Plasma Concentration of Immunoreactive Vascular Endothelial Growth Factor and Its Relation to Smoking

Taro Wasada, Reiko Kawahara, Kozo Katsumori, Mitsuhide Naruse, and Yasue Omori

We determined the plasma concentration of immunoreactive vascular endothelial growth factor (IR-VEGF) and searched for a relationship between it and the degree of microangiopathy. The plasma VEGF level was measured using an enzyme immunoassay in 110 non–insulin-dependent diabetes mellitus (NIDDM) patients with varying degrees of nephropathy or retinopathy (RP) and in 39 healthy controls and 30 nondiabetic patients for comparison. One fourth of the control subjects, 60% of whom were currently smokers, had plasma levels of IR-VEGF higher than the lower limit (15.6 pg/mL) of detection for this assay, whereas this was the case in half of the NIDDM patients. Plasma IR-VEGF was detectable in all patients with cerebral infarction, chronic renal failure, and severe infection, suggesting that tissue hypoxia might be a common cause for the elevation of plasma VEGF in these disorders. The prevalence of measurable plasma IR-VEGF levels increased in parallel with increases in the urinary albumin excretion rate ([UAER] 35.1% for UAER <30 mg/24 h, 54.8% for UAER 30 to 300 mg/24 h, and 61.3% for UAER >300 mg/24 h; $P < .05$ *v* UAER <30 mg/24 h). The mean measurable plasma concentration tended to increase with increasing UAER. However, there was no such correlation with the severity of RP. Smoking caused an acute increase of plasma IR-VEGF in only 22.6% (12 of 53) of the patients with a smoking habit. In conclusion, these findings suggest that circulating IR-VEGF may be linked to the progression of nephropathy, and smoking may facilitate this process by causing tissue hypoxia in susceptible patients.

VASCULAR ENDOTHELIAL growth factor (VEGF) is an endothelial cell–specific mitogen originally characterized as a vascular permeability factor. VEGF mRNA expression has been identified in a variety of cell types,[1] including epithelial (adrenal gland, lung, retina, and kidney glomeruli), endothelial (retina and kidney glomeruli), and mesenchymal (retinal pericytes, mesangial cells, vascular smooth muscle cells, and myocardium). At least four isoforms produced by alternative splicing have been identified.[2] It appears that VEGF121 and to a large extent VEGF165 are secreted in soluble form, whereas the two larger isoforms (VEGF189 and VEGF206) remain cell-associated, perhaps because of their greater affinity for cell-surface proteoglycans.[2] VEGF production and secretion are upregulated by hypoxia.[3] It is well known that ischemic retinal disorders precede intraocular neovascularization. Vitreous concentrations of VEGF were shown to be correlated with active neovascular proliferation in the retina.[4] From these observations, VEGF is considered as a candidate factor responsible for the pathogenesis of proliferative diabetic retinopathy (RP). Recently, increased circulating VEGF levels were first reported in patients with preeclampsia,[5] probably due to placental hypoxia. Increased vascular permeability, proteinuria, and hypertension are common features in both preeclampsia and diabetic nephropathy. In addition, hyperglycemia is known to augment protein kinase C (PKC) activity, which could act as a potent inducer of VEGF gene expression in many cell types associated with diabetic complications.[6]

In the present study, therefore, we measured plasma levels of VEGF using a highly sensitive immunoassay in non–insulin-dependent diabetes mellitus (NIDDM) patients with varying degrees of RP and nephropathy, and also examined the acute effects of cigarette smoking on the plasma concentration of VEGF, because smoking causes tissue hypoxia.

SUBJECTS AND METHODS

Subjects

The study population included 110 randomly selected NIDDM patients (82 men) aged 22 to 77 years (mean, 53.8) at varying stages of RP or nephropathy. Thirteen patients were being treated by diet alone, 70 were receiving oral hypoglycemic agents, and 27 were on insulin therapy. Their body mass index was 23.2 ± 2.3 kg/m^2 and hemoglobin A_{1c} 7.9% ± 1.3% (mean ± SD). RP was divided into three groups: no evidence of diabetic RP (no RP), only microaneurysms and/or mild hemorrhages and/or mild hard exudates (early RP), and severe hemorrhages, multiple soft exudates, intraretinal microvascular abnormalities, macular edema, or quiescent state after laser photocoagulation (late or treated RP). Nephropathy in these patients was categorized according to the mean urinary albumin excretion rate (UAER) examined on several occasions (UAER <30 mg/24 h, normoalbuminuria; UAER >30 and <300 mg/24 h, microalbuminuria; and UAER >300 mg/24 h, macroalbuminuria). Patients with azotemia, liver dysfunction, and cardiovascular disease, as well as premenopausal female patients, were excluded from the present study.

