Enhanced Recombinant Adeno-Associated Virus–Mediated Vascular Endothelial Growth Factor Expression in the Adult Mouse Retina

A Potential Model for Diabetic Retinopathy

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Diabetic retinopathy, one of the most serious complications of long-term diabetes, could clinically be divided into two stages: *1*) background retinopathy that does not cause visual impairment and *2*) proliferative retinopathy, which is a potentially blinding condition. This study aims to investigate the correlation between enhancement of vascular endothelial growth factor (VEGF) expression and neovascular changes. A binary recombinant adeno-associated virus construct producing green fluorescent protein (GFP) and VEGF under the control of the human cytomegalovirus promoter, recombinant adeno-associated virus (rAAV).VEGF.GFP, was produced and injected into the subretinal space of C57BL mice. GFP expression was tracked by fluorescence fundus photography, and VEGF expression was confirmed by immunohistochemistry and enzyme-linked immunoassay. Neovascular changes were monitored by fluorescein angiography and histology and by quantifying the number of inner retinal vessels. GFP expression was found in 100% of injected eyes, and vascular changes were detected in 9 of 10 rAAV.VEGF.GFP–injected eyes. Of these, four demonstrated microaneurysms and five showed moderate to severe leakage. There was a statistically significant increase in blood vessel number in the inner nuclear layer ($P < 0.03$) and dilatation of retinal veins ($P \leq 0.05$). This work has demonstrated that the development of different stages of diabetic retinopathy is closely correlated with an increased VEGF level in the retina. *Diabetes* 52: 857–863, 2003

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bFGF, basic fibroblast growth factor; ELISA, enzyme-linked immunosorbent assay; GCL, ganglion cell layer; GFP, green fluorescent protein; HRP, horseradish peroxidase; INL, inner nuclear layer; MMP, matrix metalloproteinase; PA, plasminogen activator; PCNA, proliferating cell nuclear antigen; rAAV, recombinant adeno-associated virus; RPE, retinal pigment epithelial; VEGF, vascular endothelial growth factor.

Diabetic retinopathy is one of the most serious complications of long-term diabetes (1–3). Clinically it could be divided into two stages: *1*) background retinopathy that does not cause visual impairment and *2*) proliferative retinopathy, which is a potentially blinding condition. In the proliferative stage of the disease, new blood vessels are not properly formed, resulting in leaky vessels and the subsequent accumulation of blood in the subretinal space (4). Compared with the general population, diabetes sufferers have an ~25-fold increase in the risk of blindness. The presence of diabetic retinopathy closely correlates with the period of time the patient has suffered from diabetes. However, >90% of diabetes sufferers eventually develop retinopathy (1–3), with ~60% of type 1 and 20% of type 2 diabetes sufferers developing proliferative retinopathy. Considering the large number of people suffering from diabetes, there is an urgent need to develop new therapies for diabetic retinopathy, as the present treatments, photocoagulation and vitrectomy, are invasive and provide only temporary protection.

Historically diabetes has been studied using monkey, dog, rat, and mouse models. Diabetes can be induced in animals by surgical pancreatectomy, the use of chemicals, such as alloxan or streptozotocin, or feeding of a high galactose diet (5–9). However, while these animals sometimes demonstrate the temporary early changes of background diabetic retinopathy observed in humans, due to the induction of diabetes they become very sick, are hard to manage, and die before advanced steady background or proliferative retinal changes are established. The lack of suitable animal models has hampered not only our understanding of the development of diabetic retinopathy, but also the development of more effective methods of treatment.

Several studies have demonstrated the temporal and spatial correlation of vascular endothelial growth factor (VEGF) and diabetic retinopathy (10). Although there is a variety of angiogenic factors that may have a role in diabetic retinopathy development, VEGF, which specifically induces endothelial cell proliferation, has emerged as the most important factor (11). Elevated VEGF levels have been measured in the vitreous of patients with diabetic

retinopathy (10–13) and in streptozotocin-induced diabetic rats (14).

In this work, an adeno-associated virus vector was used to express VEGF in the retinas of adult mice. Retinal changes resulting from increase in VEGF levels were monitored using ophthalmological and histological examinations to establish any correlation with diabetic retinopathy.

