Pericytes in the macrovascular intima: possible physiological and pathogenetic impact

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Juchem G, Weiss DR, Gansera B, Kemkes BM, Mueller-Hoecker J, Nees S. Pericytes in the macrovascular intima: possible physiological and pathogenetic impact. Am J Physiol Heart Circ Physiol 298: H754–H770, 2010. First published December 18, 2009; doi:10.1152/ajpheart.00343.2009.—The frequently observed den-endothelialization of venous coronary bypass grafts prepared using standard methods exposes subendothelial prothrombotic cells to blood components, thus endangering patients by inducing acute thrombo-embolic infarction or long-term proliferative stenosis. Our aim was to gain deeper histological and physiological insight into these relations. An intricate network of subendothelial cells, characterized by histo-logical features specific for true pericytes, was detected even in healthy vessels and forms, coupled to the luminal endothelium, a second leaflet of the macrovascular intima. These cells, and particu-larly those in the venous intima, express enormous concentrations of tissue factor and can recruit additional amounts of up to the 25-fold concentration within 1 h during preincubation with serum (intimal pericytes of venous origin activate 30.71 ± 4.07 pmol coagulation factor x·min−1·10−6 cells; n = 15). Moreover, decoupled from the endothelium, they proliferate rapidly (generation time, 15 ± 2.1 h, n = 8). Central regions of atherosclerotic plaques, as well as of those of restenosed areas of coronary vein grafts, consist almost completely of these cells. In stark contrast with the prothrombogenicity of the intimal pericytes, intact luminal endothelium recruits high concentra-tions of thrombomodulin (CD 141) specifically within its intercellular junctions, activates Protein C rapidly (42 ± 5.1 pmol/min·106 venous endothelial cells at thrombin saturation; n = 15), can thus actively prevent coagulatory processes, and never expresses histologically detectable and functionally active tissue factor. Given this strongly prothrombotic potential of the intimal pericytes and their overshooting growth behavior in endothelium-denuded vascular regions, they may play important roles in the development of atherosclerosis, thrombo-sis, and saphenous vein graft disease.

endothelium; tissue factor; atherosclerosis; thrombosis; coronary by-pass operation

IMMUNOHISTOLOGICAL STUDIES have shown that high concentra-tions of tissue factor (TF), one of the key catalysts of coagulation, are expressed specifically in the adventitial and, surpris-ingly, also the intimal region of surgically freshly explanted human saphenous veins. During intraoperative handling and temporary storage of the vessel segments in the course of bypass operations, the antigen expression level in the intima increases dramatically (52). Our laboratory has shown recently that surgical manipulation of venous bypass vessels often results in extensive endothelial lesions (73). Once this occurs, TF expressed in the subendothelial region of the intima is exposed to blood and immediately activates the respective coagulation cascades. In parallel, activated platelets and neutrophils attach firmly to extensions and to the extracellular matrix of peculiar subendothelial cells, in which these hitherto almost completely ignored intimal cells are cocooned.

Taken together, these observations imply that endothelium-denuded coronary bypass vessels, especially those of venous origin, possess substantial prothrombotic potential that may represent substantial danger to a patient. This concept offers a possible explanation for the highly unsatisfactory clinical prog-nosis for venous grafts in aortocoronary position, which, in the context of the acute phase of the so-called saphenous vein graft disease (42, 51), are threatened soon after operation by sud-denly occurring thromboembolism. A remarkable survey (51) has shown that 15–30% of such bypass grafts fail acutely in this manner within the first postoperative year. In addition, it is known that practically all remaining grafts are endangered in the longer term by primarily proliferative wall processes (which often end up also in sudden thrombotic occlusion). After 10 years some 60% of the vein grafts are occluded and practically all of the remaining grafts reveal angiographically evident severe stenoses (10, 23, 61).

The question of the identity of those intimal cells, which, by means of apparently acute inflammatory stimulation of TF expression and proliferation, are probably responsible for the above disease processes, is difficult to answer. In recent years it has become apparent that vascular endothelium does not express TF (47, 62). The extent to which the presence of cells in the subendothelium can be regarded as physiological at all has never been investigated systematically for veins, and in-frequently for the aorta (2, 54, 57, 60, 63). The earlier sugges-tion of a possible contribution of invading smooth muscle cells (SMC) to the massive TF expression in atherosclerotic plaque regions has now been refuted (24, 47, 75). Immigrating mono-cytes, the role of which has also been discussed extensively in this context, express TF only after activation and then only at very low levels that could never account for the procoagulatory potency of vulnerable plaques (46, 47). The same holds for a possible cooperation between monocytes and platelets, since the latter also express only very low amounts of TF (11). To date only a single study has mentioned intimal pericytes as important pathogenetic cells and attributes the deposition of insoluble calcium salts in atherosclerotic aortic lesions to these cells (8). According to a further histological investigation, pericyte-like cells occur not only in the intima of normal human arteries but also in that of veins (2). Current opinion,
However, still regards pericytes as pluripotent cells being restricted to the microvasculature (19).

Obviously, a significant discrepancy exists between the common knowledge that the macrovascular intima constitutes the origin of almost all severe vascular illnesses (such as saphenous vein graft disease, thrombosis, and atherosclerosis) and, as yet, our still insufficient knowledge about the cytobiologic texture and the functional roles of this particular wall layer of macrovessels in the various areas of the human circulatory system. This applies to arteries, but all the more so to the relatively scarcely investigated veins.

To gain further insight into these complex pathogenetic relations, we re-examined intimal cell-architecture in human and bovine aorta and human saphenous veins using specific microscopic techniques for the inspection of intimal structure in situ as well as specially developed intimal tissue/cell isolation and culture methods. We show that the healthy macrovascular intimal barrier to the blood comprises not only the endothelium but also a subendotheliocally closely coordinated, net-like second tissue layer made up of cells, best described as true pericytes. These hitherto largely ignored, constitutive intimal cells display rapidly inducible and crucial prothrombotic features and can proliferate rapidly in endothelium-denuded areas in contact with the locally formed serum. We therefore hypothesize that intimal pericytes are not only at the primary focus of such fateful pathophysiologic events leading to saphenous vein graft disease, thrombosis, and atherosclerotic processes but that excessively proliferating and TF-expressing cells of this type are also mainly responsible for the life-threatening symptoms at the final stages of these illnesses.

MATERIALS AND METHODS

All experiments with human tissue were done with written informed consent of the patients and with approval of the Ethical Committee of the Hospital Munich Bogenhausen or the Ethical Committee of the Ludwig-Maximilians-University of Munich according to the principles expressed in the Declaration of Helsinki.

Harvesting of Human and Bovine Tissue

Saphenous vein remnants were harvested after coronary bypass operations, resected vein grafts were obtained during cardiac reoperations, and samples of resected human aortic tissue were gained during aortic surgery. Bovine vessels were collected from euthanized young cows in the local slaughterhouse. The tissue samples were immediately transported under aseptic conditions to the laboratory in ice-cold minimal medium (see supplemental media; all supplemental material can be found with the online version of this article) +10 IU/ml heparin and fixed as described below.

Preparation of Vein Wall Extract

Human saphenous veins (2.5 g) were homogenized (0°C) for 5 min in 10 ml DMEM (Invitrogen, Karlsruhe, Germany), warmed up to room temperature, and subsequently centrifuged for 2 min at 10,000 g. The supernatant was added to SC cultures of various vascular origin to stimulate TF expression.

