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Intravitreal VEGF and bFGF produce florid retinal neovascularization and hemorrhage in the rabbit

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Abstract

Purpose. Vascular endothelial growth factor (VEGF) causes widespread retinal vascular dilation, produces breakdown of the blood-retinal barrier, and is implicated in ocular neovascularization (NV). Basic fibroblast growth factor (bFGF) also has been implicated in the production of ocular NV. This study was performed to investigate the ability of simultaneous sustained intravitreal release of both VEGF and bFGF to induce robust retinal NV in the rabbit.

Methods. Intravitreal implantation of sustained-release Hydron polymeric pellets containing both 20 µg of VEGF and 20 µg of bFGF was performed on adult male Dutch belted rabbits. In other animals either 20 µg or 50 µg bFGF-containing pellets was implanted intravitreally; also, either 20 µg VEGF or 50 µg VEGF-containing pellets was implanted. Control rabbits received either blank polymeric pellets or a pellet containing 30 µg bovine serum albumin. Eyes were examined by indirect ophthalmoscopy after surgery at 24 hrs, 48 hrs, 4 days, 7 days, 14 days, 21 days, and 28 days. Findings were documented by color fundus photography and fluorescein angiography (FA). Eyes were enucleated and prepared for histologic analysis at 28 days following intravitreal implantation of the VEGF/bFGF-containing pellets.

Results. In all eyes implanted with VEGF/bFGF pellets, dilation and tortuosity of existing blood vessels were observed within 48 hrs after pellet implantation. The progression of retinal vascular changes was rapid and occurred over the entire optic disk and medullary rays between 4 and 7 days. Hemorrhage occurred as early as 14 days after VEGF/bFGF pellet implantation. In eyes with massive hemorrhage, total traction retinal detachment developed after the second week.

The presence of abnormal tissues at the vitreo-retinal interface within 28 days was demonstrated by light microscopy while FA showed profuse leakage of dye from anomalous vessels within the first week. Neither bFGF-exposed eyes nor control eyes showed any vascular changes. Eyes that received only VEGF-containing pellets exhibited tortuosity of existing vessels, but neither hemorrhaging nor retinal detachment occurred.

Conclusions. These results demonstrate that retinal vascular changes leading to hemorrhaging is produced rapidly in the rabbit by simultaneous intravitreal release of both VEGF and bFGF. Understanding how these growth factors induce retinal NV may suggest novel therapeutic treatment strategies.

Keywords: animal model; basic fibroblast growth factor; hemorrhage; retinal neovascularization; vascular endothelial growth factor

Introduction

Retinal neovascularization (NV) is new blood vessel formation in the retina and is a major cause of blindness in the United States.[1] Pathologic retinal angiogenesis is the final common pathway leading to visual loss in retinopathy of prematurity (ROP), diabetic retinopathy, and the sequelae of ischemic branch and central retinal vein occlusion.[2,3] Diabetic retinopathy is the major cause of new blindness in developed countries within the working age group[4] while ROP is the leading cause of visual loss in newborns. Such pathologies have in common an ischemic or hypoxic retina

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that is thought to release numerous angiogenesis factors leading to the development of retinal NV.[5] Common sites for NV to occur are at the optic disc head and the major arcades of the retina.[2] Despite various treatment modalities that include panretinal photocoagulation, cryotherapy, and vitrectomy, progressive retinal NV continues to occur with subsequent severe visual loss.[6,7,8,9] Moreover, age-related macular degeneration (AMD) is the most common cause of severe visual loss in people over the age of 65;[10] and the wet or exudative form is characterized by choroidal NV.[11]

Recent studies indicate that a link exists between vascular endothelial growth factor (VEGF) and various ocular diseases and that VEGF may be an essential part of the molecular signaling cascade leading to retinal NV.[12,13,14] VEGF is elevated in both the retina and the vitreous of patients with diabetic retinopathy and other retinal disorders. Moreover, choroidal NV that is present in AMD expresses VEGF, which is elevated in the vitreous of patients with subretinal NV.[15,16,17] VEGF is an endothelial cell-specific mitogen that displays angiogenic activity,[18] induces vascular permeability, and responds to hypoxic conditions.[19] Blockade by VEGF antagonists produces partial inhibition in animal models of either retinal or iris NV thereby suggesting a major role for VEGF in ocular NV.[20,21,22] Moreover, multiple intravitreal injections of VEGF produced iris NV in primates,[23] while subsequent studies indicated that such multiple injections of VEGF could produce retinal ischemia and microangiopathy in an adult primate.[24] A previous study that utilized the albino rabbit demonstrated that sustained-release of VEGF in high amounts produced mild retinal NV for a short time period before regressing back to normal.[25]

