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Isolation, identification, and continuous culture of coronary endothelial cells from guinea pig hearts

S. Nees¹⁾, A. L. Gerbes, E. Gerlach

Department of Physiology, University of Munich/Federal Republic of Germany

J. Staubesand

Department of Anatomy, University of Freiburg i. Br./Federal Republic of Germany

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Coronary endothelial cells—endothelial cell culture—5'-nucleotidase—adenosine—adenine nucleotide metabolism— isolation of endothelial cells

Viable and homogeneous endothelial cells were obtained from isolated guinea pig hearts by application of a special perfusion technique of the coronary system with an isotonic collagenase-trypsin solution and subsequent purification of the dissociated cells by Percoll density gradient centrifugation. The coronary endothelial cells were grown in tissue culture for periods up to 7 months. Serial passage proved to be possible. During logarithmic growth, generation time was found to be 18 h; it could be reduced to 16 h by addition of thrombin to the culture medium. Light, phase contrast and scanning electron microscopy as well as autoradiography revealed that cultured coronary endothelial cells grew as strict monolayers of closely apposed, polygonal large cells. By scanning electron microscopy, it could be demonstrated that the morphology of the cultured cells changed characteristically during attachment of the cells to their substratum. The changes observed were very similar to those of proliferating endothelial cells of isolated coronary vessels kept in organ culture. According to transmission electron microscopy studies, cultured coronary endothelial cells proved to contain only an extremely small number of Weibel-Palade bodies. Nucleoside phosphorylase (EC 2.4.2.5.) and 5'-nucleotidase (EC 3.1.3.5.) were identified in freshly isolated as well as in cultured endothelial cells. Their specific and total activities proved to be much higher than in myocardial tissue, thus indicating a prominent role of nucleotide metabolism in the coronary endothelium.

Introduction

Endothelial cells are known to be involved in the regulation of transport processes across vessel walls [35, 6] and in hemostatic mechanisms [22, 23]. Structural, metabolic and functional abnormalities of the endothelium may be of particular significance in certain disorders, such as atherosclerosis [43, 44], diabetic angiopathy [9], inflammatory processes [54] and thrombosis [19, 20]. In addition, there is increasing evi-

dence that endothelial cells can participate in the local control of circulation by means of secretion [39], binding [7] or inactivation [25] of vasoactive metabolites and by their assumed ability to contract spontaneously [32, 18, 2].

Endothelial cell metabolism and its regulation is poorly understood, mainly because of methodological problems which hinder or exclude biochemical analyses of intact endothelial cells under in situ conditions. On the other hand, it has now become possible to study biochemical features of endothelial cells grown in culture under physiologically defined and carefully controlled conditions. So far successful cultivation has been reported for endothelial cells derived from human umbilical veins [12, 24], bovine vena cava [55], the portal vein of guinea pigs [4], the bovine pulmonary artery [8], and aortae from cattle [21], rabbits [30] and pigs [52]. To our knowledge, there is no report in the literature on culture and long term maintenance of identified endothelial cells from the circulatory system of isolated organs.

This paper describes a procedure for the isolation and cultivation of homogeneous coronary endothelial cells from guinea pig hearts. The cultured cells were characterized by means of light and electron microscopic techniques, by their growth behaviour and their generation times. In addition, we have determined the specific activities of two enzymes which have been previously shown to be characteristic for coronary endothelial cells in guinea pigs [46, 47]. Preliminary results from these investigations have been reported at the 51st Congress of the German Physiological Society [36] and at the 3rd International Symposium on "Purine Metabolism in Man" [37].

Materials and methods

Enzymes, chemicals, reagents, and media

Trypsin, collagenase (Cl. histolyticum, type II), medium 199, fetal calf serum (FCS), glutamine, penicillin and streptomycin were purchased from Seromed, München. Sodium salts of 5'-AMP and α -glycerophosphate, inosine and xanthine oxidase were obtained from Boehringer, Mannheim. Pure and highly polymerized DNA (calf

¹⁾ Dr. S. Nees, Physiologisches Institut der Universität, Pettenkoferstr. 12, D-8000 München 2/Federal Republic of Germany.

Tab. I. Specific and total activities of 5'-nucleotidase and nucleoside phosphorylase in freshly isolated and cultured coronary endothelial cells (secondary culture) and in ventricular myocardium of isolated perfused guinea pig hearts. Data are means from separate analyses of three series of culture dishes and 3 hearts.

	Specific activity [nmoles/min × mg protein]			Total activity [nmoles/min × g wet tissue]		
	Endothelial cells		Myocardium	Endothelial cells		Myocardium
	isolated	cultured		isolated	cultured	
5'-Nucleotidase	77	75	4	19 300	19 100	1 880
Purine nucleoside phosphorylase	232	234	3	37 000	37 400	760

thymus) was supplied by Sigma, St. Louis, USA. (Methyl-¹⁴C)-thymidine (53 mCi/mmol) and (methyl-³H)-thymidine (42 Ci/mmol) were purchased from Amersham-Buchler, England. Reagents for fixation and embedding tissue samples and cells prior to electron microscopy (glutaraldehyde; dodecyl succinic acid anhydride; osmium tetroxide; Epon 812; 2,4,6-tri(dimethyl-aminomethyl)phenol; methylnadic anhydride) and the phosphate determination kit were obtained from Serva, Heidelberg. Photoemulsion NTB-2 for autoradiography was purchased from Eastman Kodak Corp., Rochester, N. Y. Percoll and Cytodex were obtained from Pharmacia, Uppsala. The protein test kit was supplied by Biorad (Richmond, Cal.). All other chemicals of the highest available purity were purchased from Merck, Darmstadt.

Culture medium. Medium 199 was supplemented with 20% FCS, 200 U/ml penicillin, 20 µg/ml streptomycin and 2 mM glutamine.

