Regulation of Growth Factor mRNA Levels in the Eyes of Diabetic Rats

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The underlying etiology of diabetic microvascular disease remains unknown. To examine the potential contribution of basic fibroblast growth factor (bFGF), which is an angiogenic factor, and insulin-like growth factor-I (IGF-I) to the development of diabetic microvascular disease, bFGF and IGF-I mRNA levels were measured in tissues of control, diabetic, and insulin-treated diabetic rats. Diabetes was induced in rats by intravenous injection of streptozotocin (STZ) 65 mg/kg, and the rats were maintained for 21 days. bFGF mRNA levels increased threefold in the eyes of diabetic versus control rats, whereas a consistent change in bFGF mRNA levels was not observed in other tissues. In contrast, IGF-I mRNA levels decreased in the eyes and other tissues, including kidney, lung, and skeletal muscle, of diabetic as compared with control rats. Insulin treatment prevented the diabetes-induced increase in bFGF and decrease in IGF-I mRNA levels. Acidic FGF (aFGF) mRNA levels were unchanged in eyes from diabetic versus control rats. In partially purified retinas, diabetes increased bFGF mRNA levels twofold as compared with levels in control retinas, whereas IGF-I mRNA levels decreased to 58% of control levels in retinas from diabetic rats. Insulin treatment again prevented the diabetes-induced increase in IGF-I mRNA levels in the retina but had no effect on the diabetes-induced increase in bFGF mRNA levels. bFGF peptide levels were minimally increased in diabetic versus control retinas. Treatment of diabetic rats with the aldose reductase inhibitor sorbinil prevented the diabetes-induced increase in sorbitol accumulation and *myo*-inositol depletion in the lens, but it did not affect the diabetes-induced increase in bFGF and decrease in IGF-I mRNA levels in the retina. Induction of hypoinsulinemia by fasting the animals did decrease IGF-I mRNA levels but did not reproduce the diabetes-induced increase in bFGF mRNA levels in the eye. In conclusion, these data demonstrate that the effect of diabetes on growth factor mRNA levels in the eye is gene-specific, suggest that different metabolic abnormalities are responsible for diabetes-induced alterations in the production of different growth factors in the eye, and are consistent with a role for bFGF in the development of diabetic retinopathy.

DIABETES MELLITUS is associated with microvascular complications that include thickening of the capillary basement membrane and in some cases endothelial cell proliferation and neovascularization.[1] Although several biochemical abnormalities have been identified in microvasculature affected by diabetes,[2-4] the underlying etiology of the microvascular complications is not known, and more specifically, the potential role of growth factors in the development of diabetic microangiopathy has not been defined. Previous studies have demonstrated increased insulin-like growth factor-I (IGF-I) levels in the vitreous of patients with diabetic retinopathy, leading to the suggestion that IGF-I may be involved in the development of diabetic retinopathy.[5] The presence of endothelial cell proliferation and neovascularization in tissues affected by diabetic microangiopathy suggested that an angiogenic factor may be involved in the development of diabetic microangiopathy. One well-characterized angiogenic factor is basic fibroblast growth factor (bFGF), which is a mitogenic peptide that stimulates both the proliferation of multiple cell types, including endothelial cells, in culture and angiogenesis in in vivo and in vitro systems.[6-8]

To examine whether increased production of bFGF and IGF-I may contribute to the development of diabetic microangiopathy, we previously examined the effect of 4 days of severe diabetes on tissue bFGF and IGF-I mRNA levels.[9] Induction of diabetes increased steady-state levels of bFGF mRNA in a variety of tissues including the eyes, lung, brain, and heart of diabetic versus control rats, whereas IGF-I mRNA levels decreased in these same tissues.[9] These changes were prevented by insulin treatment. These data are consistent with a diabetes-induced increase in bFGF production contributing to the development of diabetic microangiopathy, but are not consistent with a role for increased IGF-I production contributing to the development of diabetic microangiopathy. To examine further the potential role of bFGF and IGF-I in diabetic microangiopathy, the present study was designed to investigate the effect of more prolonged but less severe diabetes on steady-state bFGF and IGF-I mRNA levels in a variety of rat tissues, and to begin to define the metabolic abnormalities responsible for diabetes-induced changes in bFGF and IGF-I mRNA levels.

MATERIALS AND METHODS

Animal Studies

Diabetes mellitus was induced in male Sprague-Dawley rats (190 to 230 g) by an intravenous injection of streptozotocin (STZ) 65 mg/kg body weight (Upjohn, Kalamazoo, MI) into the tail vein. Control rats were injected with a similar volume of buffer (0.9% NaCl acidified to pH 4.5 with sodium citrate). All rats were allowed free access to food and water. Induction of diabetes was confirmed 24 hours after STZ injection by the onset of hyperglycemia as determined using glucose oxidase reagent strips read on an

