Noninvasive Visualization of Blood Flow in the Choriocapillaris of the Rat

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Purpose. The rat has been used to generate models of various eye diseases. However, methods to study the choriocapillaris noninvasively have been inadequate in this species. Laser-targeted angiography was applied to generate local, repetitive angiograms of the choriocapillaris in the rat and to assess the similarity between the choriocapillaris of the rat and that of the subhuman primate.

Methods. Carboxyfluorescein was encapsulated in heat-sensitive liposomes and injected intravenously in rats. The liposome contents were then released locally in the choroid by the application of a short, noncoagulating heat pulse provided by an argon laser. Videoangiograms of the downstream spread of the bolus of dye were generated with excitation illumination provided by another output from the argon laser.

Results. Laser-targeted angiography demonstrated that the bolus of dye perfused the choriocapillaris. Clusters of choriocapillaris lobules were observed and appeared similar to those described in the primate. Dynamic filling and emptying patterns also were similar to those of the primate. Lobules were filled by a central arteriole and drained by a venous annulus.

Conclusions. This study demonstrates the feasibility of noninvasively studying the choriocapillaris of the living rat using the technique of laser-targeted angiography. It demonstrates as well the similarity between the rat and the primate choriocapillaris, thus indicating that the rat is an acceptable and convenient model for the study of physiological and pathologic changes in the choroidal vasculature. Invest Ophthalmol Vis Sci. 1996;37:312-317.

The choriocapillaris provides metabolic support to the outer neurosensory retina and the retinal pigment epithelium. Several fundus diseases are thought to be linked to abnormalities in the choriocapillaris, such as "dry" and "wet" types of age-related macular degeneration, gyrate atrophy, choroideremia, and acute multifocal posterior placoid pigment epitheliopathy. However, there is little information on its normal physiology and involvement in disease.

Some information can be gained from the study of the choriocapillaris in animal models. The rat is an appealing model because of the similarity of its retina and choroid to that of humans.

In addition, the rat has been used successfully to generate models of diabetes,1 choroidal neovascularization,2-5 retinopathy of prematurity,4 and glaucoma.5 Finally, the rat is a convenient laboratory animal because of its availability and low cost of purchase and maintenance. Information on the hemodynamics of the normal choriocapillaris could help establish a baseline for comparison with pathologic conditions. However, there is no information on the hemodynamics of the choriocapillaris in this species, probably because of the lack of an adequate experimental method.

We have developed a method of laser-targeted delivery that provides controlled local delivery of pharmacologic substances to the ocular fundus.6,7 The method consists of encapsulating the substance in heat-sensitive liposomes (lipid vesicles that can be disrupted by heat), administering the liposomes intravenously, disrupting the liposomes, and causing them to release their contents by warming the target tissue (retinal or choroidal vessels) with a mild, noncoagulating laser pulse through the pupil. One application...
of laser-targeted delivery, laser-targeted angiography, consists of encapsulating carboxyfluorescein, a derivative of fluorescein, in liposomes. The encapsulated concentration is high enough to cause fluorescence quenching, thus making the circulating liposomes invisible in the angiogram. When the dye is released from the liposomes with the local pulse of heat, it is diluted in the plasma and yields a bright fluorescent bolus that selectively highlights either retinal or choroidal vessels as the blood flow carries away the bolus. By following the progression of this bolus from the arteries through the arterioles, capillaries, and venules, selective high-quality angiograms of the retinal vasculature and the choriocapillaris have been generated in normal subhuman primates. These laser-targeted angiograms yielded visualization at a quality unsurpassed by conventional angiography and provided quantitative information on hemodynamics.

In this study, we evaluated the applicability of laser-targeted angiography to the dynamic and noninvasive visualization of blood flow in the rat choriocapillaris and assessed its similarity to that of subhuman primates.

MATERIALS AND METHODS

Principle

By using a laser whose light is absorbed by hemoglobin and melanin, one can create enough energy in the retinal pigment epithelium and choriocapillaris to cause the temperature to rise to 41°C. This temperature corresponds to the phase-transition temperature in the heat-sensitive liposomes, thereby causing the release of their contents. On the other hand, the retina overlying the irradiated area of retinal pigment epithelium does not reach the phase-transition temperature because of cooling by the high rate of blood flow in the retinal vessels and the nonabsorbing retinal tissue. This can be calculated from first principles and has been demonstrated experimentally in nonhuman primates by the release of a bolus in the choroid while the liposomes in the retinal vasculature remained intact.

