Time-Lapse Imaging of Vitreoretinal Angiogenesis Originating from Both Quiescent and Mature Vessels in a Novel Ex Vivo System

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PURPOSE. Diabetic retinopathy (DR) is an angiogenic disease that leads to severe visual loss. However, adequate animal models of vitreoretinal neovascularization in proliferative diabetic retinopathy (PDR) have not yet been described. The purpose of this study was to develop a novel ex vivo system for assessing vitreoretinal angiogenic processes that originate from both quiescent and mature vessels that could be observed with time-sequential imaging.

METHODS. The retinas of 7- to 8-week-old mice were cultured for 4 days, with or without several growth factors with novel procedures, and immunohistochemistry was performed. The retinas from Tie2-GFP mice were cultured with vascular endothelial growth factor (VEGF), and time-sequential imaging of vitreoretinal angiogenesis was acquired.

RESULTS. Vascular sprouts were induced by both VEGF and placenta growth factor, but not by insulin-like growth factor-1, basic fibroblast growth factor or angiopoietin-2. In explants with or without VEGF, perivascular mural cells were dissociated from endothelial cells, which is an important step during angiogenesis and in the progression of DR. Furthermore, use of time-lapse observations of retinal neovascularization events visualized that the first step in vascular sprout emergence from quiescent vessels was a single cell extension. The leading edges of a sprouting endothelial cell extended and retracted in a sequential manner. From newly formed vessels, additional vascular sprouts then emerged and new vessels fused to each other, resulting in vascular branching.

CONCLUSIONS. Time-lapse imaging of this system visualized the dynamic process in vitreoretinal neovascularization from quiescent and mature vessels. (Invest Ophthalmol Vis Sci. 2006; 47:5529–5536) DOI:10.1167/iovs.06-0573
MATERIALS AND METHODS

Animals

All animal procedures in this study were performed in accordance, both with the guidelines for animal experiments at the Kyoto University Graduate School of Medicine and with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. C57BL/6j mice were purchased from SLC (Shizuoka, Japan). For time-lapse imaging, Tie2-GFP mice, which were backcrossed to the C57BL/6j strain, were obtained from the Jackson Laboratory (Bar Harbor, ME).

Materials

Recombinant growth factors were all obtained from R&D Systems (Minneapolis, MN). Monoclonal antibodies were also purchased for platelet and endothelial cell adhesion molecule (PECAM)-1 (BD-Phar-Mingen, San Diego, CA), smooth muscle actin (SMA; Sigma-Aldrich, St. Louis, MO) and desmin (DAKO Corp., Glostrup, Denmark). Polyclonal antibodies against type IV collagen were obtained from Chemicon International (Temecula, CA).

Retinal Explant Cultures

We modified a method for culturing retinal explants that is described elsewhere. Briefly, the eyes were isolated from 7- to 8-week-old mice and transferred to PBS. Neural retinas attached by the vitreous were carefully dissected from the sclera, choroid, and retinal pigment epithelium and placed on a chamber filter (diameter, 30 mm; pore size, 0.4 μm; Millicell; Millipore, Bedford MA) with the ganglion cell layer positioned upward. Additional procedures were then undertaken to cover the retina with type I collagen gel (CellMatrix type1-A; Nitta gelatin, Tokyo, Japan), which contained the same concentration of horse serum and minimum essential medium (MEM; Invitrogen, Carlsbad, CA) as the culture medium. The chamber was transferred to a six-well culture plate in which each well contained 2 mL of culture medium, in the absence or presence of growth factors. Explants were cultured at 34°C in 5% CO₂ and the media were changed on every second day.

Immunohistological and Histologic Analysis

The retinal explants were first fixed in 4% paraformaldehyde in PBS for 10 minutes and permeabilized by Triton X-100. They were then subjected to immunohistochemical procedures, as described previously. The flatmounts were observed by a laser scanning microscope (model LSM5 Pascal; Carl Zeiss GmbH, Oberkochen, Germany). To generate cryosections of the retinal explants, the retinas were incubated with 25% sucrose in PBS for 30 minutes on ice, frozen in OCT compound (Sakura Finetek, Torrance, CA), and sectioned at a thickness of 16 μm. The cryosection underwent staining with hematoxylin-eosin or immunohistochemical staining with counterstaining by diaminido-2-phenylindole (DAPI).

