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 macrovascular 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>
</thead>
<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, secondary to aneurysm</td>
<td>6.5</td>
</tr>
<tr>
<td>C</td>
<td>55 M</td>
<td>Hemoptyis, 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>urgo8</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.3,6

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, Wal- tham, 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>Mean, μm ± SE (n)</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>
<td></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>
<td></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>
<td></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>
<td></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>
<td></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>
<td></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>
<td></td>
</tr>
<tr>
<td>v2</td>
<td>21.02 ± 1.61 (25)</td>
<td>50.89% ± 8.59 (25)</td>
<td>0.28 ± 0.04 (25)</td>
<td></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>
<td></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 venule side (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 foveola 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 parafovea 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-
ments between a4, a3, a2 categories were comparable ($P > 0.7$).

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...
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 a5 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. Weak 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 v5 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.
macular microvasculature, with stronger expression in the arteriole and weaker in capillary and venules; (2) αSMA-labeling intensity was not always proportional to the diameter of the artery or arterioles, with the most prominent labeling found in macular arterioles less than 60 μm in diameter and rather weakly present in arterioles larger than 60 μm in diameter; (3) Capillary αSMA labeling is also uneven and partial; and (4) The level of αSMA labeling in the arterioles was not proportional to the amount of vSMCs or endothelium present, as evident from the lack of correlation between arteriole diameter and intensity measurements.

αSMA is probably the single most abundant protein in adult vSMCs. It is thought to have a central role in regulating vascular contractility and blood pressure homeostasis. The location and presence of contractile proteins within the macular microvasculature is suggestive of its capability to regulate blood supply. From the data presented, it has been demonstrated that the most intensely labeled area is in macular small arterioles, suggestive of local regulatory sites for the regional and localized macular regions. This finding agrees with a recent report of rat retinal vasculature in which αSMA was high in arterioles and low in capillaries. It is interesting that our results show that the most intense labeling was in arterioles 20 to 30 μm in diameter, but even arterioles with smaller caliber have a very high level of labeling for αSMA.

With respect to the distribution of blood pressure and flow in the circulation, an important consequence is the relation between flow resistance and vessel radius. The rate of blood flow is directly proportional to the fourth power of the radius of the vessels. Although Poiseuille’s law (Appendix) does not apply to such small vessels, a diameter change in arterioles less than 20 μm 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 in large arterioles more than 60 μm in diameter may indicate a lesser regulatory capability via contraction. However, we cannot rule out that this weaker αSMA labeling is affected by aging, postmortem change, and other age-related pathologic factors in the human donor eyes. Another possible contributor could be insufficient penetration of the antibody through the thicker muscular layers of these large arterioles.

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 macular capillaries.

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 EF-1 can induce porcine retinal vein contraction in vitro using an isolated perfused vein preparation, with similar results also reported by another group.

One of the most fundamental principles of blood circulation is the ability of each tissue to autoregulate its local blood flow.
in proportion to its metabolic needs. The blood flow supplying each tissue is likely most optimally regulated at the level that meets the tissue’s requirements. An unusual feature of the retinal circulation is the lack of autonomic innervation, so more reliance must be placed on local vascular control mechanisms. Local regulation is the most efficient way to provide all nutrients to meet the demands of the tissues and cells to achieve neurovascular coupling. It is known that the oxygen tension in monkey fovea and some layers of the rat retina is very low, close to critical oxygen tension (5 mm Hg) in physiological condition. The regulatory control of blood flow in the eye is therefore a vital component in the maintenance of retinal homeostasis. In addition, local blood flow control can be divided into acute control and long-term control. For the macula, it is clear that controlling points closer to the capillary network, that is, smaller arterioles, are better placed to microadjust the capillary perfusion to best match metabolic demands of local tissue and cells rather than the larger vessels. Therefore, it is understandable that small arterioles could play a major regulatory role in the macular microvasculature. The topographic distribution of SMα labeling may indicate that different type, order, and size of macular microvessels may have different roles in the orchestrated control of local blood flow regulation.

The control mechanisms in the macular microvasculature are important for our understanding of macular physiology in normal and disease states, and it could be complicated. The results from this current study on the topographic distribution of SMα confirmed that major local flow control points are likely to be arterioles less than 30 μm in diameter and that further study of other contractile proteins such as calponin, myosin, and MLCK could add to our understanding of the local control mechanisms. Understanding the relationship between the expression of such contractile proteins and their function in the control of local perfusion of retinal microcompartments in normal and disease states could be the key to developing early diagnosis modality and intervention strategies.

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**APPENDIX**

Resistance is the impediment to blood flow in a vessel, which cannot be measured directly. But it can be calculated by measured blood flow and pressure difference between two 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:

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

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**