Preparation of Small Intimal Tissue Samples

Vessels were flushed free of blood with PBS and filled with prewarmed protease solution containing 0.1% wt/vol collagenase D and 0.1% wt/vol dispase II (Roche, Mannheim, Germany) in PBS. To harvest small intimal tissue sheets with a high content of pericytes, the solution was supplemented with 5.5 mM glucose. To isolate almost pure (endothelial) leaflets of the intima under hyperosmolar conditions, 55 mM glucose was added to the protease solution. After 15 min at 37°C, the filling was collected and the veins were repeatedly flushed with the respective PBS-glucose solution. Detached intimal tissue fragments were sedimented at g = 1 and immediately cultivated or histologically processed.

Tissue Culture

The composition of the following culture media and solutions is presented in supplemental media.

Purification and proliferation of intimal pericytes. Iso-osmotically harvested intimal tissue sheets were seeded in minimal culture medium and cultivated for up to 4 wk with weekly medium exchange to obtain pericyte-rich primary cultures. About 24 h after transfer into pericyte enrichment medium, the unattached cells were carefully flushed away with balanced salt solution and the remaining cells incubated for 1 h in PBS supplemented with 0.1% bovine serum albumin. The same protocol was repeated twice. Remaining pericytes were then cultivated to confluency in minimal culture medium for 2 wk with weekly exchange of medium. Cells were detached with a minimal volume of 0.1% trypsin/1% EDTA in PBS, suspended in a 10-times greater volume of minimal medium, gently sedimented at g = 25, seeded, and subcultured for 1 day with an exchange of medium on the next day, then weekly. Experiments were performed on cells until about 80% were confluent before multicellular nodules formed (~3 × 10^6) per 35-mm Petri dish.

Culture of EC. Hyperosmotically harvested intimal tissue sheets were seeded in preferential growth medium and cultivated to confluence with exchange of medium every third day. Subcultures were made analogously. Experiments were performed on completely confluent cell layers (~1.2 × 10^6) EC per 35-mm Petri dish.

All cell cultures were incubated under standard conditions (water-saturated atmosphere; 5% CO2; 37°C) and were given fresh medium 24 h before experiments. Besides standard cultures in 35-mm Petri dishes, special cocultures were created by plating intimal EC or pericytes at defined density in FCS-precultured polyester Transwell inserts (Corning, New York, NY). After 2 days (and firm attachment of the initially seeded cells), the inserts were inverted and the opposite membrane surface plated with the other intimal cell type at defined density. Then the inserts were put in normal position again and cultivation was continued with weekly exchange of medium.

Culture of cellular material from atherosclerotic plaques. The centers of plaque regions were carefully dissected from aortic or restenosed saphenous vein segments and chopped into pieces of 3 × 3 mm. These (~0.5 g) were incubated for 30 min at 37°C in 10 ml of the protease solution defined above and then repeatedly aspirated into a pipette to homogenize the suspension further. After filtration through a nylon net (mesh size, 50 μm), the obtained cell mixture was washed by centrifugation, resuspended in minimal culture medium, and cultivated for 4 days under standard conditions before histological investigation.

Histological Techniques

Tissue fixation. For immunohistochemistry, tissue cultures were prefixed for 2 min at room temperature by immersion in 0.5% glutaraldehyde (GA)/4% paraformaldehyde (PA) in PBS, stored overnight in PA at 4°C, permeabilized with 1% Triton X-100 in PBS for 30 min, washed with PBS, and incubated for 10 min with a 1% solution of NaBH4 to remove free carbonyl groups. Saphenous vein segments were treated similarly, but fixation with GA/PA was performed at a transmural pressure gradient of 100 mmHg.

For enzymehistochemistry or silver staining, all specimens were treated for 2 min with GA/PA and 15 min in PA (vein segments again at 100 mmHg), followed by three washings in PBS.

Staining techniques. A list of the various antibodies and fine chemicals used is given in the supplemental data section.
For immunohistochemistry, a minimum of eight specimens were analyzed per study, and the conclusions drawn were representative of all samples tested. Samples were first exposed to 1% bovine serum BSA in PBS for 3 h and then incubated overnight at 4°C with primary antibodies, washed with 0.1% Triton X-100 in PBS, incubated for 4 h at 37°C with secondary antibodies, and washed thoroughly with PBS. For double or triple 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. Antibody-linked alkaline phosphatase (AP) was used according to the supplier's instructions.

For enzymehistochemistry, demonstration of alkaline phosphatase or dipeptidyl aminopeptidase IV activity was essentially done according to standard procedures (73). The demonstration of intracellular oxidation of leucomyethylene blue in tissue samples and cell cultures was especially elaborated (details for these methods are given in the supplemental data section).

The silver staining of endothelial junctions method was described in detail elsewhere (73).

Preparation of Dipeptide Phosphothreonylglutamine Hydrochloride

Dipeptide phosphothreonylglutamine hydrochloride (PTG·HCl) was synthesized from Fmoc-Thr(PBzI)-OH and Fmoc-Gln(Trt)-Wang resin (100–200 mesh; Novabiochem, Schwalbach, Germany) using standard solid phase techniques (74). The trifluoroacetic acid salt of the peptide was converted into the respective chloride. Recovery was 76%, and purity was 96% (according to matrix-assisted laser desorption/ionization time-of-flight mass spectrometry, HPLC, and TLC analyses).

Hemostasiological Assays

For coagulation factors and chromogenic substrates, factors II, VII, X, and IX were contained in the prothrombin complex (PPS)-solution Octaplex 500 (Octapharma, Langenfeld, Germany). Factors V, X, and prothrombin as well as thrombin- and Xa-substrates (S-2238 or S-2222, respectively; Chromogenix) were purchased from Haemochrom Diagnostica (Essen, Germany).

Factor X activation and the assembly of the Prothrombinase complex were assayed as described previously (67, 73).

RESULTS

The intima consists of the continuous luminal endothelium and a second subendothelial leaflet formed by peculiar, contractile cells that deposit tissue factor in their vicinity.