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Accu-Check reflectance meter (Boehringer Mannheim Diagnostics, Indianapolis, IN).[9] At that time, twice-daily treatment with subcutaneous injections of Humulin NPH insulin 3 to 4 U (Lilly, Indianapolis, IN) was initiated in half of the STZ-injected rats. Twenty-one days after STZ injection, rats were anesthetized with methoxyflurane and tissues were excised, frozen immediately, and stored at −80°C. For eye preparations, the eye was enucleated and extraocular muscles and other adnexal tissue were removed. Partially purified preparations of retina were prepared by removing the lens and cornea. For studies using sorbinil (Pfizer Pharmaceuticals, Groton, CT), rats were fed standard rat chow with or without sorbinil 400 mg/kg diet. Treatment with sorbinil was initiated 3 days before induction of diabetes. For fasting studies, male Sprague-Dawley rats (190 to 230 g) were either fed ad libitum for 48 hours or fasted for 48 hours. All groups were allowed free access to water during the entire course of study. At the end of the study period, tissues were collected and stored as described earlier. For all studies, each preparation of RNA was made from tissue obtained from an individual rat, except for some eye samples in which preparations of RNA were prepared from eyes pooled from two rats. All study protocols were approved by the University of Iowa and Iowa City Veterans Affairs Medical Center Animal Care and Use Committee.

Preparation of RNA

RNA was isolated from tissues using a modification of previously described techniques.[10,11] Briefly, tissues were homogenized with a Polytron homogenizer (Brinkmann Instruments, Westbury, NY) in 7 vol (wt/vol) 5-mol/L guanidine thiocyanate, 10 mmol/L EDTA, 50 mmol/L Tris, pH 7.5, and 8% (vol/vol) β-mercaptoethanol. The homogenate was then extracted with an equal volume of 1:24 (vol/vol) isoamyl alcohol–chloroform (IAC) with (for skeletal muscle and heart) or without (for liver, brain, eye, lung, and kidney) 0.5 vol phenol saturated with 10 mmol/L Tris–1 mmol/L EDTA. In those samples extracted with IAC and phenol, the homogenate was reextracted with 1 vol IAC. The homogenate was then layered over 5.7 mol/L cesium chloride–0.1 mmol/L EDTA and centrifuged at 141,000 × *g* at 23°C for 16 hours. The resulting pellet was resuspended in H_2O and precipitated in 0.5 mol/L NaCl and 2 vol ethanol at −20°C. RNA was collected by centrifugation, washed in 70% ethanol, dried in vacuo, and resuspended in H_2O. RNA was quantified by measuring absorbance at 260 nm. Accuracy of quantitation was confirmed by size-separating 5 μg total RNA from different samples by denaturing agarose gel electrophoresis as described previously[9] and comparing intensity of the 28s and 18s ribosomal RNA bands after ethidium bromide staining of the gel.

Hybridization Probes

For solution hybridization/RNase protection assays, a 322–base pair rat IGF-I cDNA was subcloned into a pGEM-2 vector (Promega Biotech, Madison, WI).[12] The plasmid DNA was linearized with *Eco*RI to allow for transcription of antisense IGF-I RNAs. Cloning of rat bFGF cDNA has been described previously.[11] The 395–base pair bFGF cDNA was subcloned into pGEM-3Zf(+) (Promega Biotech), and the plasmid DNA was linearized with *Hind*III to allow for generation of antisense bFGF RNAs. Rat acidic FGF (aFGF) cDNA was cloned with reverse transcription of rat brain RNA and the polymerase chain reaction with oligonucleotides identical or complementary to nucleotides 290 to 309 and 606 to 625, respectively, of the previously reported rat aFGF cDNA sequence.[13] The 335-base pair–amplified product was subcloned into pGEM-3Zf(+), and identity of the amplified product was confirmed by DNA sequence analysis of double-stranded plasmid DNA using T7 DNA polymerase according to the manufacturer's instructions (United States Biochemical, Cleveland, OH). For solution hybridization/RNase protection assays, the plasmid DNA was linearized with *Hind*III.

Quantitation of IGF-I, bFGF, and aFGF mRNA Levels

IGF-I and bFGF mRNA levels were quantified simultaneously, whereas aFGF mRNA levels were quantified alone, using a solution hybridization/RNase protection assay as described previously.[9,12] Briefly, ^{32}P-labeled antisense RNAs were transcribed from the linearized plasmid DNAs and incubated with 20 μg total RNA at 45°C in 75% formamide/0.4 mmol/L NaCl. After a 16-hour incubation, the samples were digested with RNases A and T1. The protected double-stranded hybrids were collected by ethanol precipitation and electrophoresed on an 8% polyacrylamide/8-mol/L urea denaturing gel. All assays were performed in duplicate. Specific mRNA levels were quantified from autoradiograms resulting from this assay using a GS300 transmittance-reflectance scanning densitometer (Hoefer, San Francisco, CA) interfaced with a Hewlett-Packard model HP 3396A integrator (Rolling Meadows, IL).

Quantitation of Tissue bFGF Peptide Levels

Tissue bFGF peptide levels were quantified using a modification of previously described methods.[14,15] Tissue samples were homogenized in ice-cold detergent buffer containing 62.5 mmol/L EDTA, pH 8.0, 50 mmol/L Tris, pH 8.0, 0.4% deoxycholic acid, 1% Nonidet P-40 (Calbiochem, La Jolla, CA), 200 mmol/L phenylmethylsulfonyl fluoride (Calbiochem), 500 ng/mL leupeptin hemisulfate (ICN Biochemicals, Costa Mesa, CA), 500 ng/mL pepstatin A (ICN Biochemicals), and 500 ng/mL aprotinin (Sigma Chemical, St Louis, MO). The homogenates were clarified of insoluble material by centrifugation, and protein concentration of the supernatant was determined using the BCA Protein Assay Reagent (Pierce Chemical, Rockford, IL).