For comparison, 39 healthy subjects aged 24 to 38 years (23 men) and 30 nondiabetic patients (32 to 77 years) with miscellaneous diseases were included.

Measurement of Plasma Immunoreactive VEGF

Since our preliminary studies showed that plasma immunoreactive (IR)-VEGF levels were approximately the same before and after ingestion of a meal, blood samples were randomly collected in chilled tubes containing 500 KIU aprotinin and 1.5 mg disodium EDTA per milliliter of blood. After centrifugation at 4°C, the plasma was stored at −30°C until assayed. Current smokers among the patients were asked to smoke 1 cigarette within 3 minutes, and paired blood samples were drawn before and after smoking based on the following findings. We studied the acute effects of smoking on conjunctival oxygen tension in seven healthy volunteers who were smokers, using a special device consisting of an eye electrode cemented to a Clark-type oxygen sensor,

From the Diabetes Center, Department of Medicine, and the Institute of Clinical Endocrinology, Tokyo Women's Medical College, Tokyo, Japan.

Submitted January 18, 1997; accepted July 31, 1997.

Address reprint requests to Taro Wasada, MD, Diabetes Center, Tokyo Women's Medical College, 8-1 Kawada-cho, Shinjuku-ku, Tokyo 162, Japan.

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configured to fit easily into the cul-de-sac of the conjunctiva.[7] Oxygen tension transiently decreased to a nadir of 79% (66% to 89%) from the basal level (100%) within a few minutes after initiation of smoking (Fig 1). Based on these observations, we took blood samples within 3 minutes after smoking cessation.

The plasma VEGF level was measured by the quantitative sandwich enzyme immunoassay technique using a commercial kit (Quantikine; R & D Systems, Minneapolis, MN). This kit consists of human recombinant VEGF165 as a standard and the monoclonal antibody to it. There was no significant cross-reactivity or interference with various cytokines or growth factors, including interleukins, platelet-derived growth factor, fibroblast growth factors, insulin-like growth factor-I, and transforming growth factor. The detection limit of the assay was 15.6 pg/mL. The intraassay and interassay coefficients of variation were 8.3% and 10.5%, respectively.

Statistical Analysis

Statistical analysis was performed using the chi-squared test. Data are shown as the mean ± SD.

RESULTS

Plasma IR-VEGF Concentrations in Healthy Subjects and Patients With Nondiabetic Diseases

Ten of 39 healthy control subjects, six of whom were currently smokers, had plasma IR-VEGF levels higher than the detection limit (15.6 pg/mL), with a range of 17.0 to 55.0 pg/mL. Among the miscellaneous diseases, plasma IR-VEGF levels were detectable in all patients with cerebral infarction (17.6 to 61.5 pg/mL), chronic renal failure (32.9 to 82.5 pg/mL), pneumonia (36.4 to 389 pg/mL), and sepsis (91.6 pg/mL), but not in patients with nonischemic disorders (Fig 2).

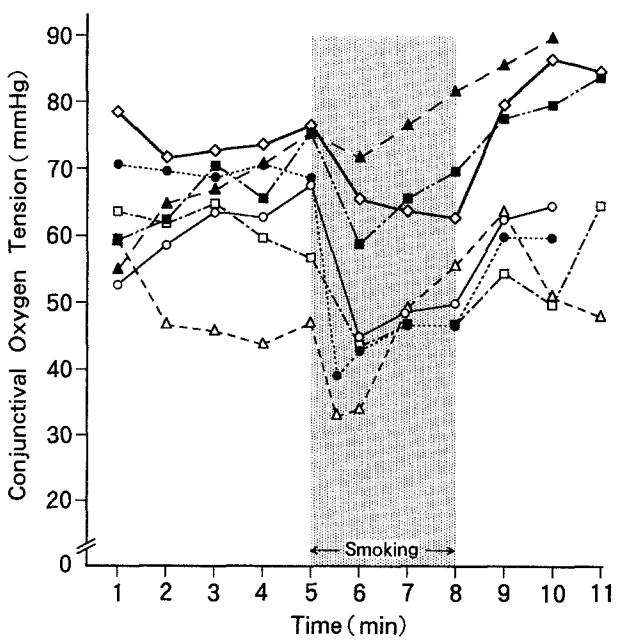

Fig 1. Changes in conjunctival oxygen tension after cigarette smoking in 7 healthy male smokers aged 28 to 41 years. A transient decrease in oxygen tension was observed within 3 minutes after initiation of cigarette smoking.

