Basic Fibroblast Growth Factor Is Neither Necessary nor Sufficient for the Development of Retinal Neovascularization

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Basic fibroblast growth factor (FGF2) is constitutively expressed in the retina and its expression is increased by a number of insults, but its role in the retina is still uncertain. This study was designed to test the hypothesis that altered expression of FGF2 in the retina affects the development of retinal neovascularization. Mice with targeted disruption of the Fgf2 gene had no detectable expression of FGF2 in the retina by Western blot, but retinal vessels were not different in appearance or total area from wild-type mice. When FGF2-deficient mice were compared with wild-type mice in a murine model of oxygen-induced ischemic retinopathy, they developed the same amount of retinal neovascularization. Transgenic mice with a rhodopsin promoter/Fgf2 gene fusion expressed high levels of FGF2 in retinal photoreceptors but developed no retinal neovascularization or other abnormalities of retinal vessels; in the ischemic retinopathy model, they showed no significant difference in the amount of retinal neovascularization compared with wild-type mice. These data indicate that FGF2 expression is not necessary nor sufficient for the development of retinal neovascularization. This suggests that agents that specifically antagonize FGF2 are not likely to be useful adjuncts in the treatment of retinal neovascularization and therapies designed to increase FGF2 expression are not likely to be complicated by retinal neovascularization. (Am J Pathol 1998, 153:757–765)

Retinal neovascularization, new blood vessel formation in the retina, is associated with a number of disease processes, including diabetic retinopathy, retinopathy of prematurity, central retinal vein occlusion, and branch retinal vein occlusion. The most common of these, diabetic retinopathy, is a major cause of new blindness in developed nations, but the other conditions also result in considerable visual morbidity. Occlusion of retinal vessels leading to retinal ischemia is a feature shared by each of these conditions, and hence they are referred to as ischemic retinopathies. The association of retinal neovascularization with ischemia or hypoxia led to the hypothesis that the development of retinal neovascularization is stimulated by one or more angiogenesis factors released by ischemic retina. Identification of the factor or factors involved is an important step toward the development of specific treatments.

Recently, attention has focused on vascular endothelial cell growth factor (VEGF). It is up-regulated by hypoxia, and its levels are increased in the retina and vitreous of patients or laboratory animals with ischemic retinopathies. Increased expression of VEGF in retinal photoreceptors of transgenic mice stimulates neovascularization within the retina, and VEGF antagonists partially inhibit retinal or iris neovascularization in animal models.

Despite the strong evidence implicating VEGF in retinal neovascularization, however, it is likely that VEGF does not act by itself. Rather, it appears to collaborate with other angiogenic factors in the stimulation of retinal neovascularization. This issue is important in terms of understanding the mechanism of new blood vessel growth in a particular vascular bed and also has relevance to the design of treatments. As noted above, VEGF antagonists can only partially inhibit experimental retinal neovascularization, and it is not known whether this is because of difficulties with delivery or potency of the therapies. This study was designed to test the hypothesis that altered expression of FGF2 in the retina affects the development of retinal neovascularization.

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antagonists or whether the residual activity is due to other angiogenic factors.

One factor that may work in combination with VEGF in stimulating retinal neovascularization in ischemic retinopathies is insulin-like growth factor-I (IGF-I). Mice with ischemic retinopathy that have low IGF-I levels due to antagonism of growth hormone have less retinal neovascularization than mice with normal IGF-I levels. Members of the fibroblast growth factor (FGF) family, particularly basic FGF or FGF2, have also been implicated. In fact, FGF2 has been a candidate retinal angiogenesis factor longer than VEGF, and there have been many studies investigating its possible role in retinal neovascularization. As with VEGF, FGF2 levels are increased in the vitreous and neovascular tissue removed during surgery from patients with retinal neovascularization due to diabetic retinopathy. However, other studies have argued against a role for FGF2. Whether or not it participates in the stimulation of retinal neovascularization remains unanswered and controversial.