Tissue extracts were incubated with prewashed heparin-sepharose (HS) CL-6B (Pharmacia, Piscataway, NJ) for 2 hours at 4°C. HS-bound protein was pelleted and washed three times in ice-cold detergent buffer, three times in ice-cold detergent buffer containing 0.5 mol/L NaCl, 20 mmol/L Tris, pH 7.4, 5 mmol/L EDTA, pH 8.0, 2 mmol/L EGTA, pH 8.0, and all four protease inhibitors listed earlier, three times in the preceding buffer containing 1.0 mol/L NaCl, and two times in the same buffer containing 0.5 mol/L NaCl. Bound proteins were eluted from HS directly into sodium dodecyl sulfate sample buffer containing 50 mg/mL dithiothreitol by incubating for 20 minutes at 65°C. The HS was pelleted, and the supernatant was fractionated by 12% sodium dodecyl sulfate–polyacrylamide gel electrophoresis.

For Western blot analysis, proteins were transferred to a nitrocellulose membrane in cold buffer containing 25 mmol/L 3-[dimethyl(hydroxymethyl)methylamino]-2-hydroxypropane-sulfonic acid (AMPSO; Research Organics, Cleveland, OH), pH 9.5, and 20% methanol. Transfers were blocked in buffer containing 10 mmol/L Tris, pH 7.5, 150 mmol/L NaCl, 5 mmol/L NaN_3, 0.35% polyoxyethylene-sorbitan monolaurate (Tween 20), and 5% nonfat dry milk. The transfers were then incubated with primary monoclonal anti-bFGF antibody (FGF3; Transduction Laboratories, Lexington, KY) at a concentration of 1 μg/mL in blocking buffer. After incubation with primary antibody, transfers were washed at 22°C in 150 mmol/L NaCl, 500 mmol/L sodium phosphate, pH 7.4, 5 mmol/L NaN_3, and 0.05% Tween 20. Transfers were then incubated in blocking buffer containing 1 μg/mL rabbit antimouse IgG (Jackson Immuno Research Laboratories, West Grove, PA), washed in 1 L of the preceding buffer, incubated in 100 mL blocking buffer containing 15 μCi ^{125}I-protein A (ICN Biochemicals), and washed

with 1 L buffer as described earlier. Signal was visualized by autoradiography using RX Fuji Medical X-Ray film (Fisher Scientific, Itasca, IL) and quantified by scanning densitometry as described earlier.

Determination of Tissue myo-*Inositol and Sorbitol Levels*

The lenses of rats were collected and weighed for determination of *myo*-inositol and sorbitol levels as described previously.[16] Lenses were boiled for 10 minutes in H_2O containing α-D-methyl-mannopyranoside as an internal standard and deproteinized with 0.5 mL 0.19-mol/L $Ba(OH)_2$ and 0.19-mol/L $ZnSO_4$. After centrifugation, the supernatant was collected, frozen, and lyophilized. The samples were derivatized, and intracellular contents were determined by gas-liquid chromatography as described previously.[16] The data were expressed as nanomoles per milligram wet weight of tissue.

Statistical Analysis

Differences between group means were assessed for statistical significance using the one-tailed multiple-comparison procedure of Dunnett when treatments were compared with control only. When single comparisons were made, Student's *t* test was used.

RESULTS

Diabetes was induced by intravenous injection of STZ 65 mg/kg, and rats were maintained for 21 days. During this period, control and insulin-treated rats gained mean of 78 and 109 g, respectively, whereas diabetic rats lost a mean of 14 g. Diabetic rats were markedly hyperglycemic, with a mean glucose level of 27.4 mmol/L, whereas control and insulin-treated rats had mean glucose levels of 5.5 and 1.9 mmol/L, respectively. Steady-state bFGF mRNA levels were determined in tissues of control, diabetic, and insulin-treated diabetic rats using a solution hybridization/RNase protection assay (Figs 1 and 2, and data not shown). The level of bFGF mRNA was significantly increased in the eyes of diabetic rats as compared with control rats, and insulin treatment prevented the diabetes-induced increase in bFGF mRNA levels in the eye (Fig 2) In contrast to the eye, bFGF mRNA levels were consistently unchanged in skeletal muscle, brain, kidney, and lung of diabetic versus control rats (data not shown), whereas in some but not all studies bFGF mRNA levels were increased in hearts of diabetic versus control rats. Interestingly, in the heart, unlike the eye, insulin treatment consistently increased, as opposed to decreased, the level of bFGF mRNA as compared with the level in hearts of control and diabetic animals (data not shown).