Instrumentation

A fundus camera (Zeiss, Oberkochen, Germany) was equipped with a video camera (Texas Instruments, Dallas, TX). The output of the camera was fed into a video image enhancer (Optical Electronics, Tucson, AZ) and stored on magnetic tape using a high-frequency videorecorder (Beta Cam, Sony, Tokyo, Japan). For the analysis, the tape was played back, and images were digitized with a frame grabber (Epix, Northbrook, IL).

Laser delivery optics were added to the fundus camera. The output of a commercial ophthalmic argon laser (Coherent Radiation, Palo Alto, CA) was divided and fed into two fiberoptic cables. Excitation of the fluorescence was achieved with the output of one fiber, which was optically coupled to a conjugate image plane of the fundus to deliver a 20° illumination at 488 nm at a power of 8 mW for a duration of 2 seconds. To cause the release of a bolus of dye from the liposomes, the output of the second fiber was coupled to another conjugate plane of the fundus to irradiate a 600-µm spot on the retina with a power of 16 mW for a duration of 200 msec. The position of the laser beam on the fundus was monitored by an aiming beam generated by visible light fed into the second fiberoptic cable.

Laser-targeted angiography was performed by aiming the visible beam at various locations on the fundus, releasing a bolus, and recording a video sequence of 2 seconds duration. On play back and digitization, the sequences of angiograms were enhanced with routine image processing algorithms. Background fluorescence was subtracted, and contrast was optimized. Background fluorescence was caused by leakage of some carboxyfluorescein (10% to 15% of the dose) out of the liposomes caused by their exposure to body temperature. The processed images were printed on photographic paper.

Liposome Preparation

As described by Ralston and coworkers, 6-carboxyfluorescein (Molecular Probes, Junction City, OR) was purified on a hydrophobic column and diluted to approximately 100 mM. Sterile Ringer’s lactate (Abbott Laboratories, Chicago, IL) was used as the diluent, and the diluted dye was filtered through a 0.2-µm syringe filter. Dipalmitoylphosphatidylcholine (DPPC) and dipalmitoylphosphatidylglycerol (DPPG) (Genzyme, Cambridge, MA) were used without further purification. Liposomes were prepared by a method described by Hope et al. Briefly, it consisted of drying the lipids (DPPC:DPPG 4:1 dissolved in methylene chloride and ethanol) to a film by rotary evaporation under vacuum. A 4% solution of carboxyfluorescein filtered through a 0.2-µm filter was mixed with the dried lipid, and the mixture was subjected to five cycles of rapid freezing at −20°C and thawing at 55°C. This was followed by forced filtration (eight passes) through a stack of two 25-mm, 0.2-µm polycarbonate filters (Poretics Corporation, Livermore, CA) placed in a thermobarrel extruder (Lipex Biomerbranes, Vancouver, Canada) to yield large unilamellar vesicles. To remove the unencapsulated dye, dialysis was performed against Ringer’s lactate at 4°C using molecular porous membrane tubings (molecular weight cut-off 14,000) (Spectrum Medical Industries, Los Angeles, CA).
The size of the liposomes was controlled because it affects their half-life in the blood and can cause clotting if it exceeds 450 nm. Size distribution was determined by quasi-elastic light scattering. A unimodal distribution with a peak at 128 nm was found. To determine the free and encapsulated concentrations of the dye, fluorophotometry was performed before and after lysis of the liposomes by heat.

**Animals and Procedures**

Institutional Review Board approval was obtained from the Johns Hopkins University School of Medicine. All animals were treated in compliance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. Twenty-five Long-Evans black-hooded rats were used. They were anesthetized with ketamine (50 mg/kg) and xylazine (10 mg/kg) intramuscularly, and their pupils were dilated with a mixture of homatropine 2% and phenylephrine 10%. Laser-targeted angiography was performed to evaluate the choroidal vasculature. A specially made silicone contact lens (Eyetech, Morton Grove, IL) was used to provide good image clarity and to protect the cornea. A 1.5 ml/kg dose of liposomes containing carboxyfluorescein was injected intravenously, yielding a dye dose of 7 mg/kg.