Phosphate buffered saline (PBS). 140 mM NaCl, 4 mM KCl and 1 mM potassium phosphate buffer, pH 7.4.

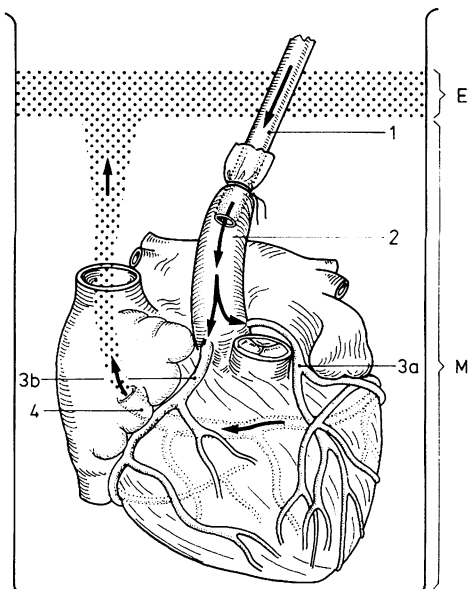


Fig. 1. Schematic representation of steps involved in the perfusion of the guinea pig heart and in the collection of dissociated coronary endothelial cells. The heart is completely immersed into culture medium containing 20% sucrose. The coronary endothelial cells are enzymatically detached by perfusion of the coronary system with isotonic collagenase-trypsin solution (aortic valves are closed). The perfusate containing the endothelial cells, overlayers the sucrose solution, thus preventing contact of the heart surface with the enzymes.—1 Cannula.—2 Aorta.—3a Left, 3b right coronary artery.—4 Coronary sinus.—Arrows direction of perfusion.—E Endothelial cell layer.—M Medium M 199 containing sucrose (20% w/v).

Percoll gradients. 70 ml Percoll and 30 ml of 3% (w/v) NaCl were aseptically mixed and centrifuged at 30 000 × g for 1 h in plastic centrifuge tubes in a Sorvall centrifuge RC2-B equipped with a SS-34 rotor.

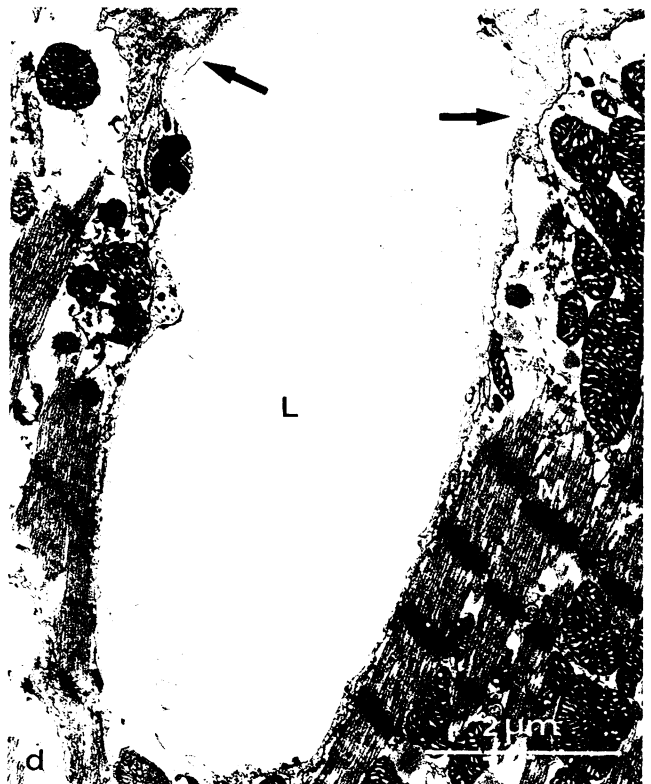
Isolation of coronary endothelial cells

All procedures were performed aseptically. Hearts from ether-anaesthetized female guinea pigs (250–300 g) were isolated and perfused by means of a peristaltic pump according to the technique of Langendorff [31]. In order to obtain a non-beating preparation part of the right atrium was dissected. After an initial perfusion with PBS for 5 min (constant volume, 5 ml/min, 40 °C) the cannulated hearts, still connected to the pump, were completely immersed into 50 ml conical tubes (Falcon Plastics, Oxnard, Cal.) filled with 20 ml medium 199 containing 20% sucrose (w/v) at 40 °C (Fig. 1). Thus contact was minimized between heart surface and enzyme solution used to dislodge the coronary endothelial cells.

Dispersion of the endothelial lining of the coronary vessels was initiated by a perfusion period of 1 min at 40 °C with dissociation medium (PBS containing 0.1% collagenase (w/v) and 0.1% trypsin (w/v)). The perfusate leaving the coronary sinus within this time overlayers immediately the more dense sucrose immersion medium. After ending the initial perfusion, the coronary system was exposed for 15 min to the enzyme solution which had been retained in the vascular space. Endothelial cells which had been dissociated from the vessel walls during the exposure to the enzymes, were then flushed out of the heart by a short perfusion with PBS devoid of enzymes. The concentrated cell suspension combined with the enzyme solution which already overlayers the sucrose medium. This crude endothelial cell harvest was aspirated and centrifuged (10 min, 250 g). The sedimented cells were washed with culture medium and centrifuged once again.

