Heterogeneity of Microvascular Pericytes for Smooth Muscle Type Alpha–Actin

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Abstract. Microvascular pericytes are believed to be involved in various functions such as regulation of capillary blood flow and endothelial proliferation. Since pericytes represent a morphologically heterogeneous cell population ranging from circular smooth muscle-like to elongated fibroblast-like morphology it is possible that regulation of blood flow (via contractility) and control of endothelial proliferation (as well as other metabolic functions) may be accomplished by different subsets of pericytes. In the present study we provide evidence for heterogeneity of pericytes at the molecular level by using two novel technical approaches. These are (a) immunostaining of whole mounts of the microvascular beds of the rat mesentery and bovine retina and (b) immunoblotting studies of microdissected retinal microvessels. We show that pericytes of true capillaries (midcapillaries) apparently lack the smooth muscle isoform of α-actin whereas transitional pericytes of pre- and postcapillary microvascular segments do express this isoform. Thus, regulation of capillary blood flow may be accomplished by the smooth muscle-related pre- and postcapillary pericytes whereas the nonmuscle pericytes of true capillaries may play a role in other functions.

The capillary wall consists basically of two different cell types, i.e., endothelial cells which form the capillary tube and pericytes which are located on the abluminal surface of the endothelial tube. Pericytes are branched cells that are wrapped by the capillary basal lamina. According to their location and morphology three types of pericytes have been distinguished (Zimmermann, 1923). These are (a) precapillary, (b) midcapillary, and (c) postcapillary pericytes. Pericytes of the pre- and postcapillary segments display gradual transitions to smooth muscle. Therefore, these pericytes have been classified as transitional pericytes (Zimmermann, 1923) to distinguish them from the pericytes of true capillaries. Precapillary pericytes have several circular branches which tend to wrap themselves around the vessel. Midcapillary pericytes are spindle-shaped, highly elongated cells (up to 300 μm in length) that extend mainly in the long axis of the vessels and have many short secondary processes (centipede-like appearance in silver stain). Postcapillary pericytes are shorter stellate-shaped cells that cover the abluminal surface of postcapillaries and postcapillary venules (for orientation, see summarizing drawing in the Discussion).

Generally, pericytes are believed to be involved in two functions (a) controlling endothelial proliferation and thereby the growth of new capillaries (Antonelli-Orlidge et al., 1989; Kuwabara and Cogan, 1963; Orlidge and D'Amore, 1987) and (b) regulating capillary blood flow via a contractile mechanism (Tilton et al., 1979; Vimtrup, 1922). Support for contractility of pericytes has been provided by the observation that pericytes in tissue culture are capable to contract collagen lattices (Kelley et al., 1987). Further indirect support for contractile features was provided by the demonstration, that certain pericytes react with antibodies to smooth muscle myosin, tropomyosin, and cyclic GMP-dependent protein kinase (Joyce et al., 1985a,b, 1986), the smooth muscle isoform of α-actin (Herman and D'Amore, 1985; Skalli et al., 1989) and desmin (Fujimoto and Singer, 1987).

In contrast to antibodies to smooth muscle myosin and tropomyosin which also react with a variety of nonmuscle cell types (Gröschel-Stewart and Drenckhahn, 1982), antibodies to smooth muscle α-actin (SM-α actin) are specific for smooth muscle and smooth muscle–related cells (Gabbiani et al., 1984; Skalli et al., 1986, 1987). Therefore, antibodies to SM-α actin have been proposed as a tool to distinguish pericytes from endothelial cells and fibroblasts in microvascular cell cultures (Herman and D'Amore, 1985).

However, the existence of SM-α actin in pericytes has so far only been demonstrated by examination of pericytes grown in cell culture and by immunostaining of tissue sections (Herman and D'Amore, 1985; Skalli et al., 1989). Since it is difficult to distinguish pre- and postcapillaries from midcapillaries in tissue sections, even at the electron microscope level, it is presently still unknown whether SM-α actin occurs only in a subpopulation of pericytes, for example in the above mentioned transitional pericytes of pre- and postcapillaries, or whether this isoform of actin is common to all types of pericytes including pericytes of true capillaries.