Quantification of Neovascular Sprouts and Statistical Analysis

On preparation of the flatmounted retinal explants, we performed five lines of incisions which produced five lobes of the retina. After organ culture, these retinas were stained with PECAM and type IV collagen. PECAM is believed to be expressed in both quiescent vessels and angiogenic vessels, whereas the expression of type IV collagen is localized to mature vessels and is not found around neovascular sprouts until they have matured. Additional characteristics of neovascularization include filopodial extensions and the formation of cord-like or tubelike structures. We then defined PECAM-positive and type IV collagen-negative cordlike or tubelike structures as vascular sprouts, as these could emerge without growth factor stimulation.

Newly formed vessels were subjected to three independent blind counts. For each treatment group, from 6 to 10 retinas were analyzed. The results shown are expressed as the mean ± SD, unless otherwise indicated. For statistical analysis, we used the Student’s t test or analysis of variance to compare quantitative data populations with normal distribution and equal variance. Data were analyzed by using the Mann-Whitney rank sum test or the Kruskal-Wallis test for populations with non-normal distributions or unequal variance. P < 0.05 was considered statistically significant.

Time-Lapse Imaging

We performed time-sequential imaging of retinal angiogenesis using Tie2-GFP transgenic mice, in which the Tie2 promoter drives eGFp expression in a vascular endothelial cell-specific manner. We prepared flatmount retinas as described earlier in this section and incubated them for several days. To observe live images, we cut out the filter membrane of the chamber filter containing the retina, and then placed it with ganglion cell layer downward on a glass-bottomed dish (Matsunami Glass, Osaka, Japan). The retinal explant on the glass was then incubated under the same conditions used for retinal explant cultures in culture medium containing 25 ng/mL VEGF. We obtained confocal images using a laser scanning microscope (LSM 5 Pascal with a 20× objective; Carl Zeiss Meditec GmbH). The 488-nm laser line was applied to GFP imaging, and images were recorded every 15 minutes. Lower laser power was used to minimize phototoxicity.

RESULTS

VEGF-Induced Vascular Sprouting from Quiescent and Mature Vessels

To establish a new observation system for vitreoretinal angiogenesis from quiescent vessels, we performed ex vivo experiments with the mouse retina. After culturing without VEGF for 4 days, retinal endothelial cells, which was immunostained with PECAM, were slightly regressed, particularly in the capillaries, and the veins were narrowed (Fig. 1C) compared with the vasculature at the start (Fig. 1A). Vascular sprouts were not observed in the retinal explants without VEGF (Fig. 1C), whereas after treatment with 25 ng/mL VEGF for 4 days, sprouting of endothelial cells, originating from quiescent and mature vessels, was found to have emerged, and the veins were enlarged in an irregular and uneven manner (Figs. 1B, 1D). To confirm whether vascular endothelial cells penetrate the vitreous, as observed during neovascularization in PDR, we used frozen sections and performed immunohistochemistry. Some PECAM-positive cells were observed to have invaded the vitreous (Fig. 1E), although all endothelial cells were observed in the retina when no VEGF had been administered (Fig. 1F).

Characterization of the Vascular Sprout

To characterize newly formed vessels in our mouse retinal explants, we focused on endothelial cells, perivascular mural cells, and the basement membrane. Both mature and quiescent vessels use the basement membrane as a scaffold, whereas newly formed vessels are thought to induce the degradation of the extracellular matrix (ECM). In our current system, we found that quiescent vessels with or without VEGF were positively stained with anti-type IV collagen antibodies, which is a common marker of the basement membrane, whereas newly formed vessels were not (Figs. 1G, 1H). In addition, sprouting endothelial cells perforated the basement membrane through its breaks (Fig. 1G).

We also assessed the interaction between endothelial cells and perivascular mural cells and found that some of the
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A Time- and Dose-dependent Response in Sprouts

We performed a time course study and found that, in retinal explants treated with 25 ng/mL VEGF, there was no neovascularization until 48 hours (Fig. 2B). Vascular sprouts then developed at 72 hours (Fig. 2C), and a subsequent increase in both number and size was observed at 96 and 120 hours (Figs. 2E, 2F). We did not find any newly formed vessels in these explants at the baseline (Fig. 2A) or at 96 hours without VEGF (Fig. 2D). We determined the number of vascular sprouts as described in the Methods section, and showed that the number of vascular sprouts was increased over the period from 72 to 120 hours (Fig. 2G). To determine the possible dose-dependent response, we then incubated the retinal explants for 96 hours with the indicated doses of VEGF. The number of retinal vascular sprouts was found to increase gradually with the concentration of VEGF, with the maximum effects observed at the 25-ng/mL dose (Fig. 2H).

