Scanning Microscopy

Volume 1 | Number 4 Article 25

6-26-1987

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Scanning Microscopy, Vol. 1, No. 4, 1987 (Pages 1749-1758)
Scanning Microscopy International, Chicago (AMF O'Hare), IL 60666 USA

0891-7035/87$3.00+.00

SCANNING ELECTRON MICROSCOPY OF VASCULAR SMOOTH MUSCLE CELLS FROM RAT MUSCULAR ARTERIES

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(Received for publication March 04, 1987, and in revised form June 26, 1987)

Abstract

The use of scanning electron microscopy (SEM) for the examination of medial smooth muscle cells (SMC) is limited by the presence of connective tissue mass in the adventitia obscuring the detailed structure of the SMC. This study deals with the description of a method which selectively removes the connective tissue, allowing three dimensional observation of SMC using SEM. A number of methods were tried. One method involving post fixation with 2% OsO_4 used in conjunction with HCl proved most successful in removing the extracellular matrix of large mesenteric arteries from spontaneously hypertensive rats (SHR) and age-matched Wistar-Kyoto normotensive rats (WKY). In both SHR and WKY, the spindle-shaped cells are circumferentially oriented, and ran parallel to one another and formed a continuous compact medial layer with a number of intercellular junctions being evident. Surface morphology of SMC changed between relaxed and contracted states. Relaxed SMC had a smooth regular surface with very fine longitudinal grooves or striations running parallel to the cell long axis. Contracted SMC possessed regular transverse folds on its surface giving a corrugated appearance. The nerve network possessed a fishnet appearance with many triangular bulges at nerve branch points. The internal elastic laminae showed longitudinal furrows and fenestrations, the number of which varied from vessel to vessel. In the superior mesenteric artery, the SMC were circular and helically arranged. Longitudinally and diagonally arranged SMC were evident at bifurcations. Mesenteric veins consisted of one layer of flattened circular SMC that ran parallel to one another and formed a compact and complete medial layer.

Key Words: Rat mesenteric artery, smooth muscle, scanning electron microscopy, internal elastic lamina, innervation.

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Introduction

A number of morphometric studies using spontaneously hypertensive rats (SHR) and the normotensive Wistar-Kyoto rat (WKY) have been done on the mesenteric arteries to elucidate the role of structural changes of medial smooth muscle cells (SMC) in the etiology of hypertension [10,20]. Of particular interest is the large mesenteric artery (LMA) in which an increase in medial SMC mass is seen at the prehypertensive and hypertensive phases which were due to SMC hyperplasia. The importance of these muscular arteries in the development of hypertension is emphasized by recent findings that large muscular arteries are also involved in the control of vascular resistance [2,25].

Scanning electron microscopy (SEM) can be quite valuable in obtaining information pertaining to LMA morphology since cross-sectional studies using transmission electron microscopy (TEM) can represent SMC in only two dimensions at any given time and thus, requires a considerable amount of time and effort to build a three dimensional reconstruction based on serial sections. SEM is useful in this regard because of its ability to incorporate a third dimension when viewing blood vessel morphology and therefore can be easier and faster than the conventional three dimensional reconstructions. SEM is also valuable in studying SMC arrangement, shape and innervation in blood vessels.

Unfortunately, direct visualization of SMC is usually not possible using SEM because of the outer connective tissue matrix of the adventitia which obscures the underlying muscle cells. A number of techniques have been used to remove connective tissue components in various tissues from many animal species [1,6,8,11,12, 15,23,24,28,32]. However, it is not known if some of these techniques can be used for the LMA of the rat.

The primary objective of this study was to find a technique that will remove connective tissue mass from the mesenteric arteries of SHR and WKY rats, allowing for direct viewing of the blood vessel architecture, including SMC surface morphology, arrangement, innervation and the morphology of the internal elastic lamina and its relationship with SMC. Of the four

techniques attempted, one was found to be very useful for the removal of connective tissues from LMA, superior mesenteric artery and mesenteric veins from SHR and WKY rats. Details on this technique, and the morphology of the SMC from these blood vessels are described in this paper.

Materials and Methods

Large mesenteric arteries (LMA) which formed the first branch from the superior mesenteric artery (SMA) of male WKY and SHR rats (12-28 wks of age) were sampled. SMA and mesenteric veins were also sampled. Blood pressure of the rats was measured using a tail cuff compression method and ranged between 180-190 mm Hg for SHR and 125-135 mm Hg for WKY. The rats were anaesthetized with sodium pentabartitol (35 mg/kg) using an intra-peritoneal injection.

Vascular Perfusion

Isolation and perfusion of the mesenteric bed has been reported elsewhere [20]. Briefly, a flow rate of 1 ml/min/100 g body weight was used throughout the perfusion procedure. Vascular beds were perfused with oxygenated Krebs (pH 7.4, 37°C) for 10-15 min followed by 20 min perfusion with 2.5% glutaraldehyde (GA) in 0.03 M phosphate buffer and 15 min of phosphate buffer wash. This procedure results in maximally relaxed vessels and the least amount of volume change in SMC [19]. Following perfusion with wash buffer, the rat was reinfused with human whole blood for 1-2 min [24].

After perfusion with whole blood, the mesenteric bed was dissected free and was further fixed in 2.5% GA for an additional 2-3 h. Subsequently, after fixation in GA the mesenteric bed was transferred to phosphate wash buffer (pH 7.4) and refrigerated overnight at 4°C.

Dissection Procedure

Dissection and removal of the adipose and connective tissue matrix encompassing the arteries were carried out the following day under a Zeiss dissecting microscope at a magnification of 50X. Initially, gross dissection was performed to remove loose adipose and connective tissue components associated with the artery. Further micro-dissection was carried out using a pair of fine forceps and probe to remove any remaining loose connective tissue. Care was taken so as not to damage the media of the blood vessel.