FGF2 also has neurotrophic effects in the retina, and exogenous administration of FGF2 or increased expression of endogenous FGF2 decreases retinal cell death after a variety of insults. However, alteration of FGF2 levels in the retina for therapeutic benefit may be hazardous if it stimulates retinal neovascularization. Therefore, it is important to clarify the role of FGF2 in the retina both to better understand the pathogenesis of retinal neovascularization and to determine whether undesired effects on the retinal vasculature preclude therapeutic uses for FGF2.

In this study, we have used mice with targeted disruption of the Fgf2 gene and transgenic mice that overexpress FGF2 in the retina to investigate the role of FGF2 in retinal neovascularization.

Materials and Methods

FGF2-Deficient Mice

Mice with targeted disruption of the Fgf2 gene were produced by homologous recombination and will be described elsewhere. Gene disruption was confirmed by Southern blotting of tail DNA and by Western blot analysis of eyes and other tissues.

Eyes from four wild-type and four Fgf2 knockout mice were dissected, frozen in liquid nitrogen, and stored at −70°C. Protein extracts were prepared by homogenizing the frozen samples in 2 ml of 20 mmol/L Tris-HCl buffer (pH 7.4) containing 5 mmol/L ethylenediaminetetraacetic acid, 1% Nonidet P-40, 0.5% deoxycholate, 2 μg/ml leupeptin, 5 μg/ml aprotinin, and 1 mmol/L phenylmethylsulfonyl fluoride using a Tissumizer (Tekmar Co.). Extracts were clarified by centrifugation in a Sorvall GS 34 rotor at 12,000 rpm for 30 minutes. Protein concentration was determined in the supernatant using the Bio-Rad protein assay reagent (Richmond, CA). Equal amounts of total protein from the wild-type and knockout extracts (20 mg) were incubated for 2 hours at 4°C with 200 μl of heparin-Sepharose CL-6B beads preswollen in the extraction buffer. Beads were collected by spinning 1 minute at 14,000 rpm in a microfuge. After three washes with 1 ml of 20 mmol/L Tris-HCl buffer (pH 7.4), the beads were boiled 5 minutes in 100 μl of loading buffer containing 100 mmol/L dithiothreitol and 0.1% sodium dodecyl sulfate and loaded on a 15% polyacrylamide-sodium dodecyl sulfate gel. Proteins were transferred to a nitrocellulose filter and analyzed by Western blot using a polyclonal antibody against human FGF2 (Santa Cruz Biotechnology, Santa Cruz, CA) and the Amersham enhanced chemiluminescence detection system (Arlington Heights, IL). Human recombinant FGF2 used as a control was a gift from Dr. David Moscatelli.

Generation of Transgenic Mice That Overexpress FGF2 in the Retina

A full-length cDNA for human FGF2 was generously provided by Dr. Judith Abraham. It was digested with EcoRI, filled in, and blunt end ligated into the BamHI site of gBR200 containing the bovine rhodopsin promoter, an intron and a polyadenylate addition site derived from the mouse protamine gene (mp1), and a eukaryotic consensus ribosomal binding site. After transformation, a clone with correct orientation was selected. DNA was double CsCl purified and cut with KpnI and SphI to provide a 3777-bp fusion gene (Figure 1). The fusion gene was purified, and transgenic mice were generated using established techniques as previously described.

Mice were screened for the presence of the transgene by either Southern blot analysis or by polymerase chain reaction (PCR) of tail DNA. Tail pieces were digested overnight at 55°C in 50 mmol/L Tris (pH 7.5), 100 mmol/L ethylenediaminetetraacetic acid, 400 mmol/L NaCl, 0.5% sodium dodecyl sulfate containing 0.6 μg/μl proteinase K. For PCR at 63°C, two primers, P1 (5’-GATAAGGAAAATTTAGGTAGCTACGTTTGGAA-3’), and P2 (5’-GATGTCGCGAGATGCTCTTGAAATCTCGTA-3’), were chosen so as to amplify 401 bp of transgene-specific sequence (Figure 1).
Reverse Transcription (RT)-PCR

