Three-Dimensional In Vivo Imaging of the Mouse Intraocular Vasculature during Development and Disease


PURPOSE. Aberrant growth of blood vessels in the eye is a major cause of vision loss, occurring as a complication of diabetic retinopathy, age-related macular degeneration, and retinal vascular occlusions, among others. Whereas in humans, in vivo angiography is routinely used to image such diseases, many animal models of ocular vascular disease and development rely on dissected tissues that may not accurately represent in vivo conditions and require enucleation of the eye, the death of the animal, or both.

METHODS. A method of three-dimensional imaging of blood vessels was used in the living mouse eye that involved scanning laser confocal microscopy and computer-aided image reconstruction.

RESULTS. This minimally invasive technique was used to collect three-dimensional images of intraocular vessels in vivo during development. The retinal and choroidal vasculature was studied during development and disease, in models of retinal degeneration, central retinal vein occlusion, and oxygen-induced retinopathy. To aid in investigations into cell-based therapies for retinal disease, two-color imaging was used to localize transplanted cells in relation to the vasculature. This technique was used to perform serial imaging of the ocular vasculature over time, when developmental regression of vessels was observed.

CONCLUSIONS. This in vivo vascular imaging approach is valuable in monitoring normal development, disease progression, and efficacy of experimental treatments in mouse models of ocular vascular disease and may have broader applications to the field of angiogenesis by using the readily visualized ocular vascular bed as a surrogate to test pro- and antiangiogenic compounds.

MUCH of what has been learned about the biology of the ocular vasculature has relied on fixed specimens collected from animal models. The retina is well suited for this type of analysis because of the relative ease with which it can be dissected from surrounding tissues and the regular arrangement and reproducible developmental course of retinal blood vessel growth.1,2 Mice are born with an avascular retina that becomes fully vascularized during the first few weeks of life through radial growth of vessels from the optic nerve head to the peripheral retina, roughly analogous to the process that occurs in the human fetus during the third trimester of development. Three parallel vascular networks within different layers of the retina are established over this period. Retinas can be wholemounted and prepared flat for effective visualization of the entire retinal vasculature.3 The inner two thirds of the retina receives its blood supply from the retinal vessels, and the outer third is nourished by the choroidal circulation. The choroidal vasculature lies beneath the retina and is of clinical interest, because several neovascular diseases of the eye, including exudative age-related macular degeneration, originate in the choroid and lead to subsequent retinal damage and vision loss.

The retinal vasculature of the mouse eye has been visualized in vivo in studies involving both fundus photography and fluorescence angiography, which have been valuable in identifying gross changes in retinal vessels but lack the resolution necessary to visualize the smallest capillaries. In addition, these images are limited to two-dimensional representations. The scanning laser ophthalmoscope has been adapted from its use in the clinic to study leukocyte trafficking and blood vessels in the retinas of living animals, giving investigators optical sectioning capabilities.4–10 Investigations into other vascular structures in the eye, such as the hyaloidal vessels, have largely depended on dissected tissue and/or histologic sectioning to visualize these vessels. Analysis of fixed tissue, however, gives only a snapshot of the complex events that occur during the development and remodeling of these vessels. Furthermore, analysis of dissected tissue may not provide an accurate depiction of the conditions in the living animal, and artifacts introduced by death, dissection, and/or fixation are possible.

In this study, we used a minimally invasive method of visualizing the vasculature of the mouse eye in the living animal that permits the study of vessels in vivo, without the need to kill the subject. These methods represent extensions of existing technologies into three dimensions and into new areas of study. Three-dimensional (3-D) renderings were created with scanning laser confocal microscopy and computer-aided reconstruction. These reconstructions are viewable online as a supplement to the article. This imaging approach eliminates artifacts associated with the use of fixed tissue and facilitates our investigation of several aspects of ocular vascular biology. In this report, we describe the details of the technique and how it was applied to examine the mouse ocular vasculature during development, in several disease models and in the study of a possible therapeutic modality involving stem cell transplantation into the eye.
METHODS