Washed cells harvested from 10 hearts were combined and suspended in 2 ml of fresh culture medium, which was then transferred to the top of a Percoll gradient [41]. A subsequent low speed centrifugation (30 min, 1000 g) resulted in the separation of endothelial cells

Fig. 2. Selective detachment of coronary endothelial cells. Photographs a and b were taken after perfusion of the coronary system with isotonic buffer. The pictures c and d were taken after perfusion of the coronary system with isotonic collagenase-trypsin solution (0.1% w/v, each).—a. Scanning electron micrograph of the luminal side of a coronary artery. The endothelial sheet built up by closely arranged individual cells is intact.—1800 ×.—b. Transmission electron micrograph of a capillary demonstrating good preservation of the microvasculature.—L Capillary lumen.—N Nucleus of a capillary cell.—M Myocardium.—16 000 ×.—c. Scanning electron micrograph of a coronary artery which reveals a complete desquamation of the endothelium. Arrows show remaining laminal structures of the intima.—1250 ×.—d. Transmission electron micrograph demonstrating efficient dissociation of a capillary. The capillary space (L) within the myocardium (M) is devoid of endothelial cells. Arrowheads indicate parts of the basal lamina.—12 000 ×.



from contaminating cells (mainly erythrocytes) and from some cell debris. The uppermost layer in the tubes contained a homogenous endothelial cell suspension. This layer was aspirated and diluted to 50 ml with culture medium. After a final centrifugation (10 min, 250 g) the purified cell harvest was resuspended in 80 ml of culture medium. This suspension contained approximately 2.5×10^4 cells/ml.

Culture conditions and subcultivation

Seeding. The final suspension of coronary endothelial cells was distributed equally among 40 Petri dishes (Falcon Plastics 3001, \varnothing 35 mm). Cultivation was performed at 40°C in a water saturated atmosphere containing 3% CO₂. After 24 h and then weekly the culture medium was replaced by a freshly prepared solution.

Secondary passage of cells. Primary cell cultures microscopically controlled and selected for subcultivation were washed with PBS and treated for 15 min at 40°C with the dissociation medium. A complete detachment of all cells was facilitated by use of a sterile PVC scraper. The suspended cells were then collected by centrifugation (10 min, 250 g) and washed twice with culture medium. Seeding and cultivation was performed as described above. Generation times were calculated according to standard procedures. Cell densities were daily documented by photomicrography using an inverted microscope (Leitz M 3 camera).

Microbead cultivation. 100 mg of Cytodex dextran beads (mean diameter about 100 μ m) were sterilized and washed according to the instructions of the manufacturer. After centrifugation the beads were resuspended in 10 ml of culture medium, which then was distributed among 5 standard culture dishes. Inoculation with about 10^5 cells and incubation for 1 week under the conditions described above resulted in the formation of endothelial monolayers covering each bead. They were used for ultrastructural studies by means of transmission electron microscopy.

Morphological characterization

Silver impregnation. Cultures were rinsed twice with PBS and twice with 5% glucose. Intercellular borders were specifically stained with 0.25% silver nitrate solution and fixed in 9% formalin as described by Garbarsch et al. [13].

Scanning electron microscopy (SEM). Guinea pig hearts automatically beating were first perfused for 10 min at constant pressure (80 mm Hg) with PBS containing 6% albumin, and then for 60 min with 2.5% glutaraldehyde solution in 90 mM sodium cacodylate buffer (pH 7.4) containing 0.5 mM CaCl₂.

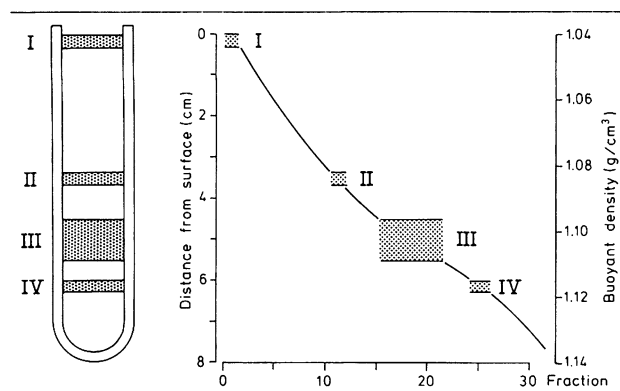


Fig. 3. Purification of a crude endothelial cell harvest by Percoll density centrifugation. — Left panel: Centrifuge tube with the respective positions of the separated cells and tissue debris. — Right panel: Buoyant density of fractions collected from the gradients. — I Pure coronary endothelial cells. — II–IV Contaminating cells and tissue debris, erythrocytes being exclusively present in band III.

Cultured endothelial cells and coronary arteries aseptically dissected and kept in organ culture for several days under the conditions described as well as freshly prepared heart slices (thickness 3 mm) were rinsed with PBS and prefixed in glutaraldehyde solution. The specimens were washed three times with cacodylate buffer containing 5% sucrose and then postfixed in 1% osmium tetroxide dissolved in cacodylate buffer. The samples were then dehydrated in graded ethanol solutions (50–100%) and “critical point dried” in CO₂ (37–40°C; 739.6 N/cm² (73 Kp/cm²)). Tissue samples were then attached to polished aluminium stubs, coated uniformly in vacuo with gold (thickness about 15 nm) and viewed immediately with a scanning electron microscope (Cambridge Instrument Co., Stereoscan).

Transmission electron microscopy (TEM). Samples of freshly isolated guinea pig hearts as well as coronary endothelial cells cultured on dextran microbeads (Cytodex, Pharmacia) were washed and fixed in glutaraldehyde and osmium tetroxide as described above. The samples were dehydrated in ethanol, stained en bloc with 0.5% uranyl acetate and 1% phosphotungstic acid in 75% ethanol, and embedded in Epon 812. Ultrathin sections were stained with uranyl acetate and lead citrate and viewed in a transmission electron microscope (Zeiss, EM 9).

Analytical procedures

Protein content of PBS-washed cultures and of enzyme preparations were measured using a dye adsorption protein assay (Biorad, Richmond, Cal.). DNA was determined according to the diphenylamine color reaction of Ashwell [1].

Preparation of enzyme extracts. Minced ventricular tissue and freshly isolated as well as cultured endothelial cells were suspended in 0.25 M Hepes buffer, pH 7.4, (about 2–10 mg tissue/ml, respectively) and disrupted by aid of a Dounce homogenizer. Ventricular homogenates were centrifuged at 200 g for 10 min and the supernatants dialysed against 0.05 M Hepes buffer, pH 7.4. Endothelial cell lysates were not centrifuged, but dialysed directly under identical conditions.

