Isolation, bulk cultivation, and characterization of coronary microvascular pericytes: the second most frequent myocardial cell type in vitro

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IN 1923, THE GERMAN ANATOMIST K. W. Zimmermann (101) published pioneering studies on the cytoarchitecture of different microvascular beds and described the distribution of a peculiar cell type in intimate contact with the endothelium in the microvascular wall. He regarded these cells, for which he coined the name “pericytes,” as typical constituents of capillaries and postcapillary venules, and he made a distinction between them and vascular smooth muscle cells (SMC; Ref. 101).

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In the subsequent 90 yr, it has become clear that this common cell type is of considerable interest, not only from the point of view of basic science, but also with regard to clinical medicine. Several reviews have described these cells in detail (19, 23, 83, 99). The following list summarizes briefly their most important functional properties: 1) their key role in regulation of different cellular processes in the vasculature, including endothelial proliferation and differentiation (3, 37); 2) the stabilization and control of permeability of blood vessels under physiological and pathophysiological conditions (93, 99); 3) their participation in vasculosclerosis and angiogenesis (6, 44, 70, 90); and 4) their abundant expression of procoagulatory tissue factor (TF) and prothrombinase; thus they provide a central contribution to protection against both bleeding and the induction of thrombotic events, and they exhibit a central involvement in atherosclerotic processes (7, 41, 65, 96); 5) as a component of the “front line” in immunological defense (4); 6) their involvement in the regulation of contractility and tone of vascular smooth muscle and hence participation in organ blood flow regulation (27, 42, 46, 47); 7) their transformation to myogenic precursor cells different from satellite cells (17); and 8) their action as resting early pluripotent adult stem cells or at least as attractors of the latter, thus the constitution via the ubiquitous microvascular networks of a system assuring the maintenance, physiological repair and regeneration of organs (10, 14).

In view of the ubiquity of the pericytes in arterioles, capillaries, and venules (63), the apparent physiological significance of these cells, and their close association with the inner lining of all blood vessels (the endothelium), it is almost a paradox that for more than four decades the annual number of publications concerned with pericytes has been almost negligible compared with the numbers of publications addressing the endothelium [e.g., the key words “endothelial cell(s)” (EC) produce 10,217 (8,783) citations in PubMed for the year 2010 and “pericytes”) only 256 (221)]. A major reason for this was certainly the publication in 1973 of a simple and generally reproducible culture method for EC of microvascular origin that preserves their major characteristics (40). In standard media (supplemented with species-heterologous FCS), these cultures are stable and apparently of homogenous composition, and they develop the typical and characteristic features of the endothelium of the large vessel from which they originate (human umbilical vein). Cultured EC can be generated in bulk, and preparations can be stored and transported in the frozen state. After reculture, they again express their typical features. These properties make EC also suitable for commercial exploitation. The saving of preparation time and effort makes such
biological products particularly attractive to potential investigators.

While considerable attempts have also been made to culture pericytes (18, 22, 35, 43, 52, 58, 60, 62, 74, 84, 95), these have not found comparably broad acceptance. These cells must be isolated from microvascular preparations, and the latter must first be separated proteolytically from their surrounding parenchyma, connective tissue, and lymphatic system, naturally always complex. The danger of substantial contamination of the cell harvest by nonpericytes (e.g., vascular and lymphatic EC, SMC, fibroblasts and myofibroblasts, macrophages, mast cells, and in preparations from the brain, microglial, and leptomeningeal cells) as well as microorganisms (bacteria, fungi, and mycoplasma) is extensive. Such extrinsic cells also tend often to proliferate more strongly in subsequent culture than the pericytes. With respect to the suitability of the resulting cultures for scientific purposes, even effective postpreparative purification procedures will remain unconvincing if the cultured cells cannot be identified unambiguously as pericytes. Indeed, a major problem in pericyte research, recognized by Zimmermann already in 1923 (101) and still valid, is the general lack of unequivocal pericyte markers. The strong degree of uncertainty experienced in attempts to grow pericytes in vitro has been exacerbated by their occasionally claimed pluripotency. Moreover, in standard culture media these cells can be overgrown by contaminating cells, or they may even die. Should a culture succeed, the cells may dedifferentiate, i.e., may express histological or functional properties completely different from their properties in vivo. This would lead rapidly to contradictory findings and errant conclusions. A recent review addresses these problems critically for pericyte cultures derived from the brain (46), while researchers increasingly emphasize the impact of the differentiation state of the pericytes on their functional performance (86).

With this information and these experimental considerations as a background, the purposes of the present investigation were to 1) isolate and purify adult microvascular pericytes from the ventricular myocardium of various species, including human; to 2) identify and characterize them histologically and functionally; and to 3) cultivate them under optimized conditions and to harvest them in bulk amounts. Extrapolating our in vitro findings to the coronary microcirculatory system implies that pericytes may play key roles in the adjustment of local coronary blood flow, the regulation of angiogenic processes, and the regulation of hemostasis in the myocardium. Their successful bulk cultivation enables now direct experimental access to the second most frequent cell type of the myocardium under defined in vitro conditions and the isolation of unique pericyte antigens for the production of highly specific antibodies.

MATERIALS AND METHODS

Materials

Pig fetal serum was from Animal Technologies (Tyler, TX); Polymorphprep was manufactured by Axis-Shield (Oslo, Norway); Biseko, a plasma substitute, was supplied by Biotest (Dreieich, Germany); Transwell polyethylene/epithelal culture devices with a surface area of 4.5 cm², a pore size of 0.4 μm, and a pore density of 10⁶/cm² were obtained from Corning (Corning, NY); Percoll was supplied by GE Healthcare Europe (Freiburg, Germany); factors Va and prothrombin as well as thrombin- and Xa-substrates S-2238 and S-2222 were manufactured by Chromogenix and purchased from Haemochrome Diagnostica (Essen, Germany); DMEM, FCS, HEPES-buffered Earle’s salt solution, streptomycin, penicillin, balanced salt solution, PBS and trypsin/EDTA solution were obtained from Invitrogen (Karlsruhe, Germany); histidine tryptophane ketoglutarate solution used by surgeons for multiorgan protection (HTK) was from Koehler Chemie (Bensheim, Germany); Di-acetylated light density lipoprotein (Ac-LDL), CellTracker CM-H2C12, calcein-AM, and secondary antibodies (highly cross-adsorbed, if available) conjugated with Alexa Fluor 488 and 546 were purchased from Molecular Probes (Eugene, OR); Octaplex 500 was purchased from Octapharma (Langenfeld, Germany); tissue culture medium EC growth medium was purchased from Promocell (Heidelberg, Germany); rabbit antibody directed against NG2 (a typical proteoglycan of pericytes), connexin 43 (Cx43), and platelet-derived growth factor receptor type-β (PDGFR-β) were obtained from Santa Cruz (Heidelberg, Germany); Ficoll 70, Gly-Pro-4-methoxy-β-naphthylamide, Fast Blue B naphthol-AS-MX-phosphate dinatium salt, Fast Blue BB ½ ZnCl₂, Fast Red TR 4’,6-diamidino-2-phenyldilute, dilactate, TRITC-labeled dextran, and several murine monoclonal IgG antibodies [clone 1A4 for labeling α-smooth muscle actin, clone hCP for labeling calponin, clone LAM-89 for labeling human laminin, clone LHT.2 for labeling human collagen IV, clone EM34.1 directed against the mouse IgM κ-chain, and rabbit polyclonal antibodies for labeling von Willebrand factor (vWF) or fibronectin] were purchased from Sigma (St. Louis, MO); and nylon membrane filters with a diameter of 90 mm and a 20-μm pore size were purchased from Sterlitech (Kent, WA).

