Immunohistochemical Identification of Arteriolar Development Using Markers of Smooth Muscle Differentiation

Evidence That Capillary Arterialization Proceeds From Terminal Arterioles

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Abstract Arteriolar growth is an important event in the adaptation of normal tissues as well as in important pathologies, but the site of origin of new arterioles remains unknown. The network pattern of arteriolar development in skeletal muscle was detected by use of a new immunohistochemical technique that is based on the observation that fully differentiated (mature) vascular smooth muscle (SM) cells express both SM α-actin and the two myosin heavy chains (MHCs) SM-1 and SM-2, whereas less differentiated (immature) vascular SM cells do not express MHC. The anterior gracilis muscle microvascular system of 4- and 9-week-old Sprague-Dawley rats was labeled with monoclonal antibodies to SM α-actin and to SM MHC. Whole transverse arteriole networks were observed, and terminal arterioles, defined as terminal segments labeled with SM α-actin, were classified on the basis of the presence or absence of SM MHC. A significantly different percentage of terminal arteriolar endings per network without SM MHC was observed in the two groups (66.1 ± 17.3% for 4 weeks and 27.1 ± 18.5% for 9 weeks), suggesting that arteriolar development is more nearly complete in the older animals. Sparsely distributed capillaries exhibited thin extensions of SM α-actin that crossed collecting venules and joined similar extensions from an adjacent transverse arteriole, effectively forming the basis for new arcade arterioles. SM α-actin and SM MHC labeling in terminal arterioles was always continuous with upstream arterioles. The pattern of labeling provides evidence that arteriolarization proceeds along capillaries from the terminal arteriolar endings, as opposed to being initiated at random sites on the developing vasculature that are distal to the arterioles. The methods described herein provide a powerful means to assess how factors such as tissue hypoxia, growth factors, and hemodynamic stresses affect arteriolar development as well as remodeling. (Circ Res. 1994;75:520-527.)

Key Words • angiogenesis • smooth muscle development • arteriolar network • microvascular growth • arteriolar adaptation

Arteriolar growth is an important event in the adaptation of normal tissues, such as exercised skeletal muscle and trained myocardium, as well as in important pathologies, including arterial collateralization following coronary artery stenosis, wound healing, and tumor growth. In skeletal muscle, new arteriolar growth is stimulated by exercise, ligation of the muscle’s feed artery, and normal maturation. The site of origin of new arterioles within the microvascular network, however, remains unknown. Although it is widely accepted that capillaries grow by sprouting into the interstitium, an angiogenic sprouting process has not been observed in arterioles. The current hypothesis for arteriolar development is that certain capillaries, selected by presently unknown mechanisms, become invested with vascular smooth muscle (SM) cells and are therefore transformed into arterioles, a process denoted here as arterialization. Nehls and Drenckhahn have suggested that pericytes, cells of mesenchymal origin located on the abluminal side of the capillaries, may be vascular SM cell precursors. It is also possible that vascular SM cells in upstream arterioles divide and migrate to the capillary that is being arterialized. Alternatively, SM cells may be derived from undifferentiated precursor cells that are present in the parenchyma. Regardless of the cell precursor phenotype, further support for the capillary arterialization hypothesis requires an experimental technique capable of identifying SM cells and distinguishing mature versus immature vascular SM cells that are in contact with the abluminal surface of capillaries. The objective of the present study was to develop an immunohistochemical technique for assessing capillary arterialization by using differentiation state-specific markers that are indicative of the maturity state of vascular SM cells in the arteriolar network.

An important consideration in these studies was to identify appropriate antibodies to SM proteins that could be used to identify and determine the relative state of differentiation of SM cells within the developing vasculature. We chose to use monoclonal antibodies to SM α-actin as well as SM myosin heavy chain (MHC). SM α-actin is the single most abundant protein in differentiated vascular SM cells and is an integral component of the contractile machinery. It is one of the earliest proteins expressed during differentiation of SM cells, and although it is expressed by a variety of mesoderm-derived cells during development, including
cardiac and skeletal myocytes, its expression in adult animals is limited to SM and SM-related cells. It thus serves as a marker of SM cells from their earliest stages of differentiation. The SM variants of SM MHC are expressed exclusively by SM cells, and in contrast to SM α-actin, appear to be markers of SM cells that are in a later or more mature phase of SM cell differentiation. Thus, it is possible to discriminate between vascular SM cells and their precursors by using antibodies to both SM α-actin and SM MHC. Although the distribution of SM MHC and SM α-actin has been examined in the arteriolar microcirculation, the expression of SM MHC in relation to SM α-actin in the intact vascular network, allowing determination of the arteriolarization pattern, has not been studied.