RESEARCH DESIGN AND METHODS

Construction and production of recombinant adeno-associated virus/green fluorescent protein and recombinant adeno-associated virus/VEGF/green fluorescent protein. The construction of recombinant adeno-associated virus (rAAV)/green fluorescent protein (GFP) and rAAV.VEGF.GFP have been previously described (15). Following the appropriate validation of the constructs, large-scale production of the viruses was performed at the Vector Core Facility (University of North Carolina, Chapel Hill, NC). Titers of the virus were 4×10^{10} transducing units/ml and 1×10^{14} particles/ml for rAAV.GFP and rAAV.VEGF.GFP, respectively.

Subretinal injection. Animals were handled in accordance with The Association for Research in Vision and Ophthalmology Statement for the Use of Animals in Ophthalmic and Vision Research. Five-week-old C57BL mice were anesthetized and subretinally injected with 1 μl solution containing 10^{11} particles of rAAV.VEGF.GFP as previously described (15). Following subretinal injection, the presence of a localized retinal detachment (bleb) of ~25–40% of the retina was confirmed by color fundus photography. Control eyes were injected with rAAV.GFP in the same way.

Clinical photography. Photography was conducted on anesthetized animals at 2, 4, 8, and 12 weeks postinjection. Animals were anesthetized by intraperitoneal injection of ketamine (50 mg/kg body wt) and xylazine (8 mg/kg body wt) before pupil dilation with a drop of 2.5% phenylephrine hydrochloride and 1% mydriacyl. Artificial tears were used on all animals to prevent corneal drying. A small animal fundus camera (Kowa Genesis, Tokyo, Japan) was used to photograph mouse fundi using Kodak Elite 200 ISO film (Kodak, Rochester, NY). Fluorescence photography was used to image GFP expression using the small animal fundus camera (Kowa Genesis) with fluorescein filter and Kodak P1600 ISO film (Kodak). Fluorescein angiography was carried out on anesthetized animals following an intraperitoneal injection of 10% sodium fluorescein. The fluorescein angiography finding was recorded using the small animal fundus camera (Kowa Genesis). On the basis of the angiograms, eyes were categorized and leakiness was defined as minor, moderate, or severe according to the size of hyperfluorescent leakage.

Measurement of blood vessel dilatation. Fluorescein angiograms taken at 4 weeks postinjection of rAAV.VEGF.GFP- and rAAV.GFP-injected eyes were scanned into PhotoShop (Adobe, San Jose, CA) then opened in Scion Image (Scion, Frederick, MD). Venous blood vessels were outlined and their area and length digitally calculated. Blood vessel area was divided by blood vessel length to give a mean diameter for blood vessels that could be compared across animals. Mean blood vessel diameters, measured in pixels, were averaged for rAAV.VEGF.GFP- and rAAV.GFP-injected eyes, and the significance of the difference was analyzed by a one-tailed Student's t test.

Histology and immunohistochemistry. Ten rAAV.VEGF.GFP-injected eyes were enucleated at 12 weeks postinjection for different histological studies. rAAV.GFP-injected eyes were enucleated at 4 and 12 weeks postinjection. The eyes were whole mounted, paraffin embedded, or snap frozen following routine protocols (16,17).

VEGF immunohistochemistry was conducted on whole mounts as previously described (16). VEGF immunohistochemistry on frozen sections was performed by incubating the sections in rabbit anti-VEGF primary antibody (1/100; Sigma, St. Louis, MO), followed by LINK secondary antibody (DAKO, Carpinteria, CA), streptavidin–horseradish peroxidase (HRP) (DAKO), then developed with AEC (3-amino-9-ethyl-carbazole; DAKO).

Von Wellibrand factor primary antibody was used to detect the endothelial cells surrounding capillaries, and proliferating cell nuclear antigen (PCNA) primary antibody was used to detect proliferating cells in four eyes. Paraffin sections were incubated in rabbit anti-human von Wellibrand factor primary antibody (1/100; DAKO) or mouse anti-human PCNA primary antibody (1/50; DAKO), washed, and then incubated in fluorescein isothiocyanate–conjugated goat anti-rabbit secondary antibody (1/50; Zymed, San Francisco, CA) or HRP-conjugated sheep anti-mouse secondary antibody (1/100; Amersham Biosciences, Buckingham, England), respectively. Paraffin sections stained with PCNA were developed with AEC (DAKO).

