Regulation of Transforming Growth Factor-Beta, Basic Fibroblast Growth Factor, and Vascular Endothelial Cell Growth Factor mRNA in Peripheral Blood Leukocytes in Patients With Diabetic Retinopathy

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In the present study, we examined the effect of glucose concentration on the expression of vascular endothelial growth factor (VEGF), basic fibroblast growth factor (bFGF), and transforming growth factor-beta (TGF-β) mRNA using reverse transcriptase–polymerase chain reaction (RT-PCR) in normal healthy leukocytes in vitro and in leukocytes from patients with type 1 diabetes mellitus. In vitro, the level of TGF-β mRNA was altered in response to the glucose concentration (maximum at 10 mmol/L), while bFGF mRNA remained relatively constant and VEGF mRNA varied with no clear correlation with the glucose concentration. Leukocytes from type 1 patients showed no difference in bFGF or TGF-β mRNA levels compared with age-matched healthy controls. However, VEGF mRNA was significantly lower in type 1 patients compared with controls ($P < .05$). When the patients were subtyped according to the severity of retinopathy, the level of TGF-β mRNA was elevated selectively in patients with evidence of active new retinal vessels ($P < .01$) and $VEGF_{121}$ mRNA was reduced in patients with mild to moderate retinopathy. Thus, leukocyte growth factor mRNAs respond to acute changes in the glucose concentration in vitro, and are differentially expressed in type 1 diabetic patients during the course of the disease.

THE WIDESPREAD and primarily vascular nature of the clinical complications of diabetes suggests that circulating factors may contribute to the pathogenesis of disease. This has initiated a number of investigations into the role of leukocytes in the manifestation of the vascular complications of diabetes.[1-7] The role of the glucose concentration has also been studied, given that clinical studies have conclusively demonstrated that the incidence and severity of retinopathy is significantly reduced if a strict glucose regimen is maintained.[8]

Diabetic retinopathy (DR) is a common complication of diabetes that affects the retinal microvasculature and is responsible for the majority of visual impairment in the working population of the Western world. It is characterized by increased vascular permeability, increased blood flow, thickening of the basement membrane, capillary occlusion, microaneurysm formation, and the appearance of new vessels (angiogenesis),[9] all of which directly or indirectly reflect changes in retinal endothelial cell behavior. Growth factors have been implicated in the modulation of endothelial cell behavior leading to the manifestation of DR, including vascular endothelial growth factor (VEGF).[10-12] Furthermore, the expression of VEGF is regulated by the glucose concentration[13,14] and in response to other growth factors, eg, insulin-like growth factor I,[15] that are believed to be associated with an increased severity of retinopathy.[16] The distribution of basic fibroblast growth factor (bFGF), a known retinal endothelial mitogen,[17] has been shown to change with the progression of DR,[18] and the level of bFGF also has been shown to change in vitro in response to changes in oxygen tension.[19] Transforming growth factor-beta (TGF-β) has the potential to play a significant role in the development of DR, given that TGF-β is known to be released from macrophages and degranulating platelets at the site of vascular injury. It is also known to induce extracellular matrix synthesis, cell adhesion, and differentiation.[20]

Co-cultures of leukocytes and endothelial cells demonstrate that endothelial cell growth is dependent on the close association with and activation of leukocytes and the release of growth factors from the leukocytes.[1,19] In vivo investigations with experimental models of diabetes have demonstrated that activated monocytes are increased in diabetic rats[2] and diabetic cats,[6] and it is possible that these activated cells provide a source of growth factors that will initiate and maintain the growth of new vessels.

We wished to determine if leukocytes produce growth factors in response to changes in the glucose concentration. Thus, we have examined the expression of growth factor mRNA in leukocytes in vitro in response to changes in glucose concentration and in leukocytes isolated from patients with a range of severity of DR.