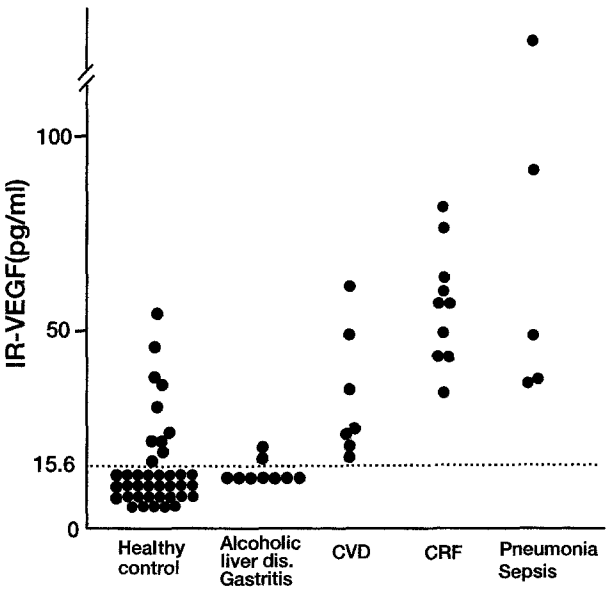

Fig 2. Plasma concentrations of IR-VEGF in healthy subjects and in patients with miscellaneous nondiabetic disorders. CVD, cerebrovascular disease (infarction); CRF, chronic renal failure; dis., disease.

Plasma IR-VEGF Concentrations in NIDDM Patients

Plasma IR-VEGF concentrations were measurable in 55 of 110 patients (50.0%). The prevalence of measurable plasma levels of IR-VEGF was twofold higher in diabetic patients than in the healthy controls (50% *v* 25.6%, $P = .084$).

Correlation Between Plasma IR-VEGF Concentrations and RP

The prevalence of measurable plasma IR-VEGF levels did not differ among the three RP groups: 52.3% (23 of 44) for no RP, 54.5% (12 of 22) for early RP, and 45.5% (20 of 44) for late or treated RP.

Correlation Between Plasma IR-VEGF Concentrations and UAER

The prevalance of measurable plasma IR-VEGF levels increased in parallel with increases in the UAER: 35.1% (13 of 37, normoalbuminuria), 54.8% (23 of 42, microalbuminuria), and 61.3% (19 of 31, macroalbuminuria; $P = .0314$ *v* normoalbuminuria). The mean measurable IR-VEGF level tended to increase with increasing UAER (37.0 pg/mL in normoalbuminuria, 52.2 pg/mL in microalbuminuria, and 51.2 pg/mL in macroalbuminuria; NS between groups) (Fig 3).

Acute Effects of Smoking on Plasma IR-VEGF Concentrations

After cigarette smoking, only 22.6% (12 of 53) of the patients had an elevated plasma level of IR-VEGF. A smoking-induced elevation of greater than 10 pg/mL for plasma IR-VEGF tended to be more frequent in patients with macroalbuminuria as compared with normoalbuminuria or microalbuminuria (33.3% *v* 16.7% or 17.6%, respectively; NS), and the magnitude of increase in plasma IR-VEGF levels also tended to be greater in patients with a higher UAER.

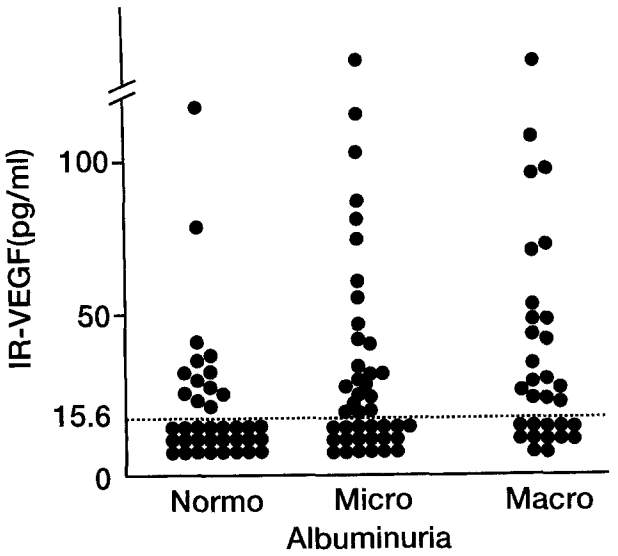

Fig 3. Plasma concentrations of IR-VEGF in different stages of nephropathy grouped by UAER: <30 mg/24 h (normo), 30 to 300 mg/24 h (micro), and >300 mg/24 h (macro). The prevalence of measurable plasma levels of IR-VEGF (>15.6 pg/mL) increased in parallel with increasing UAER.

