A new method designed to allow repeated mapping of retinal hemodynamics on a macro- and microcirculatory level was evaluated in the primate eye. The method, called “targeted dye delivery,” consists of encapsulating a fluorescent dye in temperature-sensitive liposomes, injecting the liposomes systemically, and using a light pulse from an argon laser to release a bolus of dye in a targeted retinal vessel. The follow-up of the well-defined dye front thus generated allows calculation of the blood flow and capillary transit time. Evaluation of targeted dye delivery in a monkey indicated that centerline blood velocity and the vessel diameter can be measured with a reproducibility of 10% and 4%, respectively, in vessels that are 40 μm and larger. These measurements yielded flow values that had a reproducibility of 10% on the same day and 13% on different days. The normalization of flow rate by the vessel diameter was consistent with theoretic estimates and promises to be a circulation indicator independent of variations between individual and species. The transit time across capillary beds at different locations was found to be similar, thus indicating that the method could be used to evaluate the local viability of the microcirculation. Invest Ophthalmol Vis Sci 31:2300–2306, 1990

Since many diseases of the retina result in changes in retinal circulation, information on hemodynamics can be important in the diagnosis of these diseases and the evaluation of current and new therapies. We are developing a method to map repeatedly the retinal hemodynamics on a macro- and microcirculatory level. The method, called “targeted dye delivery,” consists of encapsulating a fluorescent dye in temperature-sensitive liposomes, injecting the liposomes systemically, and releasing a bolus of dye in a targeted retinal vessel by a light pulse from an argon laser.1-3 We report here on the use of the method to assess retinal hemodynamics of the primate eye.

**Materials and Methods**

Temperature-sensitive liposomes were prepared by reverse-phase evaporation in a method that has been described previously.3-5 Dipalmitoylphosphatidylcholine and dipalmitoylphosphatidylglycerol were obtained from Avanti Polar Lipids (Pelham, AL) and used without further purification. 6-Carboxylfluorescein (Molecular Probes, Junction City, OR) was purified on a hydrophobic column and diluted to approximately 100 mM as described earlier.

3 The mean diameter of the liposomes was 0.2 μm as measured by light scattering (model 270 submicron particle sizer; Nicomp, Goleta, CA). No attempt was made to control the size distribution.

The laser delivery system (Fig. 1) consisted of a modified fundus camera (Zeiss, West Germany) to which laser delivery optics were added. A commercial ophthalmic argon laser (Coherent, Palo Alto, CA) was used to deliver a beam through a fiberoptic cable. The fiber output was magnified and projected on a plane conjugated to the retina. The beam could be moved on this plane, and consequently on the fundus, by displacing the lens with the aid of an XY translating table. The animal’s cardiac cycle was monitored using a vital signs monitor (In Vivo, Broken Arrow, OK). The operation of the system was synchronized by a microcomputer equipped with an input/output board (Data Translation, Marlboro, MA) and controlled by a data acquisition software (ASYST, Rochester, NY). Upon activation of the fundus camera switch, the computer detected the first electrocardiographic peak and activated the laser. After a preset delay, the shutter of a 35-mm camera back was activated, the flash was fired, and an angiogram was recorded on black-and-white film (T-Max 3200 ASA; Kodak, Rochester, NY). An example of the angiograms is shown in Figure 2.
SWITCH

Fig. 1. Block diagram of the laser delivery system and the fundus camera. The laser was introduced at a plane conjugate to the retina.

