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Original Article

Investigation and evaluation of an innovative retinal neovascularization staining- one click staining

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Abstract: The purpose of this study was to investigate and evaluate a fast and convenient approach to improve qualitative and quantitative assessments of retinal vessels and neovascularization (NV). Retinas of mice with oxygen-induced ischemic retinopathy at postnatal day (P) 17 were performed whole mount staining for different period of time to determine the best staining time and antibody dilution that shows retinal NV explicitly. Meanwhile we evaluated the retinal NV and the macrophages of the mice with oxygen-induced ischemic retinopathy at P13, P15, P17, P19, P21, P23 and P25 by in vitro immunostaining. The retinas from the rhodopsin promoter/vascular endothelial growth factor (rho/VEGF) transgenic mice were done as well at P21 which showed sub-retinal NV. Griffonia Simplicifolia Lectin B4 (GSA-Lectin) antibody at 1:50 staining for 45 min showed good resolution of the fine structure of retinal NV with a preferable signal/noise ratio. Double labeling with GSA-Lectin antibody and anti-F4/80 showed exquisite localization of bone marrow-derived cells with respect to the vasculature and demonstrated close association of macrophages with NV and regressing vessels. Lots of NV sprouts were shown on the outer edge of the retina in Rho/VEGF mice by this staining method, facilitating investigators to evaluate the retinal NV through either measuring the area of NV or counting the number of beansprouts-like NV. One click staining facilitates an accurate measure of retinal NV and the detailed structural analysis of retinal NV. This approach serves as a time-saving, stable and easy-to-go method in retinal NV study that is useful for anti-angiogenesis study.

Keywords: Retinal neovascularization, oxygen-induced retinopathy, rho/VEGF transgenic mice, macrophage

Introduction

Angiogenesis, which is the formation of the new vessel, is regulated by the balance of many stimulatory or inhibitory factors [1]. Physiological angiogenesis is under strict control, which is activated only under strictly defined conditions, such as development and tissue repair. However, the disruption of this balanced functioning could lead to the excessive formation of blood vessels. In particular, this pathological angiogenesis also exists within the eye [2], which is the leading cause of vision loss at all ages in a variety of clinical conditions, such as retinopathy of prematurity (ROP), diabetic retinopathy (DR), and age-related macular degeneration (AMD) [3, 4]. Macrophages are key cells both in acute and chronic inflammation. They respond to microenvironmental signals with polarized genetic and functional programs. It is reported that the sub-retinal space is physiologically devoid of macrophages (MΦ) in healthy young adult subjects, which were activated in AMD [5, 6]. ROP, first described in 1942, is one of the major clinical issues for the ophthalmologist. Partial significant retinal NV and retinal detachment characterize the severe forms of ROP [7]. This model is widely used in NV studies, including determining its molecular mechanisms, and in antiangiogenesis studies, particularly in testing antiangiogenesis trial medications. Clearly, an easy, reliable, and repeatable quantitative method is highly necessary.

Most recently, we established a method not only to quantify the retinal vasculature but also

