Topographic Distribution of Contractile Protein in the Human Macular Microvasculature

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PURPOSE. We studied the topographic distribution of contractile protein in different orders of the human macular microvasculature to further understanding of the sites for capillary blood flow regulation.

METHODS. Nine donor eyes from eight donors were cannulated at the central retinal artery and perfusion labeled for alpha smooth muscle actin (αSMA) and filamentous actin (F-actin). Confocal images were collected from the macula region, viewed, projected, and converted to a 255 grayscale for measurements. The mean intensity was measured for macular arterioles, venules, and capillary segments. The diameter of each vessel segment measured was recorded. The normalized mean intensity values from all images were ranked according to vessel types and size with a total of nine categories.

RESULTS. F-actin was present throughout the macular microvasculature whereas αSMA labeling showed variations. Overall, αSMA has a more prominent presence in the macular arterioles than in the macular capillaries and venules, and αSMA strongly labeled the smaller macular arterioles. Some capillaries also labeled positive for αSMA, including some of the capillaries in the innermost capillary ring surrounding the foveola. It was weakly present in the capillaries on the venous side and larger venules. In the larger macular arterioles closer to 100 μm in diameter, αSMA labeling was weakly present and not as ubiquitous as in the smaller arterioles.

CONCLUSIONS. Nonuniform distribution of contractile proteins in the different types, orders, and sizes of macular microvasculature indicates that these vessels may have different contractile capability and roles in macular flow regulation.

Keywords: blood flow, retinal vasculature, human
TABLE 1. Demographics for All Donor Eyes Included for This Study

<table>
<thead>
<tr>
<th>Donor Eye</th>
<th>Age and Sex</th>
<th>Cause of Death</th>
<th>Postmortem Time, h</th>
</tr>
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<tbody>
<tr>
<td>A</td>
<td>27 F</td>
<td>Bowel cancer</td>
<td>12</td>
</tr>
<tr>
<td>B</td>
<td>52 F</td>
<td>Subarachnoid hemorrhage</td>
<td>6.5</td>
</tr>
<tr>
<td>C</td>
<td>55 M</td>
<td>Hemothysis, cardiac arrest</td>
<td>20</td>
</tr>
<tr>
<td>D</td>
<td>60 F</td>
<td>Intracranial hemorrhage</td>
<td>6</td>
</tr>
<tr>
<td>E</td>
<td>60 F</td>
<td>Intracranial hemorrhage</td>
<td>6</td>
</tr>
<tr>
<td>F</td>
<td>62 M</td>
<td>Mesothelioma</td>
<td>24</td>
</tr>
<tr>
<td>G</td>
<td>64 F</td>
<td>Pneumonia, cardiac arrest</td>
<td>12</td>
</tr>
<tr>
<td>H</td>
<td>79 F</td>
<td>Motor neuron disease</td>
<td>12</td>
</tr>
<tr>
<td>I</td>
<td>90 M</td>
<td>Upper GI bleed</td>
<td>12</td>
</tr>
</tbody>
</table>

**Materials and Methods**

This study was approved by the Human Research Ethics Committee at the University of Western Australia. All human tissue was handled according to the tenets of the Declaration of Helsinki.

**Human Donor Eyes**

Nine donor eyes from 8 donors were used for this study. All eyes were obtained from the Lions Eye Bank of Western Australia following valid consent for use of such tissue for research purposes. Six eyes were received after the removal of corneal buttons for transplantation, and three eyes were received intact. None of the donors had a known medical history of ocular disease. The demographic data, cause of death, and the postmortem time to eye perfusion are as listed in Table 1.