Digestive Protocol

The following methods were tried in order that a method could be found to selectively remove the connective tissue from the vessels.

Method I. HCl and Collagenase [8].
After dissection, the blood vessels were placed in 8 N HCl for 50 min at 60°C. Following acid digestion each blood vessel was rinsed in phosphate wash buffer for 15 min and then placed in a 0.1 M phosphate buffered collagenase (pH 6.8, Sigma Type 1A) solution. Various concentrations of enzyme (10, 12, 20 mg/10 ml buffer) and digesting times (4, 8, 16 h) were studied.

Method II. KOH and Collagenase [23].
The vessels were placed in 5 N KOH at 60°C for 3 min and then washed three times in a Tris maleic buffer (pH 6.8, 800 mOsm, 23°C). The vessels were then placed in a collagenase solution (Sigma Type 1A) in Tris maleic buffer containing 0.055 mg/ml of $CaCl_2$ (pH 6.8). Various concentrations of collagenase (10, 12, 20 mg/10 ml of buffer) and digesting times (10, 17, 20 h) were studied.

Method III. HCl and Elastase [1].
The vessels were placed in 8 N HCl at 60°C for 50 min and then placed in an elastase solution (20 mg/10 ml, Sigma Type 1) in a 0.05 cacodylate buffer in 0.22 M sucrose (pH 6.8) at 25°C for 2-6 h.

Method IV. OsO_4 and HCl [28].
Following dissection, the vessels were washed in 0.1 M phosphate wash buffer and postfixed using 2% OsO_4 for 1 h at 5°C and 1-1.5 h at 40°C. The vessels were then washed with distilled water for 10 min and placed in 8 N HCl at 60°C for 20-25 min. The vessels were rinsed using distilled water for 10 min.

Processing for SEM

For Methods I-III, the vessels were rinsed in phosphate wash buffer and post-fixed in 1% OsO_4 for 1 h. The vessels from Methods I-IV were then treated with 0.5% aqueous uranyl acetate for 1 h which minimized cell shrinkage [18]. The vessels were dehydrated beginning with 70% ethanol and carried to 100% ethanol, critical point dried using liquid CO_2, sputter coated with gold, and viewed under a Philips 501 SEM.

Results

Treatment of the blood vessel segments with Methods I, II and III were unsuccessful in removing all of the connective tissue matrix from the adventitia (Fig. 1B). Compared to a control vessel without digestion (Fig. 1A), a good deal of connective tissue and fibroblasts remained after the digestive procedure. Increasing the enzyme concentration and/or digesting time did not facilitate the removal of connective tissue.

However, when Method IV was applied, total or partial removal of the connective tissue matrix was observed allowing direct visualization of SMC architecture (Figs. 1C-1F). Method IV, using the combination of OsO_4 and HCl, was the method chosen for the removal of connective tissue from mesenteric arteries and veins. Results obtained with Method IV are reported below.

Large Mesenteric Artery

Figs. 1C-1E show the arrangement and shape of the SMC around the arteries. The SMC wrap themselves circumferentially around the vessel and are orientated perpendicular to the vessel long axis. The muscle cells are spindle shaped with thick somal bodies and tapering processes that often curve inwards to disappear in the underlying muscle layers (Fig. 1D). There is no

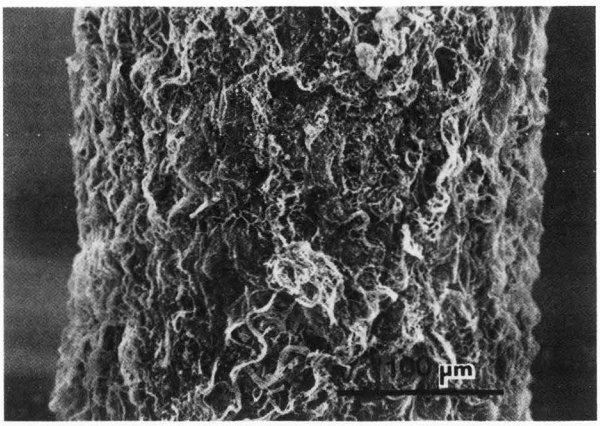

Fig. 1A. Mesenteric artery of WKY (control). Note the presence of connective tissue.

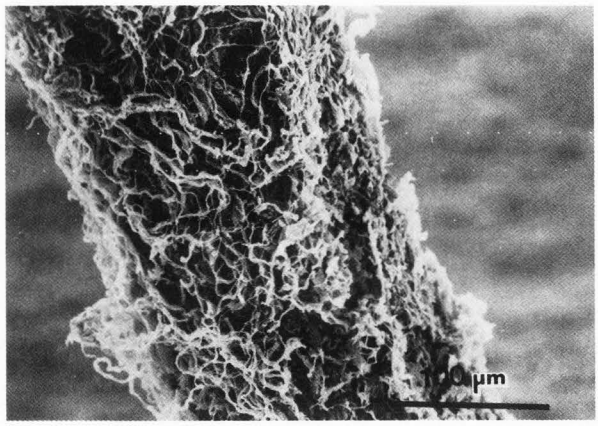

Fig. 1B. Artery from WKY using Method I. A large amount of connective tissue is still present.