1. Abbreviations used in this paper: SM, smooth muscle α.
To address this problem we have pursued two approaches. In a first series of experiments we have examined serial sections and whole-mount preparations of the rat mesentery and bovine retina microvasculature by immunofluorescence using different actin antibodies and antibodies specific for nonmuscle myosin and double-labeling techniques. In a second approach we have analyzed actin isoforms of microdissected capillaries of the bovine retina by immunoblotting. These studies have provided evidence for a switch of actin isoforms between pericytes of midcapillaries, which are negative for SM-α actin, and transitional pericytes of pre- and postcapillaries, which are positive for this isoform of actin.

Materials and Methods

Antibodies, Immunofluorescence

The following antibodies were used in this study: monoclonal mouse IgG antibody against the NH₂-terminal decapeptide of smooth SM-α actin (Progen, Heidelberg, FRG; Skaili et al., 1986), monoclonal mouse IgM antibody that reacts with all actin isoforms (Amersham International, Amersham, UK) and a polyclonal rabbit antibody against human platelet myosin (Drenckhahn and Wagner, 1986). A polyclonal rabbit antibody raised against chicken gizzard actin has been shown to cross-react with actin of muscle and nonmuscle sources (Drenckhahn and Gröschel-Stewart, 1980; Drenckhahn and Dermietzel, 1988).

For immunostaining of whole mounts of rat mesenteries 6-18-wk old Wistar rats of both sexes were anaesthetized with ether and killed by cervical dislocation. Mesenteries were stretched over Teflon rings, fixed in 2% formaldehyde for 5 min, and digested in 5 mg/ml Dispase (Boehringer Mannheim GmbH, Mannheim, FRG) in PBS (10 mM Na phosphate, 140 mM NaCl, pH 7.4) at 37°C for 30 min. Subsequently, mesenteries were fixed and permeabilized with 100% acetone at -20°C and processed for immunofluorescence using the above listed antibodies and FITC-labeled goat anti-mouse IgG (Miles, St. Louis, MO) or TRITC-labeled goat anti-rabbit IgG (Bayer Diagnostic, Munich, Germany), respectively (for detail see Drenckhahn and Wagner, 1986). Double staining was performed by incubation with a mixture of mouse IgG against SM-α actin and rabbit anti-platelet myosin. The bound primary antibodies were visualized using a mixture of the corresponding secondary antibodies tagged with TRITC or FITC, respectively.

Microcirculatory vessels of the bovine retina were freed of adherent cell layers as described previously (Kuwabara and Cogan, 1959). Briefly, neuroretinas were removed from eyeballs about 30 min after stunning, fixed with 2% formaldehyde for 5 min and digested with trypsin (1/250; Serva, FRG; 1.8 U/ml PBS) for 10-15 min. Subsequent incubation with distilled water (5-10 min) removed adhering remnants of nervous tissue. The remaining vascular tree was transferred on glass slides and allowed to dry. After permeabilization with 0.5% (vol/vol) Triton X-100 (Sigma Chemical Co., St. Louis, MO) in PBS for 1 h retinal microvessels were processed for immunofluorescence.

Dissection of Microvessels, Immunoblotting

Freshly isolated bovine retinas were fixed by immersion with 2% formaldehyde in PBS for 5 min and were then processed for isolation of the vascular system as described above. Next, microvessels were cut into small pieces with a scalpel using a dissection microscope at 30-fold magnification and dark-field illumination (Olympus SZH-ILLD, Frankfurt, FRG). Capillary fragments and fragments of larger vessels, respectively, were collected by selective aspiration using an Eppendorf pipette (100 μl tip; Brinkman Instruments Inc., Westbury, NY). Dissected microvessels were directly transferred to SDS electrophoresis sample buffer (10% sodium dodecyl sulfate). Capillaries and vessels were sonicated (12,000 g, 10 min) and the supernatant subjected to SDS-PAGE. To obtain actin bands with approximately the same amount of actin in capillaries and the large vessel fraction (as judged by density of the

