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SCANNING ELECTRON MICROSCOPY OF VASCULAR SMOOTH MUSCLE CELLS FROM SPONTANEOUSLY HYPERTENSIVE RATS

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Abstract

The fine structure of vascular smooth muscle cells from large mesenteric arteries of adult (28 weeks) spontaneously hypertensive rats and Wistar-Kyoto rats was studied using scanning electron microscopy (SEM). The effect of the digestion method for SEM on tissue size and smooth muscle cell size was also studied using morphometric analysis. A significant reduction in cell size (42-43%) was present after critical point drying, based on the reduction in volume to surface ratio of smooth muscle cells. However, percent change of volume density and volume to surface ratio of smooth muscle cells after digestion and after critical point drying, was similar between the hypertensives and normotensives. Most of the tissue shrinkage occurred during the digestion process to remove the connective tissue. Overall tissue shrinkage due to the digestion method involving OsO_4/HCl and subsequent processing for SEM, based on changes in the thickness of the medial wall, was similar between arteries from hypertensive (34.7%) and normotensive (31.4%). After compensating for the shrinkage, vascular smooth muscle cells from hypertensive animals were found to have a wider somal region (5.94 μm) than those from the normotensives (5.46 μm), suggesting cellular hypertrophy. We conclude that a significant reduction in size of tissue and smooth muscle cells took place when arteries were processed for SEM. For comparative study of vascular changes in hypertension involving SEM, cellular shrinkage due to processing should be included in the calculations in order to provide a reasonable estimate of the alterations.

Key Words: Scanning electron microscopy, vascular, smooth muscle, hypertension

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Introduction

Scanning electron microscopy (SEM) is useful for the study of surface morphology of the cells, and also the relationship between various cell types. In the blood vessels, medial smooth muscle cells (SMC) are covered by the connective tissues in the adventitial layer, therefore making the visualization of these cells difficult. A number of digestive techniques have been developed to remove the adventitial connective tissues [1,3,4,13,14,16,17]. Miller et al. using a digestive method comprising of KOH and collagenase, was able to compare SMC from arterioles from the mesenteric vascular bed of Wistar-Kyoto rats (WKY) and spontaneously hypertensive rats (SHR) [12,13]. Other digestion techniques include OsO_4/HCl method to examine vascular SMC from rat mesenteric artery [4] and dog cerebral artery [14], and acid/elastase method for the mesenteric arteries from SHR and WKY [16,17]. However, the effects of the digestion method and subsequent processing for SEM on SMC shrinkage in WKY and SHR are unknown, and must be assessed before quantitative measurements and comparison can be undertaken. The purpose of this study was to compare quantitatively the effects of the OsO_4/HCl digestion and SEM processing on tissue and SMC size from WKY and SHR.

Methods

Adult (28 weeks) SHR and WKY were obtained from the colonies of Dr. Lee in the Animal Quarters of McMaster University. At this age, systolic blood pressure of the SHR (180-190 mmHg) was significantly higher than the WKY (120-135 mmHg). The mesenteric vascular bed was isolated from WKY and SHR rats and perfusion fixed according to the method of Lee et al.[8]. This included perfusion with Krebs solution for 10-15 min followed by 20 min perfusion with 2.5% GA and 15 min of phosphate buffer wash. The following studies were carried out.

The Effect of Digestion and Processing on Cell Volume

In order to ascertain the possible effects of the OsO_4/HCl digestion method on SMC morphology and

ultrastructure, mesenteric arteries from WKY and SHR were used. Following perfusion fixation of the arteries with 2.5% glutaraldehyde and phosphate buffer wash, large mesenteric arteries from SHR and WKY were sampled from predetermined sites, according to the method of Lee et al. [8]. Each vessel was randomly divided into four segments and these segments were subjected to different treatments as described below.

Segment 1: Control Vessel. This segment was processed for transmission electron microscopy, which included post-fixation with 1% OsO_4 in cacodylate buffer, followed by treatment with 0.5% uranyl acetate. The vessel was then dehydrated beginning with 70% ethanol and carried to 100% ethanol and embedded in Spurr's resin. Previous studies [9] have shown that this procedure had minimal effect on SMC volume, so that cell size from this segment was used as the standard to compare with cells from other segments.

Segment 2: OsO_4/HCl Treatment. OsO_4/HCl digestion method to remove vascular connective tissue was applied to this segment [4]. The digestion method involved treatment of the vessel with 2% OsO_4 for one hr at 5°C and 1-1½ hr at 40°C. The vessel was then washed with distilled water for 10 min and placed in 8N HCl at 60°C for 20-25 min. After HCl digestion, the vessel was treated with 0.5% uranyl acetate, dehydrated with ethanol beginning with 70% ethanol, and subsequently embedded in Spurr's resin. This segment was used to study the effects of the digestive method on SMC size and ultrastructure.