Solutions

PBS for washing of cells before histological staining was supplemented with 1% BSA, CaCl₂-free modified Ringer solution was prepared by dissolving the following chemicals in water (in mM): 127 NaCl, 4.6 KCl, 1.1 MgSO₄, 1.2 KH₂PO₄, 25 NaHCO₃, 7.6 glucose, 2 pyruvate, 10 creatine, 20 taurine, 5 ribose, 2 aspartic acid, 2 glutamic acid, 1 arginine, and 0.5 uric acid, equilibrated with carbogen. Protease solution for disintegration of human, bovine, and pig hearts contained the following (in mg/100 ml): 90 collagenase B (Boehringer, Mannheim, Germany), 12 dispase II (Roche), and 200 highly purified BSA (Sigma); for mouse, rat, and hamster hearts: 110 collagenase B, 8 dispase II, and 200 albumin; for rabbit hearts: 37.5 collagenase B, 1.25 dispase II, and 200 albumin; for guinea pig hearts: 90 collagenase B, 3 dispase II, 1 trypsin, and 150 albumin; the proteases were dissolved in Ringer solution that was subsequently sterilized by filtration through a sterile filter (pore size of 0.22 μm). Protease solution for detachment of pericyte clumps contained 10 mg dispase II/100 ml HEPS-buffered Earle’s salt solution. Culture medium DMEM was supplemented with 10% vol/vol FCS, 200 μg/ml penicillin, and 0.2 mg/ml streptomycin. Protein-free staining buffer for dye transfer studies contained the following (in mM): 116 NaCl, 5 KCl, 1.8 CaCl₂, 1.0 NaH₂PO₄, 0.8 MgSO₄, 5.5 d-glucose, and 1.0 Na-pyruvate, adjusted to pH 7.40 before addition of 24.0 NaHCO₃ and equilibration with 5% (vol/vol) CO₂ in air.

Methods

Animal care and experimental procedures. All experiments with animal cells and organs were in accord with the Guidelines for the Care and Use of Laboratory Animals published by the National Institutes of Health and German animal protection law as approved by local authorities. The following laboratory animals were obtained from Savo medizinische Versuchstierzuchten (Kisslegg, Germany; all males, body weight given in brackets): NMRI mice (16–18 g), Sprague Dawley rats (200 g), Dunkin-Hartley guinea-pigs (250–300 g), Syrian hamsters (100–130 g), and New Zealand White rabbits (1.5–1.8 kg). Young domestic pigs and cattle were obtained from the local abattoir.

Human cell and organ preparations. All experiments with human tissue were carried out after the written informed consent of the
patients was obtained and with the approval of the ethics committee of the Ludwig-Maximilians-University of Munich according to the principles expressed in the Declaration of Helsinki. 

**Proteolytic disintegration of left ventricular myocardium of animal and human hearts.** The starting materials were hearts isolated from freshly killed laboratory animals (killed by decapitation or cervical dislocation) or from animals for slaughter (killed by a captive bolt shot), and hearts were extirpated and transported to the laboratory in ice-cold HTK solution within 1 h, as well as from explanted human hearts (donor age 30–65 yr, explantation during transplantation surgery, transported to the laboratory within 2 h in ice-cold HTK solution). Myocardial cells and microvessels were isolated proteolytically under strictly controlled aseptic conditions as outlined in the protocol in Fig. 1, employing an approach described in principle earlier (63, 69). Care was taken to compose an optimized and standardized protease solution of well-defined collagenase B, dispase II, and serum albumin preparations in modified Ringer solution (see Materials) and to suspend the myocardial tissue components as homogenously as possible. Isolated whole hearts from rodents or isolated ventricles of pig, cattle, and human hearts were perfused for 10 min at 37°C with modified, Ca²⁺-free Ringer solution gassed continuously with carbogen. The coronary system was then perfused with the protease solution for 30 min. After the left ventricles were sectioned with a sterile razor blade (pieces 3 × 3 mm), 3-g portions of the already softened tissue were suspended in 20 ml Ringer solution, gently homogenized, and dispersed with the aid of a custom-designed funnel into which carbogen was passed slowly from the narrow outlet tube. After collection of the suspended myocardial microvessels by filtration through a nylon net (mesh size: 200 nm/pixel), pericytes were detached selectively from their parent vessels on the net. For this purpose, the net was covered on both sides with further nets of smaller mesh size (50 μm), installed between two perfusable chambers in a custom built apparatus, and flushed continuously with 13 ml freshly prepared protease solution, which was pumped back and forth across the net stack in a custom-built apparatus (15 min, 37°C, 6 cycles/min).

**Enrichment of detached pericytes by Percoll density-gradient centrifugation.** After proteolytic detachment from their parent vessels, the pericytes were collected by centrifugation, resuspended in a 20% (vol/vol) isotonic Percoll solution, and fractionated by density-gradient centrifugation. The pericytes accumulated at a buoyant density of 1.057 ± 0.004 g/cm³. With the use of a custom-built high-resolution cell-separation centrifuge (63), this procedure was routine and reproducible.

**Cell culture.** Pericytes in primary and secondary cultures were grown in DMEM supplemented with 2 mM l-glutamine, streptomycin (20 μg/ml), penicillin (200 IU/ml), and 10% vol/vol species-specific fetal, or neonatal serum. In case of pericytes of bovine and porcine origin, the respective fetal sera are available commercially. For preparations from all other species (except humans), sera were prepared from blood of newborn animals of the same species. For preparations of cells of human origin, serum was obtained from placental blood. The starvation medium for the final removal of contaminating EC consisted of medium 199, supplemented with the usual antibiotics, but free of glutamine and serum. EC of arteriolar and venular origin were isolated and cultivated as described elsewhere (63).