VEGF enzyme-linked immunosorbent assay. Four eyes were enucleated at 12 weeks postinjection and were sonicated in 200 ìl of PBS/0.1% Triton X-100.

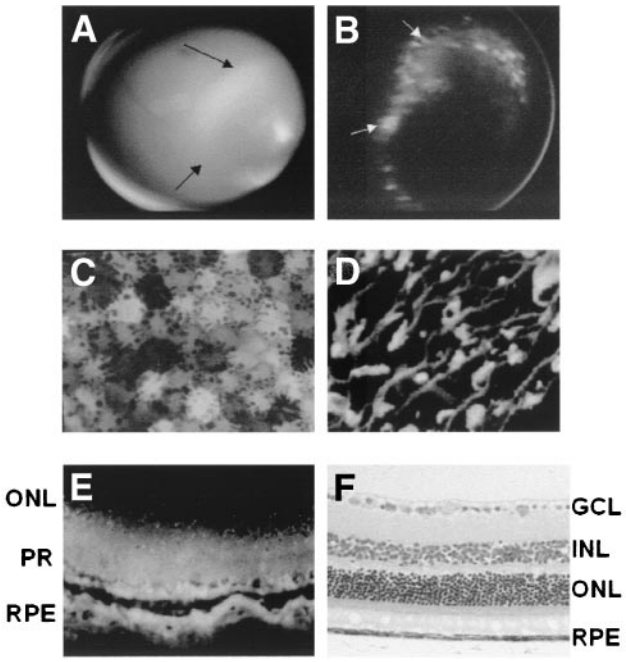

FIG. 1. Clinical photography and photomicrography of the mouse eye. *A*: Color fundus photography of a mouse eye injected with 1 μl recombinant virus. Arrows point to the edge of the subretinal bleb formed. *B*: Fluorescence fundus photography showing GFP expression in an rAAV.GFP-injected eye. Arrows point to the GFP signal. *C*: Fluoromicrograph of RPE-choroid whole mount of an rAAV.VEGF.GFP-injected eye showing expression of GFP in the RPE cells at 12 weeks postinjection. *D*: Fluoromicrograph of the corresponding neural retina showing GFP expression in photoreceptors. *E*: Fluorescence microscopy of a frozen section of a mouse eye showing strong GFP expression in the RPE cell and photoreceptor layers (PR). *F*: Photomicrograph showing morphology of an rAAV.GFP-injected retina. OS, outer segments.

After centrifugation at 1,500*g* for 10 min, the supernatant was collected and the amount of VEGF present was assayed using the enzyme-linked immunosorbent assay (ELISA) kit (R&D Systems, Minneapolis, MN).

Blood vessel counting. von Willebrand factor (DAKO)–immunostained sections were photographed at 100× magnification, and the photographs from the same section were tiled together to form a picture of the entire section. In three rAAV.VEGF.GFP-injected and two rAAV.GFP-injected eyes, capillaries interior and exterior to the ganglion cell layer (GCL) were counted in two 5-μm thick paraffin sections that were ~200 μm apart and contained retinas of equal length (18). Means and standard errors of capillary number were calculated for GCL and non-GCL areas in rAAV.VEGF.GFP-injected and rAAV.GFP-injected eyes. One-tailed Student's t tests of capillary number were conducted between the treatment and control groups in GCL and in non-GCL areas.

RESULTS

Monitoring transgene expression using GFP as a marker. All injected eyes demonstrated subretinal bleb formation (Fig. 1*A*). The subretinal fluid was absorbed by 5 days postinjection, with the disappearance of the retinal detachment. The fundus looked normal, and only a small scar remained visible at the injection site (data not shown). Fluorescence fundus photography proved to be an unreliable method for tracking rAAV-mediated GFP expression in vivo. Only 16% of rAAV.VEGF.GFP and 33% of rAAV.GFP-injected eyes (Fig. 1*B*) demonstrated GFP levels high enough to be detected by fluorescence fundus photography between 4 and 12 weeks postinjection. In contrast, 100% of whole-mounted rAAV.GFP and rAAV.VEGF.GFP-injected animals demonstrated GFP expression, as observed by fluorescence microscopy when