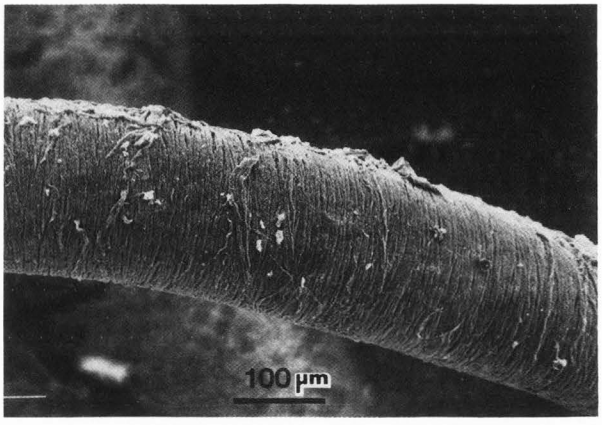

Fig. 1C. Artery from WKY (Method IV) showing vascular smooth muscle cells (SMC).

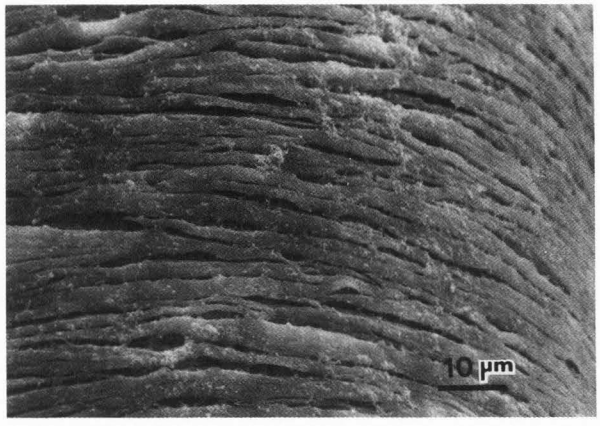

Fig. 1D. Artery from SHR showing spindle shaped SMC with thick somal bodies and tapering processes.

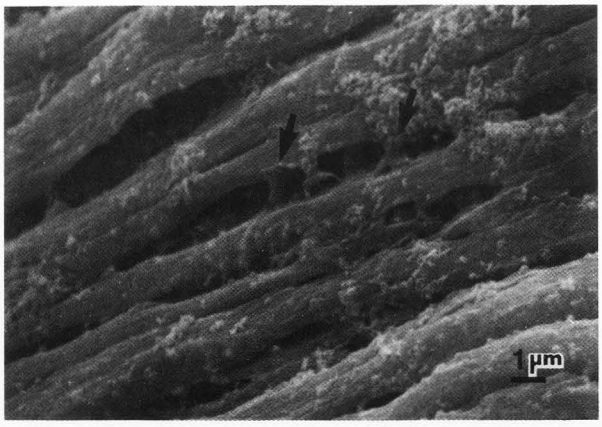

Fig. 1E. SMC from SHR displaying intercellular junctions (arrows) with lateral processes.

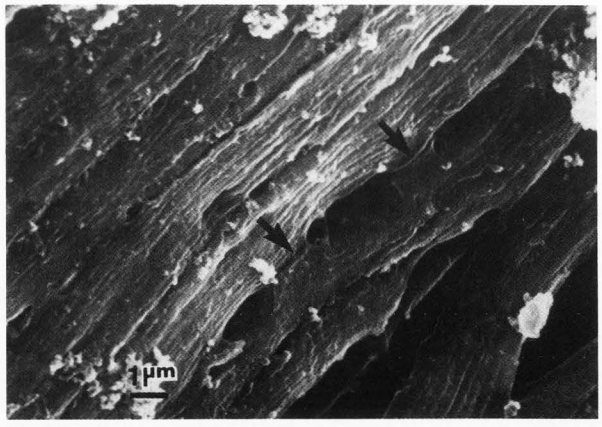

Fig. 1F. High magnification of SHR artery showing longitudinal striations on muscular cell surface and cell junctions (arrows).

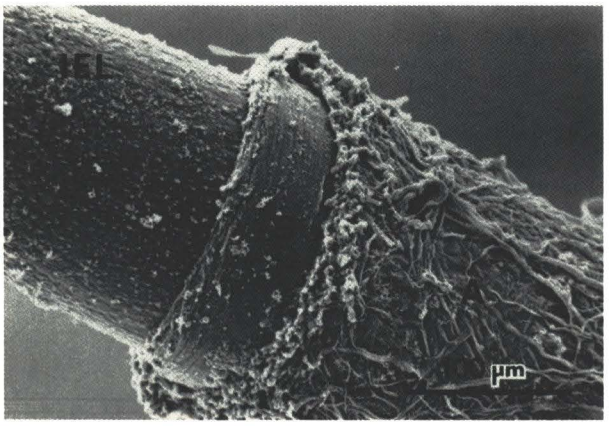

Fig. 2A. Artery from SHR displaying connective tissue matrix of adventitia (A), medial SMC (S) and internal elastic lamina (IEL).

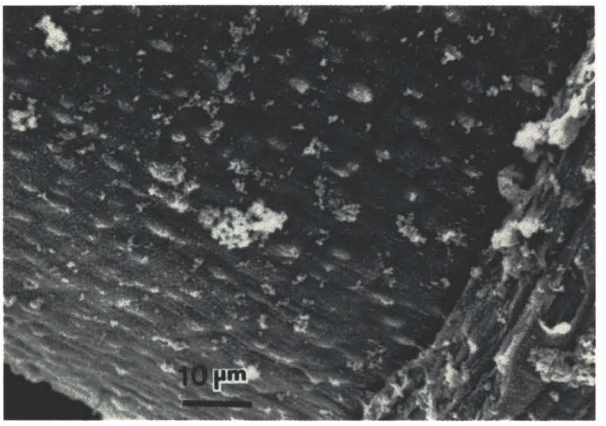

Fig. 2B. Higher magnification of SHR artery from fig. 2A showing medial SMC and IEL.