Enzyme assays

Purine nucleoside phosphorylase (EC 2.4.2.5.): A mixture of 100 μ l inosine solution (10 mM), 400 μ l potassium phosphate buffer (0.1 M, pH 7.4) and of 300 μ l enzyme extract (containing up to 100 μ g protein) was incubated for 10–30 min at 37°C. After boiling for 3 min, samples were cooled to room temperature and xanthine oxidase was added (about 0.04 U/20 μ l). Hypoxanthine was determined by differential spectrophotometry after its enzymatic conversion to uric acid according to the method of Kalckar [26].

5'-nucleotidase (EC 3.1.3.5.): A reaction mixture of 100 μ l AMP solution (24 mM), 50 μ l MgCl₂ (0.1 M), 50 μ l Hepes buffer (0.25 M, pH 8.4) and 50 μ l enzyme extract (containing up to 400 μ g protein) was incubated for 10–30 min at 37°C. After heat denaturation of the enzyme (95°C, 3 min) inorganic phosphate was determined according to Eibl [10] using a commercial phosphate determination kit (Serva). Values obtained were corrected for unspecific phosphate monoesterase activity, which was determined under identical assay conditions, but using α -glycerolphosphate (24 mM) instead of AMP-solution.

Radioactive measurements

Incorporation of ³H-thymidine into DNA. Secondary coronary endothelial cell cultures were exposed for 4 h to (methyl-³H)thymidine (40–60 Ci/mmol, 1.0 μ Ci/ml culture medium). The radioactive medium was aspirated and each culture was extensively rinsed at 0°C with PBS. After fixation with acetic acid:ethanol (1:3 v/v) for 10 min, the samples were rinsed with distilled water, extracted with 10% (w/v) trichloroacetic acid (TCA) for 10 min, followed by a second extraction with 5% TCA. After a final rinse with ethanol (96%) the cell layers were air dried and solubilized in 1 ml of 0.25 M NaOH by ul-

trasonication (Branson sonifier). Aliquots of the solution were counted in a Packard Tri Carb Liquid scintillation spectrometer, model 3380. Each sample was made up to 10 ml with scintillation fluid (5.5 g of 2,5-diphenyloxazole (PPO) and 0.15 g of 1,4-bis-2-(5-phenyloxazolyl)benzene (POPOP) in 666 ml of toluene and 323 ml of triton X 100. Counting efficiencies were 42 to 45% under these conditions.

Autoradiography. Coronary endothelial cell cultures at different growth stages were labelled with (methyl- ^3H)-thymidine and washed as described above. The air dried cell layers on the bottom of their dishes were coated with Kodak NTB-2 emulsion and exposed for 3 days at 4 °C. The counts of labelled nuclei were microscopically determined.

Results

Isolation of coronary endothelial cells

According to the endothelial classification of Bennett et al. [3] the coronary endothelium belongs to group A-1 α , which is mainly characterized by a complete and continuous basement membrane and by lack of fenestrations or pores in the capillary system. This arrangement of the coronary endothelium as a uniform monolayer of flattened cells was not altered during perfusion for 4 min with PBS (Figs. 2a, b). Exposure of the coronary system for 15 min to collagenase-trypsin solution followed by PBS-perfusion, resulted in a specific detachment of the endothelial lining, both in large and small vessels (Figs. 2c, d). The extent of this enzymatically induced desquamation of the coronary endothelial cells was found to be dependent on the concentration and grade of the digestive enzymes, their contact time with the vessel walls and the temperature. Treatment for 60 min of the vessels resulted already in a partial degradation of the vascular smooth muscle wall.

The endothelial cell harvest of usually 1 to 5×10^5 viable cells from one heart was contaminated by small amounts of red cells and leukocytes, some large or spindle-shaped cells (apparently smooth muscle cells and fibroblasts), and some tissue debris most likely originating from the elastic lamina of the larger vessels. These contaminants (specific density 1.06–1.12 g/cm 3) could easily be separated from the endothelial cells by Percoll density gradient centrifugation [41]. Due to their strikingly low specific density of only 1.03 g/cm 3 the endothelial cells remained floating on top of the gradients (Fig. 3).

Growth and culture architecture

Within 3 h after seeding, 40% of the freshly isolated endothelial cells characteristically clumped (Fig. 4a) started to flatten and spread out. The non-attached and apparently non-viable cells were removed from the culture dishes after 24 h with the first exchange of the medium. Convincing proof concerning growth and homogeneity at this stage of the culture was obtained by application of phase or interference contrast microscopy. Within the first four days small colonies of typical architecture were formed (Fig. 4b), which gradually increased and coalesced within 3 to 5 weeks. The individual cells were homogeneous, closely apposed, polygonal (\varnothing 20–30 μm) with an oval, centrally located nucleus, a granular perinuclear region and undistinct cell borders (Fig. 4c). Cul-