**Microcannulation and Perfusion Labeling**

All nine eyes were cannulated at the central retinal artery, and perfusion was labeled as previously described using microcannulation and intravascular perfusion technique.5,6

In brief, solutions were perfused through the retinal microvasculature in the following order: Ringer’s solution with 1% BSA (20 minutes), 0.1 M phosphate buffer wash (10 minutes), 10% goat serum/primary antibody (1:50 to 1:100; mouse anti-αSMA [no. A2547; Sigma-Aldrich Corp., St. Louis, MO, USA]) / 0.1% Triton X-100 (1 hour), 0.1 M phosphate buffer wash (10 minutes), 4% paraformaldehyde in 0.1 M phosphate buffer (20 minutes), 0.1 M phosphate buffer (15 minutes), secondary antibody (1:200; goat anti-mouse conjugated to Alexa Fluor 488 or 555 [A11001 and A11003, respectively; Invitrogen, City, Thermo Fisher Scientific, Waltham, MA, USA]) / Hoechst nuclei counter stain (B2261; Sigma-Aldrich Corp.) or YO-PRO-1 (Y3603; Thermo Fisher Scientific) along with phalloidin or lectin conjugated with tetramethylrhodamine or FITC (P1951 or P5282; Sigma-Aldrich) for 1 hour, followed by a final buffer wash for 30 minutes. The perfusion-labeled globes were immersed in 4% paraformaldehyde overnight for further fixation before the retinas were dissected out for flat mounting and imaging.

**Confocal Imaging**

Confocal images were collected from the macula region using ×4 (NA 0.20), ×10 (NA 0.45), and ×20 (NA 0.75) objective lenses at 1024 × 1024 pixel resolution (Plan Apo, Nikon C1 System; Nikon Corp., Tokyo, Japan). Confocal image stacks were collected using a set level of laser intensity and gain level. Images were viewed and projected using image analysis software (v.7.5.2, Imaris Software, Bitplane, Inc.; Zurich, Switzerland).

**Vessel Categories**

The arterioles are categorized by size and orders according to the modified Horton-Strahler method as described previously.5 Macular arterioles are grouped into five categories as vessels between 60 and 100 μm (a5), 50 to 60 μm (a4), 20 to 50 μm (a3), 15 to 20 μm (a2), and those that are <15 μm in diameter (a1). Macular venules are grouped into three categories, with capillaries draining directly into venule as v1 (Table 2), those <40 μm as v2, and vessels between 40 and 60 μm as v3. All other capillaries were grouped into one category and include foveal capillaries.

**Quantification**

Confocal images of αSMA labeling of each region were projected and converted to 255 grayscale for measurements. The mean intensity was measured for macular arterioles, venules, and capillaries using (Image-Pro Plus v.7.0, Media Cybernetics, Inc., Silver Spring, MD, USA), whereby the outline of the vessel segment to be measured was traced and the mean intensity value obtained using the histogram tool. The diameter of each vessel segment was also measured and recorded. The intensity measurements were normalized to the highest intensity-labeled small arteriole (a3) within each image as 100% and all other measurements from the same image expressed in proportion to it. This is for ease of comparison across images and between different samples. The normalized mean intensity values from all images were ranked according to vessel types and size with a total of nine categories as detailed in Table 2.

**Table 2. Vessel Diameter and Normalized Intensity Measurements From αSMA Label in Each Category**

<table>
<thead>
<tr>
<th>Category</th>
<th>Vessel Diameter</th>
<th>Normalized Intensity</th>
<th>CoV in Intensity</th>
</tr>
</thead>
<tbody>
<tr>
<td>a5</td>
<td>78.69 ± 4.61 (17)</td>
<td>40.70% ± 7.08 (17)</td>
<td>0.39 ± 0.07 (17)</td>
</tr>
<tr>
<td>a4</td>
<td>54.86 ± 1.02 (12)</td>
<td>74.88% ± 9.17 (12)</td>
<td>0.27 ± 0.05 (12)</td>
</tr>
<tr>
<td>a3</td>
<td>29.46 ± 0.55 (183)</td>
<td>74.74% ± 2.08 (183)</td>
<td>0.28 ± 0.01 (183)</td>
</tr>
<tr>
<td>a2</td>
<td>17.28 ± 0.15 (101)</td>
<td>79.87% ± 4.28 (101)</td>
<td>0.28 ± 0.03 (101)</td>
</tr>
<tr>
<td>a1</td>
<td>12.48 ± 0.17 (99)</td>
<td>64.14% ± 5.38 (99)</td>
<td>0.32 ± 0.02 (99)</td>
</tr>
<tr>
<td>c</td>
<td>8.90 ± 0.10 (459)</td>
<td>64.39% ± 3.06 (459)</td>
<td>0.33 ± 0.02 (459)</td>
</tr>
<tr>
<td>v1</td>
<td>10.61 ± 0.89 (9)</td>
<td>59.13% ± 16.40 (9)</td>
<td>0.32 ± 0.03 (9)</td>
</tr>
<tr>
<td>v2</td>
<td>21.02 ± 1.61 (23)</td>
<td>50.89% ± 8.59 (23)</td>
<td>0.28 ± 0.04 (23)</td>
</tr>
<tr>
<td>v3</td>
<td>53.94 ± 4.05 (8)</td>
<td>26.00% ± 6.33 (8)</td>
<td>0.31 ± 0.10 (8)</td>
</tr>
</tbody>
</table>