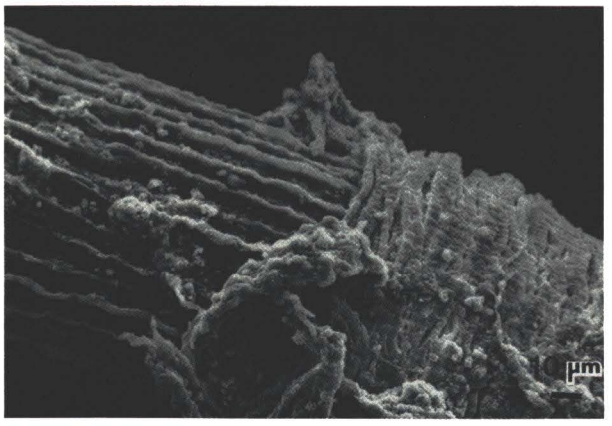

Fig. 2C. Constricted artery from WKY showing convoluted IEL and corrugated SMC.

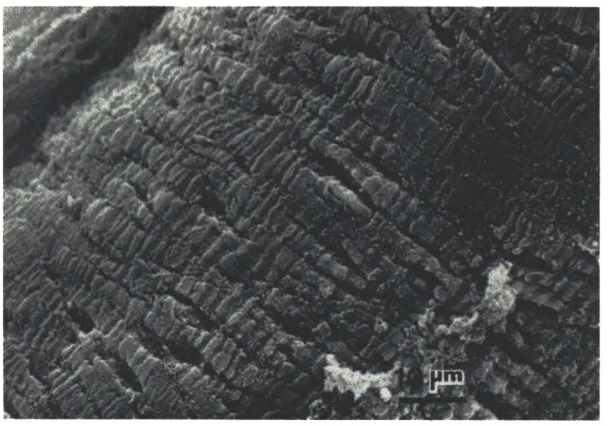

Fig. 2D. Outermost layer of SMC from a WKY constricted artery showing a corrugated appearance.

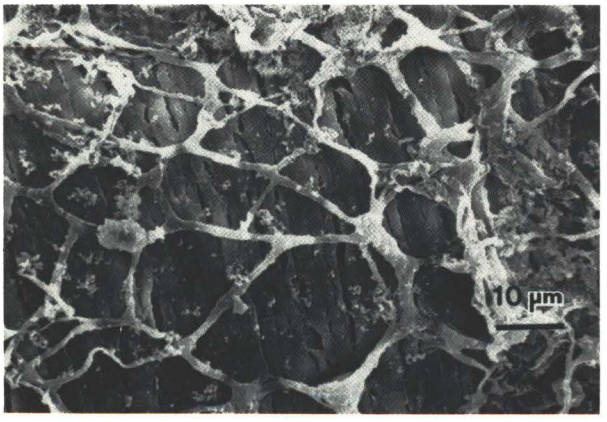

Fig. 2E. SMC innervation from SHR artery. Note the fishnet appearance of the nerve network.

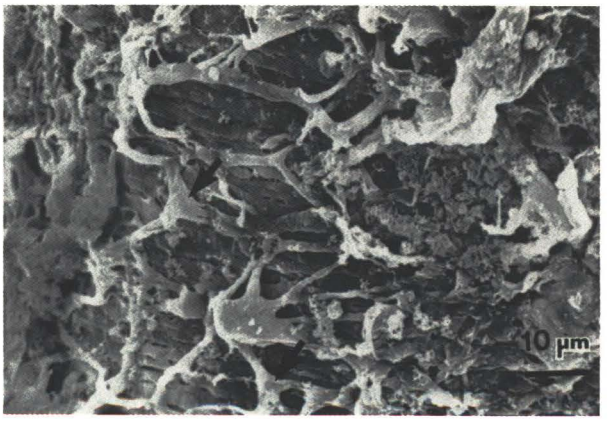

Fig. 2F. SMC innervation from SHR artery. Note triangular bulges at branch points (arrows) which may represent Schwann cell bodies.

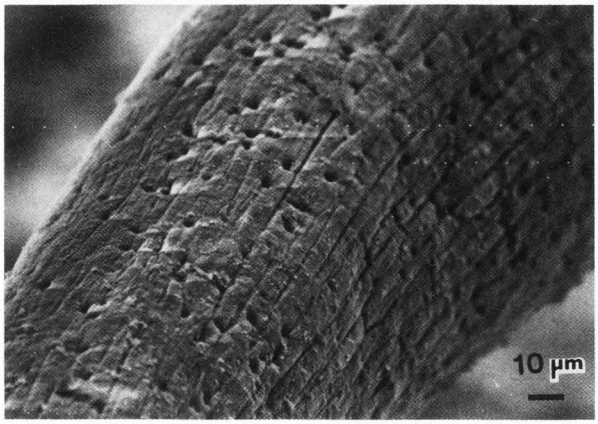

Fig. 3A. IEL from SHR artery showing longitudinal furrows and many holes or fenestrations.

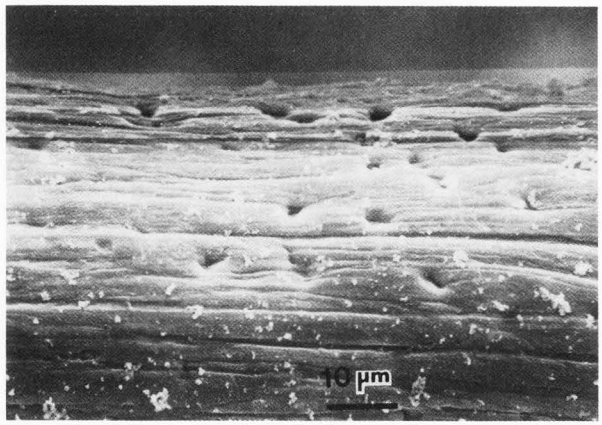

Fig. 3B. Higher magnification of IEL with longitudinal furrows and a number of fenestrations.

