the diameter of the W-P bodies (0.1–0.2 μm), their mean average length could not be accurately measured as very few of them were found lying in their entirety in the plane of an ultrathin section. Estimates made from tangentially cut W-P bodies suggested that these structures were in excess of 2 μm in length.

IN CULTURED ENDOTHELIAL CELLS: In primary culture and after 12 serial passages, the cells retained the fine structural features exhibited by endothelial cells in tissue sections (Fig. 6*a*). The mitochondria did not change in appearance and number, but there was an increase in the number of cisternae, particularly of the rough endoplasmic reticulum, and in the number of free ribosomes. Microfilaments were conspicuous, particularly when they formed a dense, often bundled, feltwork close to the part of the cell membrane in contact with the culture vessel. Pinocytotic vesicles and the Golgi apparatus remained prominent in the cultured cells. Present in the vicinity of the latter, but also frequently close to the outer cell membrane, were W-P bodies. The W-P bodies of rabbit cells had an electron-opaque core which, on cross section, was seen to contain tubular substructures running parallel to the long axis of the body (Fig. 6*a*, *inset*). Each of these tubules had an electron-translucent center surrounded by a thin

wall which is only slightly more electron-opaque than the core matrix of the W-P body. The diameter of these tubules, 8–16 of which are present in each body, was ~200 Å. The content of the W-P bodies was separated from the cytoplasm by a unit membrane-type envelope with little or no electron-translucent space between it and the core. W-P bodies were abundant in primary cell cultures, 5–10 W-P bodies per section being found in all cells. Their incidence decreased after repeated serial cultivation, but W-P bodies with features and dimensions as seen in endothelial cells *in situ* could still be identified in cells after 10 serial passages (Fig. 6). There were only one to two W-P bodies per section of one of ten cells in these cultures. It should be noted that one can obtain at least 100 sections per cell, but that the same W-P bodies would of course also appear in more than one section.

Cell Proliferation Studies

Requirements for Growth In Vitro

SERUM TYPE: The growth of primary and first passage subcultured cells in 10% heat-inactivated rabbit serum (prepared in the laboratory), calf and horse serum (Irvine Scientific Co.), and human serum (blood bank) is shown in Fig. 7. All sera supported proliferation of rabbit marginal vessel endothelial cells, but growth in commercially available rabbit serum failed to consistently equal that seen in fresh rabbit serum; moreover, there was considerable variability among batches. When fresh rabbit serum (10% in MEM) was used routinely, the cells retained their characteristic endothelial morphology for extended periods in culture. With horse serum and with some batches of fresh human serum, an increased number of lipoid granules (as occur at higher rabbit serum concentrations) appeared.

SERUM CONCENTRATION: The rate of growth of marginal vessel endothelial cells was dependent upon serum concentration (Fig. 8). In 0.5% serum, the cells failed to proliferate but remained in a quiescent state (at least for 14 d), as growth could be stimulated by the return to higher serum concentrations. The rate of cell proliferation increased with increasing serum concentration to a maximum in 10% serum, at which a population doubling time of between 72 and 96 h for first-passage cells was achieved. Higher serum concentrations (20–30%) did not further stimulate proliferation. The rate declined and dark lipoid granules (positive to oil red O stain) were formed and filled the perinuclear region.