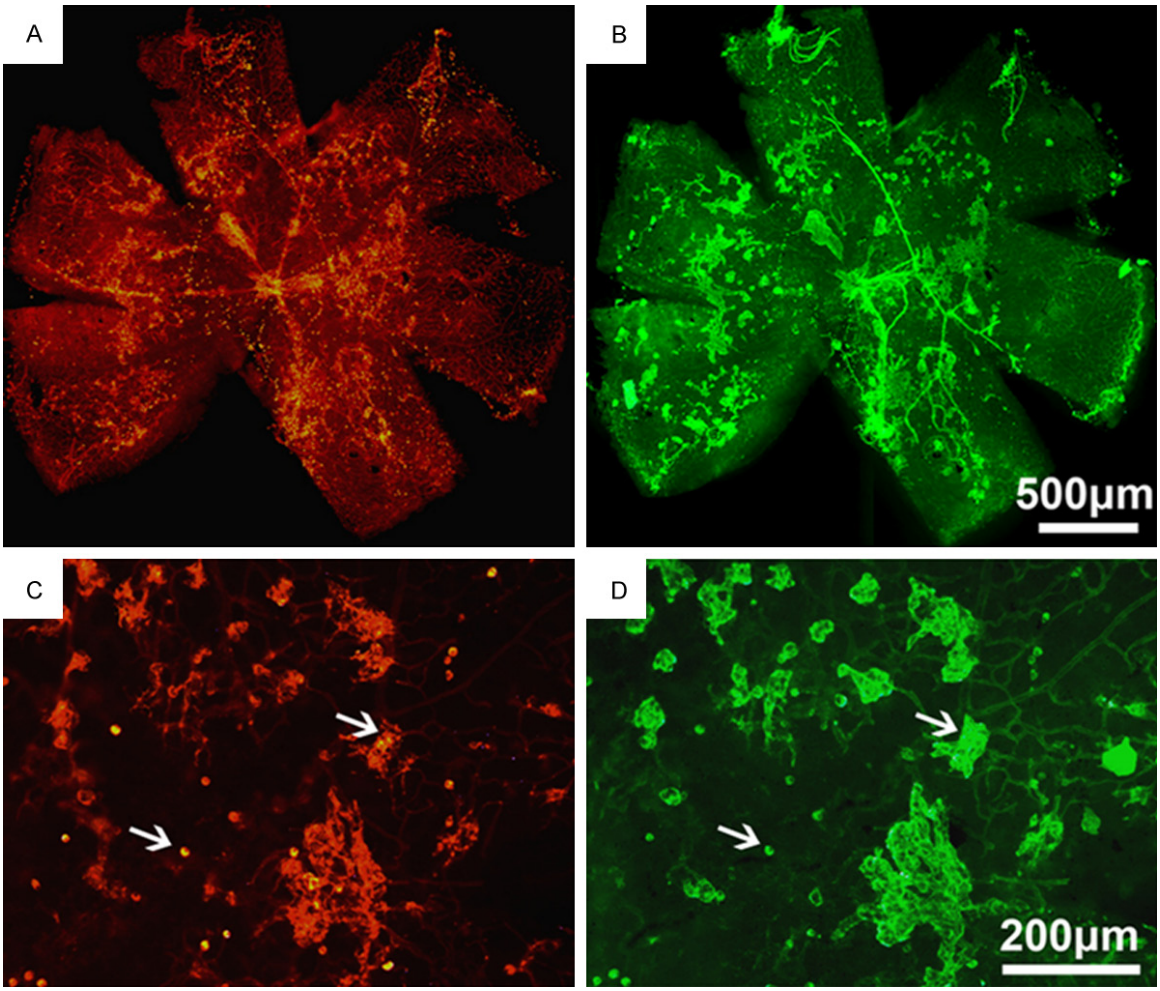


Figure 1. In vivo immunostaining with labeled anti-PECAM-1 antibody showed a close association with the in vitro immunostaining that used labeled GSA-lectin in retinas of mice with oxygen-induced ischemic retinopathy. The retinas from the mice with oxygen-induced ischemic retinopathy were in vivo immunostained with the anti-PECAM-1 antibody, which was labeled with PE (A, C is part of A), and with GSA-lectin, which was labeled with FITC (B, D is part of B). The endothelial cells and neovascularization (arrows) can be stained in the same position.

to reveal the delicate structure of retinal vessels. Briefly, ischemic-induced mice received an intravitreous injection of platelet-endothelial cell adhesion molecule-1 (PECAM-1) at P17, were anesthetized 12 hours later, and were then further stained with fluorescence-labeled secondary antibody. Retinas were flat-mounted and viewed with a fluorescence or confocal scanning microscope, which displayed a high-quality resolution of the fine structure of retinal NV [8]. However, this method requires too many antibodies, and the staining effect is highly dependent on the skills of the intravitreous injection.

As was demonstrated that VEGF increased in the inner retina of ROP mice, Rho/VEGF mice, in which the rhodopsin promoter drives the expression of VEGF in photoreceptors of the retina, develops NV in the outer retina. This model provides an excellent system to investigate early VEGF-induced changes that occur in the retina and ultimately lead to NV, as well as to test the effects of purported angiogenesis inhibitors. In addition, the increased expression of VEGF is sufficient to produce NV that originates from retinal vessels and grows into the sub-retinal space [9].

In this study, we sought to determine whether in vitro labeling, combined with retinal flat mounting and examination by fluorescent microscopy, provides advantages for the visualization of the retinal vasculature. Also, we used