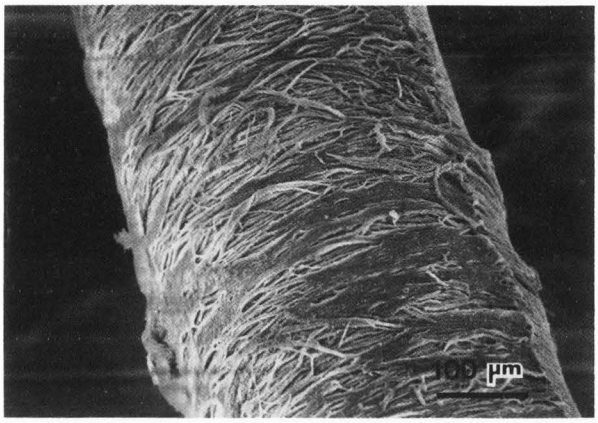

Fig. 3C. Medial aspect of superior mesenteric artery (SMA) from SHR. Note circular and helical arrangements of the SMC.

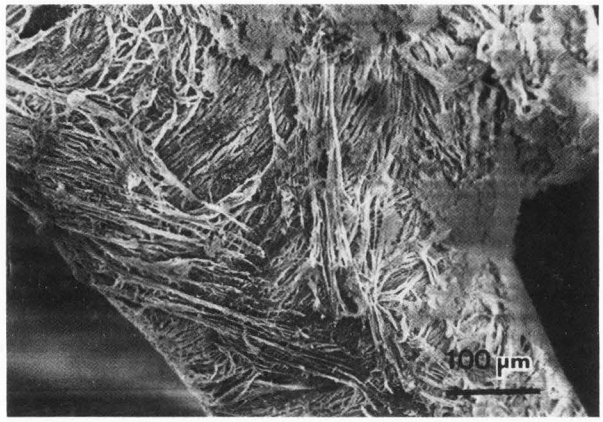

Fig. 3D. Longitudinally and diagonally arranged SMC found in medial-adventitial border of SMA at a bifurcation.

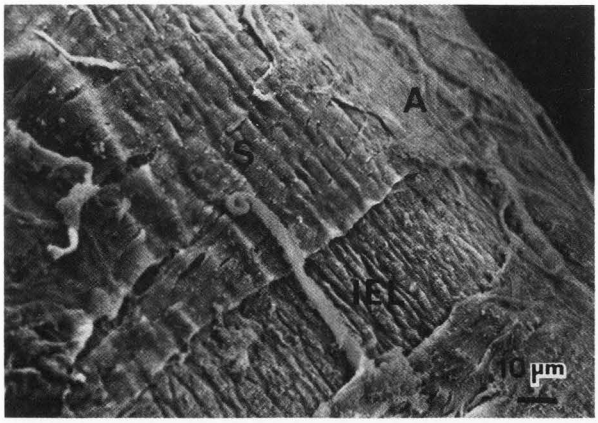

Fig. 3E. Mesenteric vein from SHR showing adventitia (A), one layer of medial SMC (S) and IEL.

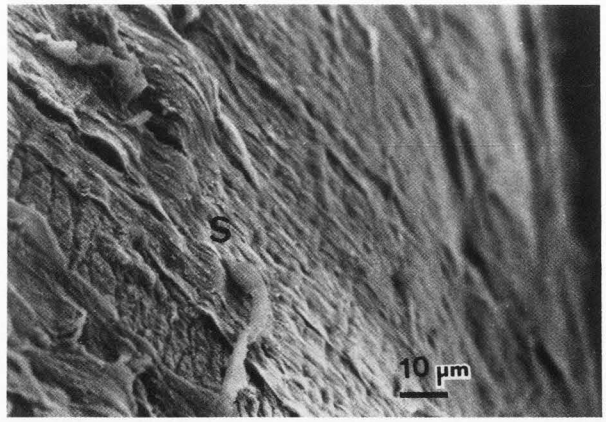

Fig. 3F. Higher magnification of a vein showing a single layer of SMC (S) and IEL containing furrows.

evidence of longitudinal muscle cells oriented parallel to the vessel long axis. Under higher magnification, intercellular contacts can be seen among neighbouring muscle cells. They appear as thin lateral processes that form a rake-like appearance along the muscle cell length (Fig. 1E).

Interestingly, the surface morphology of the muscle cells differ depending on the state of the vessel, i.e., if the vessel is fully relaxed or contracted. Fig. 2A shows a fully relaxed vessel containing three layers, the outer adventitia, the media and the internal elastic lamina (IEL). Under fully relaxed conditions the outer layer of muscle cells possesses a smooth regular surface morphology (Figs. 2A-2B). Under higher magnification, longitudinal striations in parallel with the long axis of the cell can be seen on the adventitial side of the outermost layer of SMC (Fig. 1F).

In contrast, the surface morphology of the SMC changes when the vessel is constricted. Fig. 2C shows a vessel in a contracted state since the IEL appears convoluted. The surface of the muscle cells is no longer smooth but rather possesses continuous folds giving the cell a corrugated appearance. The corrugated appearance of the muscle cells are seen in the innermost layer of muscle cells closest to the IEL (Fig. 2C) as well as in the outermost layer (Fig. 2D).

In addition to direct visualization of SMC using SEM, the OsO_4/HCl method was successful in revealing the interaction between nerves and muscle cells in some vessels. Figs. 2E-2F show a nerve network having a fishnet appearance and the outermost muscle cell layer directly under it. All nerves visualized were found within the medial-adventitial border since no nerves were found to penetrate into deeper muscle layers. Throughout the nerve network, swellings or triangular bulges can be seen at branching points between nerve fibers.

