In Vivo Immunostaining Demonstrates Macrophages Associate with Growing and Regressing Vessels

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PURPOSE. The purpose of this study was to identify ways to improve qualitative and quantitative assessments of retinal vessels and neovascularization (NV).

METHODS. At postnatal day (P) 17, mice with oxygen-induced ischemic retinopathy were injected intravitreally with one of a variety of FITC-labeled or unlabeled antibodies and humanely killed 12 hours later. Retinas were flat mounted (retinas from eyes injected with labeled antibodies) or incubated with secondary antibody and then flat mounted (retinas from eyes injected with unlabeled antibodies).

RESULTS. Retinas from eyes injected with labeled anti-platelet endothelial cell adhesion molecule 1 (PECAM1) showed good resolution of the fine structure of retinal NV, including filopodia at the tips of sprouts. New vessels originated from superficial retinal vessels, something that is widely recognized, but they also arose from deep retinal capillaries and from large retinal vessels, which is not generally known. Retinas from eyes injected with unlabeled anti-PECAM1 antibody and then incubated with labeled secondary antibody showed selective staining of retinal NV with little or no background, greatly facilitating identification and quantification of the NV by image analysis software. Double labeling with anti-PECAM1 antibody and one of three other antibodies—anti-CD45, F4/80, or anti-CXCR4—showed exquisite localization of various populations of bone marrow–derived cells with respect to the vasculature and demonstrated close association of macrophages with NV and regressing vessels. Double labeling with anti-PECAM1 antibody and anti-angiogenin growth factor (PIGF) showed high levels of PIGF in growing and regressing vessels but no detectable signal elsewhere in the retina.

CONCLUSIONS. This study describes techniques that facilitate measurements and detailed structural analysis of retinal NV and that allow identification and quantification of populations of bone marrow–derived cells and support the view that macrophages contribute to the growth and regression of vessels in the eye. (Invest Ophthalmol Vis Sci. 2007;48:4335–4341) DOI: 10.1167/iovs.07-0113

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The eye is a relatively isolated tissue compartment that provides many advantages with regard to experimental studies and development of therapies. Despite relative isolation from the systemic circulation, the critical structures of the eye, including the retina, are readily accessible and visible because they border a fluid cavity, a gel-filled cavity, or both. This facilitates local delivery of drugs and therapeutic proteins while minimizing exposure to the rest of the body. One such application, intravitreal injection of VEGF antagonists, has provided a major advancement in the treatment of several retinal vascular diseases (for a review, see Campochiaro et al.1).

The structure of the eye also allows study of the impact of experimental manipulations and potential new therapies on well-characterized vascular beds of the iris, retina, and choroid, facilitated by visualization of the various cell types involved to better define how they participate. Intravitreal microscopy provides a means by which cell types can be identified and observed in a living eye and has been used to great advantage in the study of the iris vasculature and inflammation within the anterior segment of the eye.2–6 It has also been used to study leukostasis in the retinal vasculature.7,8 However, though fine detail of labeled cells can be observed in the anterior segment, optical aberrations degrade in vivo images of the retina. Adaptive optics provides a means to correct for optical aberrations and has allowed in vivo imaging of the photoreceptor mosaic in humans.9,10 Theoretically, adaptive optics could also be used for enhanced in vivo imaging of the retina in mice, but optical aberrations are much greater in mouse eyes than in human eyes, making this a difficult task.11

In this study, we sought to determine whether in vivo labeling combined with retinal flat mounting and examination by fluorescent microscopy provides advantages for visualization of the retinal vasculature. We found a new approach that facilitates quantitative measurements of retinal neovascularization and a related approach that provides exquisite structural detail of the vasculature and bone marrow–derived cells. In addition to describing a valuable new technique, these studies confirm the close association of macrophages with regressing vessels and clearly show that they also surround new vessel sprouts.

MATERIALS AND METHODS

Reagents

Unlabeled and FITC-conjugated rat anti-mouse platelet endothelial cell adhesion molecule-1 (PECAM1) polyclonal antibody and unlabeled mouse F4/80, a pan macrophage marker, were purchased from PharMinGen (San Jose, CA). Mouse F4/80 labeled with phycocerythrin (PE) was purchased from e Bioscience (San Diego, CA). Rabbit anti-mouse CD45 was purchased from ABScience (Worcester, MA), and goat anti-placental growth factor (PIGF) polyclonal antibody (M-18) was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Secondary antibodies were goat anti-rat IgG conjugated and goat anti-rabbit IgG conjugated with fluorescent dyes (Alexa 488 and Alexa 594, respectively; 1:400; Invitrogen, Carlsbad, CA) and goat anti-rabbit IgG conju-
Intraocular Injections of Antibodies