Animal Preparation and Care

Developmental studies were performed on BALB/cByJ mice, and the oxygen-induced retinopathy (OIR) model was performed in C57BL/6J mice. Animals were anesthetized with intraperitoneal administration of 30 to 50 mg/kg ketamine and 2 to 5 mg/kg xylazine before the procedure. In most cases, fluorescently labeled dextran (2000 kDa, 50 mg/mL in sterile PBS) was injected via the tail vein, or other accessible vessels, to visualize the vasculature. The volume of dye was adjusted according to the size of the mouse and ranged from 10 μL for newborn mice to 200 μL for adults. For serial imaging, animals expressing green fluorescent protein (GFP) under the promoter for the endothelium-specific protein, Tie2, were used. Topical anesthetic (tetracaine hydrochloride 0.5%; Alcon Laboratories, Fort Worth, TX) was applied to the eyes before all procedures. Pupils were dilated with tropicamide (Mydriacyl; Alcon Laboratories) to improve imaging of the interior of the eyes before all procedures. Pupils were dilated with tropicamide (Mydriacyl; Alcon Laboratories) to improve imaging of the interior of the eye. Eyes were kept moist with artificial tears and lubricants (Visdriacyl; Alcon Laboratories) to improve imaging of the interior of the eye. Eyes were kept moist with artificial tears and lubricants (Visdriacyl; Alcon Laboratories) to improve imaging of the interior of the eye.

Retinal Disease Models

For the retinal degeneration model, rd/rd-Tie2GFP mice were used. These mice undergo rapid spontaneous retinal degeneration due to a mutation in the rod-specific gene (rd1) encoding cGMP phosphodiesterase. By 2 months of age, there is complete degeneration of the two deep retinal vascular layers and the outer nuclear layer containing the photoreceptors. For the central vein occlusion model, large retinal veins of adult BALB/cByJ mice were occluded 3 days before imaging the retina with a green diode laser at 180 mW with a 50-mm spot size, for 1 second. The OIR model used in our studies is described in detail by Smith et al. Briefly, C57BL/6J mice were placed in 75% oxygen from postnatal day 7 through 12, at which time they were returned to room air. These mice exhibit extensive central vaso-obliteration and neovascularization at the interface between perfused (peripheral) and nonperfused (central) retina. For experiments involving in vivo localization of intravitreally injected cells, bone marrow was harvested from postnatal day 2 (P2), the pupillary membrane was observed to be a highly interconnected network of vessels covering the front of the lens (Fig. 1i). Six days later at P8, evidence of regression was visible (Fig. 1j), and at P12, only a few vessels of the pupillary membrane remained (Fig. 1k). In this series, complete regression of the pupillary membrane was observed by 2 weeks of age (Fig. 1l), coinciding with when the mouse opened its eyes.

When the vessels of the hyaloid system were isolated from the complete images, we observed a dense, complex TVL, along with a significant contribution from the VHP at P2 (Fig. 1q). By P8, central pruning of these vessels was evident, with most of the branching restricted to the periphery (Fig. 1r). At P12, the number of major hyaloidal vessels emerging from the optic disc was approximately halved compared with the image at P8, and the VHP was similarly reduced in complexity (Fig. 1s). The 16-day-old mouse showed nearly complete regression of the VHP and highly pruned hyaloidal vasculature (Fig. 1t). The final phase of hyaloid regression involved relatively slow pruning of the major branches of the hyaloid artery that proceeded over the next 3 to 4 weeks (Figs. 1u–x).

As an alternative to the “digital dissection” shown in Figure 1, we used color depth coding whereby the iris, hyaloid and retinal vasculature were assigned different colors (Fig. 2a and Supplementary Video 5 at http://www iovs org/cgi/content/full/46/9/3021/DC1). This made the different vascular systems more readily distinguishable, even when viewed from within a single image.