a, macular arterioles; c, capillaries; v, macular venules; n, the number of vessels measured; SE, standard error; CoV, coefficient of variation.
above. The mean and standard errors for diameter and normalized intensities were calculated for measurements within each category. The coefficient of variation was calculated for each vessel as an indication of intensity variation within each vessel segment measured.

**Statistical Analysis**

Data were analyzed using the R language and environment for statistical computing (https://www.R-project.org/; provided free of charge from the R Foundation for Statistical Computing, Vienna, Austria). Statistical calculations were performed using linear mixed models using eye number (identity) as the random effect to account for intraeye correlation. The labeling intensity of each vessel type was compared to v3. Eight such comparisons were performed. We used a conservative Bonferroni correction to the P value to adjust for making multiple corrections, where corrected $P = 0.05/8 = 0.0063$.

**RESULTS**

Perfusion labeling of the macular microvasculature for αSMA and F-actin has been achieved in human donor eyes. The F-actin label may be seen in the cell border of endothelial cells and most strongly in the cytoplasm of vascular smooth muscle cells (vSMCs) circumferential to the major retinal arteries, whereas αSMA may be observed most strongly in the a2 to a4 vessels in the cytoplasm of vSMCs.

**αSMA Distribution Qualitative**

Overall, αSMA has a much stronger presence in the macular arterioles than in the macular venules. αSMA strongly labeled the vSMCs of the macular small arterioles (Figs. 1, 2). Capillaries also labeled positive for αSMA (Figs. 1–5) although not all capillaries are labeled, especially those draining into the venules (Figs. 2, 5). αSMA is weakly present in the larger venules (Figs. 1, 2, 4) and absent from many capillaries that drain toward the venule (Fig. 5). Only some of the capillaries in the innermost ring surrounding the fovea showed positive labeling for αSMA, but all were labeled for F-actin (Fig. 5).

In the larger macular arterioles closer to 100 μm in diameter, αSMA labeling was weakly present (Figs. 1, 4) and not as ubiquitous as in the smaller arterioles.

**αSMA Distribution Quantified**

αSMA-labeling intensity was obtained from confocal images of eight donor eyes (eight donors). One donor eye was excluded from αSMA quantification because αSMA in donor eye A was unusual, abundantly positive in labeling the macular retinal ganglion cells and obscuring the signals from the vasculature.

Measurement of αSMA labeling intensity confirmed the qualitative observation above. The mean normalized intensity is as listed in Table 2 and plotted against each vessel category as shown in Figure 6. The vessel categories are arranged in the order of large 60- to 100-μm retinal arterioles (a5) to small arterioles less than 15 μm in diameter (a1). This is followed by capillaries, venous capillaries ~8 μm (v1), venules <40 μm (v2), and retinal venule 40 to 60 μm (v3). The category of a3 vessels encompassed a broad range of vessel diameter, from 20 to 50 μm, due to the numerous a2 vessels branching off from a3 vessels as the arteriole extend from the parafoveal through to the foveal region.