Pathogen-free C57BL/6 mice (Charles River, Wilmington, MA) were treated in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research and the guidelines of the Animal Care and Use Committee at the Johns Hopkins University Medical School. At postnatal day (P) 7, litters of mice were placed in 75% oxygen for 5 days and returned to room air at P12. At P17, mice were injected intravitreally with one of the antibodies listed. Injections were performed under a dissecting microscope with a microinfusion pump (Harvard Pump Microinjection System; Harvard Apparatus Inc., South Natick, MA) and pulled glass micropipettes, as previously described.12 Micropipettes were calibrated to deliver 1 μL vehicle on depression of a foot switch. Mice were anesthetized, pupils were dilated, the sharpened tip of a micropipette was passed through the sclera just behind the limbus into the vitreous cavity, and the foot switch was depressed. For double staining, equal volumes of two antibodies were premixed. For single antibody injections, antibody was diluted 1:1 with PBS. In all cases, antibody injection amounts were 0.5 μg for PECAM1 and CD45, 0.2 μg for F4/80 and PlGF, and 0.625 mg for CXCR4. Eight to 12 hours after injection, mice were humanely killed and eyes were removed and fixed in 10% phosphate-buffered formalin for at least 5 hours. Retinas were dissected intact and washed three times with PBS containing 0.25% Triton X-100 (PBST). Retinas were then incubated with secondary antibodies (1:400 for all antibodies conjugated with fluorescent dye [Alexa 594] and 1:800 for goat anti-rabbit IgG conjugated with Cy3) on a shaker at room temperature for 45 minutes. After three washes with PBST, retinas were incubated with goat anti-rabbit fluorescent dye. Retinas were flat mounted and examined by fluorescence microscopy.

Ischemic Retina

![Ischemic Retina](image1)

Normal Retinal

![Normal Retinal](image2)

**FIGURE 1.** Detection of retinal vessels and NV by in vivo immunostaining for PECAM1. C57BL/6 mice were placed in 75% oxygen between P7 and P12 and then returned to room air. Control mice were kept in room air for the entire time. At P17, mice were given intraocular injections of 0.5 μg rat anti-mouse PECAM1, anti-mouse PECAM1-conjugated to FITC, or GSA conjugated to FITC. Mice were humanely killed 12 hours after injection, and eyes were fixed in 10% phosphate-buffered formalin for 5 hours. Retinas were dissected, and those from eyes injected with unconjugated rat anti-mouse PECAM1 were incubated with goat anti-rat fluorescent dye. Retinas were flat mounted and examined by fluorescence microscopy. Ischemic retinas from eyes injected with unconjugated anti-PECAM1 antibody and then stained with a labeled secondary antibody showed selective staining of retinal NV (A–C, arrows) and no visualization of the preexistent retinal vessels, whereas nonischemic retinas showed only faint background staining (D). Ischemic retinas from eyes injected with labeled anti-PECAM1 antibody showed preexistent and new vessels, but the staining of new vessels was stronger (E–G), and at high magnification it was possible to distinguish them from preexistent vessels (F, G, arrows). Nonischemic retinas showed weak staining of normal retinal vessels (H). Ischemic retinas injected with labeled *G. simplicifolia* lectin showed equally strong staining of new and preexistent vessels, making them difficult to distinguish (I–K). Nonischemic retinas showed strong staining of normal retinal vessels (L). Staining with the secondary antibody used in A–D, a goat anti-rat IgG conjugated with fluorescent dye, in the absence of the anti-PECAM primary antibody showed no staining in central (M) and peripheral (N) areas of the ischemic retina.
were mounted on glass slides with mounting medium (Aquamount; Polysciences, Warrington, PA) and viewed with a fluorescence microscope (Nikon Instruments Inc., New York, NY) using imaging software (SPOT RT 3.4; Diagnostic Instruments, Sterling Heights, MI). Red and green channel images were merged to assess for double labeling.