**RESULTS**

Laser-targeted angiography provided a highly selective visualization of the rat choriocapillaris. Figures 1 and 2 illustrate an angiographic sequence. At the beginning, the dye was localized at the site of release by the laser beam; the area was approximately 600 μm in diameter. Heat created by the laser caused a release of a bolus of dye in the local choriocapillaris and the underlying arterioles. Subsequent video frames show the advance of the bolus in the arterioles to the adjacent area of choriocapillaris. When the laser used for the release was turned off, the inflow of the fresh, nonfluorescent blood could be followed. This sequence is illustrated in the figures. As shown in Figures 1a and 2a, a highly fluorescent choriocapillaris appeared at the site of dye release. Discrete spots of fluorescence, away from the delivery site, appeared 17 msec later (Figs. 1b, 2b) and expanded rapidly. They were caused by the appearance of the dye released in choroidal arteries located under the site of release. When the laser was turned off, blood with free fluorescent dye was replaced gradually by fresh blood without released dye. Angiograms (Figs. 1c to 1f, 2c to 2f) obtained 17, 33, 50, and 83 msec after the end of the dye release clearly indicate a lobular pattern. Nonfluorescent blood entered from a central spot, expanded radially, and drained at the periphery. The adjacent choriocapillaris lobules thus created a honeycomb pattern clearly visible in Figures 1c, 1f, 2e, and 2f.

The lobular pattern of filling occurred with a delay that increased with the distance from the location of the laser spot. The delay resulted from the travel time in the arteries supplying each lobule. By aiming the releasing laser beam to various areas on the fundus, different clusters of lobules could be highlighted. Neighboring clusters fit together like a jigsaw puzzle. Laser-targeted angiography, performed at various locations around the disc, demonstrated the flow of dye in a radial pattern. At certain locations, the dye was found to perfuse only the local patch of choriocapillaris and a large choroidal vein. In such instances, no discrete lobular pattern developed.

The release of a bolus of dye in the choroid was not accompanied by a release in the retinal vasculature. This is illustrated in the figures by the lack of change in the fluorescence of retinal vessels.

Angiograms repeated as many as 20 times did not differ from each other, thus indicating the absence of any substantial accumulation of dye in the extravascular choroidal space or in the vessel wall. The quality of repeated angiograms remained constant for the first 20 minutes and was acceptable for a total of 45 minutes, indicating that a sufficient amount of liposomes remained in the bloodstream for this period of time.

**DISCUSSION**

A few studies have reported the use of the rat as a good model of choroidal neovascularization. These studies have had to rely mainly on histopathology and vascular casts because fluorescein angiography has not been satisfactory. Follow-up studies are very difficult because such techniques require the animals to be killed. Longitudinal studies would be required for the testing of various pharmacologic modulators that might be promising in choroidal diseases—e.g., antiangiogenic substances for choroidal neovascularization. Laser-targeted choroidal angiography not only permits selective high-quality angiograms of the choroid, it also permits such follow-up studies to be conducted.

Little is known of the physiology of the choriocapillaris, and methods currently available for study of the choroidal vasculature of rodents are limited. Labeled microspheres have been used but cannot measure blood flow in discrete areas and require the animals to be killed. They are thus inappropriate for use in experiments that require follow-up. Other methods, such as the hydrogen clearance technique, have been used to study choroidal blood flow, but they are invasive and provide information on the choroid as a whole. Laser Doppler flowmetry has been used by Riva and cowork-
Visualization of the Rat Choriocapillaris

FIGURE 1. Laser-targeted angiography of the normal rat choriocapillaris in the temporal part of the fundus. The circular area (A) is the fluorescence in the local choriocapillaries in the area of the releasing laser beam, which was approximately 600 µm in diameter. The bolus of dye appeared in the choroidal arterioles feeding the lobules (arrows, B,C) 17 to 33 msec later. After the laser used to release the bolus of dye was turned off (C), the spots rapidly were seen to spread radially, and this resulted in a lobular pattern. Angiograms (C to F) were obtained 17, 33, 50, and 83 msec, respectively, after the end of the dye release. This flow pattern corresponded to the flow in choriocapillaris lobules, which filled from a central spot (arrows, B,C) and drained along a peripheral ring (arrows, E). A honeycomb pattern was clearly visible, as seen in E and F. An outline of the lobules has been drawn by following the filling and drainage patterns from B to F and shown in E. The pre-release background fluorescence was subtracted from images A to F to highlight the dynamic changes.

ers\textsuperscript{16,17} to measure choroidal blood flow, but its use is restricted to the fovea and to animals that have a tape- tum or animals, such as the rabbit, in which the interference from retinal blood flow is minimal.