The ability of directly observing the IEL using SEM is of particular interest. Visualization of the IEL allows for categorization of vessels in the relaxed or constricted state since the IEL possessed a convoluted appearance when contracted (Fig. 2C) and a smooth cylindrical appearance when fully relaxed (Figs. 2A-2B). Figs. 2B, 3A, 3B show the outer aspect of the IEL at the medial border. The IEL exhibits longitudinally oriented furrows running parallel to the vessel long axis and a number of fenestrations or holes found randomly throughout the structure. These fenestrations were approximately 3 μm wide and their number varied according to the blood vessel sampled (Figs. 2B, 3A, 3B).

Superior Mesenteric Artery (SMA)

In the SMA, the regular array of circular muscle cells forming a uniform layer is partially disturbed (Fig. 3C). The SMC tend to have either a circular or helical arrangement around the vessel. At bifurcations with LMA, longitudinally and diagonally oriented muscle cells can be seen in the medial-adventitial border with underlying muscle layers displaying the more familiar circumferentially oriented arrangement (Fig. 3D).

Large Mesenteric Vein

Figs. 3E-3F show a mesenteric vein after using the OsO_4/HCl method. The outer adventitia, media, and IEL can be seen. The media is made up of one layer of muscle cells that possess the familiar spindle-shape appearance although they appear flatter than arterial SMC. The muscle cells are oriented perpendicular to the long axis of the vein. Similarly, the IEL contains longitudinal furrows that run parallel to the long axis of the vein.

Discussion

Since the advent of visualizing cell surfaces using HCl and collagenase [8], modifications of this method have been reported [1,6,11,12,15,23,24,28,32]. The primary task is to develop an appropriate technique that will enable direct visualization of the cell surface concerned using SEM. Although there have been many studies on the visualization of SMC in blood vessels [11,12,15,23,24,28] no method has yet been applied for the purpose of observing SMC architecture in rat LMA.

Methods I-III, although very effective in removing connective tissue from rat kidney and autonomic ganglion [8], submucosal arterioles [24] and chick pecten capillaries [1] respectively, proved ineffective in removing the connective tissue matrix of rat LMA. It is quite possible that the connective tissue density and quantity may differ in mesenteric arteries and smaller arterioles, thus a method was developed that was selective for the removal of connective tissue from LMA.

Using the combination of OsO_4 and HCl (Method IV) proved to be very effective in removing the connective tissue matrix of LMA, SMA and mesenteric veins. Other studies have shown the usefulness of OsO_4 both as a fixative and as a digestive agent to remove connective tissue elements [28,29]. The combination of OsO_4 followed by HCl digestion has proved beneficial in revealing SMC arrangement in dog brain arteries [28], mouse muscle arterioles [15] and neuromuscular junctions of the frog [6]. The present study illustrates the usefulness of this method in removing connective tissue from rat LMA. Key features of this method are outlined below.

Dissection

Micro-dissection is a critical step in the success of this method in removing connective tissue. Other studies have emphasized the importance of microdissection in the success of the method in removing the connective tissue [24]. Reinfusion with human whole blood is equally important because it gives the vessels structural rigidity and preserves their natural cylindrical shape [24]. This allows prolonged microdissection to be carried out without disrupting the SMC arrangement in the vessel media.

Digestion

Using OsO_4 both as a post-fixative and as a

removal agent of connective tissue aids greatly in fixing the SMC and thus, may minimize the alteration in SMC morphology due to prolonged exposure in HCl. Many studies have used acid digestions of 40 min or more in order to remove connective tissue elements [8,15,32]. Decreasing the acid digestion time to only 20-25 min may also help in preserving the true morphology of the SMC.

Large Mesenteric Artery

In the LMA, the muscle cells are circularly oriented with little or no pitch and are perpendicular to the vessel long axis as reported by Matthews and Gardner [22]. Previous studies have reported similar findings of circumferentially orientated SMC in other blood vessels such as human cerebral arteries [33,34] and monkey muscular arteries [4,5]. Of particular interest is the absence of diagonally and longitudinally arranged muscle cells in the medial-adventitial border of these vessels. Other studies have observed longitudinal muscle cells in the monkey mesenteric artery [12], in dog cerebral arteries [28] and rat renal arteries [26,30]. The muscle cells in LMA only partially encircle the vessel whereas muscle cells of small arterioles wrap completely around the vessel [15,17,23,24]. This study also demonstrates the presence of intercellular contacts with thin lateral processes. These findings correspond to those found in dog cerebral arteries [28] and monkey mesenteric arteries [12] using SEM. Although there are different types of arterial SMC junctions [14], these junctions probably represent either appositional type of cell junction, or gap junctions. TEM studies are necessary to identify these junctions.

The most striking finding of the study was the changes in surface morphology of SMC in relaxed and constricted states. The smooth cylindrical appearance of the IEL is a good indication of a relaxed vessel since the IEL appears convoluted when the vessel is contracted [21,27]. Consistent with the findings of Gabella [13] and Fay and Delise [9], we found that the surface morphology of the outermost muscle cells in the relaxed vessel appears smooth and uniform. Longitudinal grooves that run parallel to the cell long axis were observed on the muscle cell surface. Previous studies have reported similar findings in the mouse [15] and dog [28]. In vascular smooth muscle cells, alternating rows of surface vesicles and dense bodies were reported [7]. However, the fact that these are grooves instead of pits suggests that they may not be related to the surface vesicles. Studies on muscular arteries [12,28] have reported nodular protrusions and laminar folds on the muscle cell surface of vessels which were reported as being fixed in a relaxed state. It is quite possible that these vessels might be slightly contracted. In the contracted state, the muscle cell surface is corrugated which is consistent throughout the media since muscle cells from both the innermost and outermost layers display this pattern. Previous studies using TEM have supported the observation of isotonically contracted SMC possessing folds and myriad processes [9,13].