Figure 1. Simultaneous visualization of SM-α actin (a) and platelet myosin (b) in a 4-mm² area of the rat mesentery processed for double immunofluorescence. Arteriole A, metarteriole mA, midcapillary mC, precapillary pC, postcapillary poC, venule V. Areas shown in Figs. 2 and 3 are indicated in (a).
Figure 2. Higher magnification of area indicated in Fig. 1, stained for SM-α actin (a–c) and platelet myosin (a′–c′). Fig. 2, a–c is a slightly cropped montage of four consecutive micrographs that show continuity of an arteriole (A), that gives off two metarterioles (mA) one of which divides into two precapillaries (pC). The left precapillary gives off two midcapillaries (mC). In a′–c′ (not mounted as montage) the same areas stained for platelet myosin are shown. Note strong immunoreactivity of endothelial cells and capillary pericytes with anti-platelet myosin and the absence of immunoreactivity from smooth muscle and transitional pericytes of arterioles and metarterioles, respectively. Anti–SM-α actin does not react with endothelial cells and with no cell type of midcapillaries.
Figure 3. Higher magnification of area indicated in Fig. 1. A midcapillary (mc) is shown at its entire length extending between a precapillary (pc) and a postcapillary (poc). a and b is stained for SM-α-actin, c and d for platelet myosin. The boxed areas in a and c are shown in b and d at higher magnification. Note absence of SM-α actin reactivity from midcapillary pericytes that are visualized in d by reactivity with anti-platelet myosin (small arrows).

Coomassie blue-stained actin bands) the protein amount loaded per lane was about 15-25 μg for capillaries and 5-10 μg for larger vessels. Electrophoretograms were electrophoretically separated onto nitrocellulose filters (Schleicher & Schuell Inc., Darmstadt, FRG) which were then incubated with antibodies (primary antibodies were diluted 1/100 to 1/500 in PBS). The bound immunoglobulins were visualized using peroxidase-labeled goat anti-mouse IgG (Sigma Chemical Co.) and chloronaphthol as substrate (Nakane, 1968). Relative molecular weight standards were provided by electrophoretograms of human erythrocyte membranes.

Results

Criteria for classification of the microvascular tree are based on the branching pattern as well as on morphological and immunological features of the vascular tree (see Fig. 1 and summarizing diagram in the Discussion). Examples of immunostained areas of the mesentery and a dark-field image of the retinal microvasculature are shown in Figs. 1-4. Typically, metarterioles in the mesentery arise as lateral offshoots or terminal branches of arterioles and most frequently extend over a distance of 100–300 μm until they branch to give rise to two precapillaries. Most midcapillaries arise as lateral branches of precapillaries. As a rule, precapillaries of the mesentery terminate via an interposed midcapillary or occasionally directly in a small venule, thereby fulfilling the definition of thoroughfare channels according to Chambers and Zweifach (1944). In the retina terminal portions of metarterioles give off several pre- and midcapillaries (brush-like terminations) which further branch and join to form a dense midcapillary network (Figs. 4–7).

Immunofluorescence

Mesentery. Antibodies against SM-α actin reacted strongly with the smooth muscle layer of arteries, terminal arterioles, postcapillary venules, and veins. In the capillary bed immunostaining specific for SM-α actin was observed in pericytes of metarterioles (smooth musclelike cells), precapillaries, and many postcapillaries (Figs. 2 and 3). The SM-α actin immunofluorescence was rather weak in the bulging perinuclear portions of pericytes but was strong in the ramifying processes of pre- and postcapillary pericytes. All midcapillaries remained unreacted by this antibody. As a rule, intensity of immunofluorescence decreased gradually from arterioles via metarterioles towards terminal precapillaries (Figs. 1 and 2). In the mesenteric microvasculature the junctions between precapillaries and midcapillaries were characterized by an abrupt loss of the SM-α actin fluorescence (Figs. 2 and 3). The junctions between terminal portions of midcapillaries and beginning postcapillaries were less readily visible by the SM-α actin immunofluorescence. Fig. 3 provides an example of a mesenteric midcapillary that shows abrupt loss of immunoreactivity with anti-SM-α actin at the junctions with both pre- and postcapillary vessels. When polyclonal antibodies against gizzard actin were used (cross-reacting with muscle and nonmuscle actin isoforms) instead of the monoclonal SM-α actin antibody, all cellular components of the microvessels were brightly stained including endothelial cells, smooth muscle, and pericytes at all locations (documented for retinal capillaries; see below).