Although strong evidence indicates a causative role of VEGF in retinal NV, other angiogenic factors most likely stimulate in a parallel and concerted fashion. Members of the fibroblast growth factor (FGF) family such as bFGF have been implicated for many years in the development of retinal NV.[26,27,28] As with VEGF, bFGF has been detected in the vitreous of patients with proliferative diabetic retinopathy, while both bFGF and VEGF are present in epiretinal and choroidal neovascular membranes.[27,28]

Alternatively, other studies demonstrate that at least one form of bFGF acts as a survival factor for photoreceptors and is expressed constitutively in photoreceptors and other retinal cells.[29,30,31,32] A more recent study demonstrated that transgenic mice with either high expression or undetectable expression of bFGF did not differ significantly from wild-type mice with regard to development of NV following oxygen-induced ischemic retinopathy.[33] The authors of this transgenic study suggested that bFGF is neither necessary nor sufficient for the development of retinal NV. Thus far, the introduction of a combination of both bFGF and VEGF in an intravitreal sustained release form to induce experimental retinal NV has not been reported. Therefore, the goal of this study was to determine if sustained simultaneous release of VEGF and bFGF within the vitreous cavity would produce robust and possibly irreversible retinal NV in the rabbit.

Materials and methods

Intravitreal implantation of sustained-release pellets

Animals were treated according to the tenets of the ARVO Statement on the Use of Animals in Ophthalmic and Vision Research and to the Declaration of Helsinki and The Guiding Principles in the Care and Use of Animals (DHEW Publication, NIH 80-23). Adult male Dutch belt rabbits weighing between 2.0 and 3.0 kg were anesthetized with 35 mg/kg intramuscular injection of ketamine hydrochloride (Phoenix Pharmaceuticals, Inc., St. Joseph, MO, U.S.A.) and 5 mg/kg xylazine (Lloyd Laboratories, Shenandoah, Iowa, U.S.A.), respectively. Pupils were dilated with 2.5% phenylephrine (Alcon, Fort Worth, TX, U.S.A.) and 1% tropicamide (Baush & Lomb, Tampa, FL, U.S.A.).

Sterile preparations of both human recombinant VEGF 165 and recombinant bFGF (Pepro Tech, Rocky Hill, NJ, U.S.A.) were incorporated into a Hydron NCC polymer (Hydromed Sciences, Cranbury, NJ, U.S.A.) following specifications of the manufacturer. Also, solutions that contained either 30 μg of bovine serum albumin (BSA) or PBS alone were incorporated into the Hydron polymer to produce pellets that acted as negative controls. A total of 20 animals were utilized in this study. Intravitreal implantation of the sustained-release polymeric pellets containing both growth factors bFGF and VEGF was performed over the optic streak of the rabbits (N = 10). Moreover, 20 μg bFGF-containing pellets was implanted intravitreally (N = 2); also, either 20 μg VEGF (N = 2) or 50 μg VEGF-containing pellets (N = 2) was implanted. Control rabbits received either blank polymeric pellets (N = 3) or a pellet containing 30 μg bovine serum albumin (N = 1). Under a Zeiss operating microscope, the conjunctiva was opened; and a 2 mm incision was made in the sclera approximately 2 mm posterior to the limbus. A second minor sclerotomy was performed for insertion of a retinal pick; alternatively, a syringe with a 30-gauge $\frac{1}{2}$ inch needle was utilized. The pellet was grasped with an intraocular forcep, inserted through the sclerotomy into the vitreous cavity, and positioned in the space over the optic disk using either a retinal pick or a 30-gauge needle to maintain positioning of the pellet as the forcep was removed. The first sclerotomy then was closed with 8-0 vicryl suture. Finally, 0.3% ciprofloxacin drops (Alcon, Fort Worth, TX, U.S.A.) were applied to the ocular surface following conjunctival closure.