RESULTS

3-D In Vivo Imaging of Intraocular Vasculature during Development

In the mature eye, the visual axis is clear because of the transparency of the cornea, lens, and vitreous, all of which are normally avascular. During development, however, the growing lens is supplied by a network of vessels collectively known as the hyaloidal vasculature. Originating from the optic disc, the branched hyaloidal vessels extend toward the posterior surface of the lens. The tunica vasculosa lentis (TVL) is a major component of the hyaloidal vasculature and surrounds the lens. The anterior portion of the TVL, also known as the pupillary membrane, supplies the front of the lens. The posterior portion of the TVL lies anterior to the vasa hyaloidia propria (VHP), which branches from the hyaloid artery. Although the hyaloid system is of great interest because of the natural process of regression it undergoes, its study has been somewhat limited by the fact that it is difficult to visualize in dissected specimens. We demonstrate herein that these transient structures can be readily imaged in vivo (Fig. 1) and rendered into three dimensions, as shown in an interactive format online in Supplementary Videos 1 and 2 (http://www iovs org/cgi/content/full/46/9/3021/DC1). Different vascular structures can be digitally “dissected” from the 3-D renderings and viewed separately, as shown in Figures 1i–t and in three dimensions in Supplementary Videos 3 and 4 (http://www iovs org/cgi/content/full/46/9/3021/DC1).

We imaged mice from birth to young adulthood and selected time points from that series to demonstrate the changes occurring in the pupillary membrane (Figs. 1i–p) and hyaloid vessels (Figs. 1q–x) during their developmental regression. At postnatal day 2 (P2), the pupillary membrane was observed to be a highly interconnected network of vessels covering the front of the lens (Fig. 1i). Six days later at P8, evidence of regression was visible (Fig. 1j), and at P12, only a few vessels of the pupillary membrane remained (Fig. 1k). In this series, complete regression of the pupillary membrane was observed by 2 weeks of age (Fig. 1l), coinciding with when the mouse opened its eyes.

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In Vivo Imaging of the Ocular Vasculature in Disease Models

This method was also used to generate images in the adult mouse of the central retina, a tissue that lies approximately 3 mm deep in the eye when measured from the front of the cornea to the retina. This depth of imaging is well beyond the usual limits of light microscopy, but the transparency of the
optical axis allows effective imaging at this depth with confocal microscopy (Fig. 2b). Although the area that can be visualized is somewhat limited, high-quality images of the optic disc region are obtained. Supplementary Video 6 (http://www.iovs.org/cgi/content/full/46/9/3021/DC1) shows that more peripheral regions of the retina can be viewed on repositioning the subject. In addition to the larger vessels, retinal capillaries are also imaged, with good resolution.

Inherited retinal degenerations, such as retinitis pigmentosa, are characterized by progressive loss of retinal cells, including photoreceptors, which leads to vision loss in affected individuals. We have applied this imaging technology to study the rd1/rd1 mouse model of retinal degeneration, which from the complete images (q–x). The lens was richly supplied by the TVL and the VHP at 2 days after birth (q). These two structures were more easily distinguished at P8 (r) and P12 (s) when regression of central vessels was observed. The VHP showed complete regression by P16 (t), whereas the TVL regressed more slowly by pruning of the major vessels and completely disappeared by 5 to 6 weeks (x), with some variability in the disappearance of the last vessels.
shows, as a major aspect of the model, significant loss of the retinal blood vessels in addition to the neurosensory degeneration.\textsuperscript{15} When we imaged a 4-month-old mouse from this strain, we observed that only the major retinal vessels remained (Fig. 2c). With the loss and atrophy of retinal tissue, we were able to observe better the vessels of the underlying choroidal vasculature.

Central retinal vein occlusion (CRVO) may be caused by both local and systemic factors, and in humans the incidence increases with age. Vision loss can occur as a result of fluid accumulation or intraretinal hemorrhage in the macula.\textsuperscript{16} In a model of CRVO, a laser-induced occlusion was created near the optic disc in a mouse eye. Fluorescein-dextran was injected after the start of image capture, and images were collected as the occluded vein was slowly filled by venules (Fig. 3 and Supplementary Video 7 at http://www.iovs.org/cgi/content/full/46/9/3021/DC1). This approach identifies occluded veins in vivo that are often associated with retinal edema and ocular neovascularization in the retina and permits the study of the hemodynamics involved in retinal vascular occlusions.