**Histological methods. PROTOCOLS FOR TISSUE FIXATION.** For immunohistochemistry, tissue cultures were prefixed for 2 min at room temperature by immersion in 0.5% glutaraldehyde/4% paraformaldehyde (PA) in PBS, stored at least overnight in PA at 4°C, permeabilized in PBS containing 0.1% Triton X-100 and 0.1% Tween 20 for 30 min, washed with PBS, and incubated for 10 min with 1% NaBH₄ solution to remove free carbonyl groups. For enzyme histochemistry, cell cultures were treated for 2 min with PA followed by three washings in PBS. Very delicate structures (freshly attached isolated vessels) were fixed by setting onto an ice-cooled plate and subsequent exposure to vapor (45°C) from a 37% formaldehyde solution. For scanning electron microscopy, cell cultures and isolated blood vessels were fixed for ≥2 h in 2.5% glutaraldehyde in 0.1 M Na cacodylate buffer (pH 7).

**REMOVAL OF EXTRACELLULAR MATRIX FROM MICROVESSELS.** Respective microvessels were incubated after fixation for 8 min in 30% (wt/vol) KOH at 30°C, washed with 0.1M Na phosphate buffer, and incubated for 12 h in 0.1% collagenase D (Roche) in PBS at 37°C. Subsequently such preparations were fixed further with tannin and osmium as described elsewhere (36, 61). Finally, all specimens were washed in PBS. Very delicate structures (freshly attached isolated vessels) were fixed by setting onto an ice-cooled plate and subsequent exposure to vapor (45°C) from a 37% formaldehyde solution. For scanning electron microscopy, cell cultures and isolated blood vessels were fixed for ≥2 h in 2.5% glutaraldehyde in 0.1 M Na cacodylate buffer (pH 7).

**IMMUNOHISTOCHEMISTRY.** A minimum of six specimens were analyzed per study, and the conclusions drawn were representative of all samples tested. Specimens were incubated with antibody solution in PBS with 3% BSA (at 37°C for 1 h) and washed in PBS containing...
1% BSA and 0.1% Triton-X-100. For double staining, this procedure was repeated with different primary antibodies and appropriate secondary antibodies. Negative controls, omitting primary antibody, were made for each antibody and protocol. In contrast to this standard immunohistochemical staining procedure on fixed tissues, incubation with antibody against the 3g5 ganglioside antigen was performed on living cells in HEPES-buffered culture medium without serum for 30 min at room temperature. After being washed with medium and fixed for 5 min with PA, cells were incubated with secondary (anti-mouse κ-chain clone EM34.1) and tertiary (AlexaFluor 546 anti-rat) antibody, using solutions without Triton-X-100 and subsequently fixed for 1 h.

STAINING VIA Di-AC-LDL UPTAKE. Cell cultures were incubated in DMEM with 1% FCS and 10 μg/ml Di-Ac-LDL for 5 h at 37°C and subsequently washed and fixed.

ENZYME HISTOCHEMISTRY. Alkaline phosphatase (AP; EC 3.1.3.1.) and/or dipeptidyl amino peptidase IV activity (CD 26; EC 3.4.14.1) were visualized enzyme histochemically using standard procedures (12, 63).

SILVER STAINING. The extracellular matrix (ECM) of pericytes was silver-stained according to (5) with minor modifications. Hemostasiological assays. Factor X activation and the assembly of the prothrombinase complex were quantified as described previously (87, 96). For the preparation of vein wall extract for examining the possible inducibility of TF (41), human saphenous veins (2.5 g) were homogenized (0°C) for 5 min in 10 ml DMEM, warmed to room temperature, and subsequently centrifuged for 2 min at 10,000 g.

Dye transfer studies. Confluent cultures of bovine pericytes were washed and incubated in the protein-free staining buffer supplemented with 25 μM CellTracker CMFDA at 37°C overnight. After recovery of the stained cells in the usual DMEM medium for 4–5 h, they were loaded with calcine in protein-free staining buffer containing 10 μM calcine AM for 30 min at 37°C. The cells were then detached using trypsin-EDTA, washed in PBS by low-speed centrifugation and seeded (5–10/100 cm²) into cultures of species autologous EC, pericytes, or SMC of arteriolar origin. After 2½ h in the incubator, cultures were washed and photographed.

Statistical analysis. Data are presented as means ± SE.

RESULTS

Isolation and Purification of Pericytes from In Vitro Perfused Hearts of Eight Species

Figure 1 shows the complete procedure employed for the isolation of pericytes from the left ventricular myocardium of young mice, rats, hamsters, guinea-pigs, rabbits, pigs, and cattle and from explanted hearts from transplant patients. Important for the successful perfusion of the animal hearts was the means of death: captive bolt pistol for the large animals and decapitation for the rodents. Electric shock (used often in abattoirs for the slaughter of pigs) was unsuitable because the hearts hypercontracted irreversibly and could not be perfused. For the above reasons, we employed bovine pericytes throughout the following experiments. Nevertheless, random checks (in dishes from 3–5 hearts per species) showed that both the typical histological features of the cultivated bovine pericytes (Fig. 5) and the characteristic cell-biological and functional features described below (see Figs. 6, 7, 8, and 9 and Table 1) were present in pericyte cultures from all the other species, provided, as noted above, that these had been grown in media containing the appropriate autologous fetal or neonatal serum.

Identification and Characterization of Cultured Pericytes by Histological Features