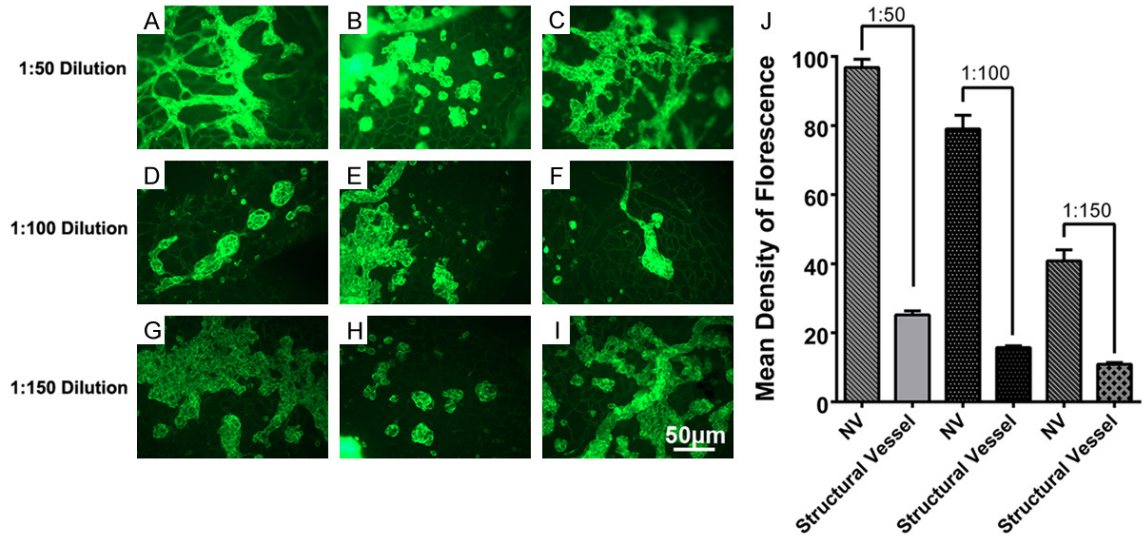


Figure 2. In vitro immunostaining with GSA-lectin (1:50) showed brightest new vessels. The retinas from mice with oxygen-induced ischemic retinopathy were incubated with the FITC-labeled GSA-lectin antibody by different concentrations (1:50 A-C, 1:100 D-F, 1:150 G-I) for 45 min. Mean density of fluorescence of both NV and structural vessels stained by GSA-lectin with 1:50 dilution were higher than 1:100 or 1:150 dilutions (J) (n=5 mice/group).

this staining method to evaluate sub-retinal NV in rho/VEGF mice and association of RNV and macrophage in ROP mouse retinas.

Materials and methods

Mice

Mice were kept under specific pathogen-free (SPF) conditions in compliance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals with the approval (SYXK-2011-0113) of the Scientific Investigation Board of Shanghai Ruijin Hospital affiliated to Shanghai Jiao Tong University School of Medicine, Shanghai, China. All approaches have been applied to minimize pains.

Mouse model of ischemic retinopathy and immunostaining

At postnatal day (P) 7, C57BL/6 mice were placed in 75% oxygen for 5 days and returned to room air at P12. At P17, mice were injected intravitreously with 1 μL of anti-mouse CD31 antibody labeled with PE (eBioscience, San Diego, CA), using a microinfusion pump as previously described [7]. Mice were euthanized 8 to 12 hours after injection. Their eyes were then removed and fixed in phosphate-buffered formalin at room temperature for at least 5 hours. Retinas were dissected intact and washed three times with PBS. Retinas were then incubated with GSA-lectin labeled with FITC (Vector Laboratory, Burlingame, CA) and anti-mouse F4/80 labeled with Phycoerythrin (eBioscience, San Diego, CA). Subsequently, retinas were washed three times with PBS for 3 min and whole-mounted on glass slides using mounting medium (Aquamount; Polysciences, Warrington, PA). Slides were viewed using a fluorescence microscope (Nikon Instruments Inc, New York, NY) with imaging software (SPOT RT 3.4, Diagnostic Instruments, Sterling Heights, MI).

To determine the best stain time, in which retinal NV will be stained but retinal structural vessel will be showed less stain, we performed a timing stain from 30 min to 3 hours. Briefly, eye fixation and retinal dissection were the same as described above. Retinas were stained with GSA-lectin (1:50), which was labeled with FITC, at RT for 30 min, 45 min, 60 min, 120 min and 180 min. After three washes, retinas were viewed by florescence microscopy using 20×. Exposures parameters are exact the same, including exposure time, gamma value, gain and white balance.

To determine the best dilution of GSA-lectin, in which retinal NV will be stained at most but reti-