Clinical evaluation of animals by ophthalmoscopy, fluorescein angiography, and fundus photography

All rabbits were examined both pre-operatively and post-operatively by indirect ophthalmoscopy, and results were documented by fundus photography. Pupils were dilated as described above, and the rabbits were anesthesized as described earlier for indirect ophthalmoscopy, fluorescein angiography (FA), and fundus photography. Eyes were examined by indirect ophthalmoscopy after surgery at 24 hrs,

48 hrs, 4 days, 7 days, 14 days, 21 days, and 28 days. In two rabbits implanted with VEGF/bFGF-containing pellets and in one rabbit implanted with a blank pellet, FA was carried out between 7 and 8 days after pellet implantation. After a rapid intravenous bolus injection of 0.5 ml 10% fluorescein sodium (Alcon, Fort Worth, TX, U.S.A.) into the rabbit ear, fundus photographs were taken over a 30-second time period. Findings also were documented by fundus photography utilizing a portable Kowa Genesis retinal camera. At 28 days after pellet implantation, animals were anesthetized as described previously. The eyes were enucleated rapidly and fixed in 10% phosphate-buffered formalin, and the animals were sacrificed immediately following enucleation by an intravenous overdose of pentobarbital. Two rabbits that did not develop traction retinal detachment were monitored up to 145 days in order to follow the natural progression of dilatation and tortuosity in existing blood vessels, subsequent hemorrhaging, reabsorption, and fibrovascular membrane formation.

Grading of retinal NV

A photographic grading system of retinal NV as described previously by two different groups[25,34] was used. Specifically, fundus photographs were arranged in a temporal sequence for evaluation on the progression of the new blood vessels over time. The photographs were evaluated in a masked manner and scored by using four grades. Grade 0 displayed no vascular abnormalities in either the optic disk or the vascularized medullary rays. Grade +1 showed marked dilation and engorged tortuosity of the existing blood vessels in both the optic disk and medullary rays. Grade +2 displayed microvascular abnormalities, which presumably reflect new capillary buds although extensive light and electro-microscopic studies have not been completed for confirmation. Grade +3 showed highly identifiable individual capillary loops growing into strands involving the optic disk and parts of the medullary rays. Grade +4 displayed total highly identifiable capillary loops growing into strands involving the entire optic disk and all of the medullary rays. Hemorrhaging occurred generally after Grade +4 was reached. In summary, stages of NV were graded as +1 (preproliferative), +2 (subtle NV), +3 (active NV), and +4 (total NV). Figure 1 shows the progression of the rabbit neovascular response over time and also displays different stages of the retinal vascular changes.

All fundus photographs were evaluated independently by two observers in a masked fashion. There was 100% agreement between the two observers (HTH and CGW) who assigned grades to the photographs. Retinal vascular changes and subsequent NV in the rabbit can be captured photographically due to the location of the lesions along the optic streak so that photographic grading is performed easily. Clinical examinations correlated highly with photographic assessments.

Pathological analysis of the eyes

The eyes were fixed immediately after enucleation in 10% phosphate-buffered formalin for at least 24 hours and then rinsed in phosphate buffer. Posterior segments of the eyes were dissected, dehydrated in progressive concentrations of ethanol-water, cleared with Histoclear (National Diagnostics, Manville, NJ, U.S.A.), and infiltrated with paraffin in a Fisher Model 166 MP tissue processor. The samples then were embedded in paraffin; and 4 um serial retinal cross-sections were cut, placed on albumin-coated glass slides, deparaffinized with Histoclear, stained in hematoxylin and eosin, and cover-slipped. Finally, the samples were photographed with an Olympus DP-10 microscope digital camera system.

Results

All animals were documented as having healthy retinas through indirect ophthalmolscopy, fundus photography, and FA prior to surgical implantation of the Hydron pellets. The sustained release Hydron pellets containing both 20 μg VEGF and 20 μg bFGF were implanted intravitreally over the optic disk of the rabbits. Pellets generally remained localized in the posterior area of the vitreous near the optic disk. The vitreous remained clear during the course of the study with no detectable retinal detachments prior to the onset of retinal NV. Eyes that were implanted with either blank pellets or a pellet containing BSA showed no identifiable abnormalities and remained unchanged from their pre-implantation appearances. Moreover, eyes that received bFGF-containing pellets displayed no vascular changes while eyes that were implanted with VEGF-containing pellets showed mild vascular changes without progression to either hemorrhaging and/or retinal detachments.