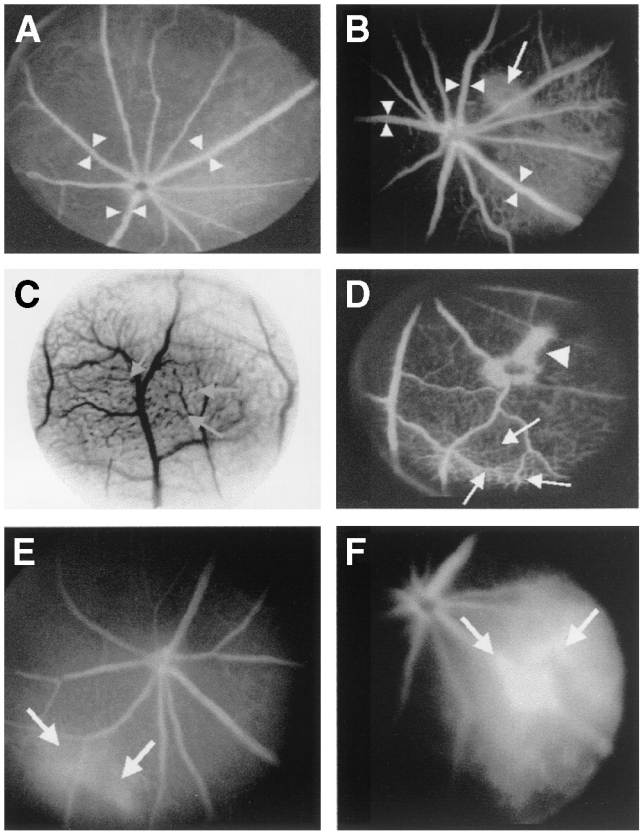

FIG. 2. Fluorescein angiogram showing (*A*) normal retinal vasculature of an uninjected control mouse eye. Arrowheads point to venous blood vessels. *B*: Dilatated blood vessels in a mouse eye injected with rAAV.VEGF.GFP. Arrowheads point to venous blood vessels. Arrow points to leakage distal to the injection site. *C*: Microaneurysms (arrows) in a mouse eye injected with rAAV.VEGF.GFP. *D*: Minor fluorescein leakage (arrows) in mouse eye injected with rAAV.VEGF. GFP. Arrowhead points to leakage at the injection site. *E* and *F*: Moderate fluorescein leakage (*E*) and severe fluorescein leakage (*F*) in mouse eye injected with rAAV.VEGF.GFP. Arrows point to site of leakage.

sampled at 2 weeks postinjection and at different time points up to 12 weeks postinjection. GFP expression in injected eyes was specific to photoreceptor and retinal pigment epithelial (RPE) cell layers. Fluorescence microscopy of whole-mounted RPE-choroid layers and neural retinas demonstrated the expression of GFP in RPE cells (Fig. 1*C*) and photoreceptors (Fig. 1*D*), respectively. The expression of GFP in the RPE and photoreceptor cells was confirmed by fluorescence microscopy of frozen sections (Fig. 1*E*). Apart from the presence of scar tissue at the injection site, retinal morphology in injected eyes was normal and no morphologic changes or infiltrating cells were observed throughout the course of the study (Fig. 1*F*).

Fluorescein angiography. Retinal vasculature was imaged by fluorescein angiography. No vascular changes were recorded in uninjected control eyes ($n = 5$) during the study period (data not shown). Eyes injected with rAAV.GFP ($n = 5$) showed normal vasculature (Fig. 2*A*). Changes in retinal vasculature were observed in rAAV. VEGF.GFP-injected eyes (Fig. 2*B*–*F*). These changes were defined as venous dilatation, microaneurysm, or leakage. Analysis of blood vessel size in fluorescein angiograms showed dilatation of venous retinal vessels following

TABLE 1
Changes in the rAAV.VEGF.GFP-injected, rAAV.GFP-injected, and control noninjected eyes at 8 weeks postinjection