Figure 1A shows a cross section of a human saphenous vein classically stained (Elastica van Gieson stain) for connective tissue (collagen, red; and elastic fibers, black). The intima (see also magnifications in Fig. 1, C, D, G, and J) is readily apparent as the narrow part of the vein wall between the internal elastic lamina and the lumen, and the adventitia as the peripheral layer reaching from the outer surface of the vessel to the external elastic lamina. The adventitia contains a dense network of nutritive microvessels (some are marked by short arrows in Fig. 1, A and B). The thin intima is surprisingly rich in (black) elastic fibers (Fig. 1C). The media, between adventitia and intima, contains many bundles of circumferentially organized smooth musculature that stain heavily for SMC α-actin (Fig. 1, B and C), calponin (Fig. 1D), caldesmon (Fig. 1E), and desmin (Fig. 1F). Antibodies against these antigens clearly also stain even the smallest arterioles and venules of the vasa venarum (as an example a few are marked by short arrows in Fig. 1, A and B). At high magnification (Fig. 1D; the internal elastic lamina as the leading structure has been demarcated by green lines), it becomes evident that α-actin-positive, contractile cells (ip) are also present in the intima, where they are preferentially organized subendothelially and form a second intimal leaflet. These contractile, also strongly calponin-positive (Fig. 1E), cells are, however, different from the typical SMC, since they contain less caldesmon (Fig. 1F) and almost no desmin (Fig. 1G). The microcirculatory system of the adventitia extends only into the outer two thirds of the media, which becomes obvious in cross sections stained for CD34, a specific endothelial marker, which stains the microvascular endothelium within the densely arranged arterioles, venules, and capillaries of the vasa venarum system (Fig. 1, H and I) as intensely as the continuous luminal endothelium (Fig. 1J). A longitudinal sectioning technique (Fig. 1K) allowed us to split the endothelial and subendothelial leaflets of the intima. An overview of such a section stained immunoenzymehistochemically for TF (red precipitate) and viewed by bright field microscopy is shown in Fig. 1L. In this section the lumen of the vessel (the black arrow indicates the subendothelial leaflet and shows also the cutting direction) was just opened and in a small area the two leaflets of the intima were separated by the blade. From this overview it is apparent that high levels of TF are present in the (microvessel-rich) adventitia and that the TF concentration declines in the media (in which the microvessel density is also reduced; compare with Fig. 1H) in the direction to the lumen and is almost zero in the inner third of the media (unspecific yellow staining). The fan-shaped organized medial feed arterioles of the vasa venarum (accidently longitudinally sectioned at the left side, side branches of them cross-sectioned at the right side, some are marked by green arrows) are classically stained dark blue for alkaline phosphatase (35).

Within the subendothelial leaflet of the intima again high concentrations of TF (red precipitate) can be recognized. Figure 1M shows a magnification of the region around the two split leaflets, and Fig. 1N shows the same detail in fluorescent light, besides the distribution of TF (as a specific marker of pericytes, red fluorescence of the enzymehistochemically produced red precipitate), now also that of von Willebrand Factor-related antigen (vWF::Ag; labeled with a green fluorescent antibody) as a specific marker of endothelium, can be recognized.

Transmission electron microscopy on representative saphenous vein preparations revealed that the subendothelial cells (SC) had some further ultrastructural features in common with SMC, e.g., they displayed focal densities of polymerized actin, attachment plaques, peg- and socket contacts, and plasmalemmal caveoleae (Fig. 2A). Generally, they were in very close contact to each other. Endothelial cells (EC) and SC shared a common extracellular matrix layer, which was partly formed by fusion of the endothelial basal membrane and the circumferentially deposited matrix of the SC (Fig. 2B), in which these cells coexist themselves. Fibronexus junctions, typical connections of myofibroblasts, were never found between the contractile cells in the intima.

Confocal microscopy of the luminal intimal surface (Fig. 3A) exemplarily shows that the α-actin-positive contractile cells in the intima of healthy adult human saphenous veins formed a net-like tissue in close proximity to the luminal endothelium (distance often less than 0.5 μm) and extends 3–20 μm deep into the connective tissue of the intima. Numerous small processes of the individual SC made direct contact with adjacent EC (Fig. 3B). Immunohistochemical staining for TF often revealed not only the cell bodies of the SC but also numerous
vesicle-like structures and abundant microparticles within their extracellular matrix. These deposits of TF (red fluorescence) in the subendothelium could obscure the immunologically demarcated cytoarchitecture of the tissue (Fig. 3C). Interestingly, thrombomodulin (CD 141), the specific receptor needed for activation of protein C (an important component of the inherent anti-thrombogenicity of the endothelium), was exclusively expressed within the endothelial clefts (blue).

Argentophilic staining was a simple means for gaining an overview of the intimal tissue texture of coronary grafts, particularly of venous origin (Fig. 3D). Such grafts were often severely lesioned or even de-endothelialized during the routinely practiced intraoperative perfusion and preservation procedures but retaining their subendothelial net of contractile cells, which was obviously much more firmly anchored to the residual intima and to the rest of the wall than the endothelium. The remaining intimal extracellular matrix always stained, and very selectively, dark brown (73).

SC also converted luminally applied and readily incorporated leucomethylene blue rapidly into visible, intracellularly trapped methylene blue. In the endothelium, however, this biochemical transformation did not proceed (Fig. 3E).

De-endothelialized areas in vein segments expressed increasingly alkaline phosphatase (AP) after exposure to serum and could thus be enzyme-histochemically differentiated from endothelialized regions (yellow-green). Expression of this enzyme activity in the lesioned venous intima was studied systematically using standardized staining procedures in 4-h periods for 4 days. Activated and thus rapidly proliferating SC in endothelium-denuded areas formed AP-positive intimal nodules in the course of 2–4 days (Fig. 3F).

The foregoing results were obtained exclusively from surgical specimens of human saphenous veins that showed no evidence of disease, and particular no varicosities or thrombotic deposits. However, since clinically silent, early stages of pathophysiologically wall processes can never be completely...
excluded in blood vessels of adult human patients, we conducted appropriate studies using saphenous veins and aortae of freshly euthanized young cows to confirm that the observed intimal network of cells below the luminal endothelium is in fact a constitutive tissue of the healthy intima not only of veins but also aorta. Proteolytically isolated intimal sheet preparations from 24 aorta and six saphenous veins from that species allowed further insights into their tissue texture. Figure 4A

Fig. 2. Transmission electron microscopic demonstration of close association between the luminal endothelium and the subendothelial cells in a representative human saphenous vein. A: subendothelial cells of the intima (SC) are continuously and closely associated with luminal endothelial cells (EC). Both cell types are separated by their extracellular matrix (ECM). Collagen bundles (C) shield the SC from deeper intimal layers. Arrow heads, polymerized SMC-α-actin in stress fibers; black arrows, caveolae; boxed arrows, attachment plaques; red arrows, peg and socket contacts. M, mitochondrion; L, lumen. B: 2 SC (1 and 2) are seen, which are only separated by a narrow gap (white arrows). The external matrix between them and EC is apparently made up of the basal membranes of the two cell types (double arrow).

Fig. 3. The highly branched contractile cells of the venous intima form a second tissue directly underneath the endothelium, which is coupled with the latter via finest extensions. Very similar results were replicated in 10 venous bypass remnants. A: overview of a typical network of SC, confocal microscopy following fluorescence labeling for α-SM-actin (green). B: delicate cell processes of SC attach in high density to the basal surface of individual EC (labeling and detection as in A). The clefts of the latter (arrow heads) were immunologically stained red for CD31 (PECAM). C: confocal micrograph, stacked images following immunofluorescence labeling of TF deposited by SC (red), endothelial vWF:Ag (in Weibel-Palade-bodies; green), and CD141 (thrombomodulin; blue). The mean distance between SC and endothelium was 0.5 μm. D: endothelial denuded intima of a saphenous vein graft after silver staining; arrow heads point to residual EC. E: detection of SC in a saphenous vein in vivo preparation following rapid uptake and selective intracellular reduction of leucemethylene blue (membrane permeable) into methylene blue (membrane impermeable and therefore intracellularly trapped). F: enzymehistochemical demarcation of exposed, endothelium-denuded SC via their AP activity (blue precipitate) after 12 h of preincubation of the venous segment in serum-containing cell culture medium ( stereo microscopic image, bright field). After further 48 h, marked proliferation of SC in the endothelium-denuded areas was visible (inset).
comprises representative results from the studies on well-preserved intimal tissue fragments of healthy aortas (tentatively similar results were obtained with corresponding tissue preparations from the bovine veins, although venous intimal tissue, due to its comparatively weakly developed extracellular matrix, is very prone to proteolytic decomposition).