We performed a histologic analysis and found that some cells were observed over the inner retinal border in the explants treated with VEGF for 84 hours (Fig. 2I). After the 120-hour incubation with VEGF, several cells appeared to be in the vitreous, the structure of ganglion cell layer and nerve fiber layer were loose and thick, and the inner retinal border was blurred (Fig. 2J), compared with the retinas without VEGF (Figs. 2K, 2L).

Angiogenic Effects of PlGF, IGF-1, or bFGF

bFGF2,20 and IGF-13,21 have been investigated previously for their role in angiogenesis and have been thought to be exacerbating factors in PDR. We investigated whether bFGF or IGF-1 increases angiogenesis in our current model system, and found that neither factor appreciably promoted retinal neovascularization (Figs. 3A, 3B), compared with the explants treated with VEGF (Fig. 2E). The extent of vascular sprouts was increased slightly by IGF-1 or bFGF exposure, but to a significantly smaller degree than VEGF (Fig. 3G). However, bFGF-, IGF-1-, and VEGF-induced neovascularization was observed to be more amplified than VEGF-induced new vessels (Figs. 3D, 3E, 3H). These data suggest that only VEGF can induce the initial step of vascular sprout and that the angiogenic effects of bFGF and IGF-1 occur only in the presence of VEGF.

Recent reports have elucidated that placenta growth factor (PlGF) exacerbates pathologic angiogenesis, without any effects on physiological cardiovascular development.22,23 We further, investigated the angiogenic potential of PlGF in our ex vivo system. PlGF was found to induce vascular sprouting, with the maximum effects observed at the 50-ng/mL dose, which was equivalent to the magnitude of the response to 25 ng/mL VEGF (Fig. 3G). However, each sprout induced by PlGF appeared to be smaller than the corresponding sprouts induced by VEGF (Figs. 3C, 3F).

Figure 1. VEGF induced vascular sprouting from both quiescent and mature vessels in retinal explants. Immunofluorescent staining was performed with anti-PECAM antibodies (A–D). Frozen sections were stained with anti-PECAM (red) and type IV collagen (green) antibodies, followed by counterstaining with DAPI (blue) (E, F). (A) Retinal explant at 0 hour. The normal architecture of the retinal vasculature is conserved. Scale bar: 200 μm. (B, E) Retinal explant treated with 25 ng/mL VEGF at 96 hours. Vascular sprouts were observed and originated from the optic disc, arteries, and veins (B). (E) Endothelial cells (arrow) migrated over the inner limiting membrane (ILM; arrowhead). (C, F) Retinal explants without VEGF at 96 hours. Retinal vessels regressed slightly, particularly the capillaries (C). Retinal vessels were found in the retina, but not the vitreous (F). (D) Magnified images of the explants treated with VEGF at 96 hours. New vessels formed cordlike or tubelike structures. (G–L) Double immunofluorescent staining for PECAM, type IV collagen, desmin, and SMA. Retinal explants treated with 25 ng/mL VEGF (G, I, K) and without VEGF (H, J, L) are shown. (G) Endothelial cells of vascular sprouts emerged through the breaks of the basement membrane in the parent vessels (arrowbeads). Newly formed vessels were not therefore sheathed by basement membrane. (H) In the explants without VEGF, the endothelial cells were completely covered with basement membrane. (I, J) Several desmin-positive mural cells were found to be dissociated from endothelial cells in the explants, with (I) or without (J) VEGF. (K, L) Several SMA-positive mural cells were also observed to be detached from PECAM-positive endothelial cells, in the presence (K) or absence (L) of VEGF. Scale bars: (A–C, K, L) 200 μm; (D–I) 100 μm.
Effect of Ang-2