RESULTS

In Vivo Immunostaining with Unlabeled Anti-PECAM1 Antibody Provides Selective Staining of Neovascularization

Mice with ischemic retinopathy were injected intravitreally with 0.5 µg unlabeled or FITC-labeled rat anti-mouse PECAM1 antibody and humanely killed 12 hours after injection. Retinas were dissected, and those from eyes injected with unlabeled anti-PECAM1 antibody were incubated with a secondary goat anti-rat IgG labeled with fluorescent dye (Alexa 488; Molecular Probes); retinas were then whole mounted. Fluorescence microscopy under low magnification showed discrete, brightly stained areas in the midperiphery of the retina (arrows) with faint background staining of the retina (Fig. 1A). High-magnification views showed new vessels on the surface of a faintly stained retina but no identifiable vessels within the retina (Figs. 1B, 1C). When the same procedure was performed in a control P17 mouse, the nonischemic whole mounted retina showed some faint staining of the edge of the retina and no staining of any vascular structures (Fig. 1D).

Retinas from eyes injected with labeled anti-PECAM1 antibody were whole mounted without additional incubation. Low-magnification views of these retinas showed large vessels and capillaries within the retina, some dark, nonperfused areas, and ill-defined hyperfluorescent areas (Fig. 1E). High-magnification views of the posterior retina showed hyperfluorescence at the optic nerve head, which likely represented remaining hyaloid vessels, and large retinal vessels emanating from the optic nerve head (Fig. 1F). Vascular sprouts (arrows) were seen protruding from a large vessel. A high-magnification view of the hyperfluorescent area seen in the upper left corner of Figure 1E showed loops and tangles of new vessels (arrows) connecting to underlying retinal capillaries (Fig. 1G). Although some new vessels were easily identified, it was difficult to be certain whether most vascular structures were preexistent or new. A nonischemic retina from a P17 mouse injected with labeled anti-PECAM1 antibody showed large vessels and capillaries, though the capillaries were not very sharp (Fig. 1H). The staining shown in Figures 1A to 1D is specific. Without injection of the primary antibody, incubation of the whole ischemic retina with the secondary antibody—goat anti-rat IgG coupled to fluorescent dye (Alexa 488; Molecular Probes)—showed no staining in the central (Fig. 1M) or peripheral (Fig. 1N) part of the retina.

Ischemic retinas from eyes injected with 0.5 µg FITC-labeled GSA, which selectively binds vascular cells, showed large retinal vessels, hyperfluorescent areas with dense vascular structures, and dark, nonperfused areas (Fig. 1I). A high-magnification view of the upper left part of Figure 1I showed a large retinal vessel leading to a brightly fluorescent area in the midperiphery and a less brightly fluorescent area in the far periphery (Fig. 1J). It is likely that the brightly fluorescent region represented new vessels superimposed on retinal capillaries, whereas the less fluorescent areas represented retinal capillaries, but it is impossible to be certain. Even higher magnification of the lower left of Figure 1I showed an area that was recognized with more certainty as retinal neovascularization, but it was still difficult to outline (Fig. 1K). A nonischemic retina from a P17 mouse injected with labeled GSA showed well-defined retinal vasculature (Fig. 1L). These data suggest that the first technique using injection of unlabeled anti-PECAM1 antibody selectively stained retinal neovascularization, whereas injection of labeled anti-PECAM1 or GSA resulted in staining of new and preexistent vessels.

In Vivo Immunostaining with Labeled Anti-PECAM1 Antibody Provides Excellent Structural Detail of Retinal NV

As noted, new and preexistent vessels were stained by injection of labeled anti-PECAM1 antibody into the vitreous cavity. With high magnification, the relationship between new and preexistent vessels could be seen in great detail. Figure 2A shows a densely packed network of NV sitting on the superficial capillary bed has the appearance of a cocoon. (B) Focusing down into the retina allows visualization of tightly packed networks of new vessels originating from the deep capillary bed that have the appearance of sacs (arrows). (C) Many small tufts of new vessels associated with superficial retinal vessels in the far periphery of the retina. (D) Large clump of NV growing from a large vessel in the posterior part of the retina has the appearance of a fungus on a tree branch. Smaller clumps (NV) are seen distally along the vessel. (E) Filopodia are seen at the tips of new vessel sprouts.
FIGURE 3. Macrophages are increased in ischemic retina and closely associate with retinal and hyaloid vessels. At P17, C57BL/6 control mice (A–D) or mice with ischemic retinopathy (E, F) were given intravitreal injections of labeled F4/80 antibody (A–C, E, F), or the retina was removed and incubated with labeled F4/80 antibody (D). In some mice, eyes were injected with a mixture of PE-labeled F4/80 and FITC-conjugated anti-PECAM1 antibody (G–J). Twelve hours after antibody injection, mice were humanely killed, and retinal flat mounts were examined by fluorescence microscopy. The number of macrophages seen in the retina of a room air control mouse 12 hours after injection of FITC-labeled F4/80 (A) was similar to that seen in the retina from an uninjected eye of a control mouse stained with F4/80 after death (D). Low-magnification view of the retina from a control mouse shows a moderate number of macrophages scattered throughout the retina with a high density at the optic nerve head (B), shown in a high-magnification view (C). Low-magnification view of the retina from a mouse with ischemic retinopathy shows a high density of macrophages throughout the retina, with many lining retinal vessels and a very high density on and around the optic nerve head (E), shown in a high-magnification view (F). Montage from an eye coinjected with PE-labeled F4/80 and FITC-conjugated anti-PECAM1 shows many macrophages closely associated with large retinal vessels and a particularly high density at the optic nerve (ON) associated with the almost completely regressed hyaloid vasculature. Some macrophages appear to be within the wall of the vessel, possibly passing from the lumen to the retina. A region of the retina containing smaller vessels stained with anti-PECAM1 (H) shows numerous macrophages (I). When visualized through red and green channels, the macrophages are seen to wrap around the vessels; some colabeled with F4/80 and anti-PECAM1 (J).