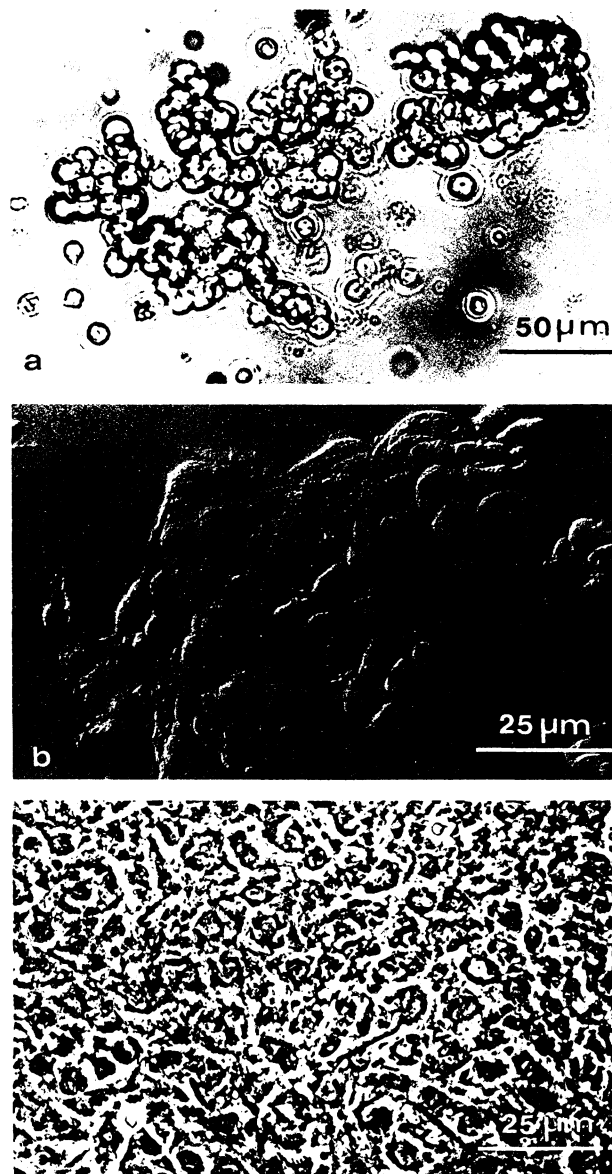


Fig. 4. Three states of coronary endothelial cell growth in tissue culture. — a. Freshly isolated, characteristically clumped cells immediately after seeding. Light microscopy. — 300 \times . — b. A small group of endothelial cells 1 week after culture initiation. Interference contrast microscopy (NOMARSKI optics). — 520 \times . — c. Confluent endothelial monolayer after about 4 weeks. Phase contrast. — 600 \times .

ture architecture proved to be uniform and did not reveal multilayering of cells, a phenomenon commonly observed in cultures of fibroblasts or smooth muscle cells. Postconfluent cultures (up to 7 months after confluency) with a density of about 5×10^5 cells/cm 2 preserved the typical monolayer arrangement of individual cells. This could be documented by silver impregnation of the cultures, which revealed silver deposits in one microscopic plane only along the cell borders.

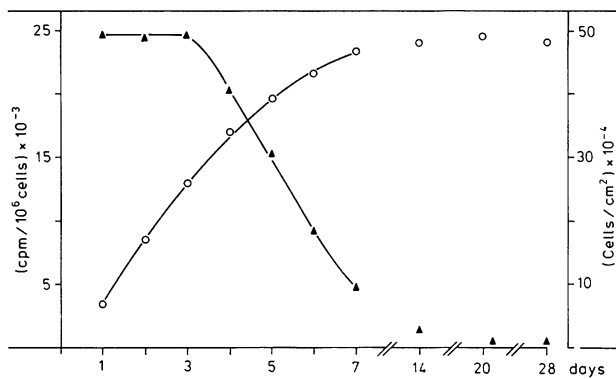


Fig. 5. (Methyl-³H)-thymidine incorporation into TCA-insoluble DNA (▲—▲) and growth curve of secondary coronary endothelial cells (○—○). Data represent the mean from measurements in 4 separate culture dishes.

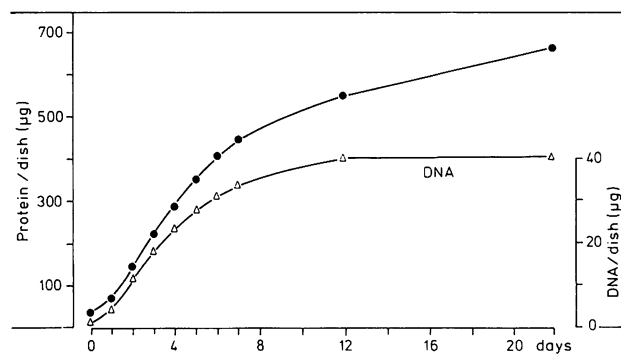


Fig. 6. DNA and protein content of coronary endothelial cells in secondary culture at different states of growth.—Starting cell density 2×10^5 cells per dish.—Density in postconfluent cultures 5.4×10^6 cells per dish. Data are the average from measurements in 3 separate dishes.

Generation time

Coronary endothelial cell growth was independent of the initial cell density of the culture. The generation time determined in proliferating cultures during three days of exponential growth proved to be 17.9 ± 0.6 h. Addition of thrombin ($1 \mu\text{g/ml}$ culture medium) resulted in a reduction of the generation time to 16.1 ± 0.4 h.

Contact inhibition in confluent cultures

The contact inhibition of coronary endothelial cell growth already microscopically observed was further demonstrated by (methyl-³H)-thymidine incorporation studies. Application of autoradiographic techniques made it possible to determine labelling indices (percentage of labelled cells in a culture) which amounted to about 90% in growing, but only to about 5% in confluent cultures. Quantitative measurements of thymidine incorporation into highly polymerized DNA (TCA-insoluble) confirmed and extended the histochemical findings. Rates of DNA synthesis declined drastically with the progression of confluency; thus minimal values were obtained in the stationary phase (G_0 phase of growth, Fig. 5). As is evident from Figure 6, DNA content in the cul-

tures did not change any more when confluency was reached. However, total protein in the cultures still increased, most likely due to the synthesis of insoluble protein fractions, which formed a kind of layer underneath the confluent cells.

Ultrastructural appearance of cultured coronary endothelial cells

Freshly isolated cells formed numerous pseudopodia (Fig. 7a). The cytoplasm contained a great number of rather long and unbranched mitochondria, as well as vacuoles, granules and a great number of plasmalemmal vesicles. The shape of the nucleus appeared to be highly irregular with deep indentations. Weibel-Palade bodies were found only occasionally. Sometimes typical junctions could be detected which are known to be characteristic features of endothelial cells.