The experiments were conducted on one rhesus monkey in compliance with the ARVO Resolution on the Use of Animals in Research. The monkey’s eyes were dilated with 10% phenylephrine and 2% homatropine, and the animal was sedated with 2.5% sodium thiamylal given intravenously. A contact lens with a radius of curvature of 5.6 mm was placed on the cornea to assure corneal clarity throughout the experiment. A 1.5 ml/kg dose of liposome suspension was given intravenously corresponding to a carboxyfluorescein dose of 14 mg/kg. The resulting blood liposome concentration was approximately 10^{12} liposomes/ml. The laser spot was positioned on an artery near the optic disc. The beam had a power of 100–150 mW, a 0.8-mm diameter, and a pulse length of 200 μsec. The size of the beam and its duration were chosen to allow enough time for the exposure of the flowing column of blood to be exposed to the laser and thereby to be heated to 41°C, the lysis temperature of the liposomes. Approximately 90% of the liposomes reaching 41°C (or about 10^9 liposomes) should lyse.\(^2,5\) Blood-vessel diameters were measured from negatives of dye-filled vessels using a dedicated microdensitometer (Fig. 3). It consisted of a 10-μm slit positioned at the focal plane of a microscope (Olympus, Japan) in front of which the negatives were placed. The negatives were rotated on the microscope stage to align the image of the blood vessel parallel to the slit. A computer controlled (via the input/output board) the motorized stage, which moved the image perpendicular to the slit. The light passing through the slit was detected by a photodiode, digitized by the board, and stored in the computer. The output was thus proportional to the transmittance of the film (Fig. 4A). A software program was developed to differentiate the curve (Fig. 4B) and measure the time delay between the maximum and minimum derivatives. The delay was converted from milliseconds into units of distance using an experimentally derived calibration parameter. Negatives selected for diameter determination fulfilled the following criteria: (1) the position to be measured was sufficiently remote from the site of laser delivery and from the dye front to ensure uniform dye filling of the vessel; (2) the position to be measured was remote from areas of capillary filling to avoid the presence of an elevated background capable of interfering with the analysis; and (3) the measured vessel was 15 μm in diameter or larger.

The dye front displacement was measured by projecting the negative on a digitizing pad (Summagraphics, Seymour, CT), aligning the projected image with a master image of the retinal vasculature, and marking the endpoint of the dye front with a cursor. A graphics software program was used to measure the length of the segment. These lengths were converted into distances on the retina with a calibration factor.
obtained from photographs of millimetric targets placed inside an optically correct model of a human eye (Eyetch, Skokie, IL). We also corrected for the difference between the axial length of the model eye and the monkey eye. 6

The blood velocity was calculated as the difference in dye front displacement between two angiograms obtained with a typical delay difference of 50 msec. Blood velocity was computed from consecutive angiograms in which the dye front remained in the same vessel branch. The value thus computed represented the centerline blood velocity in the selected segment at a particular time in the cardiac cycle.

To obtain a mean centerline blood velocity \( V_{center} \) independent of the cardiac cycle we averaged four to five measurements, taken at different intervals in the cardiac cycle.

The mean blood flow (Q) was calculated using the following formula:

\[
Q = \frac{1}{2} V_{center} \pi D^2/4
\]

where D is the vessel diameter. This formula is based on the assumption that the flow conformed to that of a Newtonian fluid and that the vessel cross-section was circular, implying that the mean velocity across the vessel was equal to \( V_{center}/2 \).

The capillary transit time, namely the time required for blood to cross a given capillary bed, was estimated from the time the dye first appeared in the precapillary arteriole to the time it reached the postcapillary venules. Figures 5A and 5B are examples of angiograms used to identify the precapillary arteriole-filling and the postcapillary venule-filling phases, respectively. Angiograms used to determine capillary transit time were taken successively with 50 msec increments in delay.

---

Fig. 4. Densitometry scan across an artery. (A) Transmittance across the negative of a vessel filled with dye. (B) Transmittance gradient: derivative of curve B.

Fig. 5. Angiograms used to determine capillary transit time. (A) The arrow indicates the location of precapillary arteriole filling. The angiogram is taken 200 msec after the beginning of the laser. (B) The arrow indicates the location of postcapillary venule filling. The angiogram is taken 400 msec after the beginning of the laser.
Table 1. Reproducibility of diameter measurements in arteries

<table>
<thead>
<tr>
<th>Approximate diameter (microns)</th>
<th>Same day*</th>
<th>Different days†</th>
</tr>
</thead>
<tbody>
<tr>
<td>80</td>
<td>4 (n = 5)</td>
<td>8 (n = 27)</td>
</tr>
<tr>
<td>40</td>
<td>4 (n = 6)</td>
<td>7 (n = 30)</td>
</tr>
</tbody>
</table>

* Parenthetical values are the number of sites tested.
† Parenthetical values are the number of pairs used in the calculation.

Table 3. Reproducibility of flow measurements in arteries

<table>
<thead>
<tr>
<th>Diameter (microns)</th>
<th>Flow (μl/min)</th>
<th>Same day*</th>
<th>Different days†</th>
</tr>
</thead>
<tbody>
<tr>
<td>105</td>
<td>7.8</td>
<td>6 (n = 4)</td>
<td>11 (n = 4)</td>
</tr>
<tr>
<td>55</td>
<td>1.2</td>
<td>10 (n = 8)</td>
<td>13 (n = 4)</td>
</tr>
</tbody>
</table>

* Parenthetical values are the number of angiograms.
† Parenthetical values are the number of pairs.