In addition, signs of inflammation such as vitreous haze and/or dilation of conjunctival blood vessels were not observed. Since the early stages of inflammation in the rabbit retina also include dilation of existing retinal vessels, this inflammatory response can be difficult to distinguish from early stages of microvascular abnormalities.

In all eyes implanted with pellets containing both 20 μg VEGF and 20 μg bFGF, grade +1 NV was observed within 48 hrs after pellet implantation. As compared to the baseline photograph which was taken prior to pellet implantation and graded as +0 (Fig. 1A), increased dilatation and tortuosity of existing retinal vessels was marked by day 4 with a grade of +3 (Fig. 1B). The implanted pellet can be seen as a whitish gray circular shadow (white arrows). The progression of retinal vascular changes was rapid and reached +4 between 4 and 7 days later with the appearance of both dilated existing capillaries and possible new vessels which occupied a position not occupied normally by retinal vessels (Fig. 1C). By day 11, the blood vessels were even more swollen (Fig. 1D). Hemorrhage from the new vessels occurred as early as 14 days after pellet implantation

(Fig. 1E), which was not observed in animals that received a blank pellet (Fig. 1F). In eyes with massive hemorrhage, total traction retinal detachment developed after the second week. These findings occurred in all 10 eyes that received pellets containing both VEGF and bFGF. However, in 2 eyes that had a lesser degree of hemorrhage, repeated cycles of hemorrhage with spontaneous absorption were observed up to 145 days along with formation of whitish fibrovascular membranes.

In addition, light micrographs of hematoxylin and eosin-stained rabbit retinal sections at 28 days after implantation of VEGF/bFGF-containing pellet are shown in Figure 2. A fibrovascular tuft can be seen arising from the optic nerve toward the adjacent retina (Magnification 10×). A higher magnification section (25×) of Figure 2 at the bottom right demonstrates the presence of previous new vessels in the pre-papillar proliferative tissue. Since all the rabbits that were implanted with VEGF/bFGF-containing pellets developed retinal NV with hemorrhaging, and those that developed massive hemorrhaging proceeded to retinal detachment, a statistical evaluation was not performed on the animals.

Fluorescein angiography (FA) was performed on two rabbits prior to implantation and within 8 days after implantation of VEGF/bFGF-containing pellets with a score of +3 NV. Prior to implantation, fluorescein angiograms displayed vessels without leakage in both the early phase (Fig. 3A) and late phase (Fig. 3B) angiograms. After VEGF/bFGF pellet implantation, definite fluorescein leakage was seen in both the early (3C) and late-phase (3D) angiograms. These blood vessels appear highly tortuous and kinky.

Discussion

This study demonstrates that sustained simultaneous release of both VEGF and bFGF within the rabbit vitreous produces robust retinal vascular changes that lead eventually to hemorrhaging, fibrovascular membrane formation, and subsequent traction retinal detachment. Histopathologic analysis demonstrates the presence of anomalous lumen within the retina while FA confirmed vascular leakage. Significant hemorrhaging of blood presumably from the more swollen retinal vessels generally occurred between 2 and 3 weeks after intravitreal implantation of the sustained-release VEGF/bFGF-containing pellets. Interestingly, the newly formed anomalous vascular lumen appear to be dilated. In diabetics, retinal vessel dilatation is a well-known phenomenon and precedes diabetic macular edema.[35] Conversely, retinal arterioles and macular arteriolar and venular branches constrict as diabetic macular edema disappears after laser photocoagulation.[36]

These results build on a previous report, which described the use of sustained high levels of VEGF for inducing short-lived retinal NV with vascular leakage in the rabbit that resulted in spontaneous regression occurring between 21 and 35 days.[25] In this study, neither sustained VEGF nor bFGF separately produced the florid neovascular response leading to hemorrhage and retinal detachment in the rabbit. The presence of sustained bFGF may provide a synergistic effect on the angiogenic properties of VEGF. Since bFGF and VEGF separately did not produce retinal NV, most likely the robust angiogenic effect of the combined growth factors is not due to a secondary effect from endotoxin which is present in low amounts of recombinant growth factor preparations. Presumably, both VEGF and bFGF within the implanted pellets were depleted within the four-week study period. However, pharmacokinetic analysis of the vitreous was not performed to determine either optimal amounts or length of time in which both VEGF and bFGF remained within the vitreous.