In contrast to dissociated and suspended cells, endothelial cells after one week in culture revealed a regular plasmalemmal surface and an ovoid nucleus very much the same as in coronary endothelial cells in situ. Their cytoplasmic architecture (Fig. 7b) directly comparable to cells in situ (Fig. 2b) and to that of freshly isolated cells as shown above (Fig. 7a) remained rather unaltered during cultivation. In addition, amorphous basal lamina-like material could occasionally be detected underneath the cells of postconfluent cultures.

Scanning electron microscopic pictures of cultured endothelial cells revealed certain morphological changes during their attachment and proliferation. Immediately after seeding, the cells were characterized by a spherical shape (Fig. 8a), whereas after an initial phase of attachment between 2 and 10 min (Fig. 8b) "jelly-fish like" forms developed (Fig. 8c and d). Flattening of the cells after 10 to 20 h was accompanied by the processes of DNA replication and cell division (Fig. 8e). In this phase all cells were characterized by numerous regularly formed granula accumulating especially in the perinuclear region. After about 48 h an unfenestrated and continuous endothelial monolayer had been formed in which some partial overlap of the individual cell boundaries became apparent (Fig. 8f).

Similar morphological features of proliferating endothelial cells not dissociated from the vascular bed could be demonstrated in dissected pieces of coronaries which were maintained in organ culture. As is shown in Figure 9, the proliferating and amoeboidically migrating endothelial cells in the immediate vicinity of the cutting line revealed similar morphological features as described above for isolated and cultured cells (Fig. 8).

Enzyme activities

In a first attempt to biochemically characterize coronary endothelial cells specific and total activities of 5'-nucleotidase and of nucleoside phosphorylase were determined. These enzymes were previously shown by histochemical techniques to be marker enzymes of the coronary endothelium in guinea pig hearts [46, 47]. As is evident from the data in Table I, both, the specific and total activities of the two enzymes proved to be rather high and almost identical in freshly isolated and cultured endothelial cells. In contrast, the corresponding activities of the same enzymes in ventricular myocardium were found to be very low.

Discussion

Isolation of pure endothelial cells from the coronary vessels is rendered difficult because of contaminations of the cell harvest with other cell types, such as pericardial and endocardial cells, pericytes, fibroblasts, and smooth muscle cells. Since all these cells are characterized by a high growth potential, long term tissue cultivation of coronary endothelial cells requires the complete elimination of all contaminants.

Several attempts were made in the past to isolate various types of cells from ventricular myocardium including coronary endothelial cells [33, 57, 27, 17, 28, 29]; however, the cell preparations obtained proved not to be homogeneous. Even a specifically developed disruption technique for myocardial tissue recently reported by Simionescu and Simionescu [51] which leaves most of the endothelial cells intact resulted in an endothelial cell harvest of only 85% purity.

The new methodological approach described in this paper is based on two subsequent steps: 1) a rather selective detachment of the endothelial cells from the intact vessel system in isolated guinea pig hearts by application of a standardized enzyme perfusion technique; 2) exclusion of the contaminating cells (<2%) and of some tissue debris by Percoll density gradient centrifugation [41], a procedure, which proved optimal because of the strikingly low specific density of coronary endothelial cells (1.03 g/cm^3).

The isolated cells were characterized by various means including studies on their morphology, viability and specific enzymatic equipment. The morphological features of freshly isolated and cultured coronary endothelial cells revealed by phase microscopy as well as by scanning and transmission electron microscopy, resembled those observed in guinea pig hearts in situ. Moreover, they were very similar to those of endothelial cells of various other origins. The extremely rare occurrence of Weibel-Palade bodies in freshly isolated and cultured cells is of special interest. While these cytoplasmic constituents have been reported to be specific markers of the endothelium from a variety of blood vessels [56], they could not be detected in the endocardium of the frog [53], in bovine coronary endothelium [16] or in partially purified endothelial cells from rabbit hearts [51].

Due to the gentle isolation procedure applied the viability of the endothelial cells in culture proved to be satisfactory. Since replication and propagation of the endothelial cells were independent of the initial cell density in the cultures (similarly as reported for endothelial cells from bovine aorta [5]), it was possible to determine the generation time in exponentially growing cultures, when contact inhibition of cell growth was negligible (G_1 phase of cell cycle). The low value of only 18 h for the endothelial cell cycle in vitro is in good agreement with findings concerning rapid repair mechanisms in various vessels in vivo after artificial lesion of the endothelium [48, 41]. In this connection it is of interest that thrombin in rather small concentrations was shown in our experiments to reduce the generation time from about 18 to about 16 h. Thus thrombin must be considered not only a mitogenic stimulator for human endothelial cells from the umbilical vein [58], but also for coronary endothelial cells. It appears likely that this function of thrombin is of general importance for a sufficiently quick repair of endothelial lesions.

Postconfluent cultures of coronary endothelial cells were characterized by uniform monolayering of contact inhibited cells. This architecture of coronary endothelial cell cultures, which was also described for endothelial cell cultures from various other blood vessels [24, 55, 4, 15], appears to be directly comparable with the arrangement of these cells in situ. All other cell types of the vessel wall hitherto successfully