SUBJECTS AND METHODS

Subjects

A total of 43 individuals were included in the study and divided among the following groups: control (C, n = 11), no retinopathy (NR, n = 7), mild/moderate (MM, n = 6), severe (SDR, n = 7), and proliferative DR (PDR, n = 12). The members of the control group were healthy subjects who were age- and sex-matched to the patient groups. All individuals in the test groups were diagnosed as having insulin-dependent diabetes mellitus according to World Health Organization criteria and were assigned to the respective groups after fundus biomicroscopic examination. The clinical evaluations were based on a modification[21] of the Airlie House classification system for DR[22]: NR, no evidence of DR; MM, microaneurysms, cotton wool spots, and retinal hemorrhage: SDR, increasing number of hemorrhages in the retina, accumulation of hard exudate, marked cotton wool spot formation, and signs of ischemia as shown by areas of avascularity and venous beading; and PDR, new vessel growth from the disc or elsewhere (Table 1).

Isolation of Peripheral Blood Leukocytes

Blood was obtained by venous puncture and diluted 1:1 with customized glucose-free RPMI 1640 (GIBCO-BRL, Paisley, UK).

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Table 1. Patient Profile (N = 43)

Parameter	Test Group				
	C	NR	MM	SDR	PDR
Age, yr (mean ± SD)	40 ± 11	40 ± 13	47.5 ± 12	41 ± 15	51.5 ± 13
Males					
No.	10	5	5	6	11
%	91	71	71	100	92
Females					
No.	1	2	2	0	1
%	9	29	29	0	8
Glucose (mmol/L)	5.3 ± 0.4	11 1 ± 6.1	20.2 ± 2.8	15.6 ± 9.4	10.4 ± 5.7
HBA_{1c} (%)	5.5 ± 0.5	9.8 ± 2.5	9.2 ± 1.4	8.5 ± 0.9	8.9 ± 2.0

NOTE. The mean HBA_{1c} (<6% is regarded as normal) and glucose concentrations at the time of sampling are shown (mean ± SD).

Leukocytes were isolated from the blood samples using Histopaque 1.077 (Sigma-Aldrich, Dorset, UK) as recommended by the manufacturer. Viability was assessed by trypan blue dye exclusion, which was routinely greater than 90%. Leukocytes isolated from healthy subjects were used for the in vitro determination of the effect of glucose concentration on growth factor mRNA expression. For these experiments, the cells were seeded at a density of 2×10^6/mL and incubated for 8 hours in a range of glucose concentrations as specified. Having determined that there was evidence of glucose-mediated regulation of growth factors, we examined the patient samples in the respective test groups.

RNA Extraction, Purification, and Reverse Transcriptase–Polymerase Chain Reaction

Total RNA was extracted from peripheral blood leukocytes using phenol-chloroform-guanidinium isothiocyanate[23] and quantified by determining the absorbance at 260 nm. A 5-μg aliquot of the respective RNA samples was reverse-transcribed using oligo dT (15 mer; Oswell, Southampton, UK) and 200 U reverse transcriptase ([RT] M-MLV; GIBCO-BRL) as described by the manufacturer. The cDNA samples were then stored at −80°C until required for amplification with the polymerase chain reaction (PCR). Sequence-specific primers for β-actin, TGF-β, and bFGF were designed using the Human Genome Mapping central database and checked for sequence similarities with other human sequences. The primers were synthesized and purified commercially (Oswell). The VEGF-specific primers were designed by Iijima et al[24] to detect the alternatively spliced forms of this growth factor (Table 2). The PCR mixture contained 5 μL cDNA in a 50-μL reaction mixture containing dNTPs (0 2 mmol/L), reaction buffer (10 mmol/L Tris hydrochloride, 1 5 mmol/L $MgCl_2$, and 500 mmol/L KCl, pH 8.3) and 3′ and 5′ primer pairs of the respective growth factors (1 μmol/L). A housekeeping gene (β-actin) was simultaneously amplified for each cDNA examined. The reaction was performed using a thermocycler (Cyclogene; Techne, Cambridge, UK) with the following PCR profile: one cycle at 94°C for 2 minutes, 55°C for 1 minute, and 72°C for 1.5 minutes; 33 cycles at 94°C for 50 seconds, 55°C for 1 minute, and 72°C for 1.5 minutes; and one cycle at 94°C for 50 seconds, 55°C for 1 minute, and 72°C for 5 minutes.The amplified products from leukocytes cultured in vitro in a range of glucose concentrations were resolved in a 1.8% agarose gel (GIBCO-BRL) containing ethidium bromide and visualized under UV light. The image of the gel was captured using Imagestore (UVP Life Sciences, Cambridge, UK), and the intensities of the corresponding PCR products were determined using the GDS5000 Gel Documentation System (UVP Systems). This method of semiquantitative analysis was used for the study of leukocytes cultured in vitro in a range of glucose concentrations.