Cultured pericytes expressed SMC-α-actin (Figs. 5, A–D) and calponin (Fig. E) in a concentration six times higher than in EC of venular origin but at only 10% of the concentration seen in SMC of aortic (or arteriolar) origin. AP expression as an ecto-enzyme of the plasmalemma was particularly characteristic for individual pericytes in a proliferating culture that had just become stationary after an initial phase of amoeboid...
motility (Fig. 5F; Supplemental Video). In postconfluent cultures, this enzyme was increasingly exported and incorporated also into the pericyte ECM (Fig. 5G), which contained abundant, lipophilic microparticles (63). The presence of the latter may be one reason for the highly selective deposition of metallic silver in the ECM of pericytes during their incubation with silver nitrate solution (black staining in Fig. 5H), a reliable criterion used successfully in the past to identify and demarcate these cells in intact vessels (5, 41, 96, 101). Furthermore, a high level of TF-antigen expression (Fig. 5I) was evident on pericytes, this not being detectable in either EC or SMC of the coronary microcirculation (63). Similar to AP, TF accumulated in the form of aggregates or as a component of microparticles at the basal side of EC in sandwich cultures (Fig. 5J and K). A characteristic ganglioside of vital microvascular pericytes that can be labeled directly by a monoclonal antibody (3g5) proved to be a further very specific marker (Fig. 5L). Moreover, pericytes in confluent cultures expressed PDGFR-β (Fig. 5M), NG2 (Fig. 5N), and Cx43 (Fig. 6, Ea and Eb). Additionally, pericytes were able to phagocytose dextran microparticles (not shown), and they endocytosed Ac-LDL via a scavenger receptor (Fig. 5O, left). In contrast to the always spindle-shaped, unbranched SMC and the EC with their paving-stone-like morphology, pericytes in culture could be identified morphologically by their long, branching (“antler-like”) processes and thin filipodia (Fig. 5O, right, and Fig. 6D; see also discussion of stained coronary microvessels in organ cultures in Ref. 63).

Identification and Characterization of Cultured Pericytes by Functional Features

Since histological markers of pericytes are in part still controversially discussed, we also examined cell-physiological and biochemical properties attributed to pericytes on the basis of in situ observations, namely cell-to-cell communication via gap junctions with other vascular wall cells, expression of procoagulatory components, and cooperation with EC during the formation of angiogenically sprouting vessels.

Intercellular communication of pericytes with each other and with EC and SMC. The studies of Zimmermann (101) already early indicated that the elongated and antler-like pericyte processes loop extensively around venules and capillaries in situ (101). We (63) have shown recently that pericytes are also constitutive in the intima of the entire arterial limb of the coronary system and completely engird the endothelial core tube. As far as we are aware, the extent to which these pericytes are interconnected by their processes has not yet been investigated. Since our new isolation procedures allowed us to prepare largely intact myocardial microvascular networks (63), such investigations appeared promising, particularly since complete functional units of the myocardial microvasculature could be stained uniformly over their entire length for the first time. Confocal microscopy allowed the rapid and sequential documentation of the staining patterns in numerous focal planes, whereby maximal intensity projections into one plane enabled quantitative evaluation. The pericytes were labeled specifically
CULTIVATION AND CHARACTERIZATION OF DIFFERENTIATED PERICYTES

Fig. 3. Final purification of highly enriched pericyte cultures by means of nutrient-deficient medium and repeated rinsing with PBS. A: cell mixture trypsinized from cultures such as that shown in Fig. 2/ after incubation for 1 day in glutamine-deficient medium. Staining for AP/DAP, pericytes (blue), and EC (red) are visible. B: culture analogous to A but 10 days older: EC are no longer seen. C: analogous culture after 2 mo in nutrient-deficient medium. The last dead EC float on the surface (bright spots). D: after normal medium was reintroduced, homogenous pericyte cultures can be obtained from such dishes. E: control experiment showing the consequences of replacing the nutrient-deficient medium by normal medium too soon (here already after 6 wk). Surviving EC began to proliferate rapidly again and form large colonies again already after 1 wk (F).

with fluorescent antibodies against α-SMC-actin, the EC with antibodies against vWF, and the nuclei (occasionally) with 4′,6-diamidino-2-phenyindole, dilactate. On this basis, we attempted to demonstrate similar interconnections between individual pericytes, and between pericytes and EC, as further evidence for their high degree of differentiation.

The representative confocal overview of an isolated terminal vessel net in Fig. 6A gives already the impression that all pericytes within the smallest functional units of the coronary system (consisting of a precapillary arteriole, its capillary net, and one or more associated postcapillary draining venules) are connected. The mean total vessel length of such units was 6,470 ± 1,360 μm/per functional unit (n = 9). In fact, vessel segments free of pericytes made up only 0.9 ± 0.3% of total vessel length (investigations at high light microscopical magnifications). The general histological condition of such preparations obtained by proteolysis implied strongly that these rare gaps were probably preparative artifacts, so that it might be claimed that a pericyte “internet” is present in the coronary microcirculation, at least with respect to anatomical facts. The average ratio of EC to pericytes in such functional units in the bovine coronary microvasculature was 2 to 3:1. In isolated vessel preparations from which the ECM had been removed proteolytically (Fig. 6B), high-resolution scanning electron microscopy showed that the intimate pericyte interconnections, already apparent using light microscopy (Fig. 6C), were in fact achieved by extremely fine, many-branched processes that not only connected the pericytes but, moreover, also the accompanying EC.

In extensive studies on cultures, high-power views showed that pericytes were generally interconnected and, in mixed cultures, also with EC (regardless of whether the latter were of venular or arteriolar origin). Figure 6, C and D, shows representative images of the fine processes in the regions of contact. Interestingly, at least in proliferating mixed cultures, these intercellular contacts were not permanent but subject to very dynamic change (see Supplemental Video). In their contact areas, the pericytes formed gap junctions between each other, and expressed Cx43 selectively (Fig. 6E) but not Cx40 or Cx37.

Further studies showed that pericytes loaded with fluorescent calcein transferred this dye readily to other autologous pericytes via long and delicate processes and often over considerable distances (up to 250 μm). A similarly rapid dye transfer to species-specific EC of arteriolar or venular origin was observed, while, dye transfer to species-autologous SMC of aortic or arteriolar origin occurred very rarely and then only very slowly (Fig. 7).

Microvascular pericytes as foci of procoagulatory reactions. Assays of the TF-catalyzed formation of coagulation factor Xa revealed that pericyte cultures expressed up to 50% of their total content of TF on their surface and obviously also in the ECM (Table 1). This follows from the marked increase in TF concentration with culture time after confluence, despite the only slow increase in cell numbers. Prothrombinase expression, on the other hand, was clearly associated with cell numbers (nuclei counts). The expression of high concentrations of both of these procoagulatory catalysts was apparently constitutive, since we never observed TF-catalyzed increases of Xa or prothrombinase-catalyzed increases in thrombin formation in the presence of extracts from human vein walls. Such extracts had proven to be very effective with respect to induction of TF in intimal pericytes of human venous and aortic origin in situ and in culture (41, 68).
Angiogenic cooperation with EC. EC in pure cultures of venular origin expressed greatly increased concentrations of typical markers like DAP and vWF after incubation in conditioned culture medium (obtained by 24-h contact with a dense mixed culture of pericytes and venular EC; Fig. 8, A and B), despite the absence of any conspicuous form changes, even during prolonged culture (over months).