Segment 3: OsO_4/HCl Method Plus Critical Point Drying. OsO_4/HCl digestion method was applied to this segment. After treatment with 0.5% uranyl acetate and dehydration in ethanol as before, this vessel segment was critical point dried, in order to evaluate the effects of critical point drying on vessel morphology. Subsequently, the vessel was placed in 100% ethanol for 24 hr. After a brief immersion in propylene oxide for 5 min, the vessel was subjected to the same infiltration procedure as described for Segment 2, involving a mixture of absolute ethanol and Spurr's resin, and the vessel was subsequently embedded in Spurr's resin.

Segments 1-3 were used for quantitative analysis using transmission electron microscopy to study the effects of the OsO_4/HCl method on SMC ultrastructure. Ultrathin longitudinal-sections of the vessels were counterstained with lead citrate. The SMC profiles appeared circular when viewed under the TEM. Vessel segments were photographed in a Philips 300 TEM using 35mm film. Magnification was selected such that the complete photograph contained only the medial component of the vessel wall.

Segment 4: Scanning Electron Microscopy. This vessel segment was processed similar to those of Segment 3 described above, except that after critical point drying, the vessel was sputter coated with gold, and examined with a Philips 501 scanning electron microscope.

Morphometry

For morphometric measurements, longitudinal-sectional profiles of the media were projected onto a screen in a Zeiss microfiche reader. A standard point-counting system [18] was used to determine the volume density (V_{smc}) and the volume to surface ratio (V/S) of the SMC. A multipurpose grid with total points (P_T) of 168 and an area of 363.5 cm^2 was used for quantification. Using three control vessel segments (segment 1), we have carried out experiments to determine the number of pictures needed to ensure that the error of measurement was less than 5% [18]. We found that values obtained with 10 photographs were not significantly different from those obtained with 20 photographs, and the probability of making an error by using 10 photographs to measure various morphometric parameters was 0.05 or less. We have therefore chosen to use 10 micrographs for each vessel in our analyses.

Points or intersects were counted separately with each series of lines or points being scanned only once. Since all SMC profiles were arranged in a similar orientation, points and intersect counts were performed twice, once with the grid lines horizontal and a second time rotated through 90°. The average of these two counts, both for points and intersects were calculated for each micrograph.

Volume density of SMC (V_{smc}) was calculated using: $V_{smc} = P_i / P_T$, where P_i = total number of points falling on SMC, and P_T = total points falling on the entire media and is expressed as $\mu m^3/\mu m^3$ of the blood vessel media. Volume to surface ratio (V/S) was also calculated to determine the changes in SMC size during digestion and critical point drying. V/S ratio was calculated using the equation:

$$V/S = \frac{P_i \times z}{4 \times I_i}$$

where P_i = total number of points falling on SMC, I_i = total number of intersect points, z = length of one test line from the grid at a specific magnification. V/S is expressed as $\mu m^3/\mu m^2$, and is used for the determination of cell size, in that an increase in V/S indicates cell size increase, i.e. cell hypertrophy [6].

Cell layers were also determined on control vessel segments at the light microscope level. Longitudinal sections of the vessels were used with counts recorded at four locations along the length of each of the two longitudinal wall profiles. Medial wall thickness was also measured using longitudinal sections of Segments 1-3 in conjunction with light microscopy in order to determine the amount of medial wall shrinkage as a result of the digestive method and critical point drying.

Student's paired t test was used to make comparisons between the control vessel (Segment 1) and Segments 2 and 3 for volume densities and V/S ratios. Student's unpaired t test was used for comparisons between SHR and WKY in order to determine if there were a difference in SMC shrinkage at any stage of tissue

preparation.

Comparison of SMC Width between Large Mesenteric Arteries of SHR and WKY

SEM was used to measure the SMC width in the large mesenteric arteries of adult SHR and WKY. Large mesenteric arteries from 4 SHR rats (4 vessels) and 3 WKY rats (6 vessels) were sampled. These vessels were subjected to OsO_4/HCl digestion and processed for SEM as described for Segment 4 above. Medial SMC from the unbranched portion of the large mesenteric arteries were photographed at a magnification of 640X and at a working distance of 12 mm. Width of the SMC at the somal region was measured from a minimum of 20 cells/vessel. Correction for shrinkage due to the digestion method was also applied, based on the change in width of medial wall thickness at various stages of processing for SEM. Comparisons between SHR and WKY were performed using Student's unpaired t test. p values at 0.05 or less were considered to be statistically significant.