The labeling technique was tested in gracilis muscles of 4- and 9-week-old rats, because precapillary arterioles grow during normal skeletal muscle maturation and vascular SM contractile protein expression increases significantly as the rat matures. Thus, it was hypothesized that the labeling procedure would demonstrate that regions containing immature vascular SM cells (ie, SM α-actin positive but SM MHC negative), presumed to be arteriolarization sites, would be more abundant in the young rat. The whole-mount aspect of the technique preserved the arteriolar network structure throughout the entire muscle so that sites of new arteriolarization could be identified.

Materials and Methods

Immunohistochemical Technique

Sprague-Dawley rats, three at 4 weeks of age (weighing 101 ± 1.7 g) and three at 9 to 10 weeks of age (weighing 220 ± 1.7 g), were anesthetized with an intramuscular injection of a 13.5% urethane and 1% α-chloralose solution. The left femoral vein was catheterized for the administration of anesthetic booster doses. The right gracilis muscle circulation was isolated by the method of Sutton and Schmid-Schönbein, with a slight modification such that the femoral artery and vein served as a single input and a single output, respectively. The animal was euthanized with an anesthetic overdose, and the blood was washed out of the gracilis muscle microcirculation by perfusion with a phosphate (FA) buffer (Difco Laboratories). Paraformaldehyde, 3% in FA buffer, was perfused through the muscle for 5 minutes to fix the gracilis muscle in its in situ position. After a thorough FA buffer perfusion wash, the anterior gracilis muscle was dissected free and digested in 3 mg/mL type I collagenase (Sigma Chemical Co) in FA buffer for 10 minutes. The muscle was washed for at least 30 minutes in FA buffer before antibody (Ab) incubation. Each Ab was diluted in a solution of 3% bovine serum albumin, 0.4% (vol/vol) Triton X-100, and 4% (vol/vol) normal goat serum in FA buffer, and each incubation lasted ~24 hours. The incubations proceeded in the following order at the given concentrations: (1) hybridoma cell supernatant containing the Ab reactive to the SM-1 and SM-2 MHCs (1:1), (2) lissamine rhodamine sulfonfyl chloride (LRSC)-labeled Fab fragments (1:100), (3) Ab to SM α-actin (1:200), and (4) fluorescein isothiocyanate (FITC)-labeled Fab fragments (1:100). The SM MHC antibody, which was produced at the University of Virginia hybridoma laboratory by G.K. Owens, showed reactivity to the SM-1 and SM-2 isoforms of SM MHC but failed to react with nonmuscle MHC (derived from rat endothelial cells), as shown in Fig 1, or with skeletal or cardiac MHC (data not shown). In addition, the Ab showed SM cell–specific immunostaining in screening of multiple tissues including rat aorta and skeletal muscle (Fig 2) and in cultured cells (not shown). The SM α-actin Ab (clone 1A4) was developed and characterized by Skalli et al and was obtained from Sigma. Secondary Abs were FITC (SM α-actin) and LRSC (SM MHC)–labeled goat anti-mouse Fab fragments from Jackson Immunoresearch Laboratories Inc. Fab fragments were used instead of whole IgG molecules as secondary Abs to prevent the second primary Ab from binding to the first secondary Ab. After labeling, the anterior gracilis muscle was mounted on a slide in a 1:1 mixture of glycerol and FA buffer with a sealed coverslip.