The effect of diabetes on IGF-I mRNA levels was simultaneously determined in the eye and other tissues from control, diabetic, and insulin-treated diabetic rats. Unlike the effect of diabetes on bFGF mRNA levels in the eye, diabetes decreased IGF-I mRNA levels in the eyes of diabetic versus control rats (Figs 1 and 2). Insulin treatment of diabetic animals at least partially prevented the diabetes-induced decrease in IGF-I mRNA levels in the eye (Figs 1 and 2). In contrast to the lack of effect of diabetes on bFGF mRNA levels in extraocular tissues, IGF-I mRNA levels were also decreased in the lung, kidney, and skeletal muscle of diabetic versus control animals (data not shown). This is

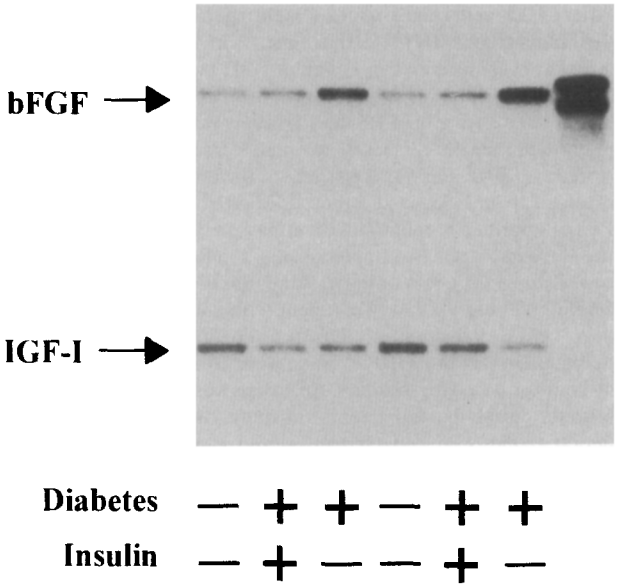

Fig 1. Autoradiogram of bFGF and IGF-I mRNA in eyes of diabetic, insulin-treated diabetic, and control rats. Each lane represents material prepared from eyes pooled from two different rats. Protected bands representing bFGF and IGF-I mRNAs are indicated. Bands in last lane at right represent undigested bFGF and IGF-I antisense RNAs.

similar to the effect of diabetes on IGF-I mRNA levels in tissues from rats in which diabetes had been induced for shorter periods.[9,17,18]

To determine whether the level of mRNA encoding another angiogenic growth factor was also increased by diabetes, aFGF mRNA level in eyes from control, diabetic,

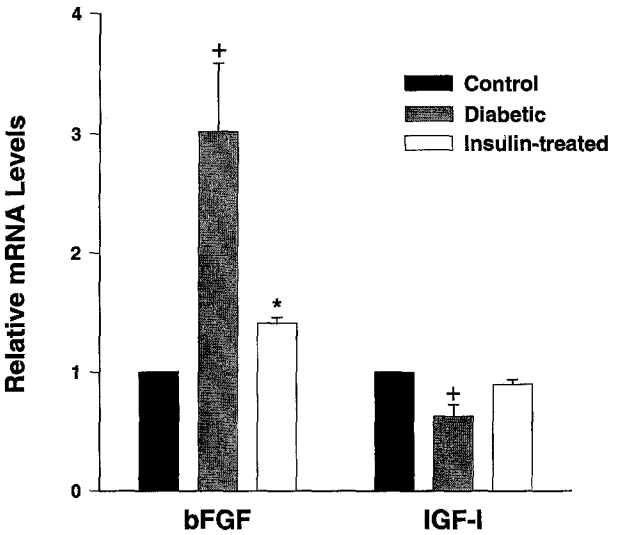

Fig 2. Quantitation of bFGF and IGF-I mRNA levels in eyes from diabetic, insulin-treated diabetic, and control rats. Values are relative levels of bFGF or IGF-I mRNA in eyes from diabetic and insulin-treated diabetic rats versus the level in control rats, which was defined as 1 for each tissue. The value for each group is the mean ± SEM of the level in three different preparations of RNA, each prepared from eyes pooled from two different rats. All samples were assayed in duplicate. †$P < .05$, *$P < .01$: *v* control.

and insulin-treated diabetic rats was determined using a solution hybridization/RNase protection assay (Fig 3). aFGF mRNA levels in the eyes of diabetic and insulin-treated diabetic rats were 118% ± 12% and 118% ± 7% (mean ± SEM, n = 3), respectively, as compared with the level in control rats, which was defined as 100%.

Given the heterogeneity of tissues in the eye, the effect of diabetes on bFGF and IGF-I mRNA levels in partially purified preparations of retina was determined. For these studies, diabetes was induced by administration of STZ 65 mg/kg and rats were maintained diabetic for 28 days. Diabetic rats lost a mean of 19 g during this period, and control and insulin-treated diabetic rats gained a mean of 136 and 141 g, respectively. Diabetic rats had a mean glucose level of 27.4 mmol/L, whereas mean glucose level in control and insulin-treated diabetic animals was 8.6 and 3.6 mmol/L, respectively. Diabetes again increased bFGF mRNA levels as compared with the level in control retinas (Fig 4). Interestingly, insulin treatment did not prevent the diabetes-induced increase in bFGF mRNA levels in the retina. IGF-I mRNA levels in these same preparations of retina decreased in diabetic versus control animals (Fig 4). In contrast to the effect of insulin on bFGF mRNA levels, the diabetes-induced decrease in IGF-I mRNA levels was prevented by insulin treatment. This demonstrates that despite its lack of effect on bFGF mRNA levels, insulin therapy was effective in preventing the diabetes-induced change in some growth factor mRNA levels in the retina.