Results

Medial surface of large mesenteric arteries from SHR and WKY displayed similar morphology when viewed using SEM. Smooth muscle cells in the unbranching portion of large mesenteric arteries of SHR and WKY were arranged circumferentially around the vessel (Figure 1A). This was the only type of SMC orientation found in all the vessels we have examined. The SMC from SHR and WKY were spindle-shaped with thick somal bodies and tapering processes that often curve inwards to disappear in the underlying muscle layers (Figure 1B). Throughout the entire unbranched region of the vessel, the SMC from both SHR and WKY displayed a homogeneous population of spindle-shaped cells. It was only at bifurcation points that some deviation from the usual spindle-shape was evident. A number of SMC possessed lateral processes with some cells displaying a forked appearance.

Volume density of the SMC (V_{smc}), i.e. the percent volume of media in the large mesenteric arteries occupied by the SMC, and V/S ratios of the SMC were similar in the control SHR and WKY (Table 1). SMC from SHR and WKY underwent similar degree of size change after digestion of the connective tissues, and also after critical point drying, because no difference was observed between SHR and WKY when the values were expressed either as absolute number, or as percent change among different groups, even though there was a tendency for the volume density and V/S ratio of SMC from SHR to be larger than WKY (Table 1). Within WKY, V_{smc} was significantly less in the digested vessels as compared to the control vessels. However, after critical point drying, V_{smc} became similar to that of control. A significant difference in V_{smc} between control segments and digested segments was also found in SHR. In contrast to WKY, a significant difference in V_{smc} was found between digestion and critical point drying in SHR (Table 1).

V/S ratios after digestion and after critical point drying were significantly decreased from control in both SHR and WKY, suggesting cellular shrinkage. Most of the cellular shrinkage occurred during digestion (31%), with a further reduction in size which took place during critical point drying (16%, Table 1).

Medial wall thickness was significantly greater in the control vessels from SHR as compared to control WKY, but the difference disappeared after the digestion and after critical point drying (Table 2). The digestion method and critical point drying significantly decreased medial wall thickness in both SHR and WKY (Table 2). The actual amount of medial wall shrinkage from control to critical point drying was similar in the SHR and WKY (Table 2).

The width of the SMC at the somal region was similar between SHR and WKY based on SEM measurements (Table 2). However, when corrected for the shrinkage due to digestion and critical point drying (34.7% for SHR and 31.4% for WKY), SMC from SHR are actually wider than those from WKY (Table 2).

Discussion

For comparative morphological studies, one of the concerns is that the procedure involved in specimen preparation may have differential quantitative and qualitative effects on the tissues from different kinds of animals, thereby introducing artifacts. Depending on the types of fixatives, dehydrating and embedding agents, and on the types of tissues, different degrees of cellular shrinkage have been reported [5]. The results from this study suggest that the degree of shrinkage due to the digestion method to remove the connective tissues in the adventitial layer, and subsequent critical point drying, was similar between the vascular SMC from SHR and WKY. However, because of the drastic reduction in cell size, the sensitivity of the method to detect any change is lost unless the degree of shrinkage was included in the final analyses.

Lee et al. [7] suggested that in routine processing of SMC for SEM (i.e. without digestion step), the majority of SMC shrinkage occurred during critical point drying. Our present study showed that when vessels were subjected to digestion method, the medial layer underwent a large degree of shrinkage after digestion such that shrinkage due to critical point drying was smaller compared to the effects of digestion. In this study, total tissue shrinkage ranged from 31-35%, which is considerably less when compared with the average shrinkage of 46.5 ± 7.7% reported for the WKY intestinal venules [2]. Differences in the digestion techniques may be one of the reasons for such difference. One possible method to reduce the degree of tissue shrinkage, is through the use of Peldri II in place of critical point drying [17]. Peldri II method has been shown to prevent cracking of the specimens during the drying process,

Table 1. Comparison of Volume Density (V_{SMC}) and Volume to Surface (V/S) Ratios of Smooth Muscle Cells from Spontaneously Hypertensive Rats and Wistar-Kyoto Rats.