Overall, this study demonstrates that irreversible robust retinal vascular changes leading to NV is produced rapidly and reproducibly by simultaneous sustained-release of both VEGF and bFGF within the rabbit vitreous. The progression of retinal vascular changes leading to NV can be monitored clinically by standard methods and graded without sacrificing the animal. Also, the size of the rabbit eye allows for intraocular injections of therapeutic agents and subsequent qualitative evaluation by standard ophthalmolscopy and fundus photography. The relatively low cost and ease of handling rabbits in comparison to either primates or pigs are additional advantages.

Moreover, quantitative evaluations of the neovascular response can be performed on digitized photographic images utilizing baseline images as background values for quantification of new blood vessels as a function of time. The highly reproducible development of these vascular changes in this rabbit model within a short time frame suggests that potential therapeutic modalities for intervention of angiogenesis can be tested readily and that potential amelioration by pharmacologic probes may be monitored *in vivo* by non-invasive micro-vascular imaging technologies. One such potential optical imaging technology involves optical Doppler tomography (ODT), which can provide simultaneous high-resolution imaging of both *in vivo* blood flow and the tissue structure surrounding the blood vessel.[37] Thus, use of this new rabbit model for the development of noninvasive vascular imaging technologies may result in a significant advance in clinical diagnosis and a more efficient design of novel retinal-specific anti-angiogenic drugs.