Angiopoietins have an important role in vascular sprouting. Ang-2 especially is assumed to induce sprouting or regression in the presence or absence of VEGF, and we evaluated the effects of Ang-2 in our ex vivo system. Ang-2 induced a few vascular sprouts (Figs. 4A–4D), whereas Ang-2 increased vascular sprouts in the presence of VEGF (Figs. 4C–4D), compared with only VEGF (Fig. 4B). Furthermore, to investigate whether Ang-2 induces regression of new vessels once they have sprouted, the explant was incubated with VEGF for 96 hours with the indicated doses of VEGF, and the neovascularization events were measured (n = 6–8). *P < 0.01 vs. 0 ng/ml. (I–L) The cryosection was stained with hematoxylin and eosin. The explants were incubated with VEGF for 84 (I) or 120 (J) hours, or the explant without growth factors for 84 (K) or 120 (L) hours.

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To analyze time-sequential phenomena in our present system, we investigated angiogenesis in retinas isolated from TIE2-GFP transgenic mice. First, we confirmed that eGFP-positive cells could be stained using anti-PECAM antibodies, in both quiescent and newly formed vessels (Fig. 5A). We then put the isolated retinas on glass-bottomed dishes after 84 hours of culturing and performed time-lapse imaging at intervals of 15 minutes (Fig. 5C). We observed time-sequential images of vascular sprouts from quiescent and mature retinal vessels. In addition, the cells containing GFP in organelles like lysosomes were moving rapidly and may be microglia (Fig. 5C).

We initially focused on the first events that occur during vascular sprouting, and determined that the single process extension of only one endothelial cell, and not the coordina-
time-sequential imaging of newly formed vessels originating from quiescent and mature vessels in the retina. In this system, we were able to describe the cytosolic leading edge and main cell body containing the nucleus in both parent vessels and angiogenic daughter vessels.

To confirm that the vascular sprouts consisted of only a single cell, we detected the GFP-positive cells by counterstaining the nucleus with propidium iodide (PI). The early vascular sprout was found to contain only one nucleus (Fig. 5B), which suggests that vascular sprouting in this system indeed begins with a single cell extension.

**Extension and Retraction of the Leading Edges.** In neovascularization of the transgenic zebrafish, filopodial extension and retraction were observed. In comparison, one leading edge after another was found to be developed in most endothelial cells during neovascularization in our present ex vivo system (Fig. 6A, Movie 4). In our current experimental model, after the extension and retraction of several leading edges, one of these generated the primary leading edge, which indicated the subsequent destination of the endothelial migration.

**Branching and Fusion of Sprouting Endothelial Cells**

Many studies have demonstrated that after the formation of the primary vascular plexus, endothelial cells experience remodeling processes in which sprouting, branching, fusion, pruning, and intussusception can be observed. We wanted to investigate the manner in which endothelial cells behave after sprouting from preexisting vessels and thus obtained time-sequential images of the branching of newly formed vessels, which had sprouted from quiescent vessels. The leading edges of the branched endothelial cells were observed to extend and retract as described in Figure 6A, until some of them proceed into the ECM and met other new vessels, which resulted in the fusion of endothelial cells (Fig. 6B and Movie 5). However, the intussusception and pruning of the new vessels are observed in vascular development, and the intussusception was reported in the preretinal neovascularization in the primate retinas treated with VEGF, whereas these processes were rarely observed in our present system.

**DISCUSSION**

The development and progress in vascular biology have elucidated the several processes of angiogenesis, especially of vascular sprouting. In our present study, we describe a new ex vivo system for studying vitreoretinal angiogenesis, and we can observe the time-sequential images of newly formed vessels from both mature and quiescent vessels. Whereas this angiogenic model has several features compared with previous models, we have to stress some noteworthy limitations to the use of this ex vivo model. These include the absence of any blood flow and the lack of circulating endothelial progenitors, leukocytes, or hormonal factors, which would play an important role in angiogenesis. Despite the limitation, we showed that both mature and low-turnover vessels in the retina possess angiogenic potential. In addition, because circulating progenitor cells could not be recruited in our model, we assumed that vascular endothelial cells in retinal quiescent and mature vessels would be sufficient for sprouting from parent vessels.