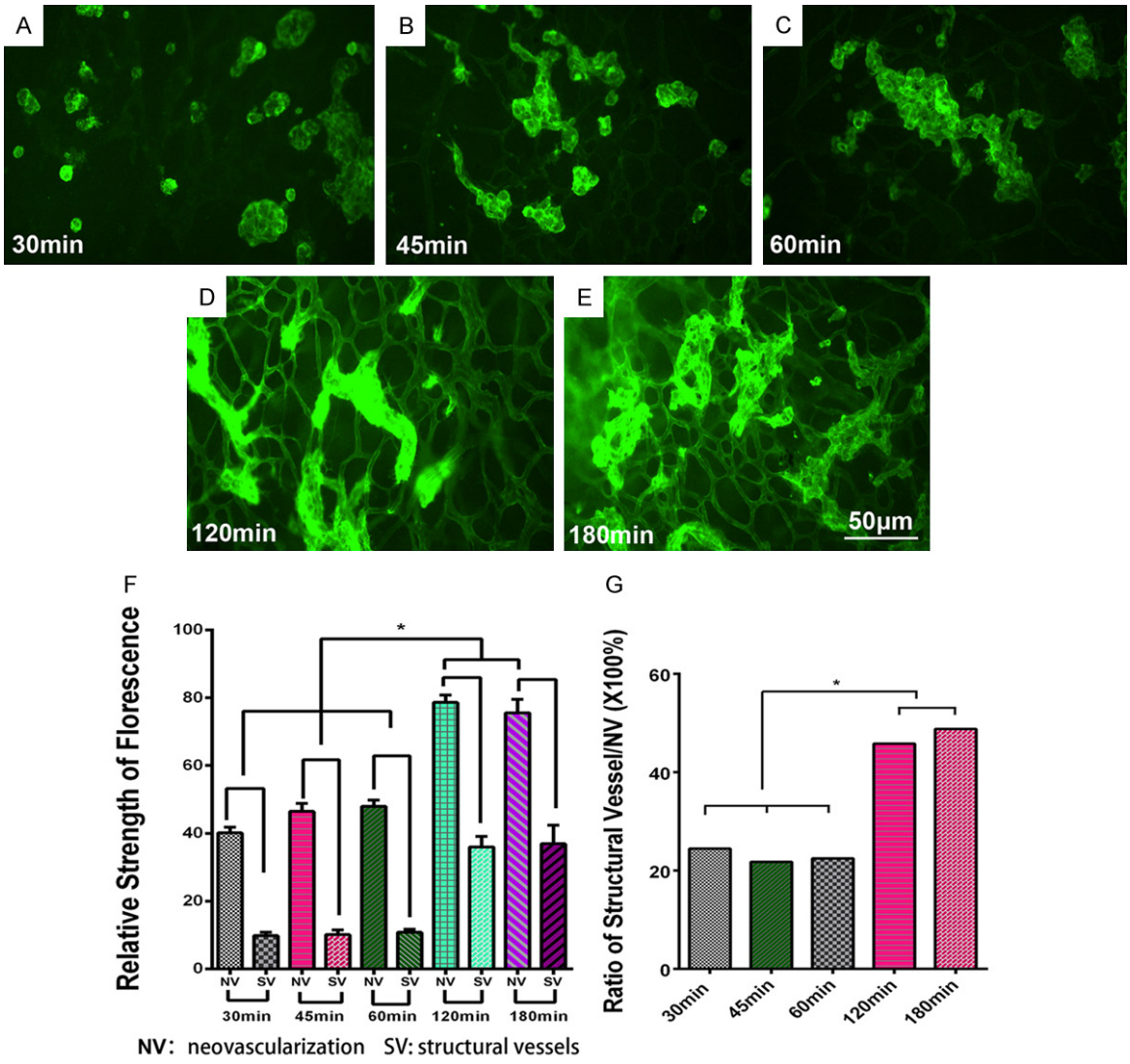


Figure 3. In vitro immunostaining with labeled GSA-lectin for 30 min, 45 min, 60 min, 120 min and 180 min. The retinas from mice with oxygen-induced ischemic retinopathy were incubated with the FITC-labeled GSA-lectin antibody for 30 min (A), 45 min (B), 60 min (C), 120 min (D) and 180 min (E). Relative strength of fluorescence and ratio of structural vessel/NV were shown in (F, G) (*P<0.05) (n=5 mice/group).

nal structural vessels will be showed less stain, we performed a serial antibody dilution from 1:50, 1:100 and 1:150. Retinas were prepared as described above, and stained by the different dilutions of GSA-lectin for 45 min.

Immunostaining of the retinas from rho/VEGF transgenic mice

Rho/VEGF transgenic mice at P21, which showed NV in the sub-retina, were euthanized. Their eyes were then removed and fixed in phosphate-buffered formalin at room temperature for at least 5 hours. Retinas were dissected and incubated with the FITC-labeled GSA-lectin (1:50) for 45 min. Other mice were received intracardiac perfusion with fluorescein-labeled dextran. Retinal flat mounts were examined by fluorescence microscopy, focusing on the NV on the outer edge of the retina [10].

Statistical analysis

Quantitative data were presented as mean ± SEM. Statistical significance was determined using two tailed Student's t-test and one-way ANOVA. *P* values of 0.05 was considered statis-

tically significant. Statistical analysis was performed with SAS9.0 software.

Results

In vivo immunostaining with labeled anti-PECAM-1 antibody showed a close association with in vitro immunostaining with labeled GSA-lectin in retinas of mice with oxygen-induced ischemic retinopathy

At P17, mice with oxygen-induced ischemic retinopathy were injected intravitreously with 1 μL of antibody (PECAM-1), which was labeled with PE. After 8-12 hours, mice were anesthetized and retinas were immunostained with FITC-labeled GSA-lectin for 45 min. In **Figure 1**, the region that was stained with PECAM-1 (**Figure 1A**, **1C** is part of A) was also stained with GSA-lectin (**Figure 1B**, **1D** is part of B), which is the site of retinal structure vessels, NV and endothelial cells.

In vitro immunostaining with GSA-lectin at 1:50 showed brightest new vessels

The retinas from the mice with oxygen-induced ischemic retinopathy were incubated with FITC-labeled GSA-lectin antibody for 45 minutes at 1:50 dilution (**Figure 2A-C**), 1:100 dilution (**Figure 2D-F**) and 1:150 dilution (**Figure 2G-I**). GSA-lectin at 1:50 dilution showed brightest NV as well as structural vessels than the other dilutions (**Figure 2J**).

In vitro immunostaining with labeled GSA-lectin for 45 min showed the preferable signal/noise ratio

The retinas from the mice with oxygen-induced ischemic retinopathy were incubated with FITC-labeled GSA-lectin antibody for 30 minutes (**Figure 3A**), 45 minutes (**Figure 3B**), 60 minutes (**Figure 3C**), 120 minutes (**Figure 3D**) and 180 minutes (**Figure 3E**). Retinas that were incubated for 30 min only showed vapory vessels, whereas retinas that were incubated for longer time showed stronger fluorescence of both new vessels and structural vessels. Relative strength of fluorescence and structural/NV ratio were shown in **Figure 3F-G**. Incubation for 45 minutes and 60 minutes resulted in significantly lower ratio of structural vessels/NV, indicating a preferable signal/noise ratio (*P<0.05).