Given the increase in bFGF mRNA levels in the eyes and retinas of diabetic rats, the effect of diabetes on bFGF peptide levels in the retinas and eyes of diabetic and control rats was determined by Western blot analysis. In the first set of studies, diabetes was induced by intravenous injection of STZ 65 mg/kg, and rats were maintained for 28 days. Diabetic rats gained a mean of 8 g, and control rats gained a mean of 152 g. Diabetic rats were hyperglycemic, with a

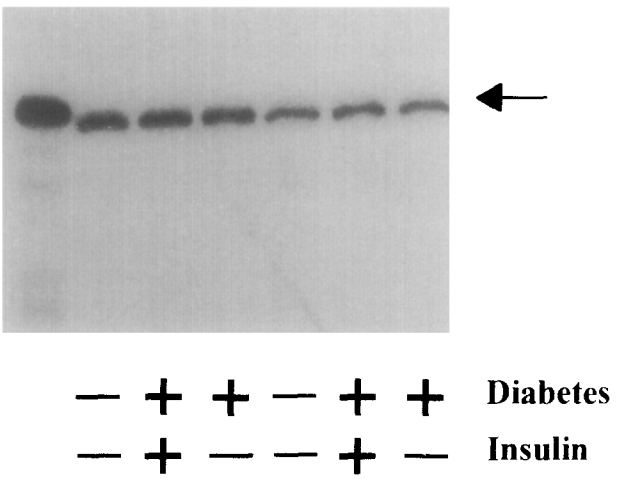

Fig 3. Autoradiogram of aFGF mRNA in eyes of diabetic, insulin-treated diabetic, and control rats. aFGF mRNA levels were determined using independent preparations of RNA, each prepared from eyes pooled from two different rats. The protected band representing aFGF is indicated. Band in the first lane represents undigested aFGF antisense RNA.

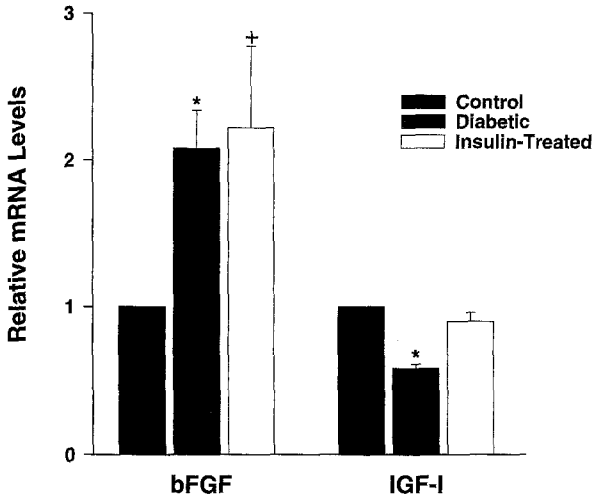

Fig 4. Quantitation of bFGF and IGF-I mRNA levels in retinas from diabetic, insulin-treated diabetic, and control rats. Values are the relative levels of bFGF and IGF-I mRNA in retinas from diabetic and insulin-treated diabetic rats as compared with the level in retinas from control rats, which was defined as 1.0. Each value is the mean ± SEM of the level in four different preparations of RNA, each prepared from one of four different rats. Each sample was assayed in duplicate. *$P < .01$ *v* control; †$P < .05$ *v* control.

mean glucose level of 23.6 mmol/L, and control rats had a mean glucose level of 8.9 mmol/L. Similar to previous studies using other tissues,[19-21] multiple isoforms of bFGF with molecular weights of 21, 20, and 18 kd were present in the retina (Fig 5). For these studies, an equal amount of protein was applied to each lane. The level of bFGF peptide in the different sets of retinas was variable, although with the exception of one control sample (Fig 5, lane h), there was a tendency for bFGF peptide levels to be increased in diabetic versus control retinas. When the level of bFGF peptide, as determined from the autoradiogram, was expressed as arbitrary densitometric units (ADU), bFGF levels in retinas from diabetic and control animals were 4.9 ± 0.5 and 3.7 ± 2.0 ADU, respectively. There was not a preferential change in a specific isoform of bFGF. In another study in which equivalent amounts of protein from whole eyes were analyzed, a similar result was obtained: bFGF peptide level in extracts from diabetic eyes increased

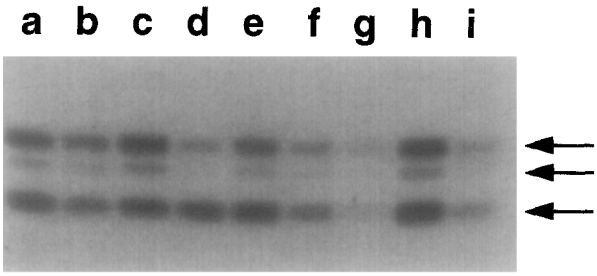

Fig 5. Western blot analysis for bFGF performed on heparin-bound materials from extracts of retinas from diabetic and control rats. Extracts were obtained from diabetic (lanes a to e) and control (lanes f to i) rats, and equal amounts of protein were analyzed. Bands that correspond to the three different isoforms of bFGF are indicated by arrows.