	Neovascular changes	No change	Total
rAAV.GFP	0	5	5
rAAV.VEGF.GFP	9	1	10
Noninjected control	0	5	5

Data are *n*. (See Table 2 for details of neovascular changes in rAAV.VEGF.GFP-injected eyes, as evaluated using fluorescein angiography.)

rAAV.VEGF.GFP injection (Fig. 2*B*). Mean venous blood vessel diameter of rAAV.GFP-injected eyes was 19.74 ± 2.03 pixels and that of rAAV.VEGF.GFP-injected eyes was 24.48 ± 2.33 pixels ($P < 0.05$). This increase represented a 24% increase in vessel diameter in rAAV.VEGF.GFP-injected eyes (Fig. 2*B*) when compared with rAAV.GFP-injected eyes (Fig. 2*A*). Fluorescein angiography performed on rAAV.VEGF.GFP-injected eyes ($n = 10$) demonstrated vascular changes, described as leakages or microaneurysm in 90% of these eyes (Table 1), appearing at 2–4 weeks postinjection. Microaneurysms were visible in four eyes, while minor to moderate leakage was observed in six eyes (Table 2). Severe leakage was noted in one animal, and one animal did not demonstrate any vascular changes (Table 2). Microaneurysms appeared as pinpoint hyperfluorescent dots that faded in the later phases of fluorescein angiography (Fig. 2*C*, arrows). In eyes where leakage was observed, the extent of leakage was defined as minor (Fig. 2*D*, small arrows), moderate (Fig. 2*E*), or severe (Fig. 2*F*), based on the intensity of fluorescein leakage.

Detection of VEGF expression in rAAV.VEGF.GFP-injected eyes. VEGF expression was monitored by immunohistochemistry and quantified by ELISA from pooled retinas. No immune reactivity to the VEGF antibody was detected in whole mounts of rAAV.GFP-injected and noninjected eyes (Fig. 3*A* and *B*). Whole-mounted retinas showed a strong immune reaction to VEGF antibody around the injection site in rAAV.VEGF.GFP-injected retinas, covering up to 30% of the total retinal surface (Fig. 3*C*). Immunohistochemistry of paraffin-embedded sections of rAAV.GFP-injected eyes demonstrated the presence of low level VEGF in the GCL and in the inner nuclear layer (INL) (data not shown). In contrast, in paraffin-embedded

TABLE 2
Neovascular changes in the rAAV.VEGF.GFP-injected eyes ($n = 10$) at 8 weeks postinjection as evaluated using fluorescein angiography

Eye number	Leakage	Microaneurysms
1	Minor	No
2	Moderate	No
3	None	Yes
4	Minor	No
5	Severe	No
6	Moderate	Yes
7	None	Yes
8	None	No
9	Moderate	Yes
10	Severe	No

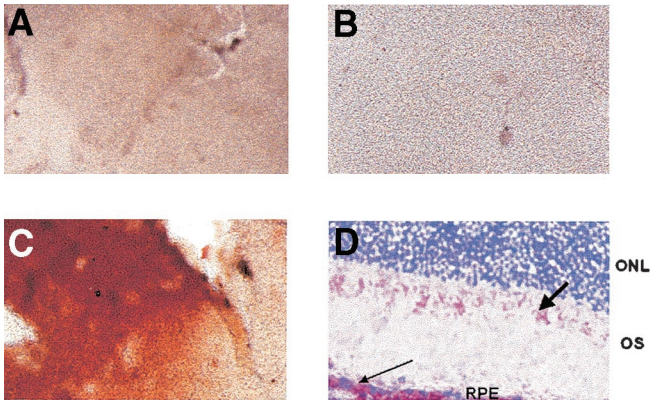

FIG. 3. Immunohistochemical staining at 4 weeks postinjection using a VEGF antibody. *A*: Uninjected eye. *B*: rAAV.GFP-injected eye. *C*: rAAV.VEGF.GFP-injected eye. Note the red staining. *D*: Frozen section from a rAAV.VEGF.GFP-injected eye. Arrows point to the VEGF immunoreactive RPE cells (long arrow) and outer segments (OS) (short arrow).

