Hypoxic Induction of Vascular Endothelial Growth Factor Is Markedly Decreased in Diabetic Individuals Who Do Not Develop Retinopathy

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OBJECTIVE — A small percentage of patients do not develop any evidence of diabetic retinopathy (DR) even after many years of diabetes. Vascular endothelial growth factor (VEGF) has been implicated in the development of DR. Therefore, we sought to determine if the regulation of VEGF differs in those patients who develop DR after many years of diabetes compared with those who do not develop DR.

RESEARCH DESIGN AND METHODS — We performed standard 7-field stereoscopic fundus photography on 95 consecutive patients with type 1 and type 2 diabetes. Patients were categorized into 3 groups according to the presence or absence of DR and the duration of diabetes: group 1 was defined by presence of DR, group 2 was defined by absence of DR after >10 years duration of diabetes, and group 3 was defined by absence of DR with long-standing diabetes (≥20 years for type 1 diabetes and ≥15 years for type 2 diabetes). Monocytes from 40 ml peripheral blood were isolated from all patients, and the hypoxic induction of VEGF was determined in vitro.

RESULTS — We found no significant difference in the basal level of VEGF in patients with and without DR. However, we did find a markedly significant difference in the hypoxic induction of VEGF between patients from group 1 and group 3 (4.35 ± 0.55 vs. 1.87 ± 0.3, P < 0.00013).

CONCLUSIONS — This study suggests that 1 mechanism for the absence of DR in patients with long-standing diabetes is a decreased hypoxic induction of VEGF.

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The natural history of nonproliferative and proliferative diabetic retinopathy (DR) has been well documented in several multicenter clinical trials (1–7). Specific stages of DR, which normally progress in an orderly fashion, are characterized by defined clinical parameters on ophthalmological examination (1–7). The prevalence and severity of nonproliferative and proliferative DR has been shown in large population studies to be related to the age of diabetes onset (8–10), the duration of diabetes (7,11,12), the type of diabetes (13), and the level of glycemic control (6,7,14–20). However, it is well recognized that considerable variability exists between individuals in the development and progression of DR (2,3,21–23). In type 1 diabetes, nonproliferative DR peaks in incidence after 12–18 years of diabetes, with ~98% of all patients demonstrating evidence of any DR after 20 years of exposure to diabetes (8,24,25). In type 2 diabetes, there is a peak in the incidence of nonproliferative DR 5–12 years after diabetes is diagnosed (11,26). The apparent shorter duration until onset of nonproliferative DR in type 2 diabetic patients may be attributed, at least in part, to the well-documented 5- to 7-year mean lag in the recognition and diagnosis of type 2 diabetes (26).

Over the past 5–6 years, a mounting body of evidence has been generated that supports a major role for vascular endothelial growth factor (VEGF) in the pathogenesis of proliferative DR (27–34). The stimulus for increased VEGF in the retina and ocular fluid of patients with proliferative DR is due, in large part, to the production of retinal ischemia and hypoxia. VEGF has also been implicated in the development of early phases of nonproliferative DR. Elevated levels of VEGF, directly resulting in an increase in vascular permeability, are increased in diabetic subjects before the onset of DR (31,35). Injection of VEGF into normal nonhuman primate eyes induces many of the hallmark changes of nonproliferative DR (36). Retinal blood flow has been shown to be significantly decreased in the diabetic patient before the onset of any DR (37), which may result in ischemia and/or hypoxia in the retina and in turn stimulate VEGF production. However, hypoxia may not be the most important stimulus for increased VEGF production in the retina of patients before the onset of nonproliferative DR (35). A wide variety of cytokines and metabolites (glucose [38–40], AGEs [41,42], IGF-1 [43–45], insulin [46], and angiotensin II [47–49]) are elevated in the fluid of patients with proliferative DR is due, for increased VEGF in the retina and ocular fluid of patients with proliferative DR (31,35). Injection of VEGF into normal nonhuman primate eyes induces many of the hallmark changes of nonproliferative DR (36). Retinal blood flow has been shown to be significantly decreased in the diabetic patient before the onset of any DR (37), which may result in ischemia and/or hypoxia in the retina and in turn stimulate VEGF production. However, hypoxia may not be the most important stimulus for increased VEGF production in the retina of patients before the onset of nonproliferative DR (35). A wide variety of cytokines and metabolites (glucose [38–40], AGEs [41,42], IGF-1 [43–45], insulin [46], and angiotensin II [47–49]) are elevated in the diabetic patient and have been suggested to promote DR via stimulation of VEGF. Furthermore, many of these cytokines and metabolites induce VEGF through a similar if not identical signal transduction pathway as hypoxia (42,46).

We have recently reported that the failure to generate new coronary artery collateral blood vessels by the process of angio-
genesis in many patients with chronic vascular insufficiency is associated with a failure to appropriately increase VEGF production in response to hypoxia or ischemia (50). In the present study, we have attempted to extend this paradigm to patients with DR. Specifically, we sought to determine whether diabetic patients with DR produce more VEGF in response to hypoxia than those diabetic patients who have not developed any evidence of DR. For this purpose, we have assessed the relationship of the VEGF response to hypoxia in monocytes harvested from diabetic patients with the presence or absence of DR. The results revealed a highly significant correlation, with increased hypoxic induction of VEGF in those patients with DR in comparison with those patients who do not develop DR, even after many years of diabetes.