1.2- ± 0.1-fold (mean ± SEM, n = 4) as compared with the level in extracts from control eyes, which was defined as 1.0 (data not shown). Consistent with the lack of effect of diabetes on bFGF mRNA levels in extraocular tissues, no change in bFGF peptide levels was observed in hearts (98.1 ± 5.2 *v* 104.0 ± 11.5 ADU, mean ± SEM, n = 5) or brains (338.9 ± 30.3 *v* 328.1 ± 18.8 ADU, mean ± SEM, n = 5) of diabetic versus control rats (data not shown).

Subsequent studies were performed to begin to define the metabolic abnormalities responsible for diabetes-induced changes in bFGF and IGF-I mRNA levels in the retina. Given the potential role of polyol, especially sorbitol, accumulation in the development of diabetic complications, the role of sorbitol accumulation in the diabetes-induced increase in bFGF and decrease in IGF-I mRNA levels in the retina was examined. For this, rats were made diabetic by intravenous injection of STZ 65 mg/kg and were maintained for 21 days; half of the control and diabetic rats were treated with the aldose reductase inhibitor sorbinil. Diabetic and sorbinil-treated diabetic groups each lost a mean of 17 g, whereas control and sorbinil-treated control rats gained a mean of 98 and 95 g, respectively. Diabetic and sorbinil-treated diabetic groups were both hyperglycemic, with mean glucose levels of 23.0 and 23.9 mmol/L, respectively. The level of bFGF mRNA in partially purified retinas of diabetic rats increased to 291% of the level in control retinas, which was defined as 100%, whereas the level in retinas of diabetic rats treated with sorbinil was 252% of control levels (Fig 6). Treatment of control rats with sorbinil did not have a significant effect on bFGF mRNA levels in the retina. Similarly, treatment with sorbinil did not have a significant effect on the diabetes-induced decrease in IGF-I mRNA levels in the retina (Fig 6). Sorbinil also had little to no effect on IGF-I or bFGF mRNA levels in other tissues (data not shown). Efficacy of sorbinil therapy was determined by measuring levels of glucose, sorbitol, fructose, and *myo*-inositol in lenses from sorbinil-treated and untreated control and diabetic rats (Table 1). Sorbinil treatment was effective, as demonstrated by the decrease in sorbitol and increase in *myo*-inositol levels in lenses of diabetic rats treated with sorbinil.

Table 1. Effect of Sorbinil on Glucose, Sorbitol, *myo*-Inositol, and Fructose Levels (nmol/mg wet weight) in the Lens

Determination	Control	Control + Sorbinil	Diabetic	Diabetic + Sorbinil
Glucose	0.5 ± 0.1	0.2 ± 0.0*	5.6 ± 0.2†	6.5 ± 0.7†
Sorbitol	0.3 ± 0.1	0.1 ± 0.1	30.3 ± 2.1†	0.9 ± 0.1*‡
myo-Inositol	2.8 ± 0.1	3.9 ± 0.4	0.1 ± 0.0†	2.4 ± 0.1†‡
Fructose	0.3 ± 0.1	0.1 ± 0.0	10.1 ± 0.5†	1.8 ± 0.2†‡

NOTE. Mean ± SEM of levels in five different lenses for each group.
*$P < .05$ *v* control.
†$P < .01$ *v* control.
‡$P < .01$ *v* diabetic.

The decrease in IGF-I mRNA levels in the tissues of diabetic animals can be reproduced in fasted animals, presumably due to the hypoinsulinemia and/or growth hormone resistance associated with both states.[18,22,23] To determine whether hypoinsulinemia induced by fasting increased bFGF mRNA levels in the eye, rats were fasted for 48 hours. Fasting decreased bFGF and IGF-I mRNA levels in the eye to 74% ± 26% and 80% ± 8% (mean ± SEM, n = 3), respectively, of the level in control eyes, which was defined as 100% (Fig 7). Efficacy of the fast was demonstrated by the decrease in hepatic IGF-I mRNA levels in fasted rats to 36% ± 5% (mean ± SEM, n = 3) of control levels, similar to previous reports.[18,22]