Table 2. Nucleotide Sequence for Primers Used in PCR

Primer	Sequence	Product Size
5′ β-actin	GTG GGG CGC CCC AGG CAC CA	
3′ β-actin	GTC CTT AAT GTC ACG CAC GAT TTC	548
5′ TGF-β	ATC AGA GCT CCG AGA AGC GGT ACC	
3′ TGF-β	GTC CAC TTG CAG TGT GTT ATC CCT G	310
5′ bFGF	TTC TCG AAC CGC TGT GTC TCC	
3′ bFGF	CAA ACA CCT CCA GCA TTT CGG	444
5′ VEGF	CGA AGT GGT GAA GTT CAT GGA TG	403
3′ VEGF	TTC TGT ATC AGT CTT TCC TGG TGA G	535
		607

Semiquantitative Analysis of Growth Factor mRNA Levels Using RT-PCR

The mRNAs for TGF-β, bFGF, β-actin, and VEGF were detected by RT-PCR (Fig 1). The specificity of the amplification was confirmed by sequencing the PCR products for TGF-β, bFGF, and β-actin and by size and restriction enzyme mapping of the VEGF PCR products (results not shown), which was consistent with previously published data using these primer pairs.[24] The conditions for amplification were optimized by confirmation that the quantitation was performed during the exponential phase of the reaction. Consequently, a 5-μL aliquot of the undiluted PCR product was used for quantitation with the GDS5000 System and a 1:10 and 1:100 dilution was used for quantitation by Southern hybridization. A dot blot was prepared using the dilutions of the respective PCR products and hybridized using the cDNA probes. TGF-β (2.1-kb *Eco*R1 fragment), β-actin (1.7-kb *Pst*1 fragment), bFGF (1.4-kb *Eco*R1 fragment), and $VEGF_{121}$ and $VEGF_{165/189}$ PCR products generated from sequence-specific VEGF primers[24] that were excised from low–melting-point agarose gel and subsequently purified using a commercial DNA clean-up kit (Nucleon; Scotlab Bioscience, Glasgow, UK). The cDNA probes were labeled with αP^{32}-dCTP (Amersham Pharmacia Biotech, Little Chalfont, UK) and hybridized as previously described.[25] The level of radioactivity on the filters was determined using a $Matrix_{96}$ (Packard, Berkshire, Glasgow, UK). The results are presented as the ratio of the level of signal (cpm) obtained with TGF-β-, bFGF-, and VEGF-specific probes to the cpm obtained using a β-actin-specific probe.

Statistical Analysis

Data were analyzed by two-tailed Student's *t* test for the in vitro culture of leukocytes and are expressed as the mean ± SD. The data for growth factor mRNA levels in the respective test groups of subjects were analyzed using one-way ANOVA and Dunnett's test. The level of significance for the analyses is indicated in the Figures. The correlation between test parameters was determined using Pearson analysis.