sections of rAAV.VEGF.GFP-injected eyes, the majority of VEGF immunoreactivity was present in the outer nuclear layer (ONL), the inner segments of the photoreceptor cells (Fig. 3*D*, thick arrow), and to a lesser degree in the RPE cells (Fig. 3*D*, thin arrow). These observations were in agreement with the transduction specificity of rAAV and confirmed that the increased level of VEGF in these eyes was the result of rAAV-mediated gene delivery. Increased VEGF immunoreactivity was correlated with an increased amount of VEGF (10.37pg VEGF/ig total protein), quantitated by ELISA, in rAAV.VEGF.GFP-injected retinas that demonstrated leakiness. Although the expression of endogenous VEGF in the retina has been previously reported (19,20), we were unable to detect endogenous VEGF in the retina of uninjected or rAAV.GFP-injected animals.

Histological examination of rAAV.VEGF.GFP-injected eyes. Histological examination of rAAV.VEGF.GFP-injected eyes ($n = 10$) demonstrated normal retinal morphology in nine eyes (Fig. 4*A*). In one eye significant fluorescein leakage was seen at 4 weeks postinjection, and retinal degeneration was observed by 12 weeks postinjection. The retinal degeneration coincided with the growth of blood vessels from the retinal vasculature into the subretinal and inner plexiform layers (Fig. 4*B*). There was no statistically significant difference ($P = 0.225$) in the number of blood vessels present in the GCL between rAAV.GFP-injected and rAAV.VEGF.GFP-injected animals (Fig. 5). However, there was a statistically significant increase ($P < 0.03$) in the number of retinal blood vessels of the INL (82.87 ± 5.09) in rAAV.VEGF.GFP-injected eyes showing microaneurysm, or minor to moderate leakage when compared with the control rAAV.GFP-injected eyes (65.00 ± 7.95) (Fig. 5). The change was most pronounced around the injection site, where the increased number of capillaries covered an area approximately the size of the retinal bleb (Fig. 4*D*). In these eyes the presence of PCNA-positive cells was also demonstrated in the INL (Fig. 4*F*). When compared with an area of the same size away from the injection site (Fig. 4*C*), the number of capillaries decreased to a level similar to that observed in rAAV.GFP-injected control eyes and there were no PCNA-positive cells present. Control rAAV.GFP-injected eyes did not have any PCNA-positive cells in the INL (Fig. 4*E*).

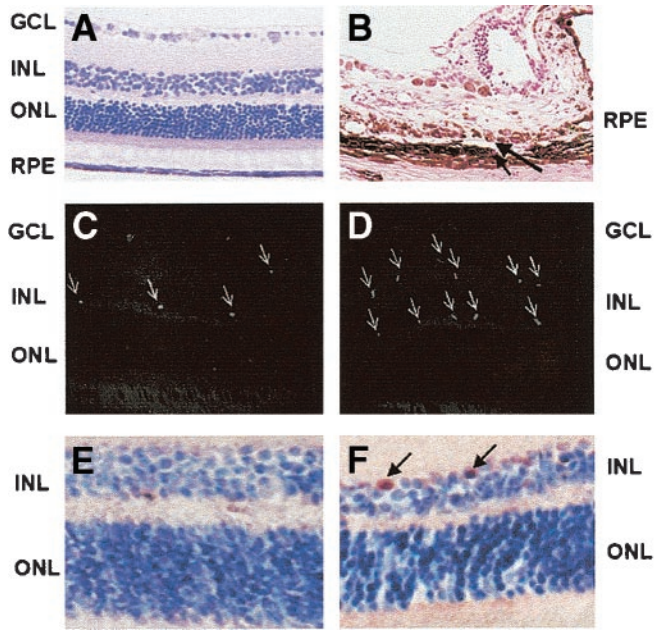

FIG. 4. Photomicrographs (*A*, *B*, *E*, and *F*) and fluoromicrographs (*C* and *D*) of the mouse eye at 12 weeks postinjection. *A*: Hematoxylin-eosin (H-E) staining of an eye that demonstrated minor changes (microaneurysms) in fluorescein angiograms. *B*: H-E staining of an eye that demonstrated severe fluorescein leakage in fluorescein angiograms. Blood vessels staining with von Willebrand factor primary antibody (white dots) in a rAAV.VEGF.GFP-injected eye opposite to the injection site (*C*) and adjacent to the injection site (*D*). *E* and *F*: PCNA staining of rAAV.GFP-injected (*E*) and rAAV.VEGF.GFP-injected (*F*) eyes. Arrows point to immunoreactive cells.