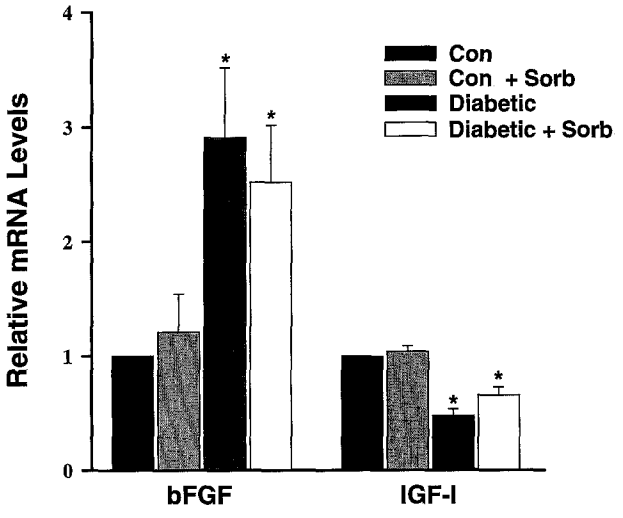

Fig 6. Quantitation of bFGF and IGF-I mRNA levels in partially purified retinas from sorbinil (Sorb)-treated and untreated control (Con) and diabetic rats. Values represent relative levels of bFGF and IGF-I mRNA in retinas from Sorb-treated Con and diabetic rats and untreated diabetic rats as compared with the level in untreated Con rats, which was defined as 1. Value for each group is the mean ± SEM of the level in five different preparations of RNA, each prepared from one of five different rats. All samples were assayed in duplicate. *$P < .01$ *v* Con.

DISCUSSION

To date, the angiogenic factor responsible for the endothelial cell proliferation and neovascularization present in

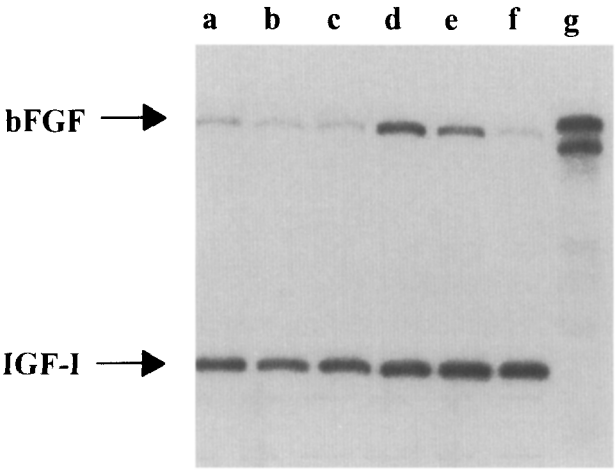

Fig 7. Autoradiogram of bFGF and IGF-I mRNA levels in eyes from fasted and fed rats. bFGF and IGF-I mRNA levels were determined using independent preparations of RNA, which were prepared from the eyes of three different fasted (lanes a to c) and fed (lanes d to f) rats. Bands in lane g represent undigested bFGF and IGF-I antisense RNAs.

proliferative diabetic retinopathy has not been identified. bFGF is an angiogenic factor that accounts for the majority of mitogenic activity present in normal bovine retinal extracts,[24] thus making it a candidate for an etiologic factor in the development of diabetic retinopathy. In a previous study, we demonstrated that severe acute diabetes in rats increased bFGF mRNA levels in a variety of tissues including the heart, lung, brain, and eyes, although the increase in the eyes was relatively modest as compared with changes in the other tissues.[9] In contrast, in the present study in which diabetes was less severe and more prolonged, there was a marked increase in bFGF mRNA levels in the eyes and retinas of diabetic rats, and these were the only tissues in which bFGF mRNA levels were consistently increased, suggesting that if bFGF does contribute to the development of diabetic microangiopathy, its role may be limited to retinopathy. This is consistent with previous observations that demonstrated differences between diabetic retinopathy and the microvascular disease present in other tissues and the subsequent suggestion that the pathogenesis of diabetic retinopathy may differ from the pathogenesis of microvascular disease in other tissues.[5,25] In contrast to the effect of diabetes on bFGF mRNA levels, the steady-state level of the mRNA encoding aFGF, which is another member of the FGF family that stimulates angiogenesis, was unchanged in the eyes of diabetic rats, whereas IGF-I mRNA levels were decreased in the eyes, retinas, and other tissues of diabetic rats. These data demonstrate that the effect of diabetes on growth factor mRNA levels in the eye and retina is gene-specific.

Studies of the potential role of bFGF in the development of diabetic retinopathy in humans are limited and inconclusive. Previous studies with small numbers of patients have demonstrated increased levels of bFGF in the vitreous of some patients with proliferative diabetic retinopathy versus patients with inactive proliferative retinopathy or other retinal diseases.[26,27] However, a subsequent study using immunohistochemistry did not demonstrate increased bFGF in eyes from patients with diabetic retinopathy, although it did demonstrate an altered distribution of immunoreactive bFGF in eyes of patients with diabetes.[28] The physiologic significance of this altered distribution of bFGF is not known. In the present study, bFGF peptide levels increased in retinas and eyes of diabetic versus control rats, although because of animal-to-animal variability in bFGF levels, the increase did not reach statistical significance. The reason for the discrepancy in the diabetes-induced change in bFGF peptide and mRNA levels is not clear, although it may relate to the ability of bFGF to bind to the heparan sulfate present in proteoglycans and to be stored in that form in the extracellular matrix.[6] Levels of heparan sulfate–containing proteoglycans are decreased markedly by diabetes.[1] The synthetic rate of bFGF may thus be increased by diabetes, but total cell-associated bFGF peptide levels may increase only minimally because of increased turnover of bFGF due to the reduced availability of heparan sulfate–containing proteoglycans. Metabolic labeling studies will be required to address this point.