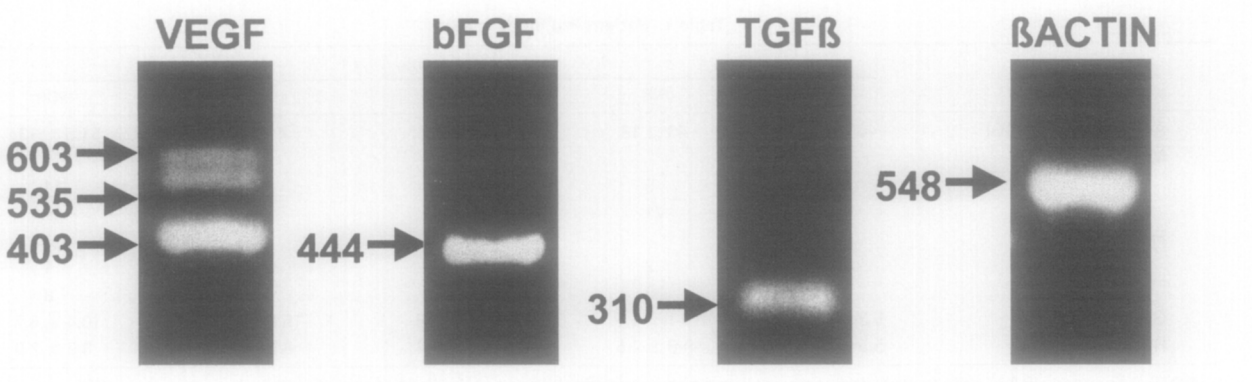

Fig 1. Agarose gel showing amplified PCR products for TGF-β, bFGF, VEGF, and β-actin. A 10-μL aliquot of the respective PCR products were analyzed at 70 V on a 1.8% agarose gel, stained with ethidium bromide, and visualized with UV light. The alternatively spliced isoforms of VEGF were 403, 535, and 607 bp for $VEGF_{121}$, $VEGF_{165}$, and both $VEGF_{189}$ and $VEGF_{206}$, respectively. The size of the amplified PCR products for bFGF, TGF-β, and β-actin was confirmed as 444, 310, and 548 bp, respectively.

RESULTS

Effect of Glucose Concentration on the Level of Growth Factor mRNA in Leukocytes From Healthy Subjects

Leukocytes isolated from healthy subjects were incubated with a range of glucose concentrations for 8 hours. RT-PCR was performed to determine the level of growth factor–specific mRNA. The mean values are calculated from six samples in each test group, and the data are representative of three experiments. Figure 2 shows the mean level of the TGF-β PCR product in the respective test groups, and indicates a significant increase in the PCR product when leukocytes from healthy subjects were exposed to 10 mmol/L glucose for 8 hours compared with 5 mmol/L ($P < .01$) and a significant decrease when leukocytes were exposed to 15 mmol/L ($P < .01$) and 25 mmol/L glucose ($P < .001$) using the two-tailed Student's *t* test. The level of bFGF is also shown for the same populations of cells and shows no significant changes over the range of glucose concentrations examined (5 to 25 mmol/L). The level of PCR products for the specific VEGF isoforms was found to be highly variable, ranging from a complete absence of product to relatively high levels, indicating high individual variability.

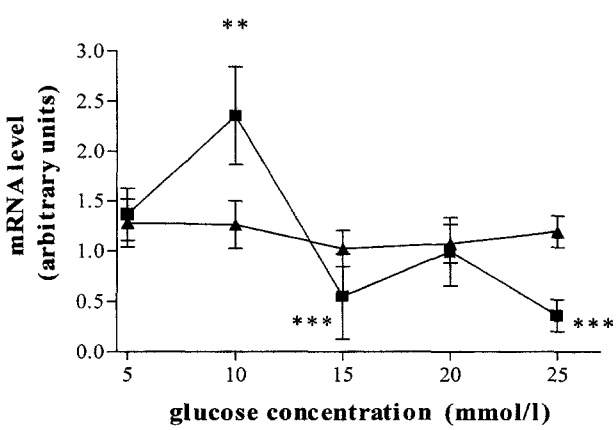

Fig 2. Level of TGF-β (■) and bFGF (▲) PCR products in leukocytes from control subjects after exposure to 5 – 25 mmol/L glucose for 8 hours. Band intensities were determined using densitometry with the Gelbase 5000 software and are given as the ratio of growth factor mRNA to β-actin mRNA. The level of TGF-β PCR product was significantly elevated in leukocytes incubated in 10 mmol/L glucose 5 mmol/L glucose ($P < .01$), and decreased in response to 15 mmol/L (**$P < .01$) and 25 mmol/L glucose (***$P < .001$). The level of amplified bFGF remained relatively constant in all glucose concentrations tested.**