In healthy intima of the bovine aorta and saphenous vein (not shown) also, an ~5-μm-thick network of SMC-α-actin-positive cells was always present. Every bovine aortic SC was characterized by bizarre antler-like branches (Fig. 4B) that contacted up to 30 EC with their smallest extensions. The area occupied by these large cells was on an average 0.13 mm² (n = 12 microscopic countings each in 4 largely intact intimal tissue fragments). In comparison, the mean size of luminal EC in an intact saphenous vein was 4.100 ± 0.011 mm². Scanning electron micrographs of the basal surface of a freshly isolated intimal tissue fragment revealed the native complexity comprised of the SC processes contacting EC. D: basal surface of some EC of an intimal tissue piece viewed with SEM. The ends of the SC processes seem to be properly fused with the EC. E: bright field view of an isolated intima preparation after 3 h of incubation in serum-containing culture medium. The SC can be demarcated after staining blue for their induced AP activity. F: intimal double sheet proteolytically detached from the inner surface of the aortic wall under hypertonic conditions after 1 day in culture; staining is as in E. G: analogous tissue isotonically separated; culture and staining are as in E.

Further Histological Characterization Identified Subendothelial Cells as Intimal Pericytes

In view of the peculiar morphology of the SC in the intima of human and bovine arteries and veins, their ultrastructure, their high content of TF, their low caldesmon and missing desmin expression as demonstrated in the intima of human saphenous veins (compare Fig. 1, F and G), and their intimate ultrastructural association with the endothelium, it seems highly unlikely that these cells were SMC, myofibroblasts, or macrophages. Rather, most of their characteristics corresponded more closely to those of classical (microvascular) pericytes (6, 19, 20, 72, 76). To substantiate this contention further, generally accepted pericyte-specific staining experiments were performed, the main results of which are summarized in Fig. 5.

Cultured SC of aortic or venous origin generally expressed high levels of SMC-α-actin and calponin (Fig. 5, A and B), but caldesmon and desmin were only weakly expressed or not detectable (not shown). Exposed to serum-containing media, AP was expressed at high levels (Fig. 5C) as a membrane-bound ectoenzyme (V max = 20 ± 2.8 nmol AMP/min·10^6 pericytes). AP particularly characterized individual intimal pericytes that became stationary after an initial phase of amoeboid motility and apparently started the production of extra-cellular matrix components in which the enzyme was also covalently incorporated after export and incorporation into microvesicles or microparticles, which were abundantly arranged directly underneath a confluent endothelial sheet (compare Fig. 8, C and D). Sporadically, cultured human intimal pericytes of venous origin developed networks of delicate, TF-positive cell processes (Fig. 5, E and F) and spread out on areas (0.14 ± 0.021 mm²; n = 25) similar to those of the...
corresponding SC in intimal tissue preparations (see above). Their filigree extensions apparently represented the terminal branches of the SC just described (compare Fig. 4, B–D), where a single pericyte could be coupled with up to 30 EC. The TF-containing microvesicles or microparticles frequently pinched off from those filament networks and attached to free areas of the bottom of the respective Petri dishes (Fig. 5, E and G; see also the corresponding video in the data supplements) or, in older mixed cultures during spontaneous morphogenesis (see below), assembled in high concentration directly below the confluent luminal endothelium [these microparticles were also AP positive (Fig. 8, C and D)]. A characteristic ganglioside of vital microvascular pericytes that could be labeled by a monoclonal antibody (3G5) proved also to be a highly specific pericyte marker of intimal pericytes. In contrast with AP, the expression of this antigen was independent of the migratory

Fig. 5. Identification of cultured subendothelial cells as intimal pericytes. Frames A–F were taken from cultures of subendothelial cells (pericytes) isolated of intimal tissue from bovine aortic origin; frames G–L show intimal pericytes from human saphenous veins (stainings were replicated in cultures from 25 or 22 independent cell harvests from bovine aorta or human venous graft remnants, respectively, and were found to be generally reproducible in routinely cultured intimal pericytes independently of their macrovascular origin). A and B: immunofluorescent labeling for SMCα-actin or calponin, respectively. C: enzymehistochemical staining for AP. D: immunofluorescent detection of TF in a SC (red), accompanying EC stained green for vWF:Ag. E: staining of a pericyte via antibody-linked AP; illustration of filamentous, net-like extensions, which occasionally and spontaneously spread out over a significant surface. Abundant vesicle or microparticle-like structures (1 indicated by red arrow head) with high content of TF were continuously pinched off and deposited in the vicinity of their parent cells (1 indicated by red arrow head). F: strongly TF-positive microfilaments of an intimal pericyte shown in E at higher magnification. G: phase contrast micrograph of a pure, confluent pericyte culture. Two red arrow heads point to suspended vesicles or microvesicles, respectively, which are abundantly pinched off. H: detection of a pericyte-specific ganglioside using 3G5 antibody (red fluorescence). I: same frame as in H stained for AP. J: demonstration of extracellularly deposited proteins in confluent pericyte cultures: collagen IV (blue), which is deposited in bridge-like structures together with fibronectin (red), in between cell islands (nuclei green). K: phase contrast picture of a mixed culture of EC and pericytes, which is just forming a double-sheeted, intima-like structure. L: same culture as in K after immunofluorescent labeling of the pericytes with antibody against their characteristic proteoglycan NG2.
behavior of these cells (AP-negative, migrating cells display the 3G5-antigen as well as the stationary, AP-positive cells; see Fig. 5, H and I). In confluent cultures, massive production, export and deposition of typical extracellular matrix proteins (Fig. 5J) were also evident, in which the pericytes literally enveloped themselves in. In addition, venous intimal pericytes expressed NG2 (Fig. 5, K and L), a characteristic proteoglycan of microvascular pericytes (3).

Rapidly Proliferating Intimal Pericytes Are Pathogenetically Significant

Cross sections of macroscopically healthy wall areas of human aorta showed repeatedly discrete staining for pericyte tissue and activated T lymphocytes directly below a seemingly continuous endothelial sheet (Fig. 6, A and B),