A number of vessels retained both their muscle and nerve arrangement. The nerve network had a fishnet appearance with triangular bulges at nerve branching points. Similar structures representing Schwann cell bodies have been reported elsewhere [32]. Although swellings can be seen throughout the nerve network, it is uncertain if these are actually varicosities, although visualization of varicosities using SEM have been reported [16,32].

In the IEL, longitudinal furrows are observed with fenestrations which are distributed randomly throughout the IEL. Similar holes or fenestrations have been seen in canine aorta [31] and in cerebral arteries [3]. Fine ridges can be seen in these arteries (Fig. 4, in ref. 31; Fig. 2 in ref. 3).

Superior Mesenteric Artery

At non-branching regions of these arteries, the muscle cells are spindle shaped but somewhat disordered, arranging themselves in a circular and helical orientation around the vessel which differs from the circular and compact layers of LMA. Interestingly, at bifurcations with LMA, the medial-adventitial border is made up of longitudinally and diagonally orientated muscle cells. Similar findings have been reported at bifurcations of human cerebral arteries [33]. Multi-directional muscle cells at bifurcations in dog cerebral artery [28] and longitudinal muscle cells in rat renal artery bifurcations [26] have also been reported.

Mesenteric Vein

In the veins, the SMC appear to retain their circular orientation running parallel with one another. However, they appear much thinner or flatter than the SMC from LMA. The medial layer is made up of only one layer of muscle cells compared to 3-4 layers for mesenteric arteries. Fujiwara et al. [11] have reported finding loose and regular SMC in the outermost medial layer of monkey mesenteric vein with an underlying circular muscle layer. Similar arrangement was not observed in the rat mesenteric vein.

The use of the OsO_4/HCl method has proven quite useful in visualizing medial surfaces of rat mesenteric arteries. Future studies comparing WKY and SHR smooth muscle cell number, size, and surface morphology will prove quite valuable in the understanding of the role muscular arteries play in hypertension. Other studies using TEM are essential to understanding the effect of the digestion method on SMC morphology.

Acknowledgements

Supported by the Ontario Stroke and Heart Foundation. We are grateful for the technical advice of Dr. B.G. Miller. We thank Mr. R. McKenzie, Mrs. T. Mok, Mrs. M. Roy, and Mrs. S. Ali for their helpful assistance and Dr. E. Mangiarua-Tassone for reading the manuscript. We also thank Ms. S.Seaman for her assistance in preparing the manuscript.

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Discussion with Reviewers

J. Holley: One of the most interesting aspects is the reorganization of smooth muscle cells (SMC) at the SMA bifurcation. Is it possible to accurately reconstruct the three dimensional organization of a "typical" mesenteric arterial junction? If so, could this information be used to predict the mechanical ability of SMC to direct blood flow by localized constrictions at these junctions?
Authors: This an interesting concept. We are not aware of any previous publications which report that in large arteries, control of blood flow can occur at the bifurcation points.

J. Holley: The mesenteric vein shows furrows along its adventitial surface, similar to the furrows which are related to constricted arteries. Since the mesenteric veins have only one layer of SMC, it seems doubtful they are constricted by the same SMC force. What is the physiological significance of the furrows in veins; will they change depending on the cardiac cycle and/or venous pressure?
Authors: Although the furrows found on the IEL from veins are similar to those found in arteries, we do not know their physiologic significance or whether they have the ability to change during the cardiac cycle and/or venous pressure.

J. Holley: The demonstration of holes or fenestrations in the internal elastic lamina is remarkable. Would you elaborate on their possible physiological role?
Authors: Holes or fenestrations could play an important role in the elastic property of the IEL and also the interaction between medial SMC and the endothelium. Therefore, a vessel with a larger number of fenestrations may have a greater communication network between medial SMC and endothelium. Furthermore, the number of holes in the IEL is probably one of the controlling factors in the passage of some blood-borne cells (e.g., leucocytes) across the vessel wall. These fenestrations may play an important role in some pathological conditions such as atherosclerosis and hypertension.

M. Roach: What proof have you (e.g., from TEM) that the structures are what you say they are?
Authors: Pertaining to SMC morphology, there is no doubt that these are SMC because of their characteristic spindle shaped appearance. Cross-sections using TEM on reimbedded specimens have substantiated this observation as well as the IEL and its convoluted appearance. Nerve profiles are somewhat more difficult to ascertain since they do undergo some deformation with the digestive method.

M. Roach: What is the rationale of perfusion with human blood? Would rat blood have the same effect?
Authors: The reinfusion of human whole blood after fixation is an important step since it gives the blood vessels structural rigidity and internal support which is needed because of the prolonged micro-dissection process. Vessels without whole blood, although fixed in a relaxed state, have a tendency to collapse after prolonged micro-dissection and digestion. We have not experimented with rat blood; however, there is no reason to suspect that the results would be different from that using human blood.

M. Roach: How do you know the convolutions in contracted arteries are not artefacts?
Authors: As mentioned in the paper, the convoluted appearance of the IEL in contracted arteries has been shown in many studies using TEM and cross-sections of the blood vessel. Cross-sections of reembedded specimens using TEM illustrated the convoluted appearance of the IEL and therefore strongly suggests that the convolutions are not artefacts.

M. Roach: Why did you not use muscle poisons to paralyze the muscle?
Authors: Muscle poisons were not used to paralyze the muscle because the fixation procedure we used is already sufficient to cause maximal relaxation of the blood vessel without causing distension.