Leukocyte Growth Factor mRNA Expression With Type 1 Diabetes and Severity of DR

The number of patients with detectable levels of VEGF, bFGF, and TGF-β were calculated for each test group. A negative sample is regarded as undetectable to the human eye in an ethidium bromide–stained agarose gel, less than 100 cpm (background levels) as determined by Southern hybridization, but shows detectable levels of β-actin PCR product (Table 3). Detectable levels of bFGF mRNA were found in all samples from the control group, while only 57% of patients with MM were positive for bFGF. The level of growth factor mRNA for VEGF, TGF-β, and bFGF was determined using β-actin as an internal housekeeping gene and compared with the control group. A significant decrease in the level of $VEGF_{121}$ ($P < .001$) and $VEGF_{165\text{-}189}$ ($P < .05$) was evident in leukocytes from type 1 patients compared with the control group by ANOVA. There was no significant difference between the level of bFGF and TGF-β mRNA between these groups (Fig 3). Type 1 patients were divided into groups according to the degree of retinal damage as already outlined (C, NR, MM, SDR, and PDR). For patients with detectable levels of VEGF, significant changes were evident in the level of VEGF in the test groups examined using one-way ANOVA and Dunnett's test. The level of $VEGF_{121}$ was significantly higher in the C group compared with

Table 3. Summary of Growth Factor mRNA Detected in Patients With Type 1 Diabetes

Growth Factor	Test Group				
	C	NR	MM	SDR	PDR
bFGF	100	71	57	67	75
TGF-β	82	100	71	100	83
$VEGF_{121}$	73	86	71	67	83
$VEGF_{165\text{-}206}$	55	57	57	67	67

NOTE. The level of mRNA for bFGF, TGF-β, $VEGF_{121}$, and $VEGF_{165\text{-}206}$ was determined using RT-PCR and Southern hybridization. The percentage of patients that were positive for the different growth factors is shown (%). A sample was regarded as positive when the cpm for the respective sample was >100.

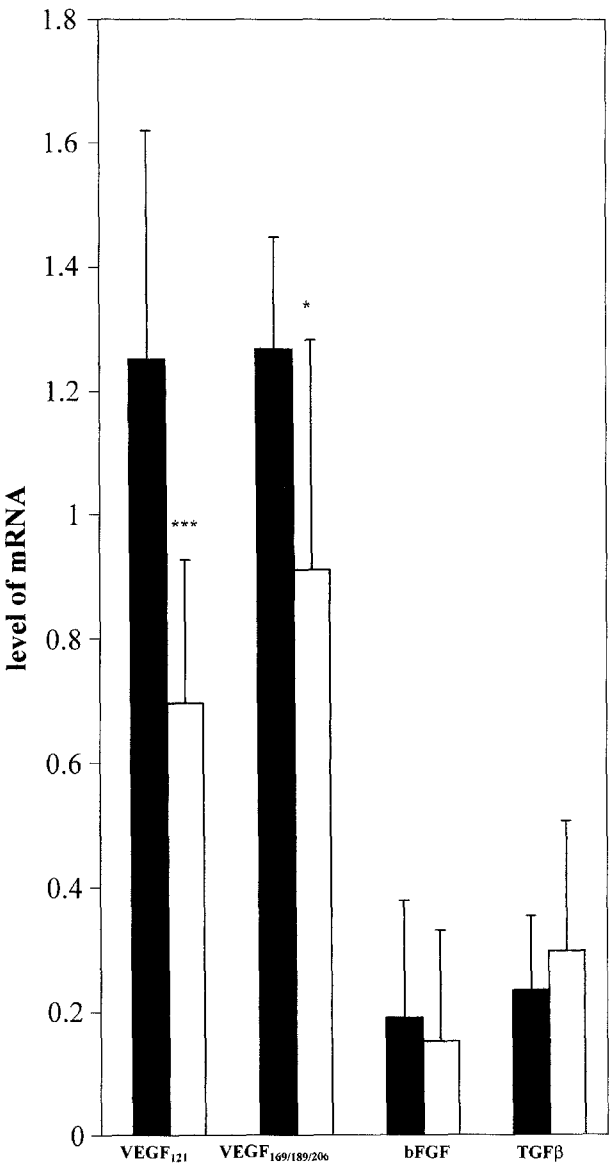

Fig 3. Level of growth factor mRNA in type 1 diabetic patients. RT-PCR was used to determine the level of mRNA for VEGF, bFGF, and TGF-β in leukocytes isolated from patients with type 1 diabetes (□) compared with an age- and sex-matched control group (■). The level of mRNA for the $VEGF_{121}$ isoform and $VEGF_{165\text{-}189}$ was significantly lower in the type 1 patient group (*$P < .001$ and *$P < .05$), although no significant change in the level of bFGF or TGF-β was evident.**