Results

Reproducibility of the Measurements

Table 1 summarizes the reproducibility of diameter measurements done on 1 day and on 2 different days. The reproducibility on the same day was derived from the coefficient of variance of measurements obtained from three different angiograms taken at the same point in the cardiac cycle. The reproducibility on different days was taken from the mean of the difference in diameter between the 2 days. The animal’s heart rate was the same for both days. The blood pressure was not controlled.

The intraobserver reproducibility of the velocity measurements was determined from the coefficient of variance of readings taken from angiograms obtained under similar conditions. The results are tabulated in Table 2. The interobserver reproducibility was calculated from the difference in readings obtained on the same angiograms by two observers.

The reproducibility of flow measurements on 1 day and on 2 different days is shown in Table 3. The reproducibility on 1 day was determined from the coefficient of variance of measurements taken from angiograms obtained under similar conditions. The reproducibility on different days was determined from the difference in flow averaged over the cardiac cycle at different locations on the 2 days. The average flow was used rather than the instantaneous one because of some difficulty in obtaining measurements at exactly the same point in the cardiac cycle on 2 different days.

Normal Blood Flow in a Primate Eye

Figure 6 is a composite map of the local flow and capillary transit time in the macula. The flow appeared to be related to the vessel diameter and the perfused area of the retina. The capillary transit time was similar throughout most of the areas measured except for the area adjacent to the fovea. These values are only estimates since the angiograms were obtained at 50-msec intervals.

For each vessel and site, the pulsatility of the vessel’s diameter was determined from the standard deviation and the range of diameter measurements obtained ten times in the cardiac cycle at three to five positions on the blood vessel (Table 4). It appears that the absolute change in vessel diameter is similar for vessels of markedly different size.

The pulsatile nature of the blood flow in a retinal artery 40 μm wide is illustrated in Figure 7. The velocity, diameter, and flow-rate values were normalized by their maximal value to indicate relative changes. The 15% change observed in the diameter of this vessel was significant since it was fourfold greater than the reproducibility.

Fig. 6. Map of retinal blood flow and capillary transit time. The flow in different blood vessel segments is indicated in microliters per minute. The arrows indicate the capillary bed for which the capillary transit time was calculated: A, 200 msec; B, 225 msec; and C, 310 msec.
The correlation between blood velocity and vessel diameter is illustrated in Figure 8. The linear regression indicated an excellent correlation (r = 0.93, P < 0.001). Moreover, we tested the linearity by a linear regression of the logarithmic values and found a good correlation (r = 0.90, P < 0.001) with a slope of 0.90. The correlation between the flow and the diameter was investigated by a power curve fit, and a high correlation was also found (r = 0.99, P < 0.001) for a power of 2.90.

Discussion

Our first goal was to assess the reproducibility of the vessel diameter and blood velocity measurements in a given eye. The reproducibility in vessel diameter of 4% reported on 1 day is similar to the 3% value obtained by different methods. Although other investigators have reported lower values, their values were the result of an average of 100 measurements obtained in vessels greater than 150 μm in diameter. The reduced reproducibility on different days was probably caused by a number of variables, such as differences in the clarity of the image (either due to the optical quality of the cornea or the degree of focus of the angiogram), difficulty in synchronizing the cardiac cycle on different days, and differences in the physiologic condition of the animal.

The similarity between intra- and interobserver reproducibilities in blood velocity for vessels 40 μm and larger indicates that the method was adequately objective. The deterioration in the reproducibility in smaller vessels suggests that a larger degree of subjectivity was present in these measurements. This was probably due to the decreased visualization caused by the less intense fluorescence in smaller vessels, the downstream dilution of the dye, and the radial diffusion across the vessel. The reproducibility in vessels 40 μm and larger is similar to that obtained with laser Doppler velocimetry by Riva et al, Feke et al, and Khoobehi et al using a method similar to ours.

The 10% reproducibility in flow measurements on 1 day is consistent with the reproducibility of diameter and velocity measurements. The reproducibility on 2 different days of 13% appears somewhat low compared with the reproducibility of diameter on different days. This is a direct result of our attempts to minimize the influence of synchronization over the cardiac cycle. Differences in physiologic condition of the animal such as blood pressure, intraocular pressure, respiratory rate, and degree of anesthesia are all variables that affect the reproducibility and were not controlled. At this point we have made no attempt to differentiate between true physiologic variation and variability due to the method, such as reduction in visibility of the dye front because of a decrease in dye concentration of the bolus.