At appropriate time points, mice were sacrificed, eyes were removed, and retinas were dissected. Retinal RNA was isolated using the guanidinium isothiocyanate method as described by Chomczynski and Sacchi.\textsuperscript{30} RT was carried out with ~0.5 μg of total RNA, reverse transcriptase (SuperScript II, Life Technologies, Gaithersburg, MD), and 5.0 μmol/L oligodeoxythymidylate primer. Aliquots of the cDNAs were used for PCR amplification at 58°C with primers for the hFgf2/mP1 fusion gene that amplify across an intron-exon border, P3 (5’-TGTTAAGT-GGCACGTGAACAG-3’) and P2 (Figure 1). The expected PCR products for the hFgf2/mP1 fusion gene fragment from genomic DNA and mRNA are 572 and 478 bp, respectively. Titrations were performed to ensure that PCR reactions were carried out in the linear range of amplification. Mouse S16 ribosomal protein primers (5’-CACTGCAAACGGGGAAATGG-3’ and 5’-TGAAGATG-GACTGTGGGATGTGG-3’) were used to provide an internal control for the amount of template in the PCR reactions.

Immunohistochemistry

Transgenic and littermate control mice were sacrificed, and their eyes were removed, fixed in 4% paraformaldehyde, and embedded in paraffin. Ten-μm sections were cut and immunohistochemically stained as previously described\textsuperscript{31,32} with a 1:50 dilution of a monoclonal anti-hFGF2 antibody\textsuperscript{33} generously provided by Dr. Thomas Reilly (Wilmington, DE). Specificity of staining was assessed by substitution of nonimmune serum for primary antibody.

Evaluation of Transgenic Mice for Retinal Neovascularization

Ten-μm serial sections were cut through the eyes of at least five mice 1 month old or older from each FGF2 transgenic line. The sections were stained with hematoxylin and eosin or histochemically stained with biotinylated griffonia simplicifolia isoelectin B4 (Vector Laboratories, Burlingame, CA), which selectively binds to endothelial cells. Slides were incubated in methanol/H₂O₂ for 30 minutes at 4°C, washed with 0.05 mol/L Tris-buffered saline, pH 7.4, and incubated for 30 minutes in 10% normal porcine serum. Slides were rinsed with 0.05 mol/L Tris-buffered saline and incubated 1 hour at 37°C with biotinylated lectin. After being rinsed with 0.05 mol/L Tris-buffered saline, slides were incubated with avidin coupled to peroxidase (Vector Laboratories) for 45 minutes at room temperature. After being washed for 10 minutes with 0.05 mol/L Tris buffer, pH 7.6, slides were incubated with diaminobenzidine (Research Genetics, Huntsville, AL) to give a brown reaction product and mounted with Cytoseal (Stephens Scientific, Riverdale, NJ).

To perform quantitative assessments, 10-μm serial sections were cut through the entire extent of each eye. The entire eye was sampled by staining sections roughly 50 to 60 μm apart, which provided 13 sections per eye for analysis. Lectin-stained sections were examined with an Axioskop microscope, and images were digitized using a 3 charge-coupled device color video camera (IK-TU40A, Toshiba, Tokyo, Japan) and a frame grabber. Image-Pro Plus software (Media Cybernetics, Silver Spring, MD) was used to delineate lectin-stained cells on the surface of the retina, and their area was measured. The mean of the 13 measurements from each eye was used as a single experimental value.