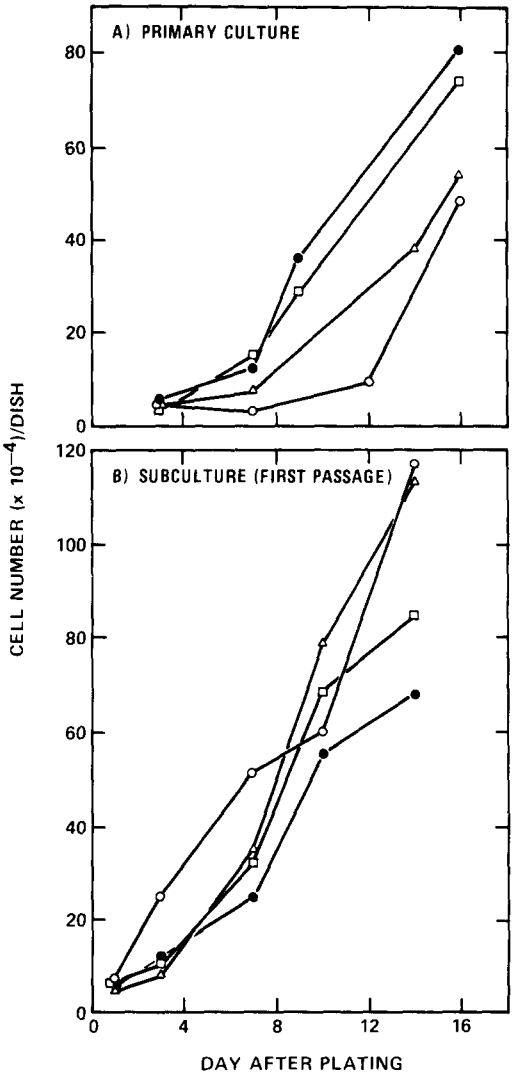

FIGURE 7 The proliferation rate of marginal vessel endothelial cells in various sera is compared. (○) 10% Rabbit serum, (□) 10% human serum, (△) 10% calf serum, (●) 10% horse serum.

MEDIA: There was no significant difference between the proliferation rate of rabbit marginal vessel endothelium in MEM or DMEM. The simpler medium MEM was used routinely thereafter.

PLATING DENSITY: Freshly isolated endothelium suspended in MEM with 10% rabbit serum was plated into 16-mm wells at various cell densities from 0.5 to 15 times those routinely used.

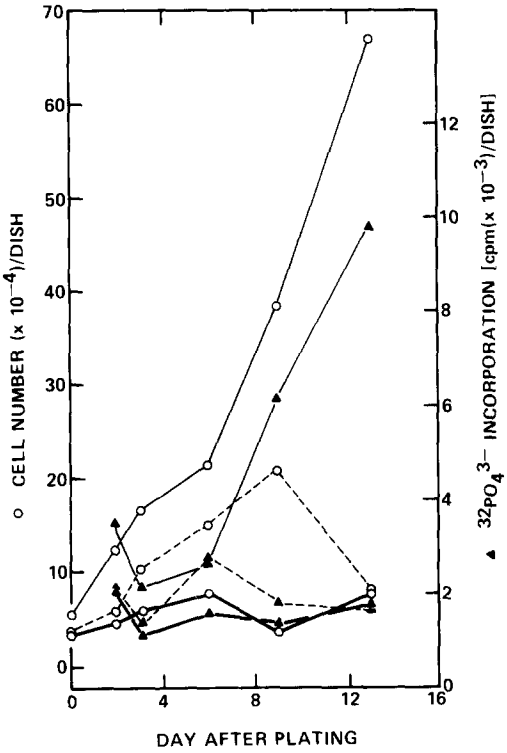

FIGURE 8 Growth of rabbit marginal vessel endothelium (first-passage subculture) at various rabbit serum concentrations. 0.5% Serum (○—○, ▲—▲), 2% serum (○- - -○, ▲- - -▲), and 10% serum (○-○, ▲-▲).

The plating efficiencies were above 80%, and the degree of cell confluence at 24 h corresponded to the plating density. The relative rate of growth was maximal at the "normal" plating density, declining for both the two highest (15 and 7.5 × normal) and the lowest (0.5 × "normal") plating densities. No other differences in the cell morphology or growth pattern of cells plated at the different cell densities were noted.

PROLIFERATION RATE IN WHOLE SERUM: The growth of primary and first-, second-, fourth-, and twelfth-passage endothelial cells in 10% rabbit serum is compared in Fig. 9. Exposure of primary cells to 0.1% trypsin and 0.33% EDTA to produce the first-passage cells stimulated the proliferative rate so that the population doubling decreased from ~5 d (rate seen after an initial lag period) to ~72 h. Thereafter the rate of proliferation decreased with each successive subculture and increased length of time in culture. Cells maintained in culture for 6 mo (twelfth passage) still contained W-P bodies but the cells failed to proliferate.

PPS: Rabbit marginal vessel endothelium was found to proliferate in MEM supplemented with 10% PPS. The growth was not so rapid as in a comparable concentration of serum (Fig. 10). Increasing the PPS concentration did not increase the rate of growth. Cells maintained in PPS failed to reach confluence, and the cell number began to decline after 10 d in culture.

PLATELET EXTRACT: The addition of platelet extract, 0.5 ml (15.0 μg protein/ml culture medium), to cells maintained in PPS increased the rate of growth above that in PPS alone (9×10^4 cells/dish on day 6 of culture, compared to 5×10^4 cells/dish in PPS alone). Early in the culture period this growth rate approached that seen in whole serum (10×10^4 cells/dish) on day 6. However, the addition of platelet extract failed to extend the proliferative period for cells maintained in PPS, and by day 9 growth in PPS plus platelet factor had fallen to 11×10^4 cells/dish compared to 21×10^4 cells/dish in whole serum.