Among the best-characterized animal models of retinal vascular disease is the OIR model. This model has features in common with retinopathy of prematurity that can affect low-birth-weight infants born prematurely who are therapeutically exposed to high levels of oxygen. In the mouse model described by Smith et al.,\textsuperscript{11} vaso-obliteration is seen in the central retina during exposure to hyperoxia which is followed by pathologic neovascularization after return to normoxia. These new vessels often leak fluid and can breach the internal limiting membrane. The utility of this imaging method is further demonstrated by generating images in vivo of oxygen-induced retinal disease in the posterior pole of the mouse eye. At postnatal day 14, 2 days after removal from hyperoxia, vaso-obliteration was visible in the central retina along with tortuosity of retinal vessels (Figs. 4d–f and Supplementary Video 8 at http://www.iovs.org/cgi/content/full/46/9/3021/DC1), which are alterations characteristic of this model. Two features that are clearly observed in vivo, but have not been emphasized in prior studies, are venous dilation and marked hyaloidal vascular tortuosity. It is presumed that the dilation observed in vivo is reversed on the death of the animal and the dissection and fixation of the retina and thus is not seen in wholemounts, whereas the altered hyaloidal vascular appearance has been not described previously because of the limitations associated with dissected tissue and/or histologic analysis. When the OIR model is followed out to P18 (6 days after return to normoxia), we observed evidence of pathologic neovascularization and vascular leak (Figs. 4g–i and Supplementary Video 9 at http://www.iovs.org/cgi/content/full/46/9/3021/DC1), both typical features of this model. The hyaloidal vessels remained tortuous and appeared dilated. Thus, the in vivo imaging method described herein is useful for analyzing retinal vascular pathology induced by hyperoxia and reveals features that may not be readily apparent from classic histologic analysis of dissected tissue.

Localization of Injected Cells In Vivo

Bone-marrow–derived lineage-negative hematopoietic stem cells have been shown in our laboratory to target the retinal vasculature and provide a protective effect from vascular and neural degeneration observed in the rd/rd mouse.\textsuperscript{12,17} Using in vivo imaging, we visualized GFP-expressing injected cells and ocular vessels simultaneously. This permitted us to verify cell targeting at the outset of long-term experiments and to follow the fate of these cells in the living animal after experimental manipulation (Fig. 5).

Serial Imaging of a Single Eye during Development

Because of the minimally invasive nature of this approach, we are able to observe single eyes in vivo over time through serial imaging. Although repeated intravenous injections of dye are possible, for these experiments we chose to use a transgenic strain of mice that expresses GFP exclusively in the vasculature under the regulation of the endothelium-specific promoter for Tie2. Mice were observed for 6 days beginning 15 days after birth to image the regression of hyaloidal vessels (Fig. 6).
the fact that the structures under observation are involved in
motion, and/or fixation. Much of the value of this method lies in
models where it was effective in visualizing important aspects
normal vascular development and in several ocular disease
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of vascular development and diseases with abnormal angiogen-
etween vessels and surrounding tissue. This becomes
significant when studying tissues such as the hyaloidal vascu-
ulation, where the relationship of these vessels to the surround-
ng vitreous, retina, and lens during development and regres-
sion cannot be appreciated by standard histologic techniques.
Recently, scanning laser confocal ophthalmoscopy has been
used to study blood vessels and leukocyte function in live animal eyes.4–10 Although the optical sectioning capabilities
have been valuable in these studies, we have extended the use
of confocal imaging in this context into three dimensions and
provided more comprehensive visualization of the intraocular
vasculature.

The imaging system described in this study utilizes an un-
usual optical path that introduces some degree of spatial aber-
ations. The light used for excitation of the fluorophores in
these experiments passes through several media with different
refractive indices and magnifications, including glass, air, mouse cornea, lens, aqueous humor, and vitreous. The light
emitted from the fluorophores then passes back through these
same media at a longer wavelength, to the detectors. This
convoluted optical path is the likely source of the altered
spatial appearance of some of the ocular structures imaged in
our studies. For example, in some 3-D images, the distance
of this age were chosen because younger mice have not yet
opened their eyes, and we sought to avoid unnecessary trauma
as a result of surgical fissure of the eyelids, which could affect
the processes under study. This age range permitted the visu-
alization of vascular regression in the developing eyes of indi-
vidual animals and demonstrates the value of this approach to
the in vivo study of ocular vasculature during development.