NR patients ($P < .01$) and MM patients ($P < .05$) (Fig 4). For the higher–molecular-weight isoforms of VEGF (165 and 189), only the NR ($P < .01$) and MM ($P < .05$) groups showed significantly lower levels than the C group. In contrast, the mean level of TGF-β for the PDR group was significantly elevated compared with the C group ($P < .01$). NR and SDR patients had significantly lower ($P < .001$) levels of TGF-β mRNA than PDR patients. The level of bFGF PCR product was comparably low for the PDR test group, and no significant differences emerged in the analysis of the different test groups (Fig 4).

Association of Glucose Control With the Level of Growth Factor mRNA

The glucose concentration and hemoglobin A_{1C} (HBA_{1C}) level were determined for each individual in the test groups at the time of sampling (Table 1). The level of PCR product for

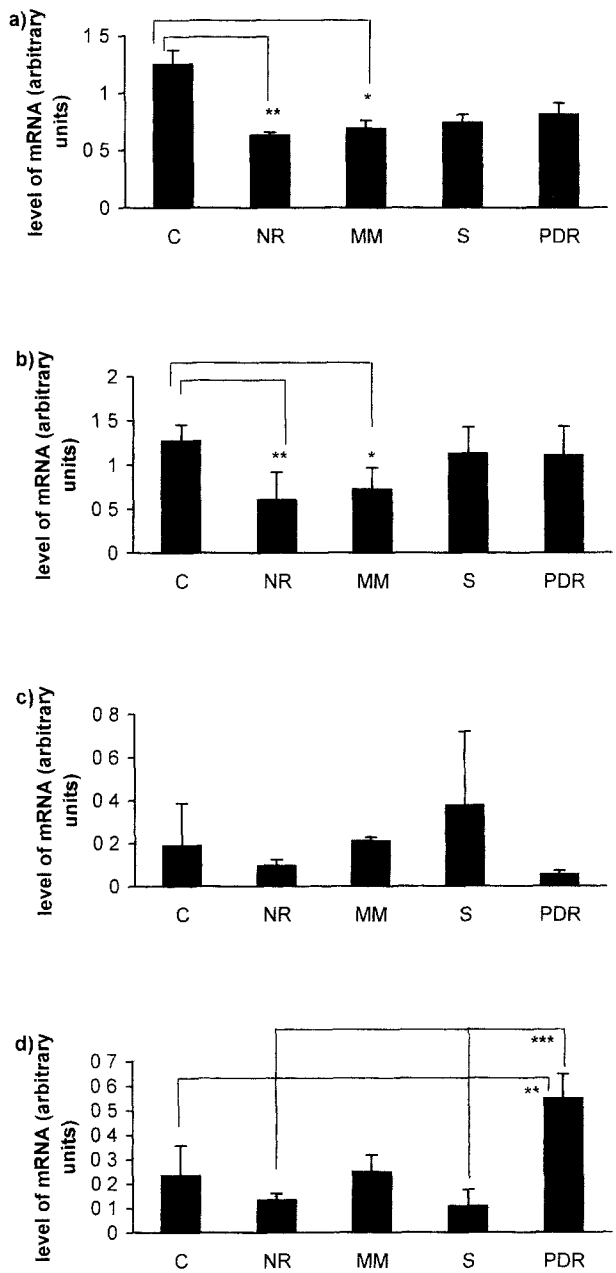

Fig 4. Histogram showing the level of TGF-β, bFGF, $VEGF_{121}$, and $VEGF_{165\text{-}189}$ mRNA in patients with varying degrees of DR. The level of each PCR product was detected using RT-PCR and is presented as the ratio of the cpm for the respective growth factors and β-actin for each patient. The level of $VEGF_{121}$ mRNA was significantly higher in the control group *v* NR patients ($P < .01$) and MM patients (*$P < .05$) (a), while for $VEGF_{165\text{-}189}$ mRNA, a similar significant change in the mRNA level was evident (**$P < .01$ and *$P < .05$) for the NR and MM groups, respectively (b). No significant changes in the level of bFGF were found (c), but a significant increase in the level of TGF-β was evident in the PDR test group *v* controls (**$P < .01$), the NR group (***$P < .001$), and the SDR group (***$P < .001$) (d).**