After seeding together with pericytes, time-lapse videocinematography showed that persistent and rapidly varying contacts between the pericyte processes and the vagrant EC were established immediately (Supplemental Video). At the same time, more and more ECM components were deposited on the floor of the culture dishes (revealed by immunofluorescence microscopy in separate studies). The dispersed EC were increasingly crowded together by the circulating pericytes, thus forming rapidly enlarging islets with the typical paving-stone cytoarchitecture. Shortly thereafter the first angiogenically activated EC emerged (Fig. 8, B and C). Exceptionally high concentrations of the characteristic endothelial markers vWF, DAP, platelet EC adhesion molecule-1, intercellular cell adhesion molecule-1, and vascular endothelial cadherin, a special proteoglycan, but not AP, were expressed in these EC. These activated EC subsequently accumulated in the vicinity of the pericyte strands, increasingly frequently resting directly on top of these (Figs. 8, D and E, and 9, A, D, and F). Scanning electron micrographs clearly showed the bizarre morphology of these cells of venular origin, which we named director cells. The richly developed lamellipodia gave these cells the appearance of small blossoms (Fig. 9, A–C), occasionally even with a stalk-like process (Fig. 9D), the first part of a forming capillary sprout. Mitotic progeny, delivered continuously from the neighboring endothelial colonies, accumulated in the immediate vicinity of the director cells and attached to the pericyte substratum or at least in direct contact with neighboring pericytes. They then began to divide systematically (Figs. 8, D and E, and 9, F and G). Daughter pericytes moving away from their parent cells nevertheless appeared to maintain permanent contact with the latter via long, straight processes (Figs. 8, J–L, and 9H). With delicate filipodia at their front (Fig. 8, F and G), these migrating cells closely resembled the morphological description of the so-called "tip cells" (15, 25, 48). When angiogenically activated EC adhered to the straight processes between these pericytes, the dividing EC orientated themselves chain-like and extended in a straight line (Figs. 8, L and P, and 9, F–H). The developing vessels increasingly showed a continuous longitudinal lumen (Fig. 8, G, black arrow, and P). These capillaries were apparently then enveloped in complex ECM, since AP, an important component of the latter, was clearly demonstrable, at least on older such tubes (Fig. 8H). In addition, highly concentrated vWF and polymerized α-SMC-actin were also demonstrable in these evolving tubes, initially in continuous threads, later themselves tube-like (Fig. 8, J–L, and N–P). Finally, pericytes migrated to the newly created vessels (Fig. 8Q) and engirded them, as in vivo, with long processes characteristic of these adventitial cells (56). The angiogenically formed, often branching vessels (Fig. 9G), which were generated spontaneously in mixed cultures without any exogenously added matrices or gel supports in our experiments, detached frequently from the floor of the culture vessel (Fig. 8R). In this they seemed to be assisted by torsion forces from the participating pericytes (Fig. 9H). Their growth proceeded subsequently at the tip, without direct pericyte contact, by means of continuously dividing, morphologically inconspicuous EC, without evidence for any sort of filaments. Numerous pseudovilli were conspicuous and gave the freshly formed capillary vessels a furry appearance (Fig. 9I). Finally, entire capillary networks formed in the culture dishes, always following the pericyte strands (Fig. 8S) and clearly definable by the endothelium-specific marker vWF (Fig. 8T).

In analogous investigations of pericytes and EC of arteriolar origin cocultivated for months, there was no evidence of any similar angiogenic development. The pericytes clumped increasingly together and both cell types grew in parallel without mutual contact of functional relevance.

DISCUSSION

The main goal of our investigations was to develop a reliable and reproducible protocol for the isolation and long-term cultivation of stable pericyte cultures. To identify these cells unambiguously, it was also necessary to characterize them both histologically and with respect to important physiological and biochemical functions.

Fig. 4. Growth curves for homogenous pericytes cultures from 8 different species in standard culture medium supplemented with 10% vol/vol FCS. A: cattle (○), human (□), domestic pig (▲), and rat (●). B: rabbit (○), mouse (■), hamster (△), and guinea pig (●). Data are means ± SE; n = 3 experiments in each group.
Heart as Starting Point for Highly Enriched Pericyte Preparations

The cardiac microcirculatory system is complex and extremely densely capillarized. In the rat, for instance, there are $9 \times 10^7$ EC/cm$^3$ tissue (53, 55), compared with only $2.7 \times 10^7$ cardiomyocytes/cm$^3$ left ventricular myocardium (53, 55). The ratio of EC to pericytes in skeletal muscle is 100:1 (3, 82). In contrast, we found a ratio of 2:1 to 3:1 in left ventricular myocardial tissue. Thus with $3.6 \times 10^7$ pericytes/cm$^3$ there are more pericytes in the heart than cardiomyocytes, and the pericyte is the second most frequent myocardial cell type.

The heart thus appeared to be an appropriate starting point for the isolation of large numbers of microvascular pericytes. Surprisingly, it seems not yet to have been used for this purpose, although there are numerous reports on the cultivation of pericytes from skeletal muscle (58), brain (22, 43), liver (95), skin (35), retina (62), lung (18), pancreas (84), kidney (74), placenta (52), and choroid plexus (60).

The isolation of microvascular pericytes from parenchymatous organs is an extremely complex separative problem that cannot be solved either by simple filtration or by the sole use of supposedly selective growth media. Immunological separation of dispersed tissue (i.e., magnetic bead separation) is also highly questionable, since the prerequisite for success with these techniques, an absolutely homogenous cellular suspension, as in the blood, is most unlikely to be fulfilled after proteolytic tissue decomposition (in our experience heterogeneous, cellular microaggregates are always unavoidable).

A particularly important aspect at the start of our studies was thus the search for suitable proteases with strictly monitored and standardized activity (see Materials), with which we were able to isolate microvessels and to obtain...
cell suspensions reproducibly, species independently, and with a high yield.