Because both primary Abs were from marine hosts, it was possible that the FITC-labeled secondary Fab fragments, which were intended to label SM α-actin, had labeled SM MHC as well. On the basis of in vitro evidence, it was assumed that immature vascular SM cells would express SM α-actin before expressing SM MHC; therefore, the order of Ab labeling was chosen such that even if SM MHC had been labeled with FITC Fab fragments, it would not affect the results, because the terminal arteriolar endings that were expressing SM MHC should be expressing SM α-actin as well. To verify that all terminal arteriolar endings expressing SM MHC were also expressing SM α-actin, a gracilis muscle from a 170-g female Sprague-Dawley rat was labeled for SM α-actin with FITC Fab fragments before labeling for SM MHC with LRSC Fab fragments. In other words, the order of labeling was reversed so that SM MHC could not have a false label. Several terminal arteriolar endings from this muscle were examined to verify that SM α-actin was always expressed in regions where SM MHC was expressed.

In separate experiments, the position of SM α-actin–positive cells relative to the abluminal surface of capillaries was determined by use of FITC-labeled Griffonia simplicifolia I (GS-1) lectin (Sigma), which has affinity for both α-methyl-D-galactopyranosyl groups and N-acetylgalactosamine in the endothelium and basement membranes of microvessels and thus clearly delineates the capillary bed topology. Three additional whole anterior gracilis muscles were dissected, fixed, and digested as previously described. After the muscle was thoroughly washed in FA buffer, it was immersed in

![Western blot analysis demonstrating the specificity of the smooth muscle (SM) myosin heavy chain (MHC) monoclonal antibody used in these studies for SM-1 and SM-2 MHCs. Cultured rat aortic endothelial cells (E.C.) or rat aortic media (A.H.) were homogenized in sample buffer, and MHC isoforms were resolved by sodium dodecyl sulfate–polyacrylamide gel electrophoresis as previously described. The gels in the left panel were stained with Coomassie blue to allow visualization of all proteins. For Western analysis, proteins were transferred to nitrocellulose and stained with the SM MHC monoclonal antibody or a control monoclonal antibody specific to the chicken sodium pump (not shown), followed by staining with an avidin-biotin-peroxidase secondary monoclonal antibody (Vectastain Kit, Accurate Chemical Co) (right panel). NM indicates nonmuscle MHC.](image-url)
FITC-labeled GS-1 lectin (30 μg/mL in FA buffer) for 30 minutes. The muscle was then incubated in the Ab to SM α-actin and labeled with LRSC Fab fragments by using the aforementioned protocol. All experimental procedures using animals were performed in accordance with institutional guidelines.

**Terminal Arteriolar Classification**

In skeletal muscle, the arcade arterioles, which are arranged in a meshlike pattern, supply blood to the dichotomous branching transverse arteriolar (TA) trees. The TA trees, in turn, supply blood to the capillaries. Initially, the entire anterior gracilis muscle was observed at low magnification (×10 objective) to identify the arcade and TAs. Whole TA trees with clearly discernible vessels, as determined by FITC-labeled SM α-actin intensity, and low background levels were then videotaped at high magnification (×40 or ×80) with a GenIIsys image intensifier (Duage-MTI Inc.), and FITC and LRSC filters were used alternately to identify the distribution of SM MHC expression with respect to SM α-actin expression. In most cases, the best image quality was at sites on the thin edges of the muscle. The ending of each terminal arteriole, defined as a terminal segment containing SM α-actin, was classified as positive if SM MHC labeling was detectable or negative if SM MHC labeling was not detectable, thus determining the maturity state of the vascular SM cell(s) at the terminal arteriolar ending. An unpaired Student’s t-test was used to determine significant differences between the percentage of negative SM MHC terminal arteriolar endings in entire TA trees in the two age groups.