FGF: FGF (50–500 ng/ml) failed to consistently stimulate the growth of rabbit marginal vessel vascular endothelium maintained in whole serum or PPS. In contrast, FGF (10–50 ng/ml) stimulated the rate of growth of quiescent 3T3 cells.

DISCUSSION

The findings presented in this report show that viable endothelial cells may be isolated from the rabbit marginal vein, artery, and capillary network

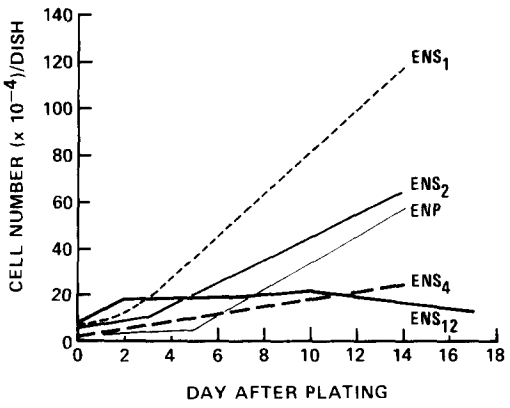

FIGURE 9 Comparison of the proliferation rate of rabbit marginal vessel endothelium in primary (*ENP*), first (ENS_1), second (ENS_2), fourth (ENS_4), and twelfth (ENS_{12}) serial passage.

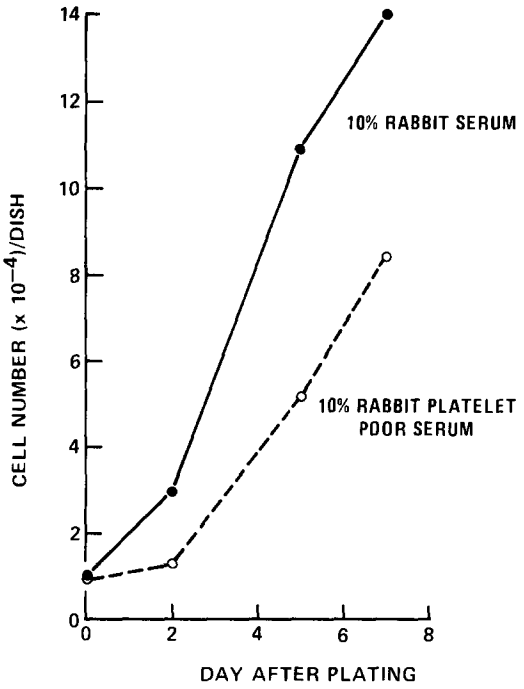

FIGURE 10 The reduced rate of growth of endothelial cells from the rabbit marginal vessels in platelet-poor plasma is compared to that seen in complete serum.

and maintained in vitro for periods up to 6 mo. These cultured endothelial cells grow to form colonies of large, polygonal cells with well-defined nuclei and indistinct cell borders similar to those seen for other types of endothelium in vitro (8, 10, 13, 17). Cell monolayers have a regular, cobblestone appearance. In comparison, fibroblasts and smooth muscle cells grow as parallel arrays of long, spindle-shaped cells. Epidermal keratinocytes form multiple layers of smaller, more cuboidal and granular cells that characteristically slough layers of flattened, mature "keratinized" cells.

Electron microscopy was used to monitor the composition and structural integrity of the cells collected from the rabbit vessels and, more importantly, their adaptation to growth in vitro. Fortunately, endothelial cells possess a unique cytoplasmic marker, the W-P body (36). These are slender, rod-shaped organelles of unknown function which, to our knowledge, occur only in endothelial cells of vertebrates; they are present in the endothelium of veins, capillaries, arteries, and endocardial cells (7, 27). In tissue sections, W-P bodies are found in highest concentration in the Golgi region of the endothelial cell. They appear in lesser numbers close to the cell membrane facing the vascular lumen and are sparse in the membranous parts of an endothelial cell. These preferential locations of W-P bodies are remarkably similar to those observed in cells growing in vitro, even after multiple passages, suggesting that they are assembled in the Golgi apparatus before they move to a position immediately beneath the cell surface. It should be emphasized that we have never observed W-P bodies in the process of merger with an outer cell membrane or of being discharged from a cell, as occurs with cell organelles in other cell types with a specific function, such as exocrine, endocrine, or neurotransmitter function. Our observations and conclusions are in agreement with those of others (2, 28).

Quantitation of W-P bodies in the numerous subcultures that were examined was not carried out, but a semiquantitative assessment of their incidence was made on selected cultures and a few cloned cell lines. After two and three passages the majority of cells usually possessed several bodies per cell. There was in general a progressive decrease in the number of W-P bodies per cell with each successive serial cultivation, particularly after the first six passages. However, it should be noted that these organelles were still present in cells after 12 subcultures. The morphologic features of the bodies in these long-term cultures were identical to those in vivo, as was their distribution within a cell.