Using different magnifications, in vitro immunostaining with labeled GSA-lectin showed the delicate structure of retinal vessels

Using different magnifications (**Figure 4**), ischemic retinas, which were stained with FITC-labeled GSA-lectin antibody, showed many small tufts of new vessels in the far periphery of the retina (**Figure 4A**). The small tufts of new vessels originated from the periphery capillary bed (**Figure 4B**). Focusing down into the retina allowed the visualization of new vessels, which originated from the deep capillary bed (**Figure 4C**). Smaller clumps of NV were observed distally along the vessels. There was even sufficient resolution to observe filopodia at the tips of new vessel spouts (**Figure 4D**). The low-magnification view of the optic nerve head was distinctly stained with FITC-labeled GSA-lectin, and endothelial cells overflowed from the hyaloid vessels (**Figure 4E**). The primary vessels and the hyaloid vessels around the optic nerve head were distinctly observed at high magnification (**Figure 4F**).

In vitro immunostaining with labeled anti F4/80 antibody showed close association with retinal NV in mice with oxygen-induced ischemic retinopathy for different days

Retinal flat mounts of P17 control mice incubated with PE-labeled F4/80 antibody and the mice with oxygen-induced ischemic retinopathy showed macrophages scattered throughout the retina (**Figure 5A**, **5B**). Comparing with the moderate number of macrophages seen in the retinas of normal P17 mice (**Figure 5B**), retinas from mice with ischemic retinopathy showed a high density of macrophages throughout the retina (**Figure 5A**). At high magnification, the exquisite structure of macrophages can be stained by both PE-labeled F4/80 antibody (**Figure 5C**) and FITC-labeled GSA-Lectin antibody (**Figure 5D**), and 5E was merged of 5C and 5D. Both the retinal NV and the macrophages on the surface of the retinas of the mice with oxygen-induced ischemic retinopathy that were euthanized at P13 (**Figure 5F**, **5K**), P15 (**Figure 5G**, **5L**), P17 (**Figure 5H**, **5M**), P19 (**Figure 5I**, **5N**), P21 (**Figure 5J**, **5O**), P23 (**Figure 5P**, **5R**) and P25 (**Figure 5Q**, **5S**) showed significant difference. The retinal NV increased with the macrophages over time, showed in **Figure 5T**, **5X**.