Fig. 6. Endothelium-denuded intimal areas give rise to intima hyperplasia, pericyte proliferation, and local thrombosis and inflammation. A: aortic intima of a 35-yr-old female with beginning hyperplasia. Intimal pericytes are stained for AP (blue), and T lymphocytes are stained for CD 26 (red-brown). B: same frame as in A; immunofluorescent staining for endothelial vWF:Ag (green). C: AP detection in the plaque area of a severely atherosclerotic aorta. D: delineation of atherosclerotic lesions after staining for AP (human aortic tissue). E: dissected patch in D (frame) was further inspected with SEM. Exposed pericytes were covered with dense fibrin deposits and adherent platelets (small white corpuscles). F–I: serial sections obtained from the same plaque area of an explanted, stenosed coronary vein graft, which had been transplanted 6 years earlier (57-yr-old male). F: staining for endothelial vWF:Ag. G: labeling for TF (violet). H: immunohistochemical demarcation of AP (blue). I: staining of activated T lymphocytes for CD 26 (brick red). J: SEM picture of an endothelium-denuded area in a residual, freshly fixed human saphenous (PMN, neutrophil; P, platelet). The red arrow heads point to exposed, branched processes of pericytes. K: SEM view of fibrin clots (F) at the contact surface of exposed IP with the blood in a lesioned coronary bypass vein graft. L: endothelium-denuded and thus AP-positive (blue) thrombogenic valve borders in a saphenous vein (42-yr-old female).
which apparently did not completely fulfill its barrier function. In the region of severe atherosclerotic lesions, AP was generally detectable within the entire considerably broadened intima (Fig. 6C), and intimal pericytes that were desmin negative but expressed AP and TF, comprised more than 90% of the cells proteolytically isolated and cultured from such wall regions (in human aortic plaques: 92 ± 7%, n = 7; in restenosed areas of explanted vein grafts: 95 ± 4%, n = 8; most of the remaining cells were T lymphocytes. For comparison, healthy intimal tissue is exclusively composed of roughly 90% endothelial cells and 10% intimal pericytes). AP had proved to be a convenient means of demarcating macroscopically atherosclerotic lesions (blue luminal surface areas in Fig. 6D). Scanning electron microscopy (SEM) revealed dense fibrin deposits and abundant platelets on the surface of the endothelium-denuded intimal pericytes in such areas (Fig. 6E). AP and TF are also expressed at high levels underneath lesioned endothelium in the core plaque areas of restenosed vein grafts, but not under adjacent intact endothelium (Fig. 6, F–H). The centers of such plaques were regularly surrounded by activated T lymphocytes that build up sharply restricted cuffs around the stenotic tissue masses (Fig. 6I). In freshly lesioned, blood-perfused venous grafts at 37°C the first interactions of intimal pericytes and platelets could be demonstrated within 5–10 s (Fig. 6J). They rapidly formed large aggregates, which catalyzed fibrin clots after about 3 min (Fig. 6K).

Exposed, and hence AP-positive (see above), intimal pericytes were also seen frequently at the outmost edges of venous valves (Fig. 6L), where they often gave rise to similar thrombotic conglomerates as shown in Fig. 6K.

Specific Isolation and Cultivation Techniques Allow the Establishment of Homogeneous Cultures of the Two Intimal Tissue Types

With the use of the osmotically controlled protocol described above for the isolation of intimal tissue rich of EC, primary cultures of venous EC could be reproducibly obtained with 95% purity and, after two passages in an optimized medium supplemented with FCS (10% vol/vol) and the growth factors epidermal growth factor (EGF; 10 ng/ml) and basic fibroblast growth factor (bFGF; 20 ng/ml), with 98% purity. The procedure for establishing homogenous cultures of intimal pericytes took advantage of the observation that these cells expressed constitutively a basal AP activity on their surface. When exposed to a specifically designed pericyte enrichment medium (for complete composition, see supplemental media) containing two essential amino acids (e.g., Gln, Thr), exclusively in the form of a readily synthetized phosphorylated dipeptide, and with glucose-6-phosphate as the only sugar, accompanying EC could not utilize these essential nutrients and died. In contrast, the intimal pericytes survived due to their potency to dephosphorylate these potential nutrients (Fig. 7).

Fig. 7. Generation of pure cultures of intimal pericytes. A: in primary cultures of intimal tissue fragments typical endothelial cell colonies (EC) and scattered intimal pericytes (IP) can be regularly recognized (phase contrast). B–G: situations in the course of 3 times repeated, 2-step incubations in serum free pericyte enrichment medium (for composition, see supplemental media) each time for 24 h (B, D, and F) following 1 h in PBS supplemented with 0.1% bovine serum albumin (C, E, and G). H: condition after recovery for 1 day in minimal culture medium (see supplemental media). I: 3 days later in the same medium, intimal pericytes rapidly spread out throughout the Petri dish. Scale bar, 250 μm.
A–G). Further cultivation in complete medium then generated rapidly 100% pure cultures (Fig. 7, H and I).

Morphogenesis of Double-Sheeted Intimal Tissue In Vitro

In mixed primary cultures of arterial or venous intimal tissue fragments (ratio of EC to intimal pericytes > 4:1), morphogenetic processes could be detected that led to spontaneous formation of double-sheeted, intima-like tissue structures within several weeks. Soon after formation of a monolayer (Fig. 8A, bottom region of this frame), the pericytes exported high amounts of AP and orientated themselves below the EC (Fig. 8B). In this state of the culture, like TF, AP was incorporated into vesicles by these cells and fixed into the extracellular matrix directly below the EC (Fig. 8, A and D). In the older areas of this developing neointima and in the course of the formation of a confluent endothelial sheet, the enzyme was gradually eliminated from the matrix and then selectively concentrated on the cell surface of the pericytes (Fig. 8A, top region). After completion of a tight endothelial sheet and substitution of the FCS in minimal growth medium by a plasma derivative (see supplemental media), the enzyme was entirely lost. As soon as an endothelial layer reached confluency, proliferation of intimal pericytes ceased and the cell count remained almost constant in both the now highly differentiated and closely associated intimal tissues in this area (Fig. 8, E and F). If the endothelial sheet was injured extensively, the pericytes in the respective area began to proliferate out of control, such that the EC could no longer cover them (Fig. 8, G and H). These proliferating pericytes of intimal origin in atheroma-like masses expressed particularly high levels of TF (Fig. 8H) and also of AP (not shown).

Growth Characteristics of Venous Intimal Endothelial Cells and Pericytes

With the utilization of the Transwell culturing technique, the growth regulation of both intimal tissue types could be investigated under defined in vitro conditions (cells from 6 to 8 independent harvests). As shown in Fig. 9A, the preferred endothelial growth medium (supplemented with FCS, bFGF, and EGF; see supplemental media) induced the shortest doubling times in homogenous endothelial cell cultures from saphenous veins (generation time, 13.8 ± 1.4 h; n = 7). In minimal medium (supplemental media) the proliferation rate was significantly lower and not influenced by a confluent pericyte layer established previously on the bottom of the Petri dish (generation time, 20.4 ± 1.8 h; n = 6). However, if the pericyte layer was established on the reverse side of the filter substrate in close proximity to the endothelial cell layer, cell proliferation rates similar to those obtained in growth factor-supplemented medium were achieved, even in the minimal medium (generation time, 14.3 ± 1.5 h; n = 7).

As illustrated in Fig. 9B, homogenous intimal pericytes seeded on the reverse side of a filter carrying a confluent layer of

![Fig. 8. Spontaneous intimal morphogenesis in mixed cultures of intimal endothelial cells and pericytes. Results were replicated from 16 to 23 culture dishes (5 independent cell harvests) and occurred (time dependently) regularly. A–D: intimal cell cultures from juvenile bovine aorta. A: widespread and diffuse distribution of AP activity (blue staining) in culture areas, which just became confluent (bottom part of the frame). In slightly older culture areas the ectoenzyme is restricted to the surface of intimal pericytes (top part of the frame). B: inspection with SEM reveals the spontaneous arrangement of the intimal pericytes below a confluent endothelial sheet (EC). C and D: segmentation and deposition of pericyte-derived microparticles. Numerous AP-positive vesicles (blue) segregate from the underlying pericyte network (C), which subsequently accumulate directly underneath the closely associated endothelial sheet (D). E–H: cultures from human saphenous vein. E: EC and pericytes seeded in a ratio of at least 4:1 form a neointima, which shows typical characteristics of the intima in intact veins: a continuous apical endothelial cell layer, green immunofluorescent staining for CD141 (thrombomodulin) selectively in endothelial clefts, and TF-positive, strictly subendothelially arranged pericytes (red). F: phase contrast picture of the identical culture area as in E. G: seeding of mixed cultures with relatively higher pericyte counts results in uncontrolled proliferation of these cells (phase contrast). H: identical region of the culture shown in G after staining of intimal pericytes for TF (red) and the EC for CD31 (PECAM; green). Like CD141 (E), the latter accumulates selectively within endothelial clefts.](http://ajpheart.physiology.org/)
Almost identical results were obtained with intimal cocultures of aortic origin (not shown).