Results

Mice Deficient in FGF2 Have a Normal-Appearing Retinal Vasculature

Mice with targeted disruption of the Fgf2 gene show no detectable expression of FGF2 in the retina by Western blot, whereas wild-type mice show a strong signal (Figure 2). Retinas histochemically stained with griffonia simplicifolia isoelectin show a reaction product localized to vascular endothelial cells providing excellent visualization of normal or abnormal retinal vessels. In wild-type postnatal day 17 (P17) mice, normal retinal blood vessels are seen as circumferential and radial tubes in the inner retina (Figure 3, A and B). At high magnification in Figure 3B, no endothelial cells are seen on the surface of the retina, although in some sections from normal mice a few endothelial cells from large retinal vessels are seen on the surface (not shown). In P17, FGF2-deficient mice, the retinal vessels are normal in appearance (Figure 3, C and D). The total area of endothelial cell staining in the retinas of FGF2-deficient mice was not statistically different from that of wild-type mice (Figure 4A, columns 1 and 3).
There Is No Difference in Retinal Neovascularization in Mice with Ischemic Retinopathy That Are FGF2-Deficient Compared with Wild-Type Mice

Wild-type P17 mice with oxygen-induced ischemic retinopathy show clumps of endothelial cells on the surface of the retina (Figure 3E, arrows) that are not seen in nonischemic retinas (Figure 3A). At higher magnification, retinal vessels are seen connecting to endothelial cell aggregates on the surface of the retina (Figure 3F, arrow). The total area of endothelial cell staining is significantly greater in the retinas of mice with ischemic retinopathy compared with those of normal mice (Figure 4A, columns 1 and 2). The area of endothelial cell staining on the surface of the retina, which has previously been demonstrated to directly correlate with the number of endothelial cells, is dramatically greater for ischemic compared with normal retinas (Figure 4B, columns 1 and 2).

FGF2-deficient mice with oxygen-induced ischemic retinopathy show clumps of endothelial cells on the surface of the retina (Figure 3, G and H, arrows), similar to those seen in wild-type mice with ischemic retinopathy (Figure 3, E and F). The total area of endothelial cell staining was significantly greater in ischemic compared with nonischemic FGF2-deficient retinas (Figure 4A, columns 3 and 4) but was not different from that in wild-type ischemic retinas (Figure 4A, columns 2 and 4). Likewise, endothelial cell staining on the surface of the retina was dramatically greater in ischemic compared with nonischemic FGF2-deficient retinas (Figure 4B, columns 3 and 4) but was not different from that in wild-type ischemic retinas (Figure 4B, columns 2 and 4).

Generation of Rhodopsin Promoter/FGF2 Transgenic Mice

Our laboratory has previously demonstrated that overexpression of VEGF in photoreceptors is sufficient to stimulate retinal neovascularization. We used a similar strategy, coupling the rhodopsin promoter to the Fgf2 gene (Figure 1) and making transgenic mice, to investigate whether overexpression of FGF2 in photoreceptors has a similar effect. Five independent transgenic founders that incorporated the rhodopsin promoter/Fgf2 fusion gene were obtained (designated bF8, bF14, bF16, bF19, and bF28). The founders were backcrossed with C57BL/6J mice to establish transgenic lines. Mice that were heterozygous at the transgene locus were used in all analyses.

Expression of FGF2 in the Retinas of Transgenic Mice

Fgf2 transgene mRNA expression was assessed by semiquantitative RT-PCR. Mice from four of the five transgenic lines showed good transgene expression (Figure 5), whereas, as expected, there was no detectable signal in transgene-negative mice (Figure 5, lane). Immunohistochemical staining for FGF2 showed reaction product within photoreceptors in the retinas of transgene-positive mice, whereas retinas from transgene-negative mice or transgene-positive mice for which nonimmune serum was substituted for primary antibody showed only background staining in the retina (Figure 6).

Transgenic Mice That Overexpress FGF2 in Photoreceptors Have Normal-Appearing Retinas with No Neovascularization

The retinas of five transgene-positive mice from each of the four lines with overexpression of FGF2 in photoreceptors were systematically examined for retinal neovascularization. Examination at high power of serial sections showed normal-appearing retinas with no evidence of retinal neovascularization in any of the eyes from the four different lines. Histochemical staining with griffonia simplicifolia isolecitin in retinas with overexpression of FGF2 in photoreceptors showed only normal retinal blood vessels (Figure 6, D and E). This contrasts with mice that overexpress VEGF in photoreceptors, which show lectin-stained retinal neovascularization in the outer nuclear layer (Figure 6F, arrows).