The next step was not only to separate the cells of interest from the enormous mass of cardiomyocytes (84% of the myocardial volume is attributable to these cells, only ~2 to 3% to all other cell types; Ref. 55) while losing as little microvesSEL tissue as possible but also to eliminate contaminating cells, for example, EC of diverse vascular or lymphatic origin, SMC,

Fig. 6. Occurrence of pericytes in the myocardial microvascular system and their intercellular connections in situ and in vitro. A: confocal image (overview, maximal intensity plot) of a proteolytically isolated microvascular network (left ventricular bovine myocardium) after labeling for vWF in EC (green) and for α-SMC-actin (red) in pericytes; art, arterioles; ven, venules. Interposed individual capillaries are not indicated separately. B: scanning electron micrograph of a capillary shortly beyond the point at which it emerges from its precapillary arteriole. ECM has been removed proteolytically from the formaldehyde fixed vessel. Cell extensions between pericytes are indicated by red arrows, connections between pericytes (P) and EC (E) by white arrows. If, in the course of the preparation, these connections broke at the EC surface, tiny holes or pores were visible (blue arrowheads). C: overview of a mixed culture of bovine pericytes and venular EC. Numerous processes of 2 elongated pericytes make contact with each other and dozens of EC (only some indicated by arrows). D: bright-field view of a pericyte after AP staining showing its finest processes in direct contact with a further pericyte at left. E: immunohistochemical demonstration of connexin 43 (Cx43) in cultivated pericytes, which is expressed in particularly high concentration in the contact zones (white arrows) between adjacent pericytes (Ec) and on the ends of their delicate processes (Eb).

Fig. 7. Calcein transfer between pericytes and other cell types in coculture. Bovine pericytes were prelabeled overnight with cell tracker (blue) in protein-free medium and, after 5-h recovery in culture medium loaded with calcein (green) for 30 min, proteolytically detached, suspended and incubated in species-autologous coculture with EC of venular origin (A–C) or pericytes (D–F) or SMC of aortic origin (G–J). Ratio of cultured cells to seeded pericytes was about 10:1 in each case. Micrographs show that within 150 min of incubation there was substantial dye transfer within homogenous pericyte tissue and in mixed cultures of pericytes and EC but only rarely and weakly in cocultures of pericytes and SMC (only ~5% of the latter were weakly labeled). A, D, and G: phase-contrast images of the starting cultures. B, E, and H: distribution of the cell tracker. C, F, and I: distribution of calcein. J: identification of α-SMC-actin in pericytes (weak labeling) and SMC (strong labeling).
Table 1. Procoagulatory activities of pericytes isolated from the bovine coronary microcirculatory system

<table>
<thead>
<tr>
<th>Pericyte Cultures</th>
<th>Activation of Factor X, nmol Xa min⁻¹·10⁻⁶ cells</th>
<th>Prothrombinase Activity, nmol IIa min⁻¹·10⁻⁶ cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>2 wk, 2.6·10⁵ cells/cm²</td>
<td>1.31 ± 0.28</td>
<td>0.48 ± 0.04</td>
</tr>
<tr>
<td></td>
<td>5.61 ± 0.49</td>
<td>0.53 ± 0.04</td>
</tr>
<tr>
<td>Surface activity</td>
<td></td>
<td></td>
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<tr>
<td>Lysate</td>
<td></td>
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<tr>
<td>4 wk, 3.3·10⁶ cells/cm²</td>
<td>2.43 ± 0.27</td>
<td>0.62 ± 0.07</td>
</tr>
<tr>
<td></td>
<td>7.29 ± 0.44</td>
<td>0.70 ± 0.06</td>
</tr>
<tr>
<td>Surface activity</td>
<td></td>
<td></td>
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<tr>
<td>Lysate</td>
<td></td>
<td></td>
</tr>
<tr>
<td>8 wk, 3.7·10⁶ cells/cm²</td>
<td>3.69 ± 0.49</td>
<td>0.81 ± 0.06</td>
</tr>
<tr>
<td></td>
<td>7.36 ± 0.50</td>
<td>0.73 ± 0.04</td>
</tr>
<tr>
<td>Surface activity</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lysate</td>
<td></td>
<td></td>
</tr>
<tr>
<td>8-wk-old culture of same density but in the presence of vein wall extract</td>
<td>3.80 ± 0.54</td>
<td>0.83 ± 0.06</td>
</tr>
<tr>
<td></td>
<td>7.94 ± 0.66</td>
<td>0.78 ± 0.06</td>
</tr>
</tbody>
</table>

Values are means ± SE; n = 12 in each experiment. Functional activity of factor VIIa-tissue factor complexes was measured by their ability to activate factor X and that of the prothrombinase complexes to generate thrombin. Vein wall extract for the activation of pericytes was prepared as described in MATERIALS AND METHODS.

fibroblasts and myofibroblasts, mast cells, macrophages, and the various residual blood cells. Most of these cells develop considerable proliferative potential during cultivation and displace the pericytes. We resolved these problems by a step-wise combination of filtration, mechanical agitation with standardized shear forces, high-performance density-gradient centrifugation, manual removal of contaminating cell colonies under microscopic control, and, finally, prolonged incubation in glutamine-free starvation medium. After density-gradient centrifugation and rinsing, our pericyte harvest was 4 ± 1.5·10⁶ cells/g myocardium, which represented ~10% of the estimated total number of pericytes in 1 g ventricular tissue.

Final Purification and Cultivation of Pericytes

Pericytes do not make major demands on the nutrient supply (82). Presumably these cells have a very complex metabolism, such that they are able to synthesize most of what they require from, for instance, the relatively few basic substances contained in DMEM. Microvascular EC, on the other hand, require considerably more complex culture media, although in mixed cultures (as in vessels in situ) the pericytes seemed to be able to supply the EC with the necessary nutrients, at least as long as they are close together (the so-called “nursing function” of the pericytes; Refs. 33, 41). The intensive and dynamic contact between EC and pericytes via the latter’s processes can be seen in the Supplemental Video. In a glutamine-deficient medium, however, the pericytes presumably no longer exercise their nursing function, and thus it was possible, by incubation in this medium, to “starve out” and hence completely remove the remaining EC from the pericyte cultures.

In contrast to the nutritional modesty shown by pericytes, they appear to have a major requirement for particular growth factors present only in fetal/neonatal/placental serum from the same species. This contention is supported by our studies on the growth of homogenous pericyte preparations obtained from the hearts of young mice, rats, hamsters, guinea-pigs, rabbits, domestic pigs, cattle, and middle-aged humans. Nonbovine pericytes cultured in media supplemented with FCS grew considerably less rapidly than bovine pericytes in the same medium, and the greater the phylogenetic distance between a given species and cattle, the lower the growth rate. Nonphysiological giant cells were frequent, as was the failure to express the typical markers and functions. Such cells were also exceptionally sensitive to centrifugal forces. The consequences of the species-autologous growth factor deficiency were particularly crucial for the guinea-pig pericytes. The latter, grown in medium supplemented with FCS, became apoptotic and died regularly after only 4 days. Of note is the fact that the guinea-pig was the only new-world species in our list.