**RESEARCH DESIGN AND METHODS**

**Patient recruitment**

This study was approved by the Human Research Ethics Committee of the Rambam Medical Center. Informed consent was obtained from all patients. Patients were recruited consecutively from outpatients at the Rambam Hospital and its clinics in the Haifa area over an 8-month period. Patients with no evidence of DR but with diabetes documented to be <10 years duration were excluded from the study.

**Patient data collection**

For each patient, a data sheet was completed with the patient’s identification code, age, sex, duration of diabetes, type of diabetes, medications, and HbA_1c. All patients had a dilated fundal examination performed by a trained retinal surgeon and 7-field stereoscopic fundal photography (51–56) performed and read by an experienced reader using standardized criteria. Determination of the presence or absence of DR was conducted in a masked fashion without any knowledge of the VEGF response. Absence of DR was defined as complete absence of macular edema, hard exudates, blot hemorrhages, microaneurysms, venous beading, intraretinal microvascular abnormalities, cotton wool spots, or neovascularization.

**Blood collection**

Mononuclear cells were isolated from peripheral blood by a procedure initially described by Boyum (58) and recently modified by Schultz et al. (50). Briefly, 40 ml blood was collected by venipuncture and used within 4 h. A 20-ml blood sample was gently layered onto 10 ml Histopaque-1077 in a fresh 50-ml polypropylene centrifuge tube. Tubes were centrifuged at 1,800 rpm for 30 min at room temperature. Plasma (8 ml) was removed from each tube and saved for later use. The middle phase (buffy coat) containing the monocytes was isolated and placed in a fresh 15-ml polypropylene tube. The isolated mononuclear cells were washed twice with sterile phosphate-buffered saline, and plated in 2 equal aliquots on 2 polystyrene 10-cm diameter tissue culture dishes, and incubated in a 95% room air 5% CO_2 incubator at 37°C for 1 h to allow for monocyte attachment. The medium from the 2 tissue culture dishes from a single patient was aspirated and replaced with 8 ml autologous plasma on each dish. One of the dishes was placed in a normoxic incubator at 21% O_2 and 5% CO_2, and the other tissue culture dish was placed in a hypoxia incubator at 1% O_2, 5% CO_2, and 94% N_2. After 20 h of exposure to either hypoxia or normoxia, total RNA was extracted from the cells.

**RNA isolation from monocytes**

Total RNA from the monocytes was isolated from monocytes with the TRI reagent (Molecular Research Center, Cleveland, OH) as previously described (50). On average, 10–20 μg RNA was obtained from both the normoxic and hypoxic monocytes.

**Measurement of VEGF mRNA by RNase protection assay**

The quantity of VEGF mRNA was determined by RNase protection assay using riboprobes to VEGF and 18S rRNA (58). Quantification of signal intensity was performed on a phosphorimag (Fujix). For each patient, a VEGF-to-18S ratio was calculated for both the hypoxic and normoxic cells. The fold induction of VEGF with hypoxia was calculated by dividing the hypoxic VEGF-to-18S ratio by the normoxic VEGF-to-18S ratio.

**Statistical analysis**

Data are reported as means ± SEM. Analysis between groups for statistically significant differences in the levels of VEGF (normoxic or fold induction) was performed using Student’s unpaired t tests.

**RESULTS**

Over an 8-month period, 95 consecutive patients were enrolled in the study. Of the 50 patients with type 1 diabetes, 25 had evidence of DR. Of 45 patients with type 2 diabetes, 38 had evidence of DR. There were 25 type 1 diabetic and 7 type 2 diabetic patients with no DR and diabetes duration of at least 10 years. Five type 1 diabetic patients with no DR were identified as having diabetes for ≥20 years. Of those type 2 diabetic patients without DR, 5 were identified as having diabetes for ≥15 years.

By assaying blood samples obtained on 2 or 3 separate occasions, we have previously demonstrated in nondiabetic volunteers that the measurement of the fold hypoxic induction of VEGF is reproducible and consistent (50). We have further validated the assay by repeating the VEGF mRNA measurement from blood drawn on 2 separate occasions in 3 of the patients included in this study, with the replicates in fold induction of VEGF mRNA being 2.4 and 2.3, 2.5 and 2.1, and 2.5 and 2.5.

In the 95 patients described here, the range of the fold hypoxic induction of VEGF mRNA was 0.7–20.9. The median value for the hypoxic induction of VEGF was 2.4, and the mean was 4.1. There was no significant association between fold hypoxic VEGF induction and sex, age, and glycemic control.