Although the anatomy of the rabbit retina differs significantly from the human retina[38,39] and lacks a macula, the induction, proliferation, hemorrhaging, cycle of regression, and traction retinal detachment in this novel rabbit model closely parallels the human clinical response to pathologic angiogenesis. Thus far, no such VEGF/bFGF-induced response in other animal models displays such parallel behavior. Studying the primary biochemical and cellular lesions in this rabbit model at the earliest stages may yield clues on the pathogenesis of diabetic retinopathy.[40] Ulti-
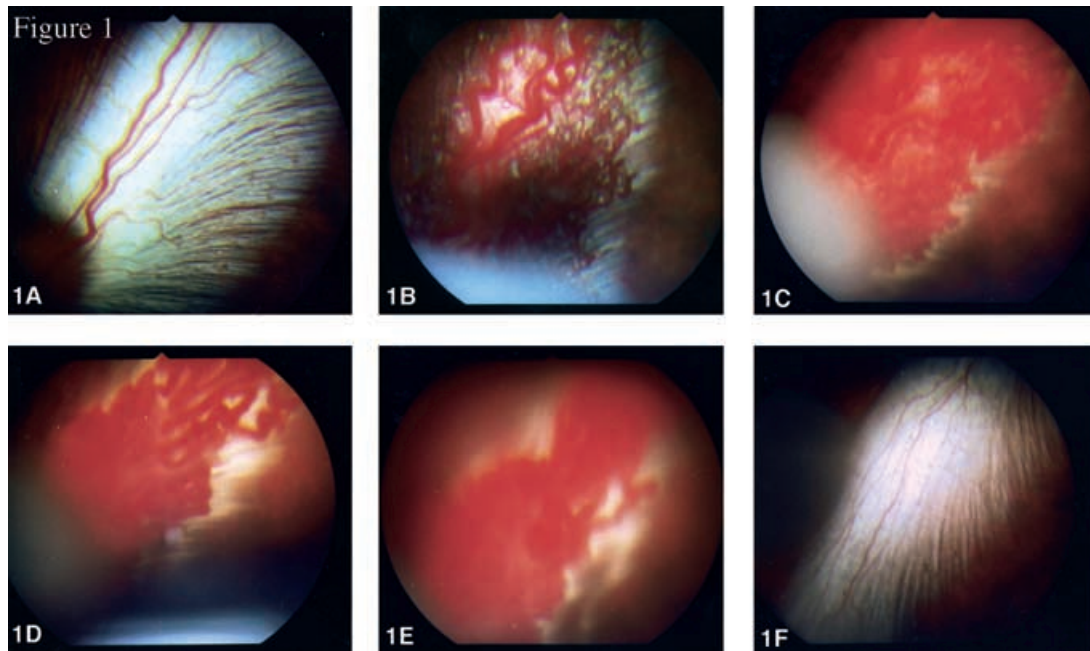
Figure 1. Stimulation of retinal neovascularization by sustained intravitreal release of both vascular endothelial growth factor (VEGF) and basic fibroblast growth factor (bFGF) in rabbits. (A) Baseline fundus photograph prior to implantation of the Hydron pellet. (B) Same rabbit as shown in (A) at 4 days after implantation of pellet containing both 20 μg VEGF and 20 μg bFGF. (C) Same rabbit as shown in (A) at 7 days after implantation of VEGF/bFGF-containing pellet. (D) Same rabbit at 11 days after pellet implantation. (E) Same rabbit at 14 days after pellet implantation. (F) Fundus photograph of another rabbit at 14 days after implantation with a blank pellet. The white arrow points to the implanted pellet.
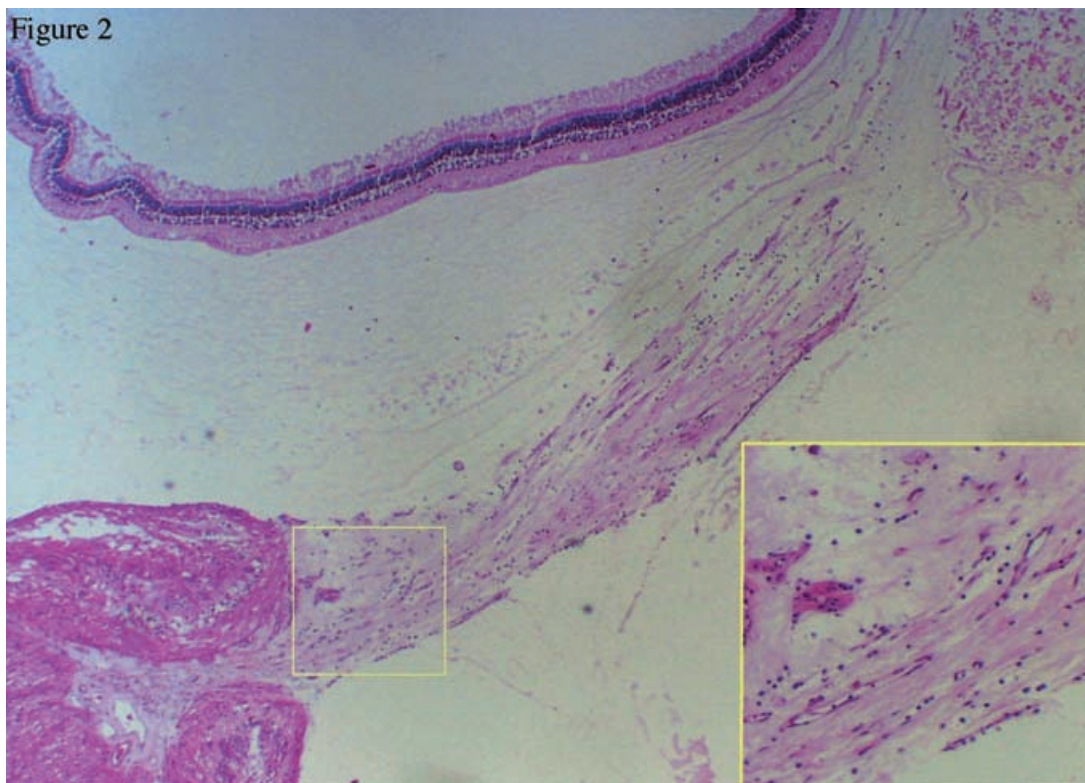