Histological Identification of Highly Purified Pericytes of Bovine Origin

Following purification and cultivation under optimized conditions, all pericyte batches were checked for the expression of typical features. Different pericyte marker techniques are available, and identification was all the more reliable with double or triple staining for multiple markers. We routinely checked the cellular expression of TF (7, 65), α-SMC-actin (23, 41), 3g5-antigen or ganglioside (35), and the synthesis of an argenophilic ECM, in which the pericytes are cocooned, as can be seen for in capillaries in situ (5), as well as in confluent cultures (Fig. 5 and Ref. 63). In native capillaries, this structure below the endothelial layers (41, 63). This is the reason why AP, a specific and readily demonstrable pericyte ecto-enzyme (17, 41, 63), can easily be mistaken for an EC marker, as has been suggested repeatedly (30, 51, 73). The presence of this enzyme in endothelial cultures should thus, from now on, be regarded simply as evidence for contamination by pericytes, which can easily be checked (63).

The identity of the cultured pericytes was confirmed further by the presence of the following antigens: NG2 (41, 52, 58), PDGFR-β (34, 58, 97), and Cx43 (49, 58). The last was expressed...
exclusively in homogenous pericyte cultures, and no Cx40 or Cx37, held to be typical markers of EC (100), was detected. Last but not least, the numerous long and branched processes of cultured pericytes (Figs. 5O, right, and 6, B–D and Eb) gave the pericytes a unique morphology that was vastly different from the spindle-shaped vascular SMC and the paving-stone-like EC.

It should also be noted, that expression of some of the histological markers in proliferating pericytes varies consider-

Fig. 8. Spontaneous angiogenic interactions of pericytes and venular EC in 7-mo-old mixed cultures. In all the following images pericytes were stained enzyme-histochemically for AP (blue) or immunologically for α-SMC-actin (red), the EC analogously either for DAP (red-brown) or vWF (green). A: homogenous culture of venular EC in conditioned medium. Some cells showed signs of activation, here, for instance, stronger expression of DAP. B: angiogenically activated cells [termed director cells (DC)] showed strong expression of vWF. This micrograph is focused in the plane of the culture in which also some elongated pericytes (arrows) are visible. C: as in B, but focused at the surface of the erected DC. D: high-power phase-contrast micrograph showing cooperation between different cells in the generation of a new vessel. Pericytes [practically the tip cells of this just-forming cellular complex (*)] can be seen, together with an angiogenically activated DC (rounded cell form with a folded surface, arrow), and several DC-precursors (already rounded up, folded surface, but lower DAP) accumulating in the immediate vicinity (lower edge of the picture). E: Bright-field image of the section in D showing the prominent EC more clearly. F: overview of a culture showing numerous pericytes (arrows) with tip-cell-like morphology at the front of sprouting capillary tubes. Also visible are numerous DC (small brown spheres). G: pericytes with tip-cell-like morphology (blue arrows: filipodia-like processes) in contact with DC (red arrow) often form vessel branching points; black arrow: developing lumen in capillary tube. H: sprouting vessels that frequently connect pericyte accumulations and EC in the immediate vicinity are soon enveloped in AP-containing ECM (blue) that is obviously bound to tiny vesicles or granule-like particles, which were attracted by EC. The closer the EC lay to the pericytes the more AP they bound (arrows). I: phase-contrast image showing an angiogenically activated cluster or row of EC. In the first phase of tube development a central guide strand became obvious and contained highly concentrated vWF (J) and α-SMC-actin (K); this structure was obviously expanded by pericytes L: overlay of fluorescence and phase-contrast showing the EC orientating themselves on the guide thread. M–P: series of images analogous to the previous showing a newly developed vessel at a later stage, in which the newly formed lumen (P) and the circumferentially arranged EC are recognizable. Q: pericytes finally enveloped the increasingly differentiated microvessels, some of which even began to grow out into the culture medium. R: at the tip of such a vessel dividing and vessel-lengthening EC can be seen (*). These did not show any conspicuous features; capillary branches (arrow) also formed spontaneously. S: in the final analysis, the entire cultures were traversed by road-like pericyte formations, along which the microvascular networks were organized (T).
ably with the phase of the cell cycle (e.g., AP, ECM, α-SMC-actin, and Cx43), while the expression of others is relatively constant (3g5-antigen, NG2, and PDGFR), as has been already noted for macrovascular pericytes of intimal origin in one of our recent articles (41).

Functional Characterization of Pericytes

Communication between pericytes and other vessel wall cells via cell processes. Zimmermann (101), the pericyte’s eponym drew attention to the bizarre, extensively ramified processes with which the pericytes engirded the endothelial tube in the capillaries and venules studied. His hand-drawn illustrations of the microscopic images indicated for the first time that pericytes are in direct contact, both with each other and with neighboring EC. He also reported that their antler-like processes often span considerable intercapillary spaces, thus connecting different capillaries with each other.

Functional considerations imply that within microvascular networks there must be a form of communication able to signal local changes in blood flow, due, for instance to metabolic processes, upstream (and ideally even to parallel capillary branches), which would thus allow appropriate adjustments to the flow resistance in the feed arterioles/arteries (77, 79–81) and even within the local capillary net. To date, interest in this aspect has concentrated primarily on intercellular communication within endothelial tissue (28, 31, 76, 78), and there is already good evidence for electrophysiological mechanisms in this respect (11, 26, 28, 58, 88, 92).

Combining the techniques described above for the preparation of largely intact myocardial microvessel networks with modern confocal and scanning electron microscopy, we were able to gain further insight into the pericyte interconnections in the coronary microcirculation. The pericytes appeared to be part of a huge vascular internet connecting all the different branches of the coronary microcirculatory system, including all branches of the arteriolar and venular trees (63). The rare cases where gaps were seen between them were most likely preparative artifacts. In addition, all the pericytes studied were also intimately connected to the neighboring endothelium. Against this background, we deemed it important to document intercellular communication between pericytes and neighboring cells in culture as evidence in support of the high degree of differentiation of the cultivated pericytes. Time-lapse video microcinematography showed clearly that pericytes actively established contact via their elongated, ramified, and filigreed processes with each other and with EC, not only those of arteriolar origin, but also those of venular origin. This cellular interaction was morphologically and cyto-architectonically highly dynamic. We formed the impression that this activity supported the rapid amalgamation of EC into a tissue (Supplemental Video).
Dye-transfer studies are an accepted means of demonstrating direct cell coupling via gap junctions (50). We observed rapid calcine transfer from loaded pericytes to both other pericytes and EC but only rarely, and then only very slowly, to SMC (of aortic and arteriolar origin). These findings have important implications for cell-cell signaling in the vessel wall, since they imply that pericytes and their associated EC form a functional unit. We have recently shown (63) that all myocardial arterioles of the muscular type (and even the feed arteries and coronaries) have an inner tube of endothelium that is separated from the vascular musculature by an intermediate layer of pericytes with a highly developed, partially hydrophobic ECM (which is also an essential part of the intima of the larger blood vessels; Ref. 41). However, as far as we are aware, there is neither functional nor electron microscopic evidence to date for direct contact between pericytes and the vascular SMC. From a functional standpoint, the muscular arterioles thus appear 1) to consist of a central double-layered tube of EC and pericytes, and 2) to have an outer tube of SMC. The very slow dye transfer between pericytes and autologous SMC in tissue culture implies further that these two blood flow regulating systems may be largely independent of each other. This contention stands in contrast to the widely held view that the arteriolar endothelium and the corresponding vascular SMC are in direct contact via myoendothelial contacts or junctions (16, 39, 50), reported to be present in particularly large numbers in the smallest arterioles (32). If it were assumed that the intermediate tube in the muscular arterioles also consisted of SMC, the concept of myoendothelial junctions could be accepted. In our view, however, the intermediate tube consists of pericytes (63) and the myoendothelial junctions in the most important resistance vessels are in fact pericyto-endothelial junctions.