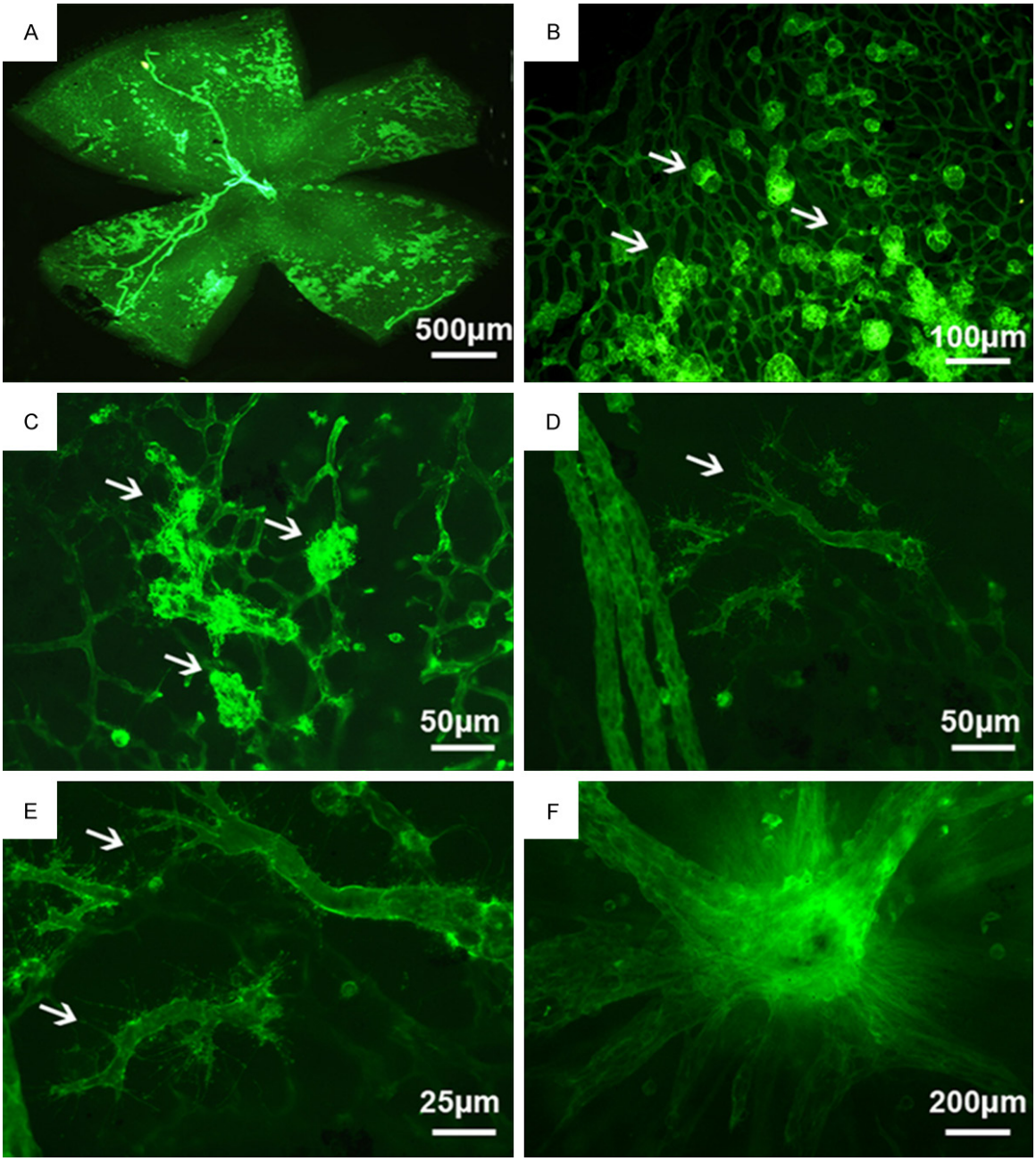
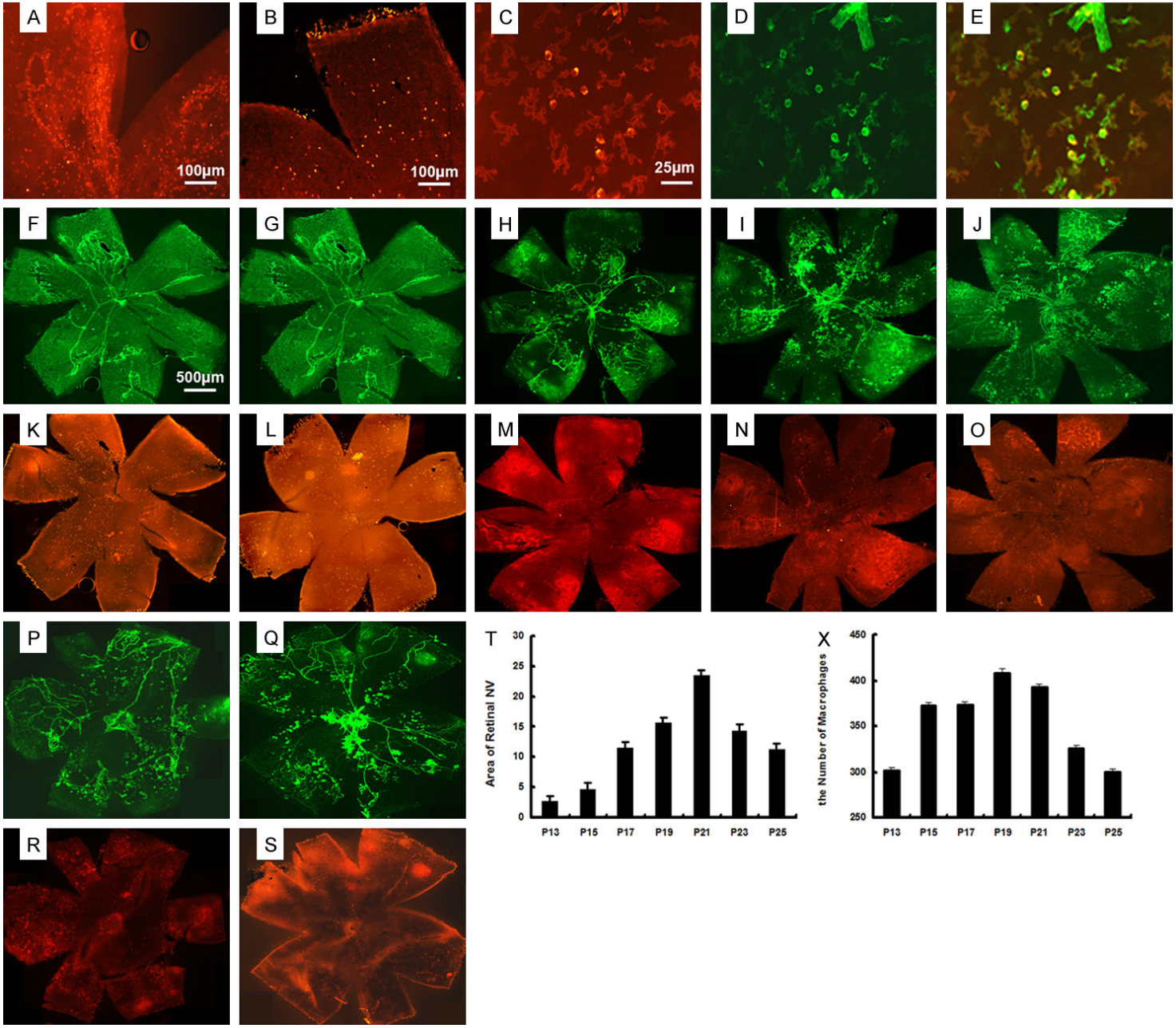