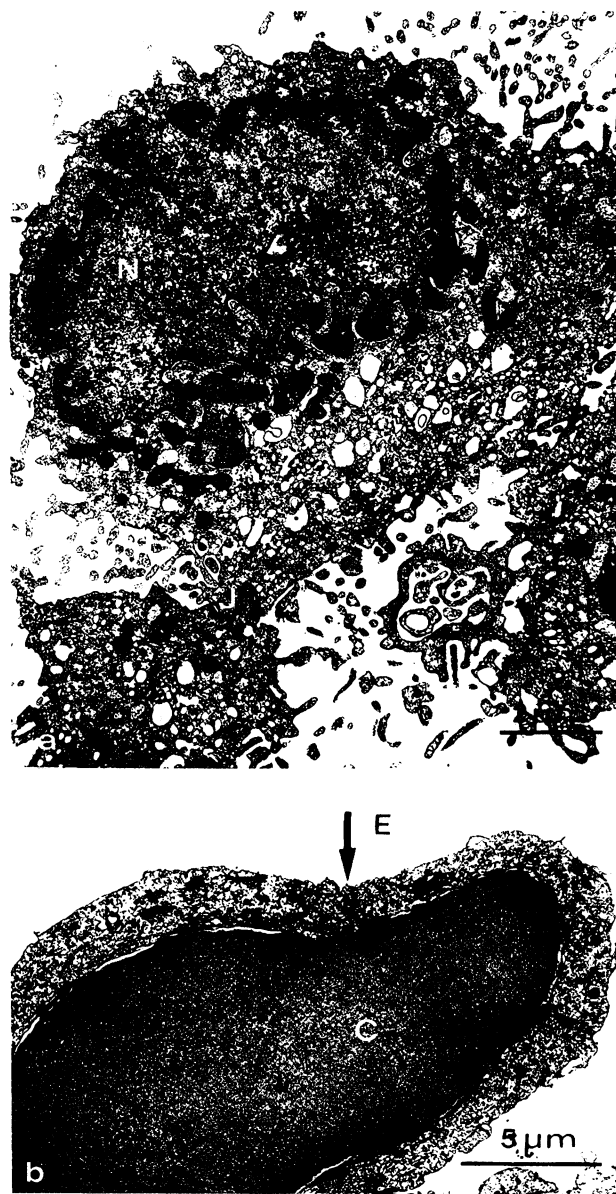


Fig. 7. Transmission electron micrographs of freshly isolated and cultured coronary endothelial cells sectioned en face.—a. Isolated coronary endothelium. The two cells shown are joined by a typical junction (J). Microvilli are present on the luminal and abluminal front. Most plasmalemmal vesicles are located within the cells. Profile of the nucleus (N) is irregular and deeply indented.—9000 \times .—b. Coronary endothelial cells grown in tissue culture on a dextran bead (Cytodex, Pharmacia). The cytoplasmic architecture compares well with that of freshly isolated cells (a) or with that of coronary endothelial cells in situ (Fig. 2b).—E Endothelial cell.—C Dextran bead.—3400 \times .

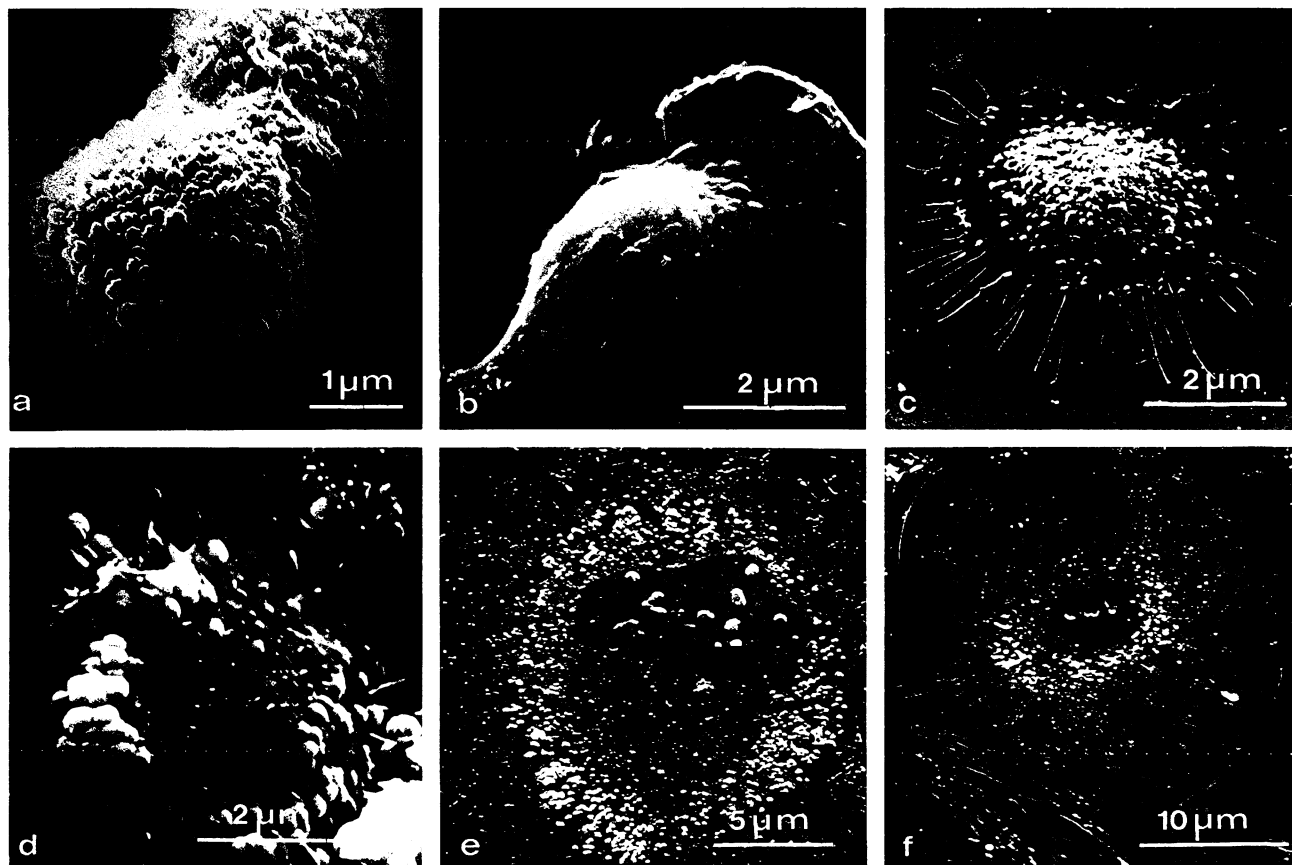


Fig. 8. Six scanning electron microscopic views of coronary endothelial cells during attachment to their substratum. — a. 2 dissociated cells, immediately after seeding. — 12000 \times . — b. A partially attached cell, 5 min after seeding. — 10000 \times . — c, d. “Jelly fish” like morphology of cells 30 and 120 min after seeding. — 8000 \times . — e. Dividing nuclei about 19 h after seeding, surrounded by an abundant number of granules. — 2900 \times . — f. Flattened cell after completion of the monolayer 48 h after seeding. Cell boundaries partly overlap those of the adjacent cells. — 1800 \times .