One of the goals of this study was to define the metabolic alterations responsible for diabetes-induced changes in bFGF and IGF-I mRNA levels. A metabolic abnormality that occurs as a result of hyperglycemia is increased conversion of glucose to the polyol sorbitol by aldose reductase, which results in sorbitol accumulation in some tissues of diabetic animals.[3,4] Sorbitol accumulation is thought to contribute to the development of diabetic complications, including diabetic microangiopathy,[2-4,5,25] although prevention of sorbitol accumulation using inhibitors of aldose reductase, eg, sorbinil,[29] in animal models of retinopathy has had variable effects on the development of retinopathy.[25] In our studies, sorbinil treatment effectively prevented some of the metabolic complications of diabetes, including sorbitol accumulation and *myo*-inositol depletion; however, it had no effect on bFGF and IGF-I mRNA levels. These data suggest that sorbitol accumulation and its consequent changes in cellular metabolism are not responsible for the increase in bFGF mRNA and decrease in IGF-I mRNA levels in retinas of diabetic rats. STZ-induced diabetes is also associated with hypoinsulinemia. Another metabolic state associated with hypoinsulinemia is fasting. In this study, we demonstrated that fasting did not reproduce the diabetes-induced increase in bFGF mRNA levels in the eye, suggesting that hypoinsulinemia is not the cause of this increase, but fasting did partially reproduce the diabetes-induced decrease in IGF-I mRNA levels in the eye, which is similar to the effect of fasting on IGF-I mRNA levels in other tissues in the rat.[18,22,23] These data demonstrate that hypoinsulinemia induced by alternative means decreases IGF-I mRNA levels, and more importantly, that the diabetes-induced metabolic abnormalities responsible for alterations in bFGF and IGF-I mRNA levels are, at least in part, different. Another interesting and unexpected difference in the effect of diabetes on bFGF and IGF-I mRNA levels was the response of these mRNAs to insulin treatment. Insulin treatment effectively prevented the diabetes-induced decrease in IGF-I mRNA levels in the eye and retina. Despite this effect of insulin on IGF-I mRNA levels in retinas, in these same retinas insulin treatment did not prevent the diabetes-induced increase in bFGF mRNA levels. These data are consistent with the clinical observation that established diabetic retinopathy often worsens with initiation of intensive insulin therapy, but are inconsistent with the ability of intensive insulin therapy to reduce ultimately the incidence and progression of diabetic retinopathy.[30,31] In that respect, one possible explanation for the lack of effect of insulin treatment on retinal bFGF mRNA levels is a difference in diabetes-induced retinal changes in rats and humans.

Other biochemical abnormalities that have been demonstrated in some tissues from rats with diabetes and that might influence tissue growth factor mRNA levels include increased activity of protein kinase C in a variety of tissues, including heart, aorta, retina, and kidney, and accumulation of advanced glycosylation end products.[2,32-35] Activation of protein kinase C increases bFGF mRNA levels and decreases IGF-I mRNA levels in cultured cells,[36,38] whereas advanced glycosylation end product–modified proteins have been shown to increase production of IGF-I by mono-

cytes,[39] although their effect on bFGF mRNA levels has not been explored. The tissue-specificity of the effect of diabetes on bFGF mRNA levels (ie, increased levels in the eye but inconsistent or no change in levels in the heart, kidney, skeletal muscle, and lung) would argue against these relatively generalized metabolic changes being responsible for the increase in bFGF mRNA levels in the eyes. However, given the existence of several different isoforms of protein kinase C, an increase in activity of an eye-specific isoform that stimulates an increase in bFGF mRNA levels remains a possibility.

The diabetes-induced increase in bFGF mRNA levels in the eye reported in this study is consistent with a potential role for bFGF in the development of diabetic retinopathy. However, given that retinal changes characteristic of proliferative retinopathy in humans do not develop in rat models of diabetes, future studies using animal models of diabetes (eg, dogs) that result in retinal changes more characteristic of those present in human diabetic retinopathy will be required to define further the role of bFGF in the pathogenesis of diabetic retinopathy. The present study also does not preclude the involvement of one of the many other recently described angiogenic factors, eg, vascular endothelial growth factor (VEGF), in the development of diabetic retinopathy. VEGF is produced by retinal pigment epithelial cells, and, consistent with the potential role of retinal ischemia in the development of diabetic retinopathy,[5] VEGF production by glial and skeletal muscle cells can be induced by hypoxia.[40,41] The effect of diabetes on VEGF gene expression and production has not been investigated to date.

In summary, diabetes has differential effects on bFGF and IGF-I mRNA levels in eyes and retinas of rats with diabetes. Induction of hypoinsulinemia by fasting partially reproduced the diabetes-induced decrease in IGF-I mRNA levels but not the increase in bFGF mRNA levels, suggesting that different metabolic abnormalities are responsible for the effect of diabetes on the production of different growth factors. Future studies will be required to define the metabolic abnormalities responsible for the diabetes-induced increase in bFGF mRNA levels in eyes and retinas of diabetic rats and the relationship of this increase to the pathogenesis of diabetic retinopathy.

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