Considering the donor eye as a random factor that could have an effect on the results, a significant difference was identified between the intensity measurements between v3 vessels and those of a4, a3, a2, a1, and c ($P < 0.0063$). Vessels a1, v1, and v2 intensity measurements were not significantly different from that of v3 ($P > 0.05$). The intensity measure-
The coefficient of variation (CoV) for all vessel categories range from 0.274 to 0.390, indicating a 27.4% to 39% spread of intensity measurements within each vessel category. The mean CoV and standard errors for each vessel category are listed in Table 2.

**F-Actin**

Six of the donor eyes were also labeled for filamentous actin using phalloidin. F-actin was labeled through all vessel levels in the macular microvasculature as previously reported. A greater labeling intensity was noted in the arterioles due to the prominent presence of smooth muscle cell layers (vSMCs). Some gaps are present in the vSMC distribution along the length of the arteriole segments, likely contributing to the inconsistencies and variation in labeling intensity. v1, v2, and v3 are scarcely labeled for αSMA. The lack of positive αSMA labeling in v3 is partly due to a loss of vSMC as reflected by the concurrent lack of Factin labeling in circumferential arrangement around the larger venules. αSMA labeling in capillaries is variable. Scale bars: A = 50 μm, B = 20 μm, C = 20 μm, D = 10 μm.

**DISCUSSION**

In this study, we studied αSMA distribution within the macular microvasculature of human donor eyes using microperfusion and labeling techniques. This microperfusion labeling technique had been carefully evaluated and validated as published in our first paper on this topic in 2010. Subsequent publications using data obtained from phalloidin and various antibody labeling of the retinal microvasculature demonstrated successful labeling of the endothelium and vSMCs in all orders of the retinal arterioles, capillaries, and venules, in both the macular and peripheral retinal microvasculature. Images of the intact retinal microvasculature labeled using this microperfusion labeling technique also served as the gold standard against which images from recent advances in label-free imaging technique such as optical coherence tomography angiography may be compared for validation.

Our major findings are as follows: (1) There is uneven topographic distribution of αSMA contractile proteins in the arterioles and capillaries.
**Figure 3.** Higher-magnification confocal projected images of the nasal region of the macula showing F-actin and αSMA labeling of different orders of arterioles. (A) Dual labeling for F-actin and αSMA are shown and the macular arterioles labeled according to vessel categories of a1 to a4. Any combination of a1, a2, and a3 vessels could branch off from larger a4 arterioles. Similarly, a1 and a2 arterioles could branch off a3 arterioles. The vessel widths of the same order of vessels are seen to reduce as they approached the foveola. (B) F-actin labeling of the macular arteriole shows fairly strong and even labeling of smooth muscle cells along the length of the a4 arteriole, which tapers from a diameter of 68 to 50 μm within this field of view. Relatively strong F-actin labeling may be seen along the a3 arterioles, which range in diameter from 27 to 44 μm for this field and also taper as they approach the foveola. (C) αSMA labeling is present strongly in the arterioles as well as in some capillaries. Variation in labeling intensity is present along different segments of the same order of arteriole as indicated by the pairs of arrowheads along the a3 and a4 vessels in this image. Weaker intensity of αSMA labeling is particularly evident in the wider portion of the a4 arteriole in the upper right corner of the image. Scale bar: 300 μm.

**Figure 4.** Higher-magnification projected confocal images of the superior region of the macula showing F-actin and αSMA labeling of different orders of venules. (A) Dual labeling for F-actin and αSMA are shown and the macular venule labeled according to vessel categories of v1 to v3. A larger macular arteriole (a5) may be seen running across the two venules converging. An obvious weaker labeling may be seen for both F-actin and αSMA in the venules when compared to the arterioles. (B) F-actin labeling of the macular venule shows relatively weak and even labeling of venule segment that broadened from 47 to 64 μm within this field of view. Even weaker F-actin labeling may be seen in v1 and v2 venules, with diameters ranging from 13 to 30 μm for this field. (C) αSMA labeling is present in a patchy manner in the venule and is relatively weak compared to that in the larger macular arteriole (79 μm diameter). Variation in labeling intensity is present along the entire length of this v3 venule: αSMA labeling intensity on the venule appears to be higher in the vicinity of the arteriole-venule crossing points (yellow triangles). Scale bar: 300 μm.
One of the most fundamental principles of blood circulation is the ability of each tissue to autoregulate its local blood flow through the thicker muscular layers of these large arterioles.