Parameter	Rat Strain	Actual Values			Percent Change Between Groups		
		Control	Digestion	CPD	Control vs. Digestion	Control vs. CPD	Digestion vs. CPD
Volume Density (V_{SMC})	SHR	0.72 ± 0.02	0.57 ± 0.05[a]	0.65 ± 0.04[c]	-20.91 ± 5.3	-10.44 ± 4.1	+13.86 ± 4.5
	WKY	0.67 ± 0.02	0.52 ± 0.03[a]	0.59 ± 0.03	-22.45 ± 2.9	-11.90 ± 6.4	+23.10 ± 4.3
	p	NS	NS	NS	NS	NS	NS
Volume to Surface Ratio (V/S)	SHR	0.68 ± 0.04	0.43 ± 0.01[a]	0.36 ± 0.03[b]	-31.59 ± 3.7	-43.05 ± 3.2	-15.93 ± 7.0
	WKY	0.59 ± 0.04	0.40 ± 0.02[a]	0.34 ± 0.03[b]	-31.39 ± 2.7	-41.97 ± 5.3	-15.65 ± 5.7
	p	NS	NS	NS	NS	NS	NS

Values are mean ± SEM. n = 4 each for WKY and SHR. CPD = Critical Point Drying. $p < 0.05$: [a]Control versus Digestion; [b]Control versus CPD; [c]Digestion versus CPD.

Table 2. Comparison of Vessel Wall Parameters between Spontaneously Hypertensive Rats and Wistar-Kyoto Rats at Various Stages of Tissue Processing for Scanning Electron Microscopy.

Parameters	**SHR**	**WKY**	**p**
Medial Thickness (μm)			
Control	22.6 ± 1.5	17.2 ± 1.5	< 0.05
Digestion	16.7 ± 1.9[a]	14.2 ± 0.9[a]	NS
CPD	15.0 ± 2.2[b]	11.8 ± 1.2[b]	NS
Percent Shrinkage after CPD as Compared with Control	34.7 ± 5.5	31.4 ± 4.0	NS
Number of Smooth Muscle Cell Layers	4.74 ± 0.08	3.55 ± 0.09	< 0.001
Smooth Muscle Cell Width (SEM Measurements)	3.87 ± 0.09	3.74 ± 0.05	NS
Smooth Muscle Cell Width (Corrected for Shrinkage)	5.94 ± 0.14	5.46 ± 0.08	< 0.05

Values are mean ± SEM. n = 4 each in SHR and WKY. CPD = Critical Point Drying.
$p < 0.05$: [a]Control versus Digestion; [b]Control versus CPD.

and gave comparable preservation of tissue structure [17]. It will be useful to evaluate whether this method will reduce some of the tissue shrinkage due to processing for SEM.

In this study, we have used SMC volume density (V_{smc}) to examine whether smooth muscle and the interstitial space shrank proportionately during tissue processing. The premise is that since it is a ratio between SMC volume and total medial wall volume, an increase in V_{smc} would indicate a decrease in interstitial space volume. V_{smc} was decreased after digestion for both SHR and WKY, suggesting that there was a larger decrease in SMC volume than interstitial space. After critical point drying, V_{smc} was increased suggesting interstitial space volume shrinkage was greater than smooth muscle at this juncture. Alteration in V/S ratio of the smooth muscle cells, on the other hand, is a useful indicator of cell size [6]. A significant decrease in V/S ratios of SMC after digestion suggests that digestion was responsible for the majority of SMC shrinkage, thus confirming our results on the medial thickness change.

At 28 weeks, large mesenteric arteries from SHR

possessed a greater number of SMC layers, suggesting SMC hyperplasia [8]. A slightly larger SMC width was found in SHR than WKY when compensated for shrinkage, which suggests cellular hypertrophy. These results were consistent with previous findings that SMC hyperplasia and hypertrophy are responsible for medial enlargement in the large mesenteric arteries of adult SHR [8]. However, the increase in the somal width of SMC from SHR was not noted unless SMC shrinkage due to processing was compensated in the calculation, therefore highlighting the importance of taking into account the effect of tissue and cellular shrinkage for comparative study of vascular changes in hypertension when SEM is used. In hypertension, an increase in vascular muscle mass due to hyperplasia is generally considered to be an important contributing factor in the development of hypertension, whereas hypertrophy of the SMC is usually an adaptive response [11], so that the differentiation of these two types of changes is important for our understanding of the disease process.

Previous morphometric studies of vascular changes in hypertension used cross-sectional profiles of the vessels to measure alterations in cell size and numbers (e.g. Lee et al.[6,8,10,15]). Because of the circumferential arrangement of the SMC in these arteries, longitudinal profiles of the SMC were presented in this type of preparation. One possible source of error, is that this type of measurement did not take into consideration the change in width of the SMC, because only the height and length of the SMC were involved in such measurement. It is conceivable that hypertrophy of SMC in hypertension may involve preferential widening of the SMC. Results from our present study indicate that this was not happening in the large mesenteric arteries from SHR, because there was only a slight increase in cell width as determined by SEM method, which seems to correspond to hypertrophy of the SMC as determined by the measurements from the cross-sectional profiles.