Subendothelial Pericytes Derived from the Intima of the Saphenous Vein are Characterized by a Particularly High and Inducible Procoagulatory Potency

Confluent human EC of aortic and venous origin never did form a functional enzyme complex with coagulation factor VIIa and could therefore not activate factor X (Table 1). In addition, they did not develop any prothrombinase activity but were able to promptly activate protein C (42 ± 5.1 pmol/min-106 venous EC and 25 ± 3.1 pmol/min-106 aortic EC; n = 8).

Intimal pericytes, however, were not able to activate protein C at all, but in stark contrast with human EC, their prothrombotic functions were exceptionally prominent, particularly in such cells of venous origin (Table 1). Pericycle cultures of both venous and aortic origin already expressed a high basal concentration of functional TF/FVIIa complexes independently upon the cell passage. When cell passage was physically disrupted for the measurement of the total TF amount of the cells in factor X activation, in the extracts of aortic pericytes four to five times, and in those of venous origin, 30 times higher concentrations of activated factor X were assayed. In an attempt to imitate the inflammatory conditions of intraoperative graft storage, we preincubated pericycle cultures of venous origin with a vein wall extract (see MATERIALS AND METHODS) and assessed factor X activation after 1 h. Under these circumstances the surface activity of the vital cells was increased about 25-fold, with correspondingly less remaining in the cells. When pericycle cultures or their lysates were preincubated with a monoclonal antibody against human TF, cleavage of FX to FXa was undetectable over 60 min by the assay [capable of detecting as little as 0.30 nmol/l FXa (data not shown)]. We also verified that the observed TF concentration was not due to endotoxin contamination of the incubation medium [Peripheral human blood monocytes, which expressed TF subsequent to endotoxin stimulation (46, 47), did not reveal functional TF activity after analogous incubations and assays].

Prothrombinase complex assembly via thrombin generation was also assessed in these cultures. In contrast with the basal TF activity, the surface prothrombinase activity was about eight times higher in venous pericytes than in the corresponding cells of aortic origin. Similar activities were noted for the prothrombinase activity in lysates of the cultures. In cultures of venous origin exposed to vein wall extract (and thus inflammatory mediators), the surface and lysate prothrombinase ac-

Table 1. Procoagulatory activities of intimal pericytes isolated from human aorta or saphenous vein

<table>
<thead>
<tr>
<th>Cell Cultures</th>
<th>Activation of Factor X, pmol Xa·min⁻¹·10⁶ cells</th>
<th>Prothrombinase Activity, pmol IIa·min⁻¹·10⁶ cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>Intimal pericytes</td>
<td>Surface Activity</td>
<td>Lyysate</td>
</tr>
<tr>
<td>Aortic</td>
<td>1.91 ± 1.11</td>
<td>7.78 ± 1.31</td>
</tr>
<tr>
<td>Venous</td>
<td>1.23 ± 0.05</td>
<td>36.2 ± 1.21</td>
</tr>
<tr>
<td>Venous, incubated for 1 h with vein wall extract</td>
<td>30.71 ± 4.07</td>
<td>36.37 ± 1.40</td>
</tr>
<tr>
<td>Endothelial cells from saphenous vein or aorta</td>
<td>Not detectable</td>
<td>Not detectable</td>
</tr>
<tr>
<td>Prothrombinase Activity, pmol IIa·min⁻¹·10⁶ cells</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Intimal pericytes</td>
<td>Surface Activity</td>
<td>Lyysate</td>
</tr>
<tr>
<td>Aortic</td>
<td>0.56 ± 0.038</td>
<td>0.34 ± 0.059</td>
</tr>
<tr>
<td>Venous</td>
<td>4.54 ± 0.48</td>
<td>6.56 ± 1.11</td>
</tr>
<tr>
<td>Venous, incubated for 1 h with vein wall extract</td>
<td>5.72 ± 1.08</td>
<td>8.56 ± 1.101</td>
</tr>
<tr>
<td>Endothelial cells from saphenous vein or aorta</td>
<td>Not detectable</td>
<td>Not detectable</td>
</tr>
</tbody>
</table>

Values are means ± SE; n = 15 in each experiment. The functional activity of factor VIIa-tissue factor complexes was measured by their ability to activate factor X, that of the prothrombinase complexes to generate thrombin. Vein wall extract for the activation of venous intimal pericytes was prepared as described in materials and methods.
activities were not significantly different from those of the normal venous intimal pericytes.

DISCUSSION

To gain deeper insight into the question of why, especially, the intima seems to be the starting point for serious vessel diseases, we re-examined its cell architecture in human and bovine aorta and saphenous vein preparations using convenient cell biological techniques. In healthy vessels a net-like subendothelial layer of cells is intimately associated with the luminal endothelium; these cells might best be described as intimal pericytes.

Given the physiological and pathophysiological importance of these cells, to date described only by a solitary group as pericyte-like cells and as a normal component of arterial and venous intima (2), it is surprising that they have aroused so little interest. The present study describes a simple procedure for the isolation of intimal tissue and the production of homogenous, highly pure intimal pericyte cultures using selective culture media. By application of these methods, a broadly based study of this interesting tissue is now possible, analogous to the situation in 1973 following Jaffe’s innovative protocol for the isolation of fetal EC (33). The demonstrated isolation of EC-rich intimal tissue fragments after gentle proteolytic pre-incubation under hyperosmotic conditions and the use of a medium supplemented with growth factors now allows the establishment of homogenous endothelial cultures also from adult vessels, free of accompanying pericytes. Indeed, the properties of the intimal pericytes observed in the present study imply strongly that contamination of EC cultures with pericytes in the past resulted in many misinterpretations, e.g., they very probably account for many earlier observations of TF expression in EC cultures and the claim that especially activated EC could trigger coagulation on contact with blood (47, 62). Given the very limited availability of specific pericyte markers, the reliable identification of this cell type in the subendothelium of large arteries and veins could be accomplished only after the detour via tissue culture. AP (EC 3.1.3.1.), a well-established constituent ectoenzyme of microvascular pericytes (18), proved to be rather an activation marker for intimal pericytes, i.e., these cells in the direct periphery of the luminal endothelium of macrovessels expressed this enzyme only after contact with serum. In mixed cultures of intimal EC and pericytes (Fig. 8, C and D) and in de-endothelialized areas of aortic or venous intima (Fig. 3F), this enzyme is not only included in the pericyte cell membrane but is also transferred into microparticles that are incorporated so abundantly in their extracellular matrix that it is difficult to recognize cell borders. Similar arguments hold for the identification of intimal pericytes in situ by staining for TF, which was demonstrated at high concentration in microvascular pericytes (9, 47). NG2, a proteoglycan expressed exclusively by mural cells during microvascular morphogenesis and which is especially useful for the identification of microvascular pericytes (3), is also expressed by the venous intimal pericytes in cultures when the latter (spontaneously) form intima-like tissue sheets (Fig. 5, K and L). A further pericyte-specific marker, a ganglioside known as 3G5-antigen (31), could only be reliably labeled in vital pericytes in venous intima using immunological processes. In fixed vessel preparations, which must be permeabilized using detergents or alcohol before labeling with antibodies, the specific ganglioside seemed to be readily washed out. Although immunological staining for SMC-α-actin or calponin could demarcate pericytes and even their finest processes in situ, these proteins were of course also typical markers for vascular SMC (21, 48) and myofibroblasts (14). Nevertheless, the use of these markers showed especially clearly that the pericytes, in contrast with the other two, always spindle-shaped cell types, have a star-like morphology, often with long, branching (antler-like) processes (Fig. 4B). This apparently generally typical morphological criterion of pericytes (39) was described originally by Zimmermann (77). In view of the only weak expression of caldesmon and the absence of desmin, two further typical SMC markers (21, 50), intimal pericytes more resemble myofibroblasts (22). However, they never display the primary characteristic, the fibronexus, of the latter (22). The only major difference between intimal pericytes and quiescent adult microvascular pericytes is the absence of desmin in the latter, whereas the former express this marker strongly in situ (32). The well-developed contractile machinery (contractile proteins, attachment plaques, gap junctions, and peg- and -socket contacts with the adjacent endothelium) of the intimal pericytes and their production of a thick, cocoon-like extracellular matrix and a common basal membrane with the endothelium argues against the possibility that these cells are monoocyte-derived dendritic cells. On the other hand, only the latter are likely to be able to develop a comparable bizarre morphology and express TF under certain conditions like the anticipated intimal pericytes. Resolution of this problem must await future studies.