We have determined some of the conditions required for the growth of rabbit marginal vessel endothelial cells in vitro. Cell growth in MEM was not significantly different from that seen in DMEM, the medium of choice for bovine aortic endothelial cells in vitro (10). Thereafter, the simpler medium, MEM, was used routinely. MEM supplemented with 10% rabbit serum appears to be the optimal growth medium. At higher rabbit serum concentrations, dark lipoid granules accumulate in the perinuclear region. These may be similar to those observed in human endothelial cells by Maruyama (17). The appearance of this granular material is indicative of unsatisfactory growth conditions, as the proliferation rate declines and the cells become more vacuolated in these cultures. PPS will support proliferation of rabbit marginal vascular endothelial cells for a limited period. A lack of response to serum growth factors has been reported for human umbilical vein endothelium in culture (12, 35), whereas 3T3 cells are known to be dependent on platelet-de-

rived growth factors (20). Rabbit marginal vessel endothelium appears to require whole serum for extended in vitro cultivation. Although this is suggestive of a platelet factor requirement, addition of one type of platelet preparation, a crude boiled extract, does not provide a serum substitute for these endothelial cells. A more purified platelet factor preparation (24) may be more effective. It is, however, likely that the growth requirements of the micro and small vessel endothelium may differ from those reported for large vessel cells such as human umbilical vein or bovine aortic endothelium. The lack of response to FGF, a potent mitogen for bovine aortic endothelial cells in vitro (11), supports such a suggestion.

The authors wish to thank M. Ellen Charlton and Irma Dachne for their technical assistance.

This work was supported by grant 19595 of the U. S. Public Health Service.

Received for publication 7 December 1979.