In conclusion, we have shown that SMC from the large mesenteric arteries of SHR and WKY underwent a significant reduction in cell size when these tissues were prepared for SEM. Even though the degree of shrinkage was similar between SHR and WKY at various steps of tissue processing for SEM, cumulatively, the ability of SEM measurements to detect SMC size change was lost unless cellular shrinkage was taken into consideration.

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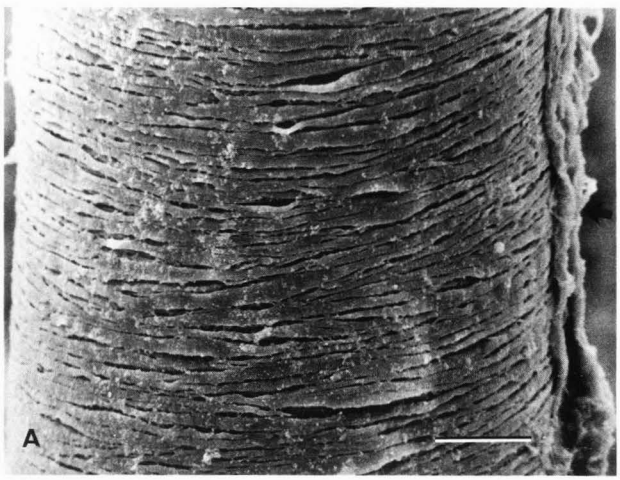

Figure 1A. Scanning electron micrograph of a large mesenteric artery from a spontaneously hypertensive rat, showing the circular arrangement of the smooth muscle cells in the medial layer. Note the presence of adventitia on one side of the artery (arrow).
Magnification bar = 30 μm.

Figure 1B. Larger magnification of 1A, showing spindle-shaped profile of the smooth muscle cells. Arrows point to the somal region of the smooth muscle cells with two tapering ends (arrowheads). Magnification bar = 15 μm.

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Discussion with the Reviewers

B.G. Miller: The control vessel segments were "dehydrated with ... ethanol and embedded in Spurr's resin." Was there also a 5 minute immersion in propylene oxide? If not, why would it be used in the other segments?

R.L. MacDonald: The control group arteries were not immersed in propylene oxide. What effect does this have on cell size?

Authors: We do not know the effect of immersion in propylene oxide on cell size. The control segments were not immersed in propylene oxide, because we wanted to follow the previous method where we have established that processing for transmission electron microscopy using that particular method did not cause significant cell size alteration [9]. In retrospect, it would have been meaningful to include another control group where the specimens were subjected to propylene oxide treatment during processing for transmission electron microscopy, in order to assess its effect on cell volume.

A.P. Evan: Would the authors recommend that each investigator perform a preliminary study to determine tissue shrinkage if they are working with a different tissue or digestion protocol than used in this study?

Authors: For quantitative morphological studies, it is essential that preliminary study to determine tissue shrinkage is carried out, even when a previously described digestive method is used, because variation in other aspects of tissue processing, such as differences in fixation procedure, and the type of dehydrant used, can significantly affect tissue or cell volume [5]. For example, the inclusion of OsO_4 and uranyl acetate treatments after glutaraldehyde treatment, has been shown to provide a better preservation of cell volume than fixation in glutaraldehyde alone [7].

L.J. McGuffee: It would have been very interesting had the authors compared the effects of critical point drying with that of Peldri II. We have also observed significant shrinkage during critical point drying of digested specimens for SEM, but found that Peldri II prevented cracking of the specimens during the drying process, and gave good preservation of the tissue [17]. Since Peldri II treated specimens may not shrink significantly, the correction factor may not be needed if this method of drying tissue for SEM is used.

Authors: It is possible that Peldri II may cause less tissue shrinkage than critical point drying, but this still needs to be established with quantitative studies. Even with critical point drying, cracking of the specimens can be minimized or prevented when the drying process is carried out at a gentle pace. Nevertheless, in future studies, it would be useful to compare the degree of tissue shrinkage between critical point drying and Peldri II, as suggested by Dr. McGuffee.

R.L. MacDonald: What measures were taken to ensure that sections were cut parallel to the long axis of the vessel?

A.P. Evan: How did the authors assure themselves that a particular ultrathin section did in deed represent a true longitudinal cut through a mesenteric vessel? An oblique cut would skew the measurements to a higher value than reality. How did the authors determine that a particular cut was at the centre of the vessel? Again, if the cut was on either side of center the measured values would be higher than the true values.

Authors: In longitudinal sections of the arteries, the thinnest section in the series usually represents the true longitudinal section of the vessel. This criterion was used in our study.