Figure 4. High-resolution imaging of retinal NV by in vitro immunostaining. A: Ischemic retinas from eyes that were stained with the FITC-labeled GSA-lectin antibody show bulbous networks of NV, which originated from retinal capillaries. B: Many small tufts of new vessels appeared in the far periphery of the retina. C: The small tufts (arrows) of new vessels originated from the periphery capillary bed. D, E: Focusing down into the retina allowed the visualization of new vessels (arrows) originating from the deep capillary bed. Filopodia (arrows) were observed at the tips of new vessel sprouts. F: The large vessels and hyaloid vessels around the optic nerve head were distinctly observed under high-magnification.

In vitro immunostaining in rho/VEGF transgenic mice showed sub-retinal NV

Twenty-one-day-old rho/VEGF transgenic mice, which showed NV in the sub-retina, were perfused with fluorescein-labeled dextran (**Figure 6A-C**) or their retinas were dissected and incubated with labeled antibodies GSA-lectin (1:50) for 40 min (**Figure 6D-F**). Retinal flat mounts were examined by fluorescence microscopy, focusing on the NV on the outer edge of the retina. Using different magnifications, the in


A
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Area of Retinal NV
30
25
20
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10
5
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X
the Number of Macrophages
450
400
350
300
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P13
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P25

Figure 5. In vitro immunostaining with labeled anti F4/80 antibody shows close association with retinal NV in mice with oxygen-induced ischemic retinopathy for different days. The retinas from the mice with oxygen-induced ischemic retinopathy and the control mice were incubated with labeled F4/80 antibody and labeled GSA-Lectin antibody for 45 min, respectively. Comparing with the moderate number of macrophages seen in the retinas of normal P17 mice (B), retinas from mice with ischemic retinopathy showed a high density of macrophages throughout the retina (A). At high magnification, the exquisite structure of macrophages can be stained by both PE-labeled F4/80 antibody (C) and FITC-labeled GSA-Lectin antibody (D), and (E) was merge of (C and D). Both the retinal NV and the macrophages on the surface of the retinas of the mice with oxygen-induced ischemic retinopathy that were euthanized at P13 (F, K), P15 (G, L), P17 (H, M), P19 (I, N), P21 (J, O), P23 (P, R) and P25 (Q, S) showed significant difference. The retinal NV significantly increased from P17, reached a peak at P21, then decreased (T, $P<0.05$). The macrophages were not significantly increased as NV was (X). They significantly increased at P19 and P21 ($P<0.05$) (n=5 mice/group).

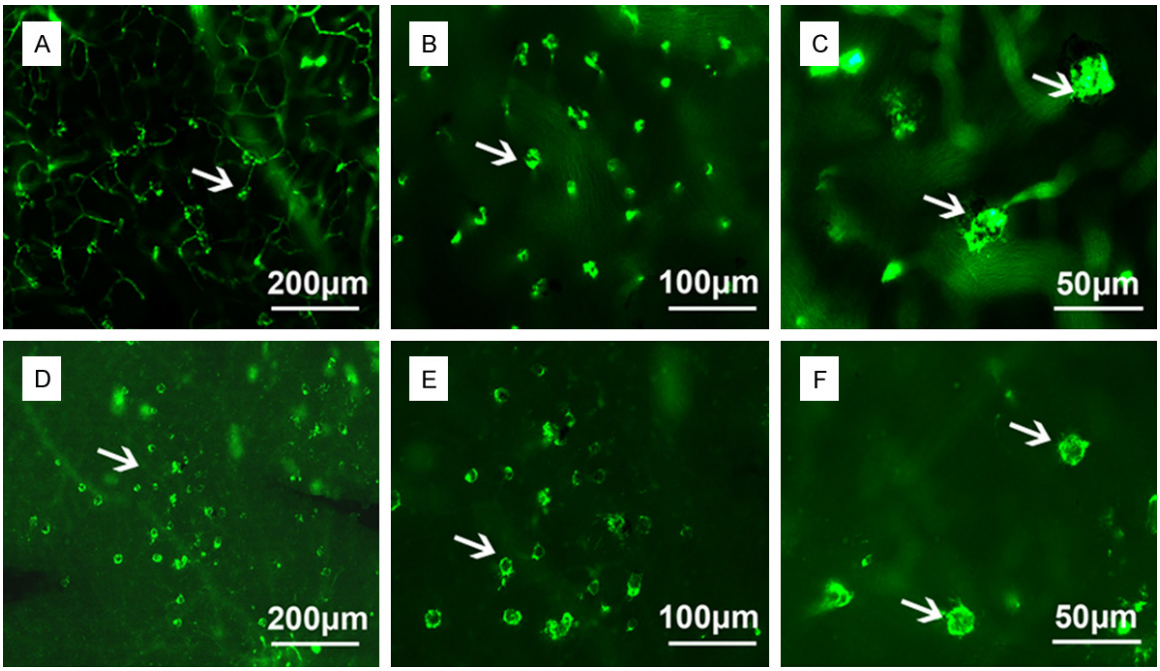

Figure 6. In vitro and in vivo immunostaining in rho/VEGF transgenic mice shows sub-retinal NV. Twenty-one-day-old rho/VEGF transgenic mice were perfused with fluorescein-labeled dextran (A-C) or retinas were whole-mounted and incubated with the labeled antibody GSA-lectin (1:50) for 45 min (D-F). Both methods showed clear differentiable NV sprouts (arrows) from the outer edge of the retina, allowing easy identification and delineation.

vitro staining also showed the detailed structure of the sub-retinal NV.