Central role of pericytes in the induction of hemostasiologic processes. The immunological demonstration of TF (Fig. 5, I–K) at the surface of pericytes and on microparticles of their ECM, together with the functionally effective, constitutive procoagulatory activity of the pericytes (Table 1) emphasizes the recently recognized central role of this cell type in hemostasiologic processes (7, 41, 96). TF-containing microparticles are present in plasma (54, 59) and are thought to be responsible for the initiation of thrombotic processes and for the danger emanating from arteriosclerotic plaques (75), in particular from those described as “vulnerable” (2, 57, 66). These, by virtue of the enormous concentrations of TF, can suddenly induce acute vessel occlusion by fibrin emboli (91). As observed elsewhere, the TF-positive microvesicles in plasma and the prothrombotic nature of many arteriosclerotic plaques very probably originate from the subendothelial pericytes of the intima (41) rather than from EC, as currently believed (8, 21). Relevant in this context is our finding that pericytes obviously possess a scavenging receptor for rapid uptake of Ac-LDL (Fig. 5O). Thus we believe that intimal pericytes (rather than macrophages or altered SMC) are not only the focus of dangerous sudden thrombotic processes at the surface of arteriosclerotic plaques (41) but also the precursors of the foam cells in these lesions.

Key participation of pericytes in angiogenic processes. Angiogenic sprouting of new blood vessels is a very complex process in which the newly formed vessels are initially not connected to the circulatory system (1, 9). The process requires that the participating EC and pericytes associate spontaneously and produce vascular endothelial growth factor (VEGF; Refs. 29, 64, 67, 70–72), whereby the ECM also appears to play important roles (24, 46). Strong experimental evidence has suggested repeatedly that EC of venular origin are most important with respect to angiogenesis (20), and our observation that arteriolar EC never showed angiogenic activity in cocultures with their respective pericytes is in good agreement with these reports. In recent years, the nature and function of the so-called tip cells, which determine the direction of vessel growth, have attracted much interest. In studies of angiogenesis in the brain and retina, these cells have been regarded as highly specialized EC with fine filopodia (15, 25, 48).

There are diverse model systems that facilitate these angiogenic processes in vitro. These rely generally on the presence of an appropriate substrate (fibrin or collagen gels, Matrigel) to support newly formed vessels and to increase the motility of participating cells (89). Since we did not use such auxiliary supports in the present study, the spontaneous occurrence of angiogenic activity in the mixed cultures of venular origin can be seen again as good evidence for the high degree of differentiation of the individual cellular components under our culture conditions. As noted above, spontaneous angiogenesis was observed frequently in month-long cocultures but, in agreement with the observations cited above, only in presence of pericytes. This angiogenesis was comparable in morphological detail to the processes observed in situ. An unexpected finding was, however, the existence of apparently specialized EC expressing very high levels of the typical EC markers vWF, DAP, platelet EC adhesion molecule-1, intercellular cell adhesion molecule-1, and vascular endothelial cadherin but not AP. These cells appeared to play a key role in myocardial angiogenesis; therefore, we named them director cells. Instead of filopodia these cells had well-developed lamellipodia, the role of which, in addition to the probable recruitment of membrane functions (such as expression of VEGF receptors; Ref. 38), is possibly to achieve increased mobility. Collective motility of interacting cells is a poorly understood, but fundamental, prerequisite during the development of new vascular systems (13, 85). Director cells always accumulated in the immediate vicinity of, or on pericytes, and it is tempting to speculate that they anchor by binding to structure-bound VEGF in the pericyte matrix. The director cells might also be responsible for the local accumulation of VEGF-A. The pericytes then began to divide and to assume guideline morphology and function. In the immediate vicinity of such angiogenically activated pericytes, although not in direct contact with the director cells, further EC accumulated and rapidly began to divide. In our cultures, the role of the tip cell was assumed not by EC, but by the pericytes on which the continuously dividing EC unified to form a tube in which a longitudinal capillary lumen was finally developed. These pericytes elongated in certain directions and at their front extended often long processes that may be regarded as filopodia (98). Particularly elongated processes or filaments that remained in contact with previously emigrated pericytes appeared to define primarily the direction of movement and extension of these cells and hence secondarily the direction too in which the sprouting and spontaneously branching (15) vessels grew. In this context, tensional forces between the migrating pericytes obviously played an important role, as suggested earlier (45, 85). Finally, the newly formed capillaries
Cultivation and Characterization of Differentiated Pericytes

were invaded by pericytes, which engirded their parent vessels with their long extensions, as is usually seen during angiogenesis in situ (64).

Final Considerations and Prospects

In the foregoing, we described methods for the isolation, purification, and culture of pericytes of bovine origin, and the cells obtained have been shown to be both morphologically and functionally highly differentiated. The initial preparative effort is considerable but worthwhile, since the cells grow well and can be subcultured many times and stored frozen without loss of their typical features. Our methods and results would thus seem to offer a good starting point, not only for further investigations into the physiology and pathophysiology of pericytes but also for bulk production of these cells, which is a prerequisite for isolation of pericyte-specific antigens for production of the corresponding antibodies. Using these cells in sandwich coculture with EC of defined microvascular origin, we have begun to construct in vitro models of the precapillary arteriolar and postcapillary venular walls for studies on the important barrier functions of these vessels (41a, 63).

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DISCLOSURES

All authors have no possible conflicts of interest with the publication of this article.

REFERENCES


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