REFERENCES

1. ANTONIADES, H. N., and C. D. SCHER. 1977. Radioimmunoassay of a human serum growth factor for Balb/c-3T3 cells: Derivation from platelets. *Proc. Natl. Acad. Sci. U. S. A.* **74:**1973–1977.
2. BURRI, P. H., and E. R. WEIBEL. 1968. Beeinflussung einer spezifichen cytoplasmatischen Organelle von Endothelzellen durch Adrenalin. *Z. Zellforch. Mikrosk. Anat.* **88:**426–440.
3. CRUICKSHANK, C. N. D. 1965. *In* Cell and Tissues in Culture. E. N. Willmer, editor. Academic Press, Inc., London. 549–568.
4. DAVISON, P., and M. A. KARASEK. 1978. Factors affecting the growth and morphology of rabbit skin endothelial cells in vitro. *Clin. Res.* **26:** 208A.
5. ERLANDSON, R. A. 1964. A new Maraglas, D. E. R. (R)732, embedment for electron microscopy. *J. Cell Biol.* **22:**704–709.
6. FOLKMAN, J., and R. COTRAN. 1976. Relation of vascular proliferation to tumor growth. *Int. Rev. Exp. Pathol.* **16:**207–248.
7. FUCHS, A., and E. R. WEIBEL. 1966. Morphometrische Untersuchung der Verteilung der Spezifischen Cytoplasmatischen Organelle in Endothelzellen der Ratte. *Z. Zellforsch. Mikrosk. Anat.* **73:**1–9.
8. GIMBRONE, M. A., R. COTRAN, and J. FOLKMAN. 1974. Human vascular endothelial cells in culture. *J. Cell Biol.* **60:**673–684.
9. GORDON, G. B., L. R. MILLER, and K. B. BENSCH. 1963. Fixation of tissue culture cells for ultrastructural cytochemistry. *Exp. Cell Res.* **31:** 440–443.
10. GOSPODAROWICZ, D., J. MORAN, D. BRAUN, and C. R. BIRDWELL. 1976. Clonal growth of bovine vascular endothelial cells in tissue culture: Fibroblast growth factor as a survival agent. *Proc. Natl. Acad. Sci. U. S. A.* **73:**4120–4124.
11. GOSPODAROWICZ, D., G. GREENBURG, H. BIALECKI, and B. R. ZETTER. 1978. Factors involved in the modulation of cell proliferation in vivo and in vitro. The role of fibroblast and epidermal growth factors in the proliferative response of mammalian cells. *In Vitro (Rockville).* **14:**85–118.
12. HAUDENSCHILD, C. C., D. ZAHNISER, J. FOLKMAN, and M. KLAGSBRUN. 1976. Human vascular endothelial cells in culture. Lack of response to serum growth factors. *Exp. Cell. Res.* **98:**175–183.
13. JAFFE, E. A., R. L. NACHMAN, C. G. BECKER, and R. MINICK. 1973. Culture of human endothelial cells derived from umbilical veins. *J. Clin. Invest.* **52:**2745–2756.
14. LOWRY, O. H., N. J. ROSEBROUGH, A. L. FARR, and R. J. RANDALL. 1951. Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* **193:**265–275.
15. LUDATSCHER, R. H. 1978. Ultrastructure of human dermal blood vessels with special reference to endothelial filaments. *Virchows Arch. B. Cell. Pathol.* **27:**347–357.
16. LUI, S. C., and M. A. KARASEK. 1978. Isolation and serial cultivation of rabbit skin epithelial cells. *J. Invest. Dermatol.* **70:**288–293.
17. MARUYAMA, Y. 1963. The human endothelial cell in tissue culture. *Z. Zellforsch. Mikrosk. Anat.* **60:**69–79.
18. PALADE, G. E. 1952. A study of fixation for electron microscopy. *J. Exp. Med.* **95:**285–298.
19. PANULA, P., F. JOÓ, and L. RECHARDT. 1978. Evidence for the presence of viable endothelial cells in cultures derived from disassociated rat brain. *Experientia (Basel).* **34:**95–96.
20. PLEDGER, W. J., C. D. SCHER, P. MARTIN, H. N. ANTONIADES, and C. D. STILES. 1977. Growth requirements of normal and neoplastic cell cultures for platelet factor. *J. Cell Biol.* **75**(2, Pt. 2)**:** 401 *a*.
21. RICHARDSON, K. C., L. JARRETT, and E. H. FINKE. 1960. Embedding in epoxy resins for ultrathin sectioning in electron microscopy. *Stain Technol.* **35:**313–323.
22. RYAN, T. J. 1975. Pathophysiology of skin capillaries. *Int. J. Dermatol.* **14:**708–721.
23. RYAN, T. J. 1976. The blood vessels of the skin. *J. Invest. Dermatol.* **67:** 110–118.
24. ROSS, R., and A. VOGEL. 1978. The platelet-derived growth factor. *Cell.* **14:**203–210.
25. SABATINI, D. D., K. BENSCH, and R. J. BARRNETT. 1963. Cytochemistry and electron microscopy. The preservation of cellular ultrastructure and enzymatic activity by aldehyde fixation. *J. Cell Biol.* **17:**19–58.
26. SANDISON, J. C. 1928. The transparent chamber of the rabbit's ear giving a complete description of improved technique of construction and introduction and general account of growth and behavior of bovine cells and tissues as seen with the microscope. *Am. J. Anat.* **41:**447–473.
27. SANTOLAYA, R. C., and F. BERTINI. 1970. Fine structure of endothelial cells of vertebrates. Distribution of dense granules. *Z. Anat. Entwicklungsgesch.* **131:**148–155.
28. SENGEL, A., and P. STOEBNER. 1970. Golgi origin of tubular inclusions in endothelial cells. *J. Cell Biol.* **44:**223–225.
29. SIMIONESCU, M., N. SIMIONESCU, and G. E. PALADE. 1975. Segmental differentiations of cell junctions in the vascular endothelium. *J. Cell. Biol.* **67:**863–885.
30. STEINSIEPE, K. F., and E. R. WEIBEL. 1970. Elektronemikroskopische Untersuchungen an Spezifischen Organellen von Endothaelzellen des Frosches (Rana temporaria) *Z. Zellforsch. Mikrosk. Anat.* **108:**105–216.
31. THORGEIRSSON, G., and A. L. ROBERTSON. 1978. The vascular endothelium pathobiologic significance. *Am. J. Pathol.* **93:**803–847.
32. VAN DEN BRENK, H. A. S. 1959. The effect of ionizing radiation on capillary sprouting and vascular remodelling in regenerative repair blastema observed in the rabbit ear chamber. *Am. J. Roentgenol. Radium Ther.* **81:**859–884.
33. VENABLE, J. H., and R. COGGERSHALL. 1965. A simplified lead citrate stain for use in electron microscopy. *J. Cell Biol.* **25:**407–408.
34. WAGNER, R. C., and M. A. MATTHEWS. 1975. The isolation and culture of capillary endothelium from epididymal fat. *Microvasc. Res.* **10:**286–297.
35. STRIKER, G. E. 1978. Factors influencing endothelial cell proliferation in vitro. *J. Cell Physiol.* **96:**203–214.
36. WEIBEL, E. R., and G. E. PALADE. 1964. New cytoplasmic components in arterial endothelia. *J. Cell Biol.* **23:**101–112.