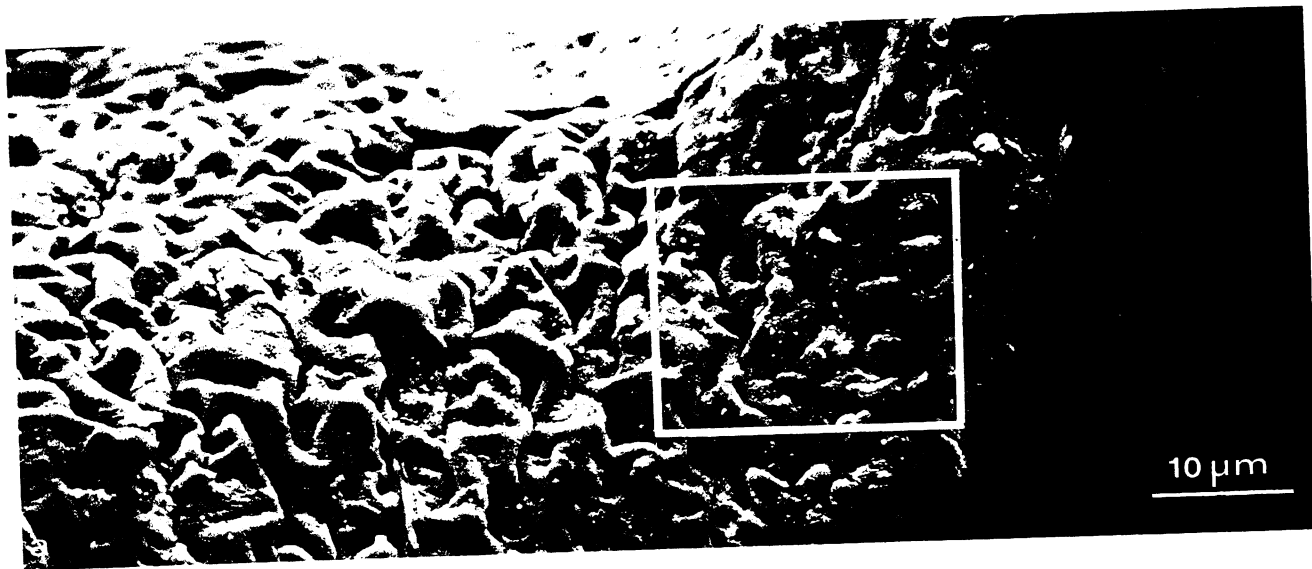
grown in tissue culture (smooth muscle cells [45], fibroblasts [11]) have been shown to form multilayers of individual cells not subjected to contact inhibition.

Only a few biochemical marker substances of vascular endothelium have been identified as yet. These include clotting factor VIII antigen and blood group antigens which exist on the plasmalemmal surface of the human umbilical vein endothelium [24], as well as some ectoenzymes [50, 34]. Promising candidates for a first biochemical characterisation of cultured endothelial cells from the coronaries seem to be 5'-nucleotidase and nucleoside phosphorylase, since both these enzymes were shown by electron histochemical techniques to be prominent markers of the capillary endothelial cells of guinea pig hearts [46, 47]. The rather high specific and total activities of both enzymes in cultured coronary endothelial cells compared with the relatively low activities of these enzymes in ventricular homogenates are in good agreement with the histochemical findings. Because the enzyme activities were shown to be almost identical both in freshly isolated and in cultured coronary endothelial cells, it appears

reasonable to conclude that enzyme perfusion of isolated hearts for endothelial cell dissociation and the conditions of cell culture did not result in prolonged metabolic alterations of the cells.

Based on the assumption that total endothelial tissue contributes about 2% of the wet weight of the myocardium [51], about 20% of the total 5'-nucleotidase activity and almost 98% of the total nucleoside phosphorylase activity in heart tissue can be considered to originate from the coronary endothelium. The remarkably high enzyme activities deserve particular attention with respect to nucleotide metabolism in these cells. According to preliminary results [36–38] adenine nucleotide content of coronary endothelial cells proved to be extraordinarily high (about 15 μ moles/g cells (wet weight)). In addition, these cells were shown to contain considerable amounts of adenosine. This vasoactive nucleoside known to originate from the breakdown of adenine nucleotides is assumed to be involved in the metabolic regulation of coronary blood flow [14, 48]. At present it is not yet clear whether the

Fig. 9. Scanning electron micrograph of proliferating endothelial cells of a dissected coronary artery after 4 days in organ culture. — a. Overview showing in the left part the vascular surface, in the right part media and adventitia of the crosssectioned vessel. — 1600 \times . — b. The marked area in a (5fold enlarged) reveals proliferating endothelial cells in the process of coating the crosssectioned media of the vessel. The typical morphological features of these cells at different stages of the cell cycle closely resemble those observed on cultured coronary endothelial cells (Fig. 8). — 8000 \times .



peculiar distribution pattern of adenine nucleotides as well as of adenosine in coronary endothelial cells is of functional importance, for instance with respect to the interaction of endothelium with platelets as recently suggested [40], or regarding the metabolic regulation of coronary flow. Future studies devoted to a further elucidation of these possible interrelationships, however, will be greatly facilitated by using routinely the techniques and procedures described in this paper.

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