Figure 2. Light micrographs of hematoxylin and eosin-stained retinal sections at 28 days after implantation of VEGF/bFGF-containing pellet in the rabbit eye. A fibrovascular tuft can be seen arising from the optic nerve toward the adjacent retina (Magnification 10×). A higher magnification section (25×) at the bottom right displays the presence of previous new vessels in the pre-papillar proliferative tissue.
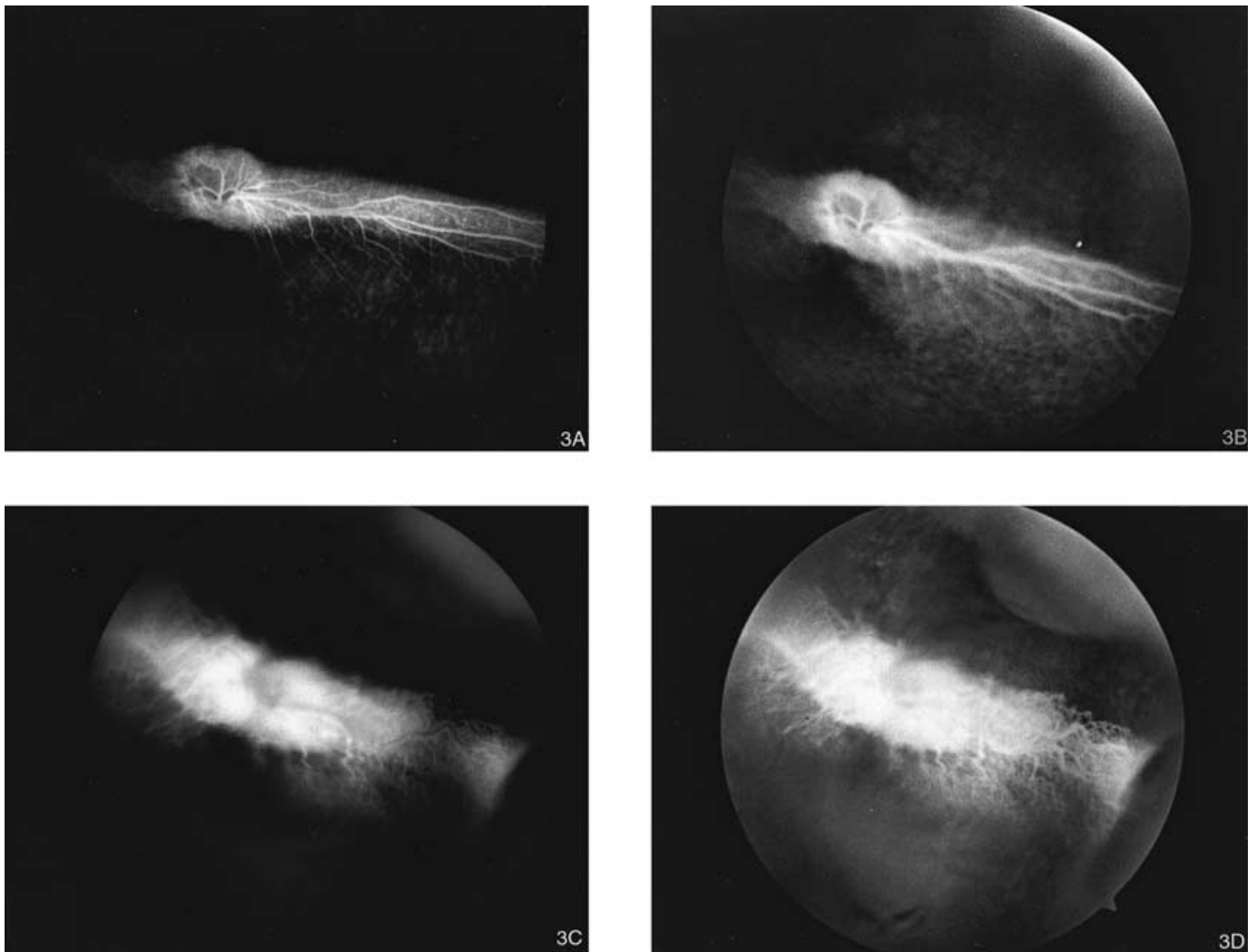
Figure 3. Fluorescein angiography (FA) on the same eye prior to implantation and at 8 days after implantation of a sustained-release pellet containing both 20 μg VEGF and 20 μg bFGF. Both the early phase (Fig. 3A) and the late phase (Fig. 3B) of the angiogram prior to pellet implantation are shown. At 8 days after implantation of a VEGF/bFGF-containing pellet, the early phase (Fig. 3C) and late phase (Fig. 3D) angiograms demonstrate leakage, tortuosity and kinkiness of the blood vessels.

mately, understanding the intricate molecular interplay of different angiogenic and anti-angiogenic[41,42,43] factors and their respective receptors will provide further understanding on the development of new vascular beds in the retina. Such studies presumably will lead to the design of novel therapeutic agents for the clinical treatment of neovascular diseases such as diabetic retinopathy and selected forms of AMD.

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