FIGURE 4.

FIGURE 5.
new vessels are seen protruding from a large retinal vessel, giving the appearance of fungus growing on a tree branch (NV; Fig. 2D). Smaller clumps of NV are seen distally along the vessel. There is even sufficient resolution to see filopodia at the tips of new vessel spouts (Fig. 2E).

**Macrophages Are Increased in Ischemic Retina and Closely Associate with Retinal and Hyaloid Vessels**

Retinal flat mounts prepared 12 hours after intravitreous injection of 0.2 μg FITC-labeled F4/80 antibody in P17 control mice showed macrophages scattered throughout the retina (Fig. 3A) that appeared, on removal and incubation of the retinas with labeled F4/80 antibody, of similar density and morphology (Fig. 3D). Compared with the moderate number of macrophages seen in the retinas of normal P17 mice (Fig. 3B), retinas from mice with ischemic retinopathy showed a high density of macrophages throughout the retina, with many lining retinal vessels (Fig. 3E). In ischemic (Fig. 3F) and nonischemic retinas (Fig. 3C), the highest density of macrophages was seen in close association with regressing hyaloid vessels around the optic nerve head. This high density of macrophages at the ON is seen in panoramic view in the montage shown in Figure 3G. In this eye, phycoerythrin (PE)-labeled F4/80 and FITC-labeled anti-PECAM1 were both injected, allowing visualization of vessels and macrophages and demonstrating close association of macrophages with large retinal vessels. Many of the macrophages appeared to adhere to or within vessel walls, sometimes both. In more peripheral retina, macrophages appeared to wrap around small retinal vessels (Figs. 3H–J, arrows). Injection of CD45 or F4/80 showed collections of bone marrow–derived cells completely coagulating regressing hyaloid vessels (Figs. 4A, 4B). A subpopulation of these bone marrow–derived cells expressed CXCR4 (Figs. 4C–F), illustrating that this particular cell surface receptor is identifiable in vivo immunostaining.

**New Vessels and Regressing Hyaloid Vessels Contain High Levels of PlGF**

PlGF is a member of the VEGF family, which binds VEGF receptor 1, located on bone marrow–derived cells and on endothelial cells. Double labeling for PlGF and PECAM1 showed that endothelial cells within neovascularization and regressing hyaloid vessels contained high levels of PlGF (Fig. 5). It was not detectable in other areas of the retina, including normal retinal vessels.

**DISCUSSION**

Retinal NV occurs as a complication of ischemic retinopathies, a group of diseases in which there is damage to retinal vessels, causing them to close and resulting in retinal ischemia. The most prevalent member of the group is diabetic retinopathy, but retinopathy of prematurity (ROP) and retinal vein occlusion are other types of ischemic retinopathies that lead to vision loss. Regardless of the inciting event leading to retinal vessel closure, once a sufficient area of the retina becomes hypoxic, hypoxia inducible factor-1 (HIF-1) levels become elevated, which is sufficient to cause retinal NV. HIF-1 stimulates the expression of genes that contain a hypoxia response element (HRE) in their promoter region, and two HIF-1–regulated genes that are necessary for sprouting of retinal NV are Vegf and angiopoietin-2. Several studies that helped to establish this were performed in the murine model of oxygen-induced ischemic retinopathy. Many other molecular targets involved in retinal NV have also been identified in this model. These studies require quantitative comparisons of the amount of retinal NV in eyes with ischemic retinopathy exposed to different experimental conditions. To quantify NV, it is necessary to distinguish new from preexisting vessels. Normally, there are no vessels above the inner limiting membrane (ILM), so any vessels above the ILM must represent NV. One approach is to perform serial sections, stain every tenth section with a marker for vascular cells, and measure the average vascular cell area above the ILM per section per eye. Given that the only cells invading beyond the ILM are vascular cells, some investigators have counted nuclei above the ILM. These approaches provide a high level of confidence in the measurements but are labor intensive and time consuming, primarily because of the need to cut serial sections through the entire eye. Another approach is to dissect retinas intact, stain whole retinas for vascular markers, and mount the whole retina. Because the normal retinal vasculature is generally stained along with neovascularization, a masked observer must distinguish new from preexistent vessels and outline the new vessels for measurement.