Conventional fluorescein angiography has been used to a certain extent,\textsuperscript{2,18} but it is limited in its usefulness in evaluating the choroid. Because the dye rapidly fills the major choroidal vessels as well as the retinal vessels, visualization of small vascular beds, such as the choriocapillaris, usually is hampered by the lack of contrast caused by the greater fluorescence emanating from the major choroidal vessels. Moreover, abnormal as well as normal choroidal vessels exposed for a prolonged time to fluorescein allow the dye to leak into the extravascular space. Dye that has leaked further hampers the visualization of small vascular beds, such as choriocapillaris neovascularization. A technique based on the subtraction of consecutive dynamic angiograms in subhuman primates has shown promising results in the effort to minimize the large background fluorescence of the major choroidal vessels,\textsuperscript{19} but the visualization totally depends on the relative dynamics of the vascular beds, and slowly perfused ones, such as choriocapillaris neovascular membranes, are not imaged.

Laser-targeted angiography, on the other hand, provides a tool to study the physiology of the chorio- capillaris in different regions of the posterior pole and in different animal species. The application in the rat and in the primate indicates that laser-targeted angiography has several advantages. First, the local release of a fluorescent bolus permits selected vascular beds, such as the choriocapillaris, to be visualized without interference from the fluorescence of overlying or underlying beds. Second, the short pulsed release, accompanied by rapid washout, ensures that the dye does not accumulate outside the vessels perfused by the bolus. Third, the angiograms can be repeated for at least 45 minutes (i.e., as long as the liposomes are circulating in the blood). This provides opportunities to correct errors in alignment of the camera and to perform angiography of both eyes.

Application of laser-targeted angiography in the rat showed a lobular pattern of flow in the choriocapill-
FIGURE 2. Laser-targeted angiography of the normal rat choriocapillaris in the nasal part of the fundus. The circular area (A) is the fluorescence in the local choriocapillaries in the area of the releasing laser beam, which was approximately 600 μm in diameter. The bright spot seen in the center of images A and B is an artifact caused by a reflection (arrow, A). After the laser used to release the bolus of dye was turned off (C), the spots rapidly were seen to spread radially, and this resulted in a lobular pattern. Angiograms (C to F) were obtained 17, 33, 50, and 83 msec, respectively, after the end of the dye release. This pattern corresponded to the flow in choriocapillaris lobules, each of which drained along a peripheral ring (arrow, E). A honeycomb pattern was clearly visible as seen in E and F. An outline of the lobules has been drawn by following the filling and drainage patterns from B to F and shown in E. As in Figure 1, the pre-release background angiogram was subtracted from images A to F to highlight the dynamic changes.

laris. The filling pattern of the choriocapillaris was demonstrated to be from the center of the lobules, with drainage of the lobules from the periphery. The same filling and drainage pattern was obtained with multiple releases of bolus of dye. This pattern of flow in the choroid of the rat, as demonstrated by laser-targeted angiography, was similar to that observed in the primate. Laser-targeted choroidal angiography in the primate revealed the presence of well-defined clusters of choriocapillaris lobules, each fed by a branch of the posterior ciliary artery.

The minimal lateral diffusion of dye between adjacent lobules, as observed in the primate, also was seen in the rat, indicating that there was no functional communication between adjacent vascular clusters under normal physiological conditions. Because the choriocapillaries form a continuous bed anatomically, the segmental pattern demonstrated by laser-targeted angiography is created by the dynamics of circulation. The pressure gradient between the arteriole and the venules prevents blood from reaching the capillaries of the adjacent lobule.

Similarities in structure and function of the rat choroid to those of the primate makes the rat a convenient and appropriate model for the study of choriocapillaris function. The availability of a tool for the selective angiographic study of the choroid expands the possibilities of research in choroidal vascular physiology and pathophysiology.

Key Words
angiography, choriocapillaris, laser, liposomes, rat

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