On the basis of such experience and considerations we were able to evaluate and interpret the histological results after analogous stainings of intimal tissue preparations and of the vascular intima in situ, despite the special technical and cell biological difficulties to demonstrate the pericyte-specific features and markers behind the tight luminal endothelial barrier of intact blood vessels as mentioned above. In addition, the traditional silver stain of the intimal surface proved to be of considerable value for the quantitative evaluation of endothelial lesions and for the visualization of the spatial extent of pericyte nets in vein segments explanted before bypass operations (73). It became apparent that the circumferential extracellular matrix, which acts like a cocoon for the intimal pericytes, specifically stained dark brown to black as has been reported for brain microvascular pericytes (5). This also allowed the clear visualization of the intercellular clefts of the luminal endothelium, particularly under unfavorable incubation conditions that led to their opening, for instance in surgical vessel preparations during intraoperative storage (73).

Adenosine is specifically trapped in EC and degraded to uric acid (44). Against this background, the selective labeling of pericytes after ready uptake of leucomethylene blue and its intracellular oxidation to membrane impermeable methylene blue can be explained. Apparently, these cells lack an enzyme, such as xanthine oxidase of the endothelium (30), that can immediately reconvert the trapped blue compound to its membrane permeable leuco-form.

The demonstration of the presence of highly differentiated pericytes in the subendothelium of large arteries and veins requires that the role of these cells be reconsidered. Traditionally, pericytes have been regarded solely as typical constituents
of microvessels (19, 59). In the retina, they exercise survival functions with respect to the maintenance of microvascular architecture and in particular the endothelium (28). Specialized pericytes in other organs are also well known and have been thoroughly studied under other names, e.g., the stellate (58) or Ito-cells (66) in the liver and the mesangial cells of the renal glomerulus (65). More recently, the roles of microvascular pericytes in blood flow and blood pressure regulation, maintenance of the blood-brain barrier, immune defense, inflammatory processes, hemostatic reactions, and angiogenesis have attracted attention (6, 9, 19, 20, 72, 76). The intimate association and interaction between the endothelium and the pericytes in microvessels (4), in addition to the similarly close correlation of EC and pericytes in the intima of large vessels reported in the present study, suggests that a general feature of pericytes is their support, both structurally and functionally, of endothelium, regardless of the vessel type. In other words, the role of the pericyte, to expand its Latin derivation, is that of a necessary endothelium-embracing satellite cell, which is not restricted to the microvasculature.

The existence of pericytes in the intima is of fundamental medical relevance, particularly with respect to the pathogenesis of atherosclerotic disease and the feared fulminant thromboembolic closure of many such affected vessels during the final stage of the respective illnesses [including venous bypass grafts transplanted into arterial positions (73)]. Our results show that luminal EC and their pericyte companions exert a strong mutual influence on each other with respect to growth and proliferation. The rate of division of proliferating EC and the formation of a confluent layer thereof is enhanced strongly by direct contact with their satellites, providing the ratio of EC to pericytes is at least 4:1. In such cell cultures we observed the spontaneous formation of a neointima (Fig. 8) characterized by a confluent luminal endothelial layer and a loose subendothelial network of pericytes, whereby the individual cells of both layers remain in the G0 phase of growth, as in the intima of healthy vessels. This observation is consistent with the fundamental roles attributed to microvascular pericytes with respect to vascular development, stabilization, maturation, and remodeling (4). Interestingly, if the local pericyte concentration in a mixed intimal cell culture is raised beyond 1:4, nodular clumps of intimal pericytes form and the growth of the resulting tumor-like excrescences can no longer be covered and inhibited by the endothelium. This phenomenon observed in vitro resembles very closely the observation that the core region of atherosclerotic plaques is no longer covered by endothelium, where pericyte-like masses are present (Fig. 6, D–K). Very probable also is that the unrestricted proliferation of pericytes in such wall regions of arteries (or coronary venous grafts) is determined not only by the absence of the inhibitory influence of the endothelial layer but also by the now unrestricted access of growth factors from the intravascular space. Particularly important in this context is the presence of platelets, which, by virtue of their special adhesion structures, can anchor particularly well in endothelium-denuded intimal regions [Fig. 4B (15, 73)]. They release not only growth-promoting factors but also proinflammatory and prothrombotic peptides and proteins (25), which probably also activate the intimal pericytes in their close vicinity (Fig. 6, E and J).

These considerations imply a direct contribution of the intimal pericytes to increasingly stenotic vessel wall thickening in plaque regions, regardless of whether the vessel is an artery or a graft segment of venous origin. Given common histological markers in SMC and pericytes and the limited possibility for discrimination between the two in the past, it is feasible that the subendothelial cells characterized in the present study have been mistaken for SMC (56, 57). The role played by medial myocytes invading the intima in such proliferative wall processes, a proposal that still receives considerable support (12, 45, 53), remains unclear. It must be emphasized, however, that more than 90% of the proliferating cells were identified by us as intimal pericytes, when aortic plaques and stenotic tissue masses in venous bypass grafts were dissected, proteolytically dissociated into their constitutive cells and subsequently maintained in tissue culture. T lymphocytes, which have in the past also been seen as major plaque formers (29, 54), accumulate almost exclusively in the border zones of the atherosclerotic regions, like monocytes or more specifically the macrophages generated therefrom (13, 16).

Broadly accepted now is the fact that it is not so much the degree of stenosis that endangers the atherosclerotic patient (27), but rather the rapid activation of inflammatory and thrombotic processes in plaque regions that may seem innocuous angiographically, but which can rupture rapidly (17, 34, 37, 71). This realization led to the creation of the term vulnerable plaque (1, 40, 49).