In this study, we sought to determine whether labeling of retinal cells before death facilitated their study in retinal flat mounts. We found that injection of a labeled anti-PECAM1 antibody stained endothelial cells in new and preexistent vessels. Vascular sprouting was observed from superficial vessels, as is widely recognized to occur, and also occurred from vessels deep within the retina. Quantification of intraretinal NV in ocular sections has been previously described. High magnification provided exquisite structural detail, showing bulbous networks of vessels emanating from capillaries and large vessels. Even filopodia, previously described using high-magnification confocal microscopy, were visualized at the tips of sprouts using standard fluorescence microscopy. The fine detail allows easy distinction between new and preexistent vessels by experienced observers, but not by image analysis software. However, injection of unlabeled anti-PECAM1 antibody into the vitreous, followed by postmortem incubation of whole retinas with secondary antibody, resulted in selective staining of new vessels with minimal staining of preexistent vessels or other background. It appears that use of a labeled primary antibody provides greater sensitivity and stains all vessels, whereas use of an unlabeled primary antibody is less sensitive, which allows it to recognize something that is different about the new vessels compared with preexistent vessels. Perhaps...
the upregulation of PECAM-1 or a structural difference allows for greater accessibility, but whatever the difference that allows for selective staining of new vessels, it is a major advantage given that it allows software recognition of NV and completely objective measurement of its area.

In addition to hypoxia-induced gene expression in endogenous retinal cells, mounting evidence indicates that circulating cells from outside the eye, particularly hematopoietic progenitor cells and leukocytes, may also contribute to retinal NV. The role of macrophages in the pathogenesis of ischemia-induc ed retinal NV is uncertain, though it has been postulated that they to contribute to choroidal NV. Coinjection into the vitreous of labeled anti-PECAM1 and anti-F4/80 showed that, compared with normal retinas, ischemic retinas show an increased number of macrophages that localize to regions of vascular sprouting but also to areas of vascular regression. These data support previous studies implicating macrophages in the regression of hyaloid vessels, and they suggest that macrophages may also participate in the growth of new vessels in the eye. In other settings, subpopulations of macrophages have been shown to promote opposing effects, a similar phenomenon may help explain the apparent paradox of opposing macrophage-induced effects on different vessels in proximity in the eye. The fine detail provided by in vivo labeling will be useful to explore potential differences in these two populations of macrophages. The in vivo immunostaining technique can also be used to evaluate for cell surface receptors, as shown by staining for CXCR4, which showed labeling of a subpopulation of the bone marrow-derived cells.

Localization of secreted proteins can be particularly challenging because their constant release from the cells producing them may reduce levels below the limit of detection. However, in vivo immunostaining for PlGF showed a strong signal that colocalized with endothelial cells in new vessels and regressing vessels. Because VEGFR1 is upregulated on endothelial cells participating in NV, the increase in staining for PlGF could be attributed to its interaction with the increased population of VEGFR1 on these cells or PlGF produced by the cells. It is intriguing, however, that PlGF, a chemoattractant for bone marrow-derived cells, localizes to growing and regressing vessels surrounded by bone marrow-derived cells. Additional studies are needed to determine whether PlGF contributes to the dense infiltration of macrophages in these locations.

In summary, we have described a new technique of in vivo immunostaining in the eye that will facilitate quantification of retinal NV and a related technique that shows exquisite structural detail of new vessels. This has allowed us to show that new vessels sprout from deep and from superficial capillaries in ischemic retina and that increased numbers of macrophages associate with growing and regressing vessels. The improved resolution enhances localization of cell surface and secreted proteins, suggesting that molecular characterization of macrophages may determine whether different populations of macrophages are involved in these opposing effects.

References