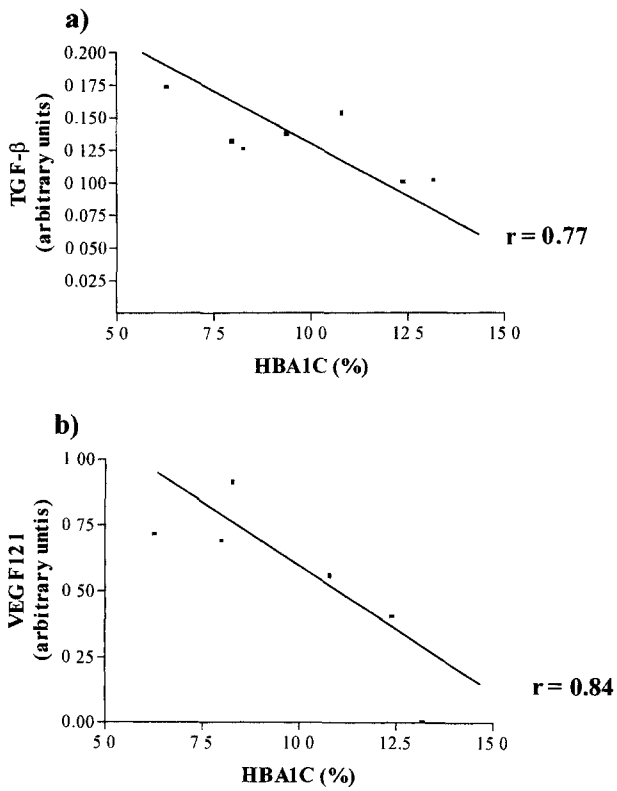

Fig 5. Correlation between TGF-β and $VEGF_{121}$ levels with HBA_{1c} level (%) in NR patients. A significant correlation was observed for both TGF-β ($P = .044$) and $VEGF_{121}$ ($P = .037$) with HBA_{1c}. As HBA_{1c} increased, TGF-β and $VEGF_{121}$ declined. This trend was not observed with respect to bFGF levels and was not found in the control group or any of the patient groups with signs of retinal disease.

TGF-β, VEGF, and bFGF was compared with the HBA_{1C} and glucose concentrations for each of the test groups (Table 3). A significant correlation was found between TGF-β and HBA_{1C} levels in NR patients ($r = .77$) and also between HBA_{1C} and $VEGF_{121}$ levels ($r = .84$) in the same patient group using Pearson analysis (Fig 5 and Table 4). No other correlations with the glucose concentration at the time of sampling, age, sex, or duration of diabetes and the levels of growth factor mRNAs were found.

DISCUSSION

In the present study, we have examined the hypothesis that activated leukocytes may provide a source of growth factors for retinal endothelial cells during the process of neovascularization. This study presents evidence for specific changes in the level of leukocyte growth factor mRNA for patients with type 1 diabetes mellitus. The level of $VEGF_{121}$ and $VEGF_{165\text{-}189}$ mRNA was significantly lower in patients with diabetes. The increased expression of VEGF in the endothelial cells and vessel walls of the eye in patients with diabetes has previously been described,[26] and the role of VEGF in retinal vascular proliferation has also been demonstrated.[27] The lower level of VEGF mRNA in this study may reflect an increase in the level of transcription of the mRNA that would result in the higher level of growth factor expression. However, if we divide the patient groups according to the severity of DR, then it is at the early stages of disease when significant changes in the level of VEGF mRNA are evident. This is consistent with the increased expression of VEGF recorded in an experimental rat model of diabetes before any observable retinal changes[28] and may reflect the increased translation of VEGF mRNAs. Studies have shown that the level of VEGF in intravascular leukocytes within the retinal endothelium is similar in eyes from diabetic and nondiabetic subjects but changes did occur in the choroidal vasculature.[26] This may be of significance to the developing pathology of DR and to the identification of changes in VEGF mRNA in peripheral blood leukocytes.