The 15% pulsatility in vessel diameter was significant since it was fourfold greater than the reproducibility. Hill and Crabtree observed, in humans, a pulsatility in diameter of 4–12% in two thirds of the arteries, but Delori and Fitch reported a value of only 3% in humans. The reason for the difference is not clear. It may be related to the variation in techniques or in species or may have been induced by anesthesia. The threefold pulsatility in the velocity is
consistent with the values reported by others in humans and animals.10,14,15

We obtained a significant linear correlation between blood velocity and vessel diameter. The power of 0.90 (r = 0.99, P < 0.001) obtained from our data yields a value of 2.9 for the power dependence of flow on diameter. This is very close to the theoretical value of 3 derived from Murray’s law.16 These results provide an indirect validation of our method. They also suggest that the relation of flow to the vessel diameter could be a reliable indicator of hemodynamic normality that is less affected by individual or even species differences. As such, this parameter is useful in comparing our data with those of Riva et al,7 obtained in humans with laser Doppler velocimetry, yielding a power of 2.76 (r = 0.52, P < 0.001).

The absolute blood velocities we obtained and those of others are compared in Figure 9. Our measurements show good agreement with those of Bulpitt et al15 which were obtained by cineangiography in pigs and primates. Our results are somewhat higher than the measurements Riva et al7 obtained in humans. These investigators calculated Vcenter from Vsys and Vdias. In our measurements Vcenter represents the average of five velocity measurements taken at equal intervals throughout the cardiac cycle. The difference may also be species related. Khoobehi et al11 used a version of our liposome-dye delivery system in rhesus monkeys and obtained similar values in vessels close to 100 μm. Due to the small number of data points, it is not easy to evaluate the significance of the discrepancy at low diameters.

In addition to providing information on blood flow of large vessels, targeted dye delivery appears to be capable of assessing the microcirculation by providing measurements of capillary transit time and of blood flow in vessels about 20 μm in diameter. The preliminary data shown in Figure 6 indicate that the capillary transit time is relatively constant in the macular region. This observation implies that this parameter could be used to detect an abnormality in the local microcirculation. Semiquantitative evaluation of the microcirculation in the parafoveal area, obtained by blue-field entoptoscopy, has yielded clinically interesting findings.17 Targeted dye delivery would have the advantage of providing objective measurements over large areas of the posterior pole.

In conclusion, the preliminary application of targeted dye delivery to the study of retinal hemodynamics in primates indicates that the method is capable of yielding values for blood flow that are consistent, in vessels larger than 40 μm, with results obtained by other methods. In addition, targeted dye delivery allows the assessment of the local microcirculation by providing measurements of capillary transit time and of blood flow in vessels about 20 μm in diameter. The ability to map repeatedly retinal hemodynamics should permit the detection of local and temporal changes in retinal hemodynamics due to physiologic or pathologic alterations.

Key words: blood flow, microcirculation, liposomes, fluorescein angiography, retina

Acknowledgments

The authors thank Kathleen Louden who edited the manuscript, Mary Lynn Dietsche who typed it, and Norm Jednock and Michael Heneghan who provided photographic services.

References

7. Riva CE, Grunwald JE, Sinclair SH, and Petrig BL: Blood flow in vessels about 20 μm in diameter. The preliminary data shown in Figure 6 indicate that the capillary transit time is relatively constant in the macular region. This observation implies that this parameter could be used to detect an abnormality in the local microcirculation. Semiquantitative evaluation of the microcirculation in the parafoveal area, obtained by blue-field entoptoscopy, has yielded clinically interesting findings.17 Targeted dye delivery would have the advantage of providing objective measurements over large areas of the posterior pole. In conclusion, the preliminary application of targeted dye delivery to the study of retinal hemodynamics in primates indicates that the method is capable of yielding values for blood flow that are consistent, in vessels larger than 40 μm, with results obtained by other methods. In addition, targeted dye delivery allows the assessment of the local microcirculation by providing measurements of capillary transit time and of blood flow in vessels about 20 μm in diameter. The ability to map repeatedly retinal hemodynamics should permit the detection of local and temporal changes in retinal hemodynamics due to physiologic or pathologic alterations.

Key words: blood flow, microcirculation, liposomes, fluorescein angiography, retina

Acknowledgments

The authors thank Kathleen Louden who edited the manuscript, Mary Lynn Dietsche who typed it, and Norm Jednock and Michael Heneghan who provided photographic services.

References