**DISCUSSION**

3-D in vivo imaging of the ocular vasculature permits the study
of vascular development and diseases with abnormal angiogen-
esis in living animals, providing new insights into the dynamics
of vascular growth and regression. Such in vivo imaging under
physiologically relevant conditions is critical to obtaining valid
assessments of the effects of various drugs on vascular beds, in
situ. Although there is direct application to validating angio-
genic and angiostatic therapeutics for the treatment of neovascu-
lar eye diseases such as age-related macular degeneration,
diabetic retinopathy, and retinopathy of prematurity, there are
also substantial indirect benefits to be had by using the ocular
vasculature as surrogates for other vascular beds in the body.
The utility of this imaging method was validated in models of
normal vascular development and in several ocular disease
models where it was effective in visualizing important aspects
of the models studied. In some cases, this method creates
opportunities to observe features not possible with other
methods because of alterations resulting from death, dissec-
tion, and/or fixation. Much of the value of this method lies in
the fact that the structures under observation are involved in
several important retinal vascular diseases, and they remain
relatively undisturbed during observation.

In vivo angiography of retinal and choroidal vessels is rou-
tinely used in the management of ocular vascular disease in
humans and has also been applied to the study of animal
models of retinal disease. Fluorescein angiography, with mod-
dified digital or analog human fundus cameras, has been the
principal technique used to image rodent ocular vascula-
ture. However, this type of imaging is limited to analysis in
two dimensions, making it difficult to appreciate the relation-
ship between vessels and surrounding tissue. This becomes
significant when studying tissues such as the hyaloidal vascu-
ulation, where the relationship of these vessels to the surround-
ng vitreous, retina, and lens during development and regres-
sion cannot be appreciated by standard histologic techniques.

**FIGURE 4.** Imaging a model for oxygen-induced retinopathy in vivo. Mice were imaged at P14, after exposure to normal oxygen conditions (a–c) or hyperoxia (d–f). Hyperoxia induced retinal vaso-obliteration and vascular tortuosity (f), as previously described, but also visible in vivo were an alteration in retinal vein-to-artery size ratio, or venous dilation, and marked tortuosity of the hyaloid vessels (compare b with e). At P18 (g–i), evidence of the retinal NV that characterizes this model was observed (i), along with persisting hyaloid tortuosity (h). NV is indicated by the enlarged regions of the retinal vessels. These enlarged regions are likely to be sites of dye extravasation from the neovessels that have been reported to be prone to leak fluid. See Supplementary Videos 8 and 9 online. Scale bar, 500 μm.

**FIGURE 5.** Localization of intravitreally injected cells in vivo. GFP-expressing bone marrow–derived, lineage-negative hematopoietic stem cells were injected into the vitreous of mice that received intravenous infusion of red dye. (a) Injected cells (green) concentrated near the optic disc area from which the hyaloid vessels (red) emerged. (b) Two weeks after injection, GFP+ cells persisted in the vitreous and, in some cases, targeted and adhered to segments of the regressing hyaloid vasculature. Scale bar, 200 μm.
between the iris vasculature and the hyaloidal vessels appears inadequate to accommodate the lens. We are currently developing algorithms to perform corrections for these optical aberrations automatically. Another potential improvement in this technique is the application of multiphoton excitation. The use of longer-wavelength infrared light to excite fluorophores potentially reduces photodamage than the shorter wavelengths commonly used for fluorescence microscopy. These longer wavelengths also have better penetration into tissues, allowing for deeper imaging. These properties are well suited to in vivo imaging in the eye. We expect that multiphoton excitation will enhance the quality of the images obtained and minimize potential adverse effects of the laser light used.

The ability to observe biological processes in their undisrupted, natural context is critical to facilitating translational research in several fields and would significantly advance our understanding of basic, underlying mechanisms of the angiogenic process. A major gap in this progression is the need for injected dyes. Arrows: vessels from each eye that persist over the course of the experiment. Scale bar, 200 μm.

Figure 6. Serial imaging of single eyes over time. Mice expressing GFP in vascular endothelial cells were used to observe the regression of blood vessels in the eye. (a, b) Serial images of the hyaloid vasculature obtained from two mice are shown. Imaging spanned P15 to P21 and relied on the intrinsically fluorescent vasculature eliminating the need for injected dyes. Arrows: vessels from each eye that persist over the course of the experiment. Scale bar, 200 μm.

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References