B. Miller: Figs. 1A and 1B were presented as examples, respectively, of a control vessel without digestion and of the other "unsuccessful" methodologies. What dissection, if any, was attempted after osmication? The relative lack of OsO_4 uptake by the connective tissue would facilitate its differentiation from the underlying vessels and would allow more precise extraction of obscuring tissues. Do you feel this phenomenon could contribute to the success of this method?
Authors: All micro-dissection was done prior to osmium treatment. At this stage of the process, any possible amount of the loose connective tissue was removed using a dissection microscope and a pair of fine forceps. Subsequently after osmication, only a very fine layer of connective tissue remained which was not possible to remove using mechanical dissection. In some instances loose connective tissue remained on the vessel and was dissected free. It is quite possible that osmication may separate the medial and adventitial layers resulting in a greater success of HCl to remove the collagen.

B. Miller: Other studies have demonstrated that vessels could be viewed over the entire circumference [23, 24, Miller et al (1987) Hypertension 9, 59-68]. Was this capacity demonstrated by this method? Given the fact that these vessels have multiple layers of vascular smooth muscle (VSM) cells, what technique(s) would you employ with this method to address the next step-quantitative analysis of the vascular smooth muscle?
Authors: This method does allow for complete

viewing of the entire vessel circumference. Some vessels however, do retain some of their connective tissue elements which obscure some portions of the vessel. It is difficult to achieve an exact measurement of SMC length since in most cases the tapered ends disappear into deeper muscle layers. To incorporate a technique for quantitative analysis of SMC a number of parameters would first have to be considered including cell length, number of cell layers and the amount of shrinkage the cells undergo with this method.

B. Miller: The authors suggested that the OsO_4-HCl method will better preserve the "true morphology" by reducing the amount of exposure to HCl. Other studies, using greater (8) or less exposure [23, Moore et al (1985) Blood Vessels 22, 265-277] to HCl, demonstrated the preservation of ultrastructure with TEM of the vessels. By what methodologies was the ultrastructural preservation of the OsO_4-HCl method compared to that of Moore and other techniques [8,24]? Is there a difference in this observation between various exposure techniques? How do the changes in ultrastructure of similar cells compare among the various methods of exposure? What criteria did you use to rank these methods for this result?
Authors: We have found that the most appropriate acid digestion time is between 20-25 min for the successful removal of connective tissue mass from the LMA of the rat. A number of artefacts are introduced when the acid digestion time is increased to 30 min or more. These artefacts include the formation of small blebs on the SMC surface, an increase in the separations between cells and the disruption of SMC arrangement which could be due to the digestion of intercellular elements that bind the SMC together. TEM assessment of various methods awaits further studies.

B. Miller: Why were some medial muscle cells noticeably separated from their neighbours and underlying tissues (Figs. 1D, 1E, 3C, 3D), yet others (Figs. 2A, 2B, 3C, 3D) were not?
Authors: In some vessels larger separations can be found between SMC than other vessels suggesting a greater amount of SMC shrinkage took place. This could be due to a greater digestion time of 25 min used for older arteries (28 wk old rats) vs 20 min for younger arteries (12 wk old rats). A quantitative study is needed in order to find out exactly how much the SMC shrink using this digestive method.

B. Miller: Fig. 1A suggested that the adventitial connective tissue as having a wavy but basically longitudinal orientation. The adventitial connective tissue in Fig. 2A appeared less thick, more compacted, and more randomly oriented. Was this a result of digestion or dissection? Might the adventitia be "peeled back" and inverted over itself?
Authors: Fig. 1A shows a control vessel in which digestion was omitted and dissection was kept to a minimum in order that the reader may see the amount of connective tissue mass that must be removed before SEM viewing of the SMC can be accomplished. In Fig. 2A, the method involving OsO_4 and HCl as described in the paper was applied to this vessel and is responsible for the thin and more compacted adventitial surface. Although the method applied to this vessel was not totally successful it is a useful figure since it illustrates the relationship between the IEL, medial SMC and the adventitia.

B. Miller: Was the dissection or the digestion process responsible for the disruption and removal of the muscle cells from Figs. 2A and 3A?
Authors: We are not sure which stage of the process (i.e., micro-dissection, digestion, critical point drying) is responsible for the disruption and removal of the SMC. Subsequently after CPD a separation can be seen between the IEL and medial SMC suggesting different shrinking patterns between the two. It is quite possible that a portion of the medial SMC may have been separated during some stage of the method and subsequently slid off the IEL during processing of the vessels.

B. Miller: Figures 1F and 2E show little or no residual basal lamina. Fig. 2B has residual basal lamina/basement membrane, in the form of a diffuse coating of globular material. In your estimation, how much of a complication is this residual material?
Authors: It is not possible to predict if the basal lamina will remain or not until the vessels are viewed under the SEM. It seems that vessels from older rats (28 wk) retain their basal lamina much more readily than younger rats (12 wk) and therefore a longer digestion time of 25 min is needed.

B. Miller: It was implied in the Abstract that a "smooth regular surface" which also has "longitudinal grooves or striations" are normal surface morphology for relaxed vascular smooth muscle (VSM) cells. The only specimen demonstrating this phenomenon was Fig. 1F, depicting a SHR artery. The presence of these grooves was described only for the outermost muscle cells of the LMA in the Results. Given that these are relaxed vessels, might the presence of some VSM cells grooving be related to maximally stretched muscle cells? Why are the grooves not evident in all specimens? How have you eliminated the probability that this phenomenon is an artifact of tissue processing?
Authors: Longitudinal grooves running parallel to cell long axis can also be seen in Fig. 1E from a different vessel. In some cases these grooves cannot be seen since the basal lamina was retained or from the deposition of connective tissue debris after digestion. At present we are looking at longitudinal cross-sections of the vessels using TEM in order to ascertain what the significance of these grooves are.