Discussion

New blood vessel formation or NV is essential for normal eye development. However, this process can also cause severe ocular disease. In the retina, NV is associated with several disease processes. The occlusion of retinal vessels, which leads to retinal ischemia, is a feature that is shared by most diseases in which retinal NV occurs. This observation led to the hypothesis that the development of retinal NV was stimulated by one or more angiogenesis factors, which are released by the ischemic retina [11]. Previously, experimental animal models of retinal NV have been well designed and established, which allowed investigators to observe the high-resolution images of NV and qualitatively assess the extension of NV formation. Oxygen-induced retinopathy in the mouse is a highly reproducible animal model of ROP, which is based on the hyperoxia-induced vaso-obliteration of capillaries in mouse pups and their subsequent return to room air [12].

Several methods have been utilized since the establishment of this model. Briefly, these methods are classified into three different approaches: perfusion, section staining and whole-mount staining. For the section staining,

a commonly used method is the quantification of the nuclei or lumens of vascular cells to reflect the retinal NV. Using image-analysis software, retinal NV was determined by measuring the vessel protruded into the vitreous cavity in at least 10 sections in one eye. This method is particularly time-consuming, and there are too many artificial factors. Additionally, the inner limiting membrane of the retina must be identified. Another commonly used method is perfusion using India ink, fluorescein isothiocyanate-dextran (FITC-dextran), or Evans Blue. FITC-dextran perfusion can show the entire retinal vascular structure, including the vessel structures and NV, and remains a good method to distinguish the ischemic areas. The traditional whole staining method [13] is time-consuming and shows too many structural vessels, making it more difficult to distinguish structural vessels from NV.

In the retina, neovascular sprouting was observed in superficial vessels, which is widely recognized to occur, and in deep vessels within the retina [14]. Based on our previous introduction to in vivo staining for qualitatively and quantitatively assessing retinal vessels and NV, in this study, we described the further developed in vitro staining techniques, which have been applied to improve the identification and evaluation of the measurement method for the area of the NV. The sprouts protrude in the surface of the retina from the inner limiting membrane; thus, it is easy to control staining time and to show the new NV. With different magnifications, the new method using labeled GSA-lectin showed the delicate structure of retinal structure vessels and NV, which the anti-PECAM-1 antibody stains. Using this staining method, it was demonstrated that RNV in OIR mice showed close association with macrophages as angiogenesis progressing. This staining technique displayed a large clump of NV growing from large deep vessels in the posterior part of the retina. In addition, the appearance of a fungus on a tree branch and smaller clumps (NV) were observed distally along the vessels. Even filopodia, which were previously described using high-magnification confocal microscopy, were visualized at the tips of sprouts using this simple staining technique and standard fluorescence microscopy.

For several years, laboratory studies have demonstrated that a few of the molecular and cellular players in the tumor/microvascular microenvironment, for instance, vascular endothelial growth factor (VEGF), placental growth factor (PlGF), basic fibroblast growth factor (bFGF) [15-17] and so on, play central roles in several retinal vascular diseases. Clinical trials are now confirming the importance of VEGF in disease pathogenesis and are providing new treatments for diseases that we could not treat previously. The demonstration that VEGF is upregulated by hypoxia [18, 19] and that levels of VEGF are increased in the retina and vitreous of patients [20-23] or laboratory animals [23, 24] with ischemic retinopathies suggests that VEGF is a potential mediator of retinal angiogenesis. This hypothesis is supported by studies that VEGF antagonists partially inhibit retinal or iris NV in animal models. There are also many data indicating that VEGF can stimulate NV in other tissues [25-28].

In summary, we have adopted a new strategy and staining technique, which allows the direct observation of retinal vessel structures and NV in detail. In the present study, we also examined the preferable antibody concentration and signal/noise ratio so as to improve qualitative and quantitative assessments of retinal vessels and NV, which facilitates the determination of the effects of gene therapy or other biological agents in pre-clinic animal experiments. This approach provides a high level of confidence in measurements, does not require labor-intensive and time-consuming tasks, and can reduce experimental expenses. Due to the notable split-second work, the entire staining work takes less than one hour and is tentatively named “One Click Staining”.

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Disclosure of conflict of interest

None.

Authors’ contribution

Conceived and designed the experiments: B.X, L.Z., Performed the experiments: Y.Z., Q.L., Y.G.

Analyzed and interpreted data: Q.J., Y.C, X. S. and drafted the paper: Y.Z., Q.L. Contributed reagents/materials/analysis tools: Y.Z., X. S.

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