The level of $VEGF_{121}$ mRNA in leukocytes from NR patients was significantly correlated with the HBA_{1C} level, which was not evident in patients for whom signs of DR were evident. The significant decrease in VEGF mRNA and the correlation with HBA_{1c} in NR patients may be indicative of an attempt to regulate growth factor production in conditions of increased glucose concentration. This regulation may be lost in the later stages of retinal disease. This pattern is also evident from clinical studies that have demonstrated a clear association between glucose control and progression of retinopathy.[8] However, the outcome of an intensive insulin regimen to normalize HBA_{1c} is also significantly associated with the duration of diabetes.[29] Therefore, chronic exposure to high concentrations of glucose may initiate other factors, eg, advanced-glycation end products,[30] that influence disease progression.

In vitro culture of leukocytes indicated that the level of TGF-β mRNA changes in response to the glucose concentration, with the highest level observed at 10 mmol/L and the lowest at 25 mmol/L. Interestingly, the HBA_{1c} for type 1 NR patients was significantly correlated with the level of TGF-β mRNA for the respective patients. As the disease severity

Table 4. Correlation Between the Level of Growth Factor mRNA and HBA_{1c} (%)

Growth Factor	Test Group: C		NR		MM		SDR		PDR	
	r	*P*	*r*	*P*	*r*	*P*	*r*	*P*	*r*	*P*
bFGF	−.61	.27	−.59	.16	−.68	.21	−.68	.14	−.19	.60
TGF-β	−.52	.37	−.77	.04	−.75	.14	−.51	.30	−.07	.85
$VEGF_{121}$	−.43	.47	−.84	.04	−.24	.70	−.51	.30	−.34	.33
$VEGF_{165\text{-}206}$	−.58	.30	−.53	.22	−.46	.44	−.19	.72	−.13	.72

NOTE. The level of PCR product for TGF-β, VEGF, and bFGF was correlated with the level of HBA_{1c} in the different patient groups. A positive correlation was shown with HBA_{1c} and TGF-β levels and $VEGF_{121}$ levels using Pearson correlation (*r*). The level of significance for the correlation was determined by a 2-tailed test (*P*). A positive correlation was found for $VEGF_{121}$ and TGF-β in the NR group ($P < .05$).

progresses, the regulation of TGF-β may be more substantially controlled by additional factors that we cannot reproduce in vitro that are associated with progressive vascular damage, thereby losing any glucose-specific regulatory effect. The significantly increased level of TGF-β mRNA in the proliferative phase of the disease (PDR) may be indicative of a specific stimulus to the activated, growing endothelial cells. Thus, there may be features of poor glucose control that damage the retinal vasculature either directly by affecting the endothelium or indirectly by leukocyte activation which is subsequently independent of the glucose concentration. The role of TGF-β may be significant with respect to the known colocalization of adhesion molecules with increased expression of TGF-β[31] and the known role of TGF-β in the migration of monocytes into ischemic areas.[32] Taken in concert with the increased endothelial cell activation in patients with diabetes as indicated by elevated levels of soluble E-selectin (sE-selectin),[33] which is also associated with metabolic control,[33,34] these observations may have a potentially significant impact on our understanding of the events that occur in the retina as DR progresses. The fact that, when type 1 patients were considered as a group, no significant changes in the level of TGF-β mRNA were evident illustrates that peripheral changes in the regulation of growth factors are indicative of a changing disease status in the retina, which also has been shown to be a feature of the level of E-selectin,[33] which was found to be highest in patients with severe nonproliferative DR. Furthermore, despite the lack of any significant changes in the bFGF mRNA level between the control group and type 1 patients, there were changes within the DR patient groups. Comparison of the bFGF level in patients with MM or SDR indicated that these patients had a significantly higher level of bFGF mRNA than patients with active new vessel growth (PDR), which may reflect modification or compensatory mechanisms that are involved as the disease severity progresses.

This study demonstrates that the glucose concentration is able to regulate growth factor production both in vitro and in vivo. The specific patterns of growth factor mRNA levels that were evident with different severities of retinal disease highlight the involvement of peripheral factors in the progression of disease in the retina. Further study to extend these observations would greatly facilitate our understanding of the mechanism of glucose-induced microvascular abnormalities and consequently enable us to develop meaningful predictors and methods of disease prevention.

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