We found that there was no significant difference between patients with and without DR in the mean basal (normoxic) level of VEGF, even when patients were grouped according to type and duration of diabetes. The mean normoxic VEGF mRNA level of type 1 and type 2 diabetic patients with DR was 0.042 ± 0.015 and 0.044 ± 0.010, respectively. The mean normoxic VEGF mRNA level in type 1 diabetic patients without DR for >10 and 20 years was 0.028 ± 0.004 (P < 0.37) and 0.031 ± 0.012 (P < 0.58), respectively. The mean normoxic VEGF mRNA level in type 2 diabetic patients without DR for >10 or 15 years was 0.036 ± 0.009 (P < 0.56) and 0.032 ± 0.010 (P < 0.42), respectively. There was no significant difference in the mean normoxic level of all patients with DR compared with those patients without DR for long duration (type 1 diabetes ≥20 years and type 2 diabetes ≥15 years [0.043 ± 0.008 vs. 0.032 ± 0.008, P < 0.31]).

The mean hypoxic VEGF induction of type 1 and type 2 diabetic patients with DR was 3.7 ± 0.8 and 4.8 ± 0.8, respectively. Compared with type 1 diabetic patients with DR, there was no significant difference in the hypoxic induction of VEGF mRNA in type 1 diabetic patients without DR for
In patients with type 1 diabetes and no DR after >10 years (3.84 ± 0.6, \( P < 0.6 \)) (Fig. 1A). In patients with type 2 diabetes and no DR after >10 years of diabetes, there was a decrease in the hypoxic induction of VEGF of borderline statistical significance (1.98 ± 0.5, \( P < 0.076 \)) (Fig. 1A). In patients with type 2 diabetes and no DR after >10 years of diabetes, there was a decrease in the hypoxic induction of VEGF of borderline statistical significance (2.44 ± 0.5, \( P < 0.063 \)) (Fig. 1B). In patients with type 2 diabetes and no DR after >15 years of diabetes, there was a statistically significant decrease in the hypoxic induction of VEGF compared with type 2 diabetic patients with DR (1.76 ± 0.2, \( P < 0.0004 \)) (Fig. 1B). The difference in the mean hypoxic induction of VEGF in all patients with DR (\( n = 63 \)) versus all patients without DR and long-standing diabetes (type 1 diabetes ≥20 years and type 2 diabetes ≥15 years, \( n = 10 \)) was highly statistically significant (4.35 ± 0.55 vs. 1.87 ± 0.3, \( P < 0.00012 \)) (Fig. 1C).

Patients with DR can be further subdivided into those with or without proliferative DR. Of the patients with evidence of DR, 31% were found to have active proliferative DR. Patients with proliferative DR were found to have a significantly longer duration of diabetes compared with those patients with only background DR (23.4 vs. 18 years, \( P < 0.04 \)), but they were not significantly different in terms of HbA1c, age, sex, or type of diabetes. There was no significant difference in the mean normoxic level (0.053 vs. 0.041, \( P < 0.53 \)) or in the fold hypoxic induction of VEGF (3.0 vs. 4.5, \( P < 0.12 \)) in patients with or without proliferative DR.

**CONCLUSIONS** — We have shown that patients with evidence of any DR have
a significantly higher hypoxic induction of VEGF in their monocytes compared with patients with no evidence of any DR and a long duration of diabetes. Patients with no evidence of any DR and a relatively short duration of diabetes were not found to have a significantly different VEGF hypoxic induction compared with patients with DR. One reason for this may be that the vast majority of these patients without evidence of DR will eventually develop DR, as shown by numerous epidemiological studies (8,11,24–26).

Interindividual differences in the regulation of VEGF might be expected to influence the natural history of many disease processes involving VEGF, such as ischemic vascular disease (50,59), tumor angiogenesis (60–62), rheumatoid arthritis (63), ovarian hyperstimulation syndrome (64), inflammatory bowel disease (65), and wound healing (66). For example, we have shown that patients who are high inducers of VEGF with hypoxia grow more coronary collaterals in the setting of coronary occlusive disease (50). In the present study, we show that individuals who are low inducers of VEGF with hypoxia are less likely to develop any DR in the setting of diabetes.

It remains to be determined why individuals differ in their hypoxic induction of VEGF. Many of the nonhypoxic modulators of VEGF expression, such as insulin and IGF-1 (45), hyperglycemia, and AGEs (41), appear to be synergistic with hypoxia. Thus, interindividual differences in the plasma levels of these modulators may account for a portion of the observed interindividual heterogeneity in the hypoxic induction of VEGF in monocytes cultured in vitro in autologous plasma.

The present study has a number of important limitations. First, we have not studied the hypoxic regulation of VEGF in the retina of diabetic patients. However, the hypoxia-sensing mechanism and the signal transduction pathways leading to an induction of VEGF has been demonstrated to be similar in all cell types examined to date. Second, the stimulus for the expression of VEGF in nonproliferative DR may not involve hypoxia. Other mediators implicated in stimulating VEGF, such as the AGE proteins, regulate VEGF via a signal transduction pathway that is similar, if not identical, to that of hypoxia. Therefore, the individual heterogeneity seen in VEGF with hypoxia in this study remains relevant to the discussion of mechanisms involved in the development of nonproliferative DR.

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VEGF and diabetic retinopathy