**Results**

TA networks were observed in anterior gracilis muscles of 4- and 9-week-old Sprague-Dawley rats. In the 4-week-old group, 980 terminal arterioles in 32 TA networks were classified. In the 9-week-old group, 516 terminal arterioles in 21 TA networks were classified. Figs 3 and 4 are confocal microscope images that depict terminal arteriolar endings, defined as terminal segments labeled for SM α-actin, that are either positive for SM MHC or negative for SM MHC. Although arterioles throughout each muscle were brightly labeled for both SM α-actin and SM MHC, some terminal arterioles could not be observed because they were
obstructed by other arterioles. In some cases, the terminal arterioles were obscured by high background levels due to muscle autofluorescence and neighboring vessels. No terminal arteriolar endings were positive for SM MHC and negative for SM α-actin in the 4- and 9-week-old animals. This was also true for the 170-g rat anterior gracilis muscle in which labeling was done in reverse order to specifically verify that SM MHC was never expressed before SM α-actin. A total of 323 terminal arteriolar endings were observed in the 170-g rat anterior gracilis muscle, none of which were positive for SM MHC but negative for SM α-actin. In the 4-week-old animals, 66.1 ± 17.3% (n = 32 TA trees) of the observable terminal arteriolar endings per TA network were negative for SM MHC. In the 9-week-old animals, this percentage (27.1 ± 18.5%, n = 21 TA trees) was significantly less (P < .05).

On a qualitative level, three important observations of the network pattern of SM α-actin and SM MHC labeling were made. The first observation was that in the muscles that were double-labeled with GS-I lectin and the Ab to SM α-actin, SM α-actin-positive cells were, without exception, in close physical contact with the abluminal surface of microvessels as delineated by the lectin label. Fig 5 is a confocal microscope image that depicts a region of muscle that has been labeled with GS-I lectin and the Ab to SM α-actin. Hansen-Smith et al22 have also shown that when SM α-actin is present, it is colocalized with GS-I-stained microvessels in skeletal muscle. Since SM MHC was always colocalized with SM α-actin in the present study, SM MHC-positive cells were also in physical contact with the abluminal surface of microvessels. The second observation was that isolated patches or islands of SM α-actin and/or SM MHC were never seen. In other words, the SM α-actin and/or SM MHC labeling was always continuous from the arcade arteriole to the TA tree and finally to the terminal arteriole. This continuity was present even when the labeling at the terminal arteriolar ending was narrower than a capillary diameter and located several hundred micrometers lateral from the nearest TA tree. Occasionally, small gaps in the SM α-actin and/or SM MHC labeling of no more than 5 μm were observed at the ending of a terminal arteriole when viewed in a single focal plane. In the majority of cases, these apparent discontinuities were not observed when specimens were examined in multiple focal planes, suggesting that the labeled cells were helically coiled around the vessel. In a very few instances, however, discontinuities were evident even when viewed in multiple focal planes, but it appeared that labeled cells at the terminal arteriolar endings had been damaged by the digestion and permeabilization procedures. Even in these cases, the distribution of labeling always followed the projected longitudinal axis of the upstream vessel. The slight discontinuities due to cell damage were never extensive enough to give the impression that SM α-actin and/or SM MHC–labeled cells were not in contact with a terminal arteriole. The third observation was that sparsely distributed capillaries exhibited exceptionally long (300- to 700-μm) extensions of SM α-actin. An example of this labeling pattern is shown in Fig 6. In some instances, these long SM α-actin extensions crossed an adjacent collecting venule (CV) tree without connecting directly to the CV tree and joined a similar extension from another TA tree. This type of SM α-actin–labeling pattern, which almost always occurred in TA trees at the edges of the muscle, indicated that a new arcade arteriolar loop was being formed. A general schematic diagram (Fig 7) has been included to highlight the typical regions in which new arteriolar development was detected and to depict the process by which new arcade arterioles appear to form. Short extensions of SM α-actin labeling, connected to developing arcade arterioles, were often observed on cross-connection capillaries that lie perpendicular to the muscle fiber direction. These SM α-actin–positive cross-connection capillaries may be the genesis of TA trees supplied by the new arcade arteriole.