In contrast to the actin antibodies, antibodies to platelet myosin did not react at all with either vascular smooth muscle (see also Larson et al., 1984) or pericytes of metarterioles (Fig. 2). However, anti-platelet myosin stained the endothelial layer of the entire vascular tree and, in addition, reacted with pericytes of precapillaries, midcapillaries, and
postcapillary venules (Figs. 1 and 3). Thus, the staining pattern of anti-platelet myosin was almost complementary to that of SM-α actin in that the intensity of immunofluorescence increased towards the smaller vessels and was weakest in arterioles (Fig. 1). Only in precapillaries, postcapillaries, and postcapillary venules both antibodies overlapped in that they stained the transitional smooth musclelike pericytes that cover these particular pre- and postcapillary segments (shown for precapillaries in Figs. 2 and 3).

**Retina.** Basically, similar results were obtained with whole mount preparations of the microvasculature of the bovine retina (Figs. 4-7). The most intense fluorescence specific for SM-α actin was observed in association with the smooth muscle layer of small arteries, arterioles, and venules. Metarterioles were still moderately labeled whereas the wall of precapillaries (Fig. 7) was virtually not stained with the SM-α actin antibody. Capillaries and even early postcapillaries remained completely unstained (Fig. 8 b). On the other hand, the polyclonal antibody against gizzard actin which has been shown to cross-react with virtually all actin isoforms (Gröschel-Stewart and Drenckhahn, 1982) stained the entire vascular wall of the retinal microcirculation including endothelial cells and midcapillary pericytes (Fig. 6). The same was true for anti-platelet myosin (Fig. 7 a) which, however, did not react with the smooth muscle layer of arteries, arterioles, and veins.

That pericytes are abundant in retinal capillaries was independently shown by a simple Giemsa stain of whole mounts, demonstrating that the procedure applied for isolation of the retinal microvasculature by trypsin digestion did not cause any loss of pericytes (Fig. 5).

**Immunoblotting Studies**

To further confirm that SM-α actin is not present in detectable amounts in capillaries a different experimental approach was undertaken in which we microdissected the retinal microvasculature and separated the microvessel fragments in a capillary fraction and a fraction containing larger vessels such as arterioles and venules (Fig. 9). These fractions were subjected to SDS-PAGE and immunoblotting. Since the relative amount of actin in capillaries was about three- to fourfold lower than in the fraction of larger vessels (as judged by the density of the 42-kD band) we adjusted the protein concentration of both fractions to give actin bands of similar density in SDS-PAGE and immunoblots. This is shown in Fig. 10 in which both fractions of retinal microvessels show a similar density of the 42-kD band in immunoblots incubated with the monoclonal IgM-antibody of Amersham Corp. reacts with all actin isoforms. In addition to the predominating 42-kD actin band breakdown products of actin...
Double immunofluorescence of the bovine retinal microvasculature stained with antibodies to platelet myosin (a) and SM-α actin (b). Arrow in a indicates perinuclear portions of pericytes. Note restriction of the SM-α actin stain to the wall of a dividing terminal metarteriole (mA). Midcapillaries (mC) and even precapillaries (pC) do not react with anti-SM-α actin, but react strongly with anti-platelet myosin. The postcapillary (poC) in the lower left corner was identified by following it towards its junction with a venule which was located outside the area shown on this micrograph.

The fraction of larger vessels. In contrast, the antibody specific for SM-α actin did virtually not react with actin in the capillary fraction but displayed strong immunoreactivity with actin in the fraction of larger retinal vessels, clearly showing that capillaries do not contain any detectable amount of SM-α actin.