DISCUSSION

This work has demonstrated that long-term specific increase of VEGF expression in the adult retina could induce limited vascular changes resembling those of diabetic retinopathy. VEGF is a potent vascular permeability factor (21–23), and VEGF upregulation has been linked to neovascular eye diseases including diabetic retinopathy. VEGF-induced neovascular changes have previously been demonstrated on animal models based on increasing VEGF levels through implants (24), recombinant adenovi-

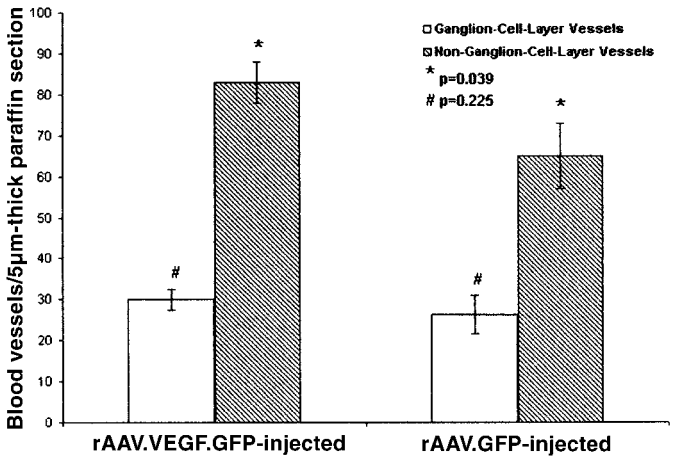

FIG. 5. Graphical presentation of the number of blood vessels in the ganglion cell layer (□) and in nonganglion cell layer (▨) areas of rAAV.VEGF.GFP-injected and rAAV.GFP-injected eyes. *$P = 0.039$, #$P = 0.225$.

rus-mediated VEGF expression (25,26), or transgenic technologies (27,28).

Studies in which VEGF protein is directly administered require the use of complicated delivery systems, as the half-life of the VEGF protein is relatively short (29). Recombinant adenovirus-mediated delivery has successfully overcome this problem by providing consistent VEGF production (25,26). However, recombinant adenoviral vectors elicit an immune response resulting in transient transgene expression, and in first-generation recombinant adenoviruses the transgene expression cannot be expected beyond 2 weeks. In addition, the inflammatory response may enhance the neovascular response, thus influencing the final outcome (30). The use of an adeno-associated viral vector could overcome the problem, as it could maintain long-term VEGF expression without evoking an immune response (31). Previous studies demonstrated GFP expression for up to 18 months (32,33) without any signs of retinal toxicity or inflammation. In this work, the simultaneous rAAV-mediated expression of GFP and VEGF was demonstrated for up to 3 months without any significant change in GFP signal intensity. One of the advantages of the binary virus complex (rAAV.VEGF.GFP) was the ability to monitor the expression of the biologically active transgene, VEGF, through noninvasive monitoring of GFP expression (34,35). Although, the sensitivity of our in vivo set up was not always sufficient to reflect the level of GFP expression, the system has provided valuable information for experimental follow-up.