There Is No Difference in Retinal Neovascularization in Mice with Ischemic Retinopathy That Overexpress FGF2 in the Retina Compared with Wild-Type Mice

Oxygen-induced ischemic retinopathy was induced in the offspring of transgene-positive bF14 and wild-type mice, and after the amount of retinal neovascularization was quantitated, the mice were genotyped. Transgene-positive mice with ischemic retinopathy showed no significant increase in the area of endothelial cell staining in the retina compared with wild-type mice with ischemic retinopathy (Figure 7).

Discussion

Numerous studies have examined the expression pattern and possible roles of FGF2 in the retina (for reviews, see
Figure 3. Assessment of retinal blood vessels and neovascularization in wild-type and FGF2-deficient mice with and without ischemic retinopathy. Retinal frozen sections were histochemically stained with the endothelial cell-selective lectin, griffonia simplicifolia I, using the peroxidase-antiperoxidase technique. Retinal blood vessels within the retinas and neovascularization on the surface of the retinas are stained with reaction product, and other retinal cells are counterstained. A and B: Normal retinal vessels in nonischemic P17 wild-type mice. C and D: Nonischemic P17 FGF2-deficient mice also show normal retinal vessels and no identifiable retinal abnormalities. E and F: Wild-type mice with ischemic retinopathy show clumps of endothelial cells on the surface of the retina due to neovascularization. G and H: FGF2-deficient mice with ischemic retinopathy also show numerous endothelial cells on the surface of the retina. Bars: 0.5 mm (A, C, E, and G) and 50 μm (B, D, F, and H).
Because FGF2 stimulates angiogenesis in chick chorioallantoic membranes, it has been viewed as a possible retinal angiogenesis factor that is involved in the growth and maintenance of normal and abnormal retinal blood vessels. This hypothesis has been investigated in several studies and, although some have been supportive, others have not.

In this study, we have investigated this hypothesis by using genetically engineered mice to examine the effect of altered expression of FGF2 on the retinal vasculature. Mice deficient in FGF2 show no difference in morphology or total area of retinal vessels compared with wild-type mice, and when their retinas are made ischemic, they develop the same amount of retinal neovascularization.

This indicates that FGF2 is not necessary for the normal development of retinal vessels, nor is it required for the occurrence of retinal neovascularization. Also, in contrast to mice with increased expression of VEGF in retinal photoreceptors, which develop intraretinal and subretinal neovascularization,12 mice that overexpress FGF2 in photoreceptors do not develop any neovascularization. Furthermore, mice that overexpress FGF2 and have oxygen-induced ischemic retinopathy show no significant difference in the amount of retinal neovascularization compared with wild-type mice with ischemic retinopathy. Therefore, FGF2 is neither necessary nor sufficient for retinal neovascularization to occur in ischemic retinopathy. In contrast to these data, a recent study used a similar strategy with genetically engineered mice that have altered expression of growth hormone and IGF-I to demonstrate that IGF-I contributes to retinal neovascularization.17

Is it possible that FGF2 stimulates vascular endothelial cell proliferation, migration, and proteolytic enzyme production in vitro and its exogenous administration stimulates neovascularization in some vascular beds, but it does not play a substantial role in retinal neovascularization? It is reasonable to consider this possibility, because the surrounding microenvironment modulates the effects of angiogenic stimuli,40 and therefore the effects of FGF2 in vitro or in other vascular beds in vivo may not be predictive of its effects in the retina. For instance, glial cells provide an important influence on retinal vessels that is not present for vessels not associated with the nervous system. Glial cells induce barrier characteristics in microvessels41 and may also act to inhibit retinal neovascularization.42 There are also differences between retinal and brain microvessels, with the former having a greater amount of pericyte coverage,43 and pericytes have been demonstrated to inhibit endothelial cell proliferation.44 Therefore, inhibitory influences in the retina may prevent retinal vascular endothelial cells from responding to FGF2 in the same way they do in other tissues or when isolated and placed in culture.
These same inhibitory influences also modulate the effects of VEGF and IGF-I, and yet it has been possible to demonstrate that VEGF and IGF-I are important stimulators of retinal neovascularization. This suggests that there may be other control mechanisms that are unique or stronger for FGF2. The \( \text{Fgf2} \) gene does not have a signal sequence,\(^{45} \) and although FGF2 gets out of cells by a mechanism that has not been clearly defined, the amount that gets out may be substantially less than the amount of VEGF that is secreted or the amount of circulating IGF-I.