**Discussion**

An immunohistochemical technique capable of identifying arteriolarization sites within intact microvascular networks by distinguishing the vascular SM differentiation state was developed and tested. Using
this technique, we have demonstrated that the cells on the abluminal surface of terminal arteriolar endings in rat gracilis muscle are frequently positive for SM α-actin, but negative for SM MHC, and that 4-week-old rats exhibit a significantly greater number of negative SM MHC terminal arteriolar endings per TA network (66.1 ± 17.3%) compared with 9-week-old rats (27.1 ± 18.5%). It is unlikely that cells in terminal arteriolar endings were negative for SM MHC because of poor primary or secondary antibody penetration, since these cells were labeled for SM α-actin. Furthermore, terminal arteriolar endings that were negative for SM MHC were never spatially isolated from regions where SM MHC labeling was abundant, such as neighboring terminal arterioles, the root TA, or another TA tree. Since there is strong evidence that SM MHC expression is a marker of mature differentiated SM cells, these results indicate that relatively immature SM cells can be identified at the leading edge of arteriolarization and establish the utility of SM MHC expression as a means of identifying newly developing arterioles. The observation that the frequency of SM MHC-positive terminal arteriolar endings increased between 4 and 9 weeks of age but did not approach 100% in the older age group argues that completion of capillary arteriolarization occurs relatively late in development (ie, 10 weeks or later). This result is consistent with the findings of Borrione et al.,20 who demonstrated that SM MHC expression increases during maturation. Determination of the exact time at which arteriolar development is complete will require examination of additional age groups and microvascular beds. Whereas the present study focused on SM α-actin and SM MHC, alternative markers of SM differentiation state, such as heavy caldesmon26 and SM α-tropomyosin,27 may have similar utility.

The labeling technique appears to be capable of identifying immature vascular SM cells, but it is not capable of determining the origin of these cells. It is possible that the precursors of immature vascular SM cells in the anterior gracilis muscle microvasculature are pericytes, other vascular SM cells, or cells of an unknown phenotype. On the basis of observations that SM MHC-positive cells were always in direct contact with

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**Fig 5.** Photomicrograph of a region of a gracilis muscle that has been labeled for all small microvessels with fluorescein isothiocyanate-conjugated *Griffonia simplicifolia* I lectin (green) and for smooth muscle (SM) α-actin with lissamine rhodamine sulfonyl chloride (red). The two images have been superimposed to demonstrate that regions of SM α-actin labeling are always in physical contact with microvessels. Terminal arteriolar endings are depicted with arrows (bar=40 μm).

**Fig 6.** Photomicrographic montage of a small transverse arteriolar tree in a whole-mount gracilis muscle that has been immunohistochemically labeled for smooth muscle (SM) α-actin by use of fluorescein isothiocyanate. The thin extension of SM α-actin labeling gradually tapers to capillary diameter about 100 μm before the distal end of the labeled region and is similar to the terminal arterioles that cross the collecting venules and join terminal arterioles from an adjacent transverse arteriolar tree, effectively forming new arcade arterioles (bar=100 μm). See Fig 7 for schematic diagram.
upstream arterioles, it is likely that SM cells in developing arterioles are derived from differentiated vascular SM cells in upstream terminal arterioles. However, we cannot rule out the possibility that other precursor cells are recruited to the site of arteriolarization. Pericytes have several vascular SM characteristics and may be capable of differentiating into vascular SM cells. Nehls and Drenckhahn\cite{Nehls1994} and Joyce et al\cite{Joyce1996} have demonstrated that the expression of SM contractile proteins in pericytes increases as the diameter of the microvessel increases from capillary to arteriolar dimensions. Ultrastructural studies have indicated that pericytes of the arterioles and venules exhibit other SM-like characteristics, such as increased numbers of myofilaments and dense bodies, that are not found in the capillary pericytes.\cite{Nehls1994} Nehls and Drenckhahn, using a whole-mount immunohistochemical protocol for rat mesentery similar to ours, were able to demonstrate that precapillary pericytes express SM \(\alpha\)-actin in vivo but that midcapillary pericytes do not. SM MHC has also been localized in the pericytes of various rat microvascular beds, including skeletal muscle, but the spatial relation of the pericyte-containing vessels to the arteriolar network was not determined because only cross sections were used.\cite{Joyce1996}

Based on our results that SM MHC distribution is continuous in the arteriolar microcirculation, the findings of Joyce et al may be interpreted to imply that all SM MHC-positive pericytes observed in their cross sections were in contact with upstream arterioles. Furthermore, since the SM \(\alpha\)-actin distribution reported by Nehls and Drenckhahn was also continuous and SM \(\alpha\)-actin was not located on the midcapillaries, all SM \(\alpha\)-actin-positive pericytes are also in contact with arterioles. Therefore, the present study is consistent with these previous studies regarding the spatial distribution of contractile protein-positive cells within the arteriolar network, irrespective of whether these cells are denoted as "precapillary pericytes" or "immature vascular SM cells."