Transgenic mouse models with high-level VEGF expression were reported to develop leaky blood vessels and intraretinal endothelial cell proliferation (27,28,36). Subsequently, these animals rapidly developed severe proliferative retinopathy and retinal detachment. In our model, the process of new vessel development was relatively more gradual and less pronounced in rAAV.VEGF.GFP-injected adult mice than in any of the known VEGF transgenic models. This difference closely correlated with the lower level of ocular VEGF detected in rAAV.VEGF.GFP-injected animals. In the majority of our animals, VEGF protein expression was sufficient to manifest features of background retinopathy but not those of proliferative retinopathy. Considering the long-term presence of increased VEGF concentration, the subtle angiogenic changes induced in 80% of injected eyes were surprising. Although the venous blood vessels were dilatated with a 24% ($P < 0.03$) increase in vessel diameter in rAAV.GFP.VEGF-injected eyes and there was a statistically significant increase in the number of retinal blood vessels in the deep capillary of the retina, the majority of eyes showed no signs of histological or morphologic changes in the retina. These observations suggest that although a high level of VEGF would induce complete angiogenesis, VEGF is only one of the angiogenic factors participating in the neovascular response. Like all biological processes, angiogenesis results from subtle and complex interactions between regulatory and effector molecules. VEGF is one of the regulatory cytokines along with IGF, basic fibroblast growth factor (bFGF), and platelet-derived growth factor (13,37–39). Extracellular proteinases and their inhibitors, such as matrix metalloproteinases (MMPs) and plasminogen activators (PAs), are affected by these regulatory proteins (40). MMPs and PAs are responsible for the degradation of the extracellular matrix, an essential process initiating endothelial cell proliferation, thus enhancing the neovascularization process (41).

From a circulatory point of view, diabetes is a disease characterized by microvascular abnormalities resulting in hypoxia. In the eye, one of the earliest signs of diabetic retinopathy is retinal capillary occlusion, blocking blood flow and generating capillary-free areas (42). Hypoxic conditions could develop in these capillary-free areas, and this in turn could induce the upregulation of angiogenic factor production, such as VEGF, bFGF, and intercellular adhesion molecules (43,44). The increased concentration of angiogenic factors would then cause vascular changes including vascular dilatations, tortuous blood vessels, microaneurysms, and endothelial cell proliferation. Subsequently, over an extended period of time, these changes could result in the development of poorly matured leaky vessels (23,45,46). The most characteristic features of rAAV.VEGF.GFP-injected eyes were dilatation of venous blood vessels, microaneurysms, and minor to moderate leakages. Dilatation of the retinal venous blood vessels is among the most common and earliest manifestations of diabetic retinopathy and can be present in the absence of other manifestations of the disease (47). Fluorescein angiograms of 8 of 10 rAAV.VEGF.GFP-injected eyes demonstrated microaneurysms and/or minor to moderate fluorescein leakage. Previous histological studies have demonstrated a strong correlation between endothelial cell proliferation, pericyte loss, and the development of microaneurysm (48). Incidentally, VEGF, which is a known factor of endothelial cell proliferation, has also been shown to promote pericyte detachment and loss (49). Although, our present understanding of microaneurysm development remains incomplete, VEGF has recently been directly linked to the formation of these vascular structures. Similar to the mouse model described here, thin-walled hypercellular structures resembling diabetic microaneurysms were described in a monkey model of VEGF-induced retinopathy (50). Although by 12 weeks postinjection there was a statistically significant increase in the number of retinal vessels, these blood vessels were present only at the area corresponding to the injection or VEGF expression site, and even within this region they were limited to the INL. The lack of new vessel formation in the GCL might be due to the uneven distribution of VEGF across the retina. Retinal fluid flow, dominated by the movement of material from the GCL toward the RPE, is expected to reduce secreted VEGF concentration in the GCL layer. Spatially, with increasing distance from the injection site, changes were limited to dilatation and microaneurysm. Considering that rAAV-mediated gene delivery can last for the lifetime of the animal (32), rAAV-mediated gene delivery can potentially provide a suitable mouse model to study the long-term effect of early vascular changes on retinal function.

It is interesting to note that 1 of 10 eyes did not demonstrate an angiogenic response. In contrast, another eye showed a strong neovascular response with severe fluorescein leakage. The GFP signal in vivo and in whole mounts was similar in these eyes, suggesting that the efficacy of gene delivery was comparable with the other

eight eyes. Histological examination of the eye demonstrating severe leakage showed extensive endothelial cell proliferation and retinal neovascularization resulting in retinal detachment and the development of a neovascular membrane. This variation in angiogenic response could be due to some experimental complications, such as bleeding, that might induce an initial inflammatory response enhancing the effect of VEGF. Alternatively, it might be due to the response to VEGF. Further studies using larger numbers of animals will be required to investigate this possibility.

In summary, this work has confirmed the essential role of VEGF expression in diabetic retinopathy and demonstrated that the development of different stages of diabetic retinopathy is closely correlated to the presence of VEGF.

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