Comparison of the amounts of VEGF and FGF2 externalized from retinal cells under baseline and hypoxic conditions demonstrate much higher levels of VEGF.\(^{46} \) Also, binding to proteoglycans in extracellular matrix\(^{47-49} \) and to soluble FGF receptors, which are present in the retina and vitreous in substantial quantities,\(^{50,51} \) may sequester extracellular FGF2 and make it inaccessible to retinal vessels.

Recently, Zhou et al.\(^{52} \) demonstrated that \( \text{Fgf2} \) knockout mice have decreased vascular smooth muscle contractility, low blood pressure, and thrombocytosis. How-

Figure 6. Staining for FGF2 or griffonia simplicifolia I in the retinas of mice overexpressing FGF2 or VEGF. A: A 2-month-old FGF2 transgene-positive mouse shows immunohistochemical staining for FGF2 selectively in retinal photoreceptors. B: Staining is absent on a serial section when nonimmune serum is substituted for primary antibody. C: A transgene-negative mouse shows faint diffuse staining for FGF2 throughout the retina but no selective staining in photoreceptors. D to F: Staining with griffonia simplicifolia I of the retina from a 4-month-old (D) or a 2-month-old (E) FGF2 transgenic mouse shows normal retinal blood vessels and no neovascularization, whereas the retina of a 1-month-old VEGF transgenic mouse shows a large clump of neovascularization (F, arrows). Bar: 50 \( \mu \text{m}. \)
ever, they could not identify any abnormalities in angiogenic processes occurring during development, and the mice had normal female reproductive activity, which is dependent on angiogenesis. In addition, although it was previously thought that FGF2 plays an important role in the repair of intravascular injury, they found that FGF2-deficient mice have a normal hyperplastic response after mechanical intra-arterial injury. These results, and those of the present study, suggest that it may be necessary to change our thinking with regard to the role of FGF2 in angiogenesis and wound repair.

Disruption of FGF signaling in photoreceptors by expression of a dominant-negative FGF receptor results in photoreceptor degeneration.\(^{52}\) This indicates that at least one FGF is a survival factor for photoreceptors. FGF2 is likely to play such a role in photoreceptors and other retinal cells. It is constitutively expressed in photoreceptors and other retinal cells,\(^{54-58}\) and its expression is increased by several types of insults, including retinal trauma, light exposure, inherited retinal degenerations, or optic nerve transection.\(^{59-62}\) This may represent an adaptation to limit cell damage, because these same insults decrease cell death from subsequent insults.\(^{63,64}\) Also, injection of FGF2 into the vitreous cavity of rats decreases cell death from an inherited retinal degeneration, constant light exposure, or ischemia.\(^{23-25}\) Increased expression of endogenous FGF2 by administering an \(\alpha\)-adrenergic agent also decreases cell death from constant light exposure.\(^{26}\) Therefore, there is reason to believe that increasing FGF2 expression in the retina is a potentially useful tool for several types of pathology. Our data suggest that this approach is unlikely to be limited by FGF2-induced retinal neovascularization.

References


Figure 7. Quantitation of the area of endothelial cell staining in retinal sections of wild-type or FGF2 transgenic mice with and without ischemic retinopathy. \(N = 7\) for each group. Using Student’s unpaired \(t\) test, there is no significant difference between ischemic wild-type versus ischemic FGF2 transgenic mice.