Based on evidence from the present study, a general hypothesis for the arteriolarization process can be constructed. Since SM \(\alpha\)-actin-positive cells were always located on the abluminal surface of a microvessel that connected to other downstream microvessels (as shown by the GS-I label), it appears that arterioles do not sprout. Furthermore, regions of immature vascular SM cells were located at the junctions of terminal arterioles and capillaries. Isolated regions of immature vascular SM cells were never observed. Taken together, the above observations provide strong evidence that new arterioles are formed when existing capillaries become invested with SM. The SM investment begins at the
junction of a terminal arteriole and a capillary and proceeds along the capillary.

Snyder and Coelho have demonstrated that total arcade arteriolar length increases in chick anterior latissimus dorsi muscle that has been stimulated by exercise. The increase in total arcade arteriolar length was attributed to the growth of new arcade arterioles rather than a lengthening of existing arterioles, because the distance between arcade arteriolar nodes in the stimulated muscle was unchanged from the control value. Our observations of the network arteriolarization pattern demonstrate that new arcade arterioles form in normal muscle maturation as well. It appears that new arcade arteriolar formation occurs at the edges of the anterior gracilis muscle when two terminal arterioles from adjacent TA trees join across a CV tree without directly connecting to the CV tree. Arterializing cross-connection capillaries that originate from recently formed arcade arterioles were identified as new TA tree root vessels. The observed network arteriolarization patterns imply that the framework for the developing arcade arteriolar microcirculation is present in the existing capillary network, with back-connection capillaries being potential arcade arterioles and cross-connection capillaries being potential TA root vessels. This pattern of arcade arteriolar formation may provide evidence for the relative contributions of hypoxia and hemodynamic stresses in arteriolar development. Perhaps the most peculiar feature of the developing arterioles is that a terminal arteriolar segment may be several hundred micrometers in length, whereas a neighboring terminal arteriole on the same TA tree may contain an SM α-actin–labeled region of only a few micrometers in length. To account for this pattern of arteriolarization, a hypoxia-induced growth stimulus would need to be localized enough to select a certain capillary for arteriolarization without stimulating neighboring capillaries and to act at the junction of an existing terminal arteriole and a capillary. If a hemodynamic stress stimulus is responsible for arteriolarization, the arteriolarizing capillaries may be exposed to a unique stress state, such as elevated wall shear or circumferential stress. The capillaries that become arcade arterioles, the “back-connection” capillaries, do not directly connect to a CV and thus are conduits between two high-pressure TA trees. The back-connection capillaries would be exposed to relatively high circumferential wall stresses, because the intraluminal pressures would be high, relative to that in neighboring capillaries. The pathways of highest shear stress in the capillary network are along the capillaries that directly connect the high-pressure TA tree to the low-pressure CV tree and do not appear to be involved in arcade arteriolar development. Since the back-connection capillaries are connected to TA trees of approximately equal pressure, the flow and wall shear stress values are likely rather low in the back-connection capillaries. These observations are consistent with the hypothesis that arteriolar development is stimulated by elevated levels of circumferential wall stress. Further experiments and network modeling studies are warranted to elucidate the possible role of hemodynamic stress in arteriolar development and capillary arteriolarization. Arteriolar development may also be dependent on tissue hypoxia; however, the observed network pattern of arteriolarization along discrete capillaries appears to be a highly localized event.

Summary

An immunohistochemical method that is capable of detecting new arteriolar development within intact microvascular networks in whole-tissue preparations is introduced. The results suggest that new arteriolar formation is initiated at preexisting terminal arteriolar endings and proceeds along capillary pathways, effectively arterIALIZING selected capillaries as contractile cells invest the capillary wall. The origin of the new contractile cells remains unknown. Comparison of skeletal muscle from 4- and 9-week-old rats showed that the number of arteriolar development sites is reduced as the animals mature. This new technique should permit the study of mechanisms of arteriolar growth during development and physiological adaptation, as well as in vascular disease, and facilitate the investigation of the relative roles of tissue hypoxia, growth factors, and hemodynamic stresses in the arteriolarization process.

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References


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