Discussion

In the present study we provide biochemical and immunocytochemical evidence for molecular heterogeneity of microvascular pericytes. In both the bovine retina and rat mesentery pericytes of true capillaries (midcapillaries) did not contain the smooth muscle isoform of α-actin (SM-α actin). Transitional pericytes of pre- and postcapillary segments of the mesentery displayed weak to strong reactions with antibodies to SM-α actin. In the retina even postcapillaries and the majority of precapillaries were negative for SM-α actin. Zimmermann (1923) defined pre- and postcapillary pericytes as transitional elements sharing characteristics of both vascular smooth muscle and pericytes of true capillaries. Support for this classification of pre- and postcapillaries is absent from pericytes of capillaries that are brightly stained with anti-platelet myosin (small arrows). P, platelets stained with anti-platelet myosin.
Figure 9. Phase-contrast micrographs of vascular fractions analyzed by immunoblotting. (a) Capillary fraction still containing few pieces of metarterioles and precapillaries; (b and c) fraction of larger vessels.

By immunohistochemistry at the electron microscopic level, Skalli et al. (1989) have demonstrated pericytes of microvessels in a variety of tissues to be positive for SM-α actin. Two aspects may help to explain the apparent discrepancies between the observation of Skalli et al. (1989) and our findings. Firstly, it is difficult to distinguish midcapillaries from pre- and postcapillaries in tissue sections, even at the ultrastructural level. Therefore it is well conceivable that the positive sectional profiles of pericytes observed by

Figure 11. Heterogeneity of smooth muscle and pericytes in microcirculation. Schematic drawing of the main segments of the microvascular bed of the rat mesentery illustrating morphological and immunological (SM-α actin, nonmuscle [platelet] myosin) heterogeneity of pericytes and smooth muscle. In the retina even precapillaries and postcapillaries are negative for SM-α actin.
Skalli et al. (1989) may represent pericytes of pre- and postcapillary segments which we found to be positive for SM-α actin in the rat mesentery. As a matter of fact, three of the four electron micrographs shown by Skalli et al. (1989) speak in favor of this possibility: figures 4 a and b of that study were interpreted as sections of "capillaries or venules" and Fig. 5 is a sectional profile of a typical venule. Secondly, the anatomy and hemodynamics of the capillary system of different tissues may differ profoundly from each other as shown in the present study for the differences between the capillary system of the mesentery and the retina. It is well conceivable that expression of SM-α actin and the morphology of pericytes may be significantly modified by hemodynamic factors such as the transcapillary hydrostatic pressure gradient which is well known to vary in different tissues (Michel, 1984). This view is supported by studies on lung capillaries which have shown that pericytes proliferate during pulmonary hypertension and develop a morphological phenotype similar to that of smooth muscle cells (Meyrick and Reid, 1983).

Our finding that all capillary pericytes of the bovine retina are devoid of SM-α actin is in obvious contradiction to the study of Herman and D'Amore (1985), who, using polyclonal muscle-specific actin antibodies, described musclelike actin as a diagnostic marker for bovine retinal pericytes both in situ (only mentioned but not shown) and in tissue culture. The expression of muscular actin in cultured pericytes may be explained in two ways: firstly, the cultured pericytes may be derived from vascular smooth muscle cells or smooth musclelike cells of metarterioles and venules rather than from pericytes of true capillaries. Secondly, the nonphysiological conditions of tissue culture may cause dedifferentiation of pericytes towards a primitive smooth musclelike phenotype. In favor of the latter possibility Tontsch and Bauer (1989) found expression of SM-α actin even in cultured cerebral microvascular endothelial cells when the cells were grown in the absence of growth factors and heparin. This effect was fully reversible when the cell cultures were again incubated with complete growth medium. In this perspective it would be important to develop in vitro conditions that suppress expression of SM-α actin in pericytes and/or avoid proliferation of contaminating microvascular smooth muscle cells. This would be an important step forward in studying the cell biology and biochemistry of capillary pericytes in vitro.

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