We also found that αSMA labeling in venules was weaker than that in capillaries, but was not absent. Some weak contractile capability may be suggested. In fact, we recently demonstrated that potent contractile agents such as ET-1 can induce porcine retinal vein contraction in vitro using an isolated perfused vein preparation, with similar results also reported by another group.

Our data also demonstrated relatively weaker αSMA labeling in the capillaries and venules when compared with small arterioles. Only partial capillaries were positively labeled in our study. It may indicate that there is some regulatory capability in the capillaries and venules. This finding agrees with a recent report of rat retinal vasculature in which αSMA was high in arterioles less than 60 μm in diameter and even arterioles with smaller caliber could still play a significant role in retinal capillary perfusion, not only by changing the flow resistance, but also by altering the Fahraeus-Lindqvist effect (a phenomenon describing the change in viscosity of blood with the decrease in diameter at the level of microcirculation).

It has been reported that the oxygen tension in the tissues could affect the degree of opening and closing of these smallest arterioles. Oxygen distribution in the retina is known to be significantly heterogeneous in the different retinal layers and regions such as the macula and peripheral retina. Weaker αSMA labeling is variably present in foveal capillaries as indicated by the open arrowheads. Venous capillaries tend to label weakly for αSMA (blue arrows). Scale bar: 150 μm.

Figure 5. High-magnification projected image of foveal segment vasculature showing detail labeling of αSMA and F-actin at the capillary level. (A) Dual labeling of F-actin and αSMA in this region shows variable level of αSMA at the capillary level. Two terminal ends of the macula arterioles (a), a small macula venule (v1), and numerous interconnecting capillaries (c) may be seen in this field. Part of the foveola (*) is visible in this field. Two green triangles point to position of the foveal capillary ring. (B) F-actin labeling shows the intact and connected microvasculature. Stronger labeling was observed in the arteriole compared to the venule. Generally, F-actin labeling in capillaries is relatively even along each capillary segment but appears to be weaker on the venous side as well as at the foveal capillaries. Solid white arrows point to capillaries of arteriole origin and open arrows point to capillaries draining toward the venous side. (C) αSMA labeling is strongly present in the arteriole as well as capillaries from arteriole origin (red solid arrows). αSMA is variably present in foveal capillaries as indicated by the open arrowheads. Venous capillaries tend to label weakly for αSMA (blue arrows). Scale bar: 150 μm.
the macular venules. \( v_3 \) has significantly lower intensity measurements per vessel category compared against \( v_3 \) vessel category is as marked with an asterisk. \( v_3 \) has significantly lower intensity of zSMA labeling when compared against \( a_4 \) to \( c \) categories. Vessel categories: \( a_5 \) = macular arterioles 60 to 100 \( \mu \)m; \( a_4 \) = macular arterioles 50 to 60 \( \mu \)m; \( a_3 \) = macular arterioles 20 to 50 \( \mu \)m; \( a_2 \) = macular arterioles 15 to 20 \( \mu \)m; \( a_1 \) = macular arterioles <15 \( \mu \)m; \( c \) = capillaries, \( v_1 \) = capillaries draining into venous side; \( v_2 \) = macular venules < 40 \( \mu \)m; \( v_3 \) = macular venules 40 to 60 \( \mu \)m; * denotes \( P < 0.0063 \).

This equation gives the flow \( (Q) \) through a cylindrical tube as a function of the driving pressure difference along the tube \( (\Delta P) \), the tube radius \( (r) \) and length \( (l) \). The dynamic viscosity \( (\eta) \) is a material property of the fluid, which describes its internal resistance to shearing motions, in which different parts of the fluid move with different velocities.

### References


10. Pittman RN. Oxygen supply to contracting skeletal muscle at points in a vessel. *Poiseuille investigated the passage of simple fluids through long, narrow-bore tubes. His main findings can be represented by the equation, known as Poiseuille’s law.*

\[
Q = \frac{\pi \Delta P \ r^4}{8 \eta l}
\]