For all these reasons, particular attention is being paid to elucidating the mechanisms behind the sudden accumulation of procoagulatory components in the region of such plaques (68, 71). The earlier suggestion of a possible contribution of the luminal endothelium or invading SMC to TF expression in the plaque region has now been refuted (24, 47, 62, 75). In good agreement with these reports, during the present study TF expression could not be demonstrated in the inner third of the media (void of nutritive microvessels and therefore void of pericytes) or in the luminal (endothelial) leaflet of the of venous intima (Fig. 1, L–N). TF is also not present in cultured EC of aortic or venous origin (Fig. 5D and Table 1). On the other hand, venous EC expressed CD141 (thrombomodulin), especially in the region of the intercellular clefts (Fig. 8E). This newly detected, meaningful location allows them, in stark contrast with the pericytes, to activate protein C rapidly and to counteract subendothelial induced coagulation processes promptly within their junctions (26). This agrees well with the expanded knowledge about the central antithrombogenic roles of vascular endothelium, not only through the expression of various anticoagulatory (coagulation inhibiting) but also of antiaggregatory (platelet inhibiting) and profibrinolytic (fibrinolysis promoting) activities (7, 64, 69, 70).

Small amounts of TF activity are detected on the surface of calcium ionophore-stimulated monocytes, which are the precursors of the macrophages that accumulate in abundance in the border zone of extensive atherosclerotic lesions. TF is also present intracellularly in monocytes, and this is thought to be decapitated after interaction with platelets (46, 47). Blood plasma has been shown to contain microparticles (38, 41), a subfraction of which contains active TF thought to be derived from platelets. The transfer of such microparticles from platelets to plaque macrophages has also been proposed to explain the rapid accumulation of TF in activated plaques (55). However, the notion of blood-borne TF in general, and as an integral component of platelets or of mononuclear cells in
Fig. 10. Scheme of the 2 hypotheses developed in the present study. A: structure of the healthy intima. The intima consists of a highly prothrombogenic subendothelial leaflet characterized by TF expression on pericytes and extracellular TF-positive microparticles, and the luminal leaflet, consisting of continuous endothelium. The latter constitutes physiologically an anticoagulatory system by virtue of its thrombomodulin and heparan expression, together with plasma protein C and antithrombin III. B: endothelium-denuded intima as a focus for proliferative and thromboembolic stenotic processes. Intimal pericytes underneath a diseased or vastly lesioned endothelial sheet proliferate increasingly rapidly and can no longer be covered by endothelium. As a consequence, blood platelets adhere and aggregate. Together with the prothrombotic pericyte membrane, now exposed to the blood, this results in catalysis of fulminant thrombotic and inflammatory processes [see elsewhere for experimental details and results (73)].
blood in particular, is in conflict with recent data suggesting that rather the platelets may be important in decrypting monocyte TF activity in a process entailing transfer of TF from these cells to activated platelets (11, 46, 47).

In conclusion, present explanations for the high thrombogenicity of vulnerable plaques are complex, complicated, and not totally convincing. The results of the present study, showing the extreme expression of immunologically detectable and functionally active TF at the surface and in the vicinity of intimal pericytes (Fig. 1, L–N), however, suggest a simpler and more coherent mechanism. Loss of the continuously antithrombogenically active endothelial barrier results in direct contact between plasma and these TF- and prothrombinase-expressing cells. The resulting activation of the coagulation cascades leads to local formation of serum, which in turn activates further subendothelial pericytes that then recruit TF from intracellular stores and pinch off numerous TF-containing microparticles (Fig. 5, E–G). Microvascular pericytes have repeatedly been described as cells expressing particularly high concentrations of TF (9, 47), and, correspondingly, we observed high concentrations of TF in the adventitia of saphenous veins (full of microvessels commonly rich in pericytes; Fig. 1, H and I), and a high TF concentration in the subendothelial leaflet of the intima (Fig. 1, L–N), where the corresponding resident pericytes are located. Given this intimal pericyte net directly underneath the endothelial coat of the inner surface of the vessel and the continuous pinching off of TF-containing vesicles from cultured pericytes observed by us (Fig. 5, D–F, and video in the data supplements), it is not improbable that the most of the TF-containing microparticles found in plasma (38, 41) originate in intimal pericytes.

An interesting corollary to these notions is the so-called saphenous vein graft disease, a serious complication after bypass operations using venous segments (36, 42, 43, 51). Our laboratory has shown elsewhere that the methods, employed routinely for intraoperative explantation and storage of such grafts, result in severe endothelial damage (73). In such vein segments rapid and massive adhesion of platelets and neutrophils can be observed within a few minutes and specific coagulation tests show that dangerously high concentrations of TF are exposed on the inner surface of the substantially de-endothelialized vessels. On reperfusion of the grafts with native blood, it is not surprising that fulminant coagulation processes are induced. Dense clots were deposited on the endothelial surface, which cause, under the influence of the pulsating arterial shear forces, the complete endothelium to be torn away and washed into the coronary microcirculation. Our laboratory (73) has concluded that the presence of a readily proliferative and strongly thrombogenic intimal pericyte network is probably the reason for the initiation of many of the acutely and chronically developing, atherosclerosis-like disease processes in such vessel segments and the resulting, still very unsatisfying, clinical prognosis especially of venous grafts (36, 42, 43, 51).

Taken together, our findings show that the, before the background of its decisive medical importance, astonishingly narrow intima of the human saphenous vein and the human and bovine aorta consists of two tissue leaflets (Fig. 10A). The abluminal, subendothelial leaflet is made up by a finely branched network of special pericytes, each coupling via abundant tiny attachment points to ~30 endothelial cells above them. These cells are embedded in their strongly developed extracellular matrix, which not only anchors this intimal tissue very firmly to the rest of the vessel wall but also, in the luminal direction, serves to affix the endothelium. The pericytes synthesize TF, which is not only expressed on the cell surface but is also incorporated in microparticles derived from the cell membrane and densely interspersed throughout the entire extracellular matrix. The subendothelial leaflet of the vascular intima is thus strongly procoagulatory.

In contrast, the luminal leaflet consists of a continuous and physiologically very tight layer of endothelial cells. These express highly concentrated thrombomodulin selectively in the region of their well-developed junctional complexes, so that protein C can be promptly activated, if thrombin is present in the local intercellular environment of the intima. This would result in marked anticoagulatory activity. Moreover, as our laboratory has shown elsewhere (73), the venous endothelium expresses constitutively antithrombogenicity by virtue of complexing and thus activating antithrombin III, probably by means of the high heparan concentration in its important glyocalyx.

Figure 10B illustrates our second hypothesis based on the high prothrombogenicity of the intimal pericytes shown in the present study and in a recently published clinical study from our laboratory (73). In addition, we have now characterized the remarkable proliferative potency of the intimal pericytes if they are decoupled from the endothelium. Furthermore, in histological studies on stenotic wall regions of human atherosclerotic aorta and in restenosed aortocoronary grafts of venous origin, marked accumulation of pericyte-typical markers has been demonstrated. We thus hypothesize that highly activated intimal pericytes play a causal role in both the frequently observed acute induction of intravasal thrombosis in transplanted bypass vessels and, in the longer term, in the late symptoms of atherosclerotic disease. The latter includes the still incompletely understood pathogenetic development of vulnerable plaques.

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DISCLOSURES

No conflicts of interest are declared by the author(s).

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INTIMAL PERICYTES: POSSIBLE ROLE IN VASCULAR DISEASE