The induction of adenoviruses encoding VEGF into the rodent vitreous has been found to induce iris neovascularization but not retinal neovascularization, but we observed vitreoretinal angiogenesis in our system. Among the several reasons for the differences, we focused on endothelial cell-mural cell (EC-MC) interactions, and found that mural cells are dispersed from endothelial cells, which may partially depend on the absence of blood flow. The hemodynamic forces are thought to increase transforming growth factor (TGF)-β and platelet derived growth factor (PDGF)-BB, which may play a key role in the recruitment and maintenance of mural cells. It is thought that the first step in vascular sprouting...
or DR progression is the disruption of the EC-MC interaction. Now, we could speculate that both the disturbance of EC-MC interaction and the presence of VEGF are necessary for endothelial cells to sprout from the parent vessels in vitreoretinal angiogenesis.

Several time-sequential angiogenic models in vascular development have already been developed. In these models, vascular development is documented, whereas our current model showed active neovascularization originating from both mature and quiescent vessels. In addition, time-lapse imaging enabled us to observe the initial events in classic angiogenesis. The first step of neovascular sprouting from the adult mouse retinal vasculature was found to be a single cell extension, which has not been elucidated previously. The leading edge of
endothelial cells, but not quiescent vessels, were fused to each other and lumenized. The neovascularization events in our retinal explants seemed to be disordered, although retinal vascular development was observed to be well-organized and amply coordinated. These differences may therefore depend on the distribution of VEGF. In our current model, VEGF was diffusely distributed. These differences may therefore depend on the distribution of VEGF. In our current model, VEGF was diffusely distributed. These differences may therefore depend on the distribution of VEGF. In our current model, VEGF was diffusely distributed. These differences may therefore depend on the distribution of VEGF. In our current model, VEGF was diffusely distributed.

The extension and retraction of the leading edges and branching and fusion of sprouting endothelial cells. (A) One after another, the leading edges of a sprouting endothelial cell (arrowhead) were observed to extend and retract, and one of these edges was then followed by the migration of the main cell body. After 84 hours of organ culture, the explant was analyzed with time-lapse imaging, which is available as Movie 4. The images shown were selected from individual frames of this movie and are labeled with the time from the start of the time-lapse imaging. (B) Time-lapse images showing that from one sprout, another sprouting endothelial cell emerged and formed a branching structure (arrowhead). Two sprouting tips of endothelial cells, but not quiescent vessels, were fused to each other (arrow). The first frame of this movie was taken after 84 hours of organ culture. The entire time-lapse movie is available as Movie 5. Scale bar: 50 μm.

The neovascularization events in our retinal explants seemed to be disordered, although retinal vascular development was observed to be well-organized and amply coordinated. These differences may therefore depend on the distribution of VEGF. In our current model, VEGF was diffusely prevalent in the retina, whereas in the developing retina, VEGF is thought to increase in the avascular zone and decrease rapidly as this region becomes vascularized. Also, there was endothelial sprouting from parent vessels, whereas we rarely observed intussusception of neovascular vessels in our ex vivo system without hemodynamic forces. Both of them have been reported to induce the arborization of the vascular network.

We investigated whether IGF-1 and bFGF would increase the levels of vascular sprouting in our system and found that neither of them induced many sprouts. However these factors could promote neovascular sprouting when induced by VEGF. These data suggest that stimulatory events that are specific to VEGF induce the sprouting of endothelial cells from parent vessels and that IGF-1 and bFGF may impact on neovascular endothelial cells, which could exacerbate the extent of diabetic retinopathy. In contrast to these factors, PlGF also induced vascular sprouts in our system. We speculate therefore that this process of vascular sprouting may depend on Flt-1 stimulation, as PlGF and VEGF share this receptor in common.

The effects of Ang-2 in our system were complicated. Ang-2 increased vascular sprouts in the presence of VEGF, whereas the regression induced by Ang-2, which was shown in the previous report, was not evident. The Ang-Tie2 system modulates both sprouting in endothelial cells and EC-MC interaction, and the latter was disturbed in our system. It remains to be investigated how the Ang-Tie2 system affects vascular sprouting and regression both directly and indirectly.

In conclusion, we have developed for the first time, a new ex vivo system of vitreoretinal neovascularization in adult murine retinas which can be observed by time-sequential imaging. We are also therefore the first to observe time-lapse imaging of vascular sprouting from both quiescent and mature vessels, for which the initial step was a single cell extension only. The leading edges were found to protrude into the ECM and then retract one after another. Further detailed analysis of vitreoretinal angiogenesis will be important for the future development of new therapeutic targets in ocular angiogenic diseases, such as PDR.

References