DISCUSSION

Circulating IR-VEGF concentrations were measurable in miscellaneous nondiabetic disorders such as cerebral infarction, chronic renal failure due to glomerulonephritis or hypertensive nephrosclerosis, and severe infectious diseases. Tissue hypoxia may be a common cause for elevation of plasma IR-VEGF concentrations in these disorders. About 25% of healthy controls also showed detectable levels, and 60% of them were currently smokers. Therefore, the elevation of plasma IR-VEGF is not specific for diabetes or its vascular complications. Even taking these findings into consideration, an association between plasma IR-VEGF and the degree of albuminuria found in the present study seems relevant clinically. None of our NIDDM subjects had active diseases other than RP or nephropathy. Although circulating plasma IR-VEGF may emerge from undefined ischemic lesions, the association of the plasma IR-VEGF concentration with the degree of albuminuria, but not with the degree of RP indicates that the kidney is the major site accounting for plasma levels of IR-VEGF in these diabetic patients. The higher plasma IR-VEGF concentrations in patients with increasing albuminuria cannot be explained by the impairment of renal clearance, because the plasma levels of IR-VEGF were comparable to those in chronic renal failure of nondiabetic origin.

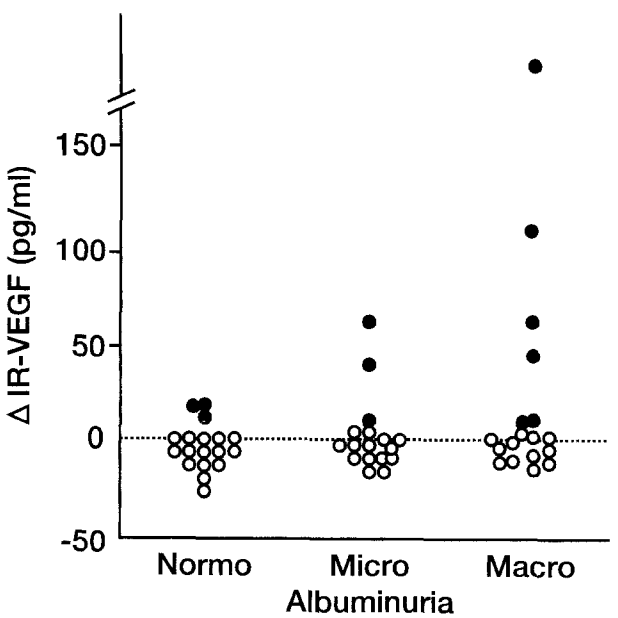

Fig 4. Increment (△) of plasma IR-VEGF greater than 10 pg/mL after cigarette smoking in the graded nephropathy groups.

Increased expression of VEGF mRNA has been identified within glomeruli, which may result in glomerular hyperpermeability for circulating macromolecules and may lead to proteinuria.[8] In addition, a number of reports have indicated that a high glucose level augments PKC activity in mesangial cells and other cell types.[6,8] Since PKC is a potent inducer of VEGF gene expression, the proteinuria and increased vascular permeability in the diabetic state may be related to PKC-induced overexpression of VEGF.[8] Alternatively, glycosylated hemoglobin shows a higher affinity for oxygen, and thus, poorly controlled diabetes may cause tissue hypoxia. However, there was no relation between hemoglobin A_{1C} and IR-VEGF levels in our study (data not shown).

On the other hand, the lack of an association between plasma IR-VEGF and the degree of RP was unexpected, but it may be explained by the fact that almost all of our patients in the late RP group had been treated with laser photocoagulation. Aiello et al[4] reported that vitreous concentrations of VEGF were increased in the eyes of subjects with active neovascularization, but not those with simple RP or quiescent stage resulting from laser photocoagulation therapy. Patients who had received laser photocoagulation showed a decrease in the intraocular VEGF concentration of 75%.[4] Therefore, further studies would be interesting to determine whether untreated active proliferative RP or occlusive retinal vessel disease could alter the plasma level of IR-VEGF.

An increase in plasma IR-VEGF in response to smoking was found in a small fraction of the patients. However, an incremental response tended to be seen more frequently in patients with macroalbuminuria as compared with normoalbuminuria or microalbuminuria. We found no correlation between the cigarette nicotine content and the magnitude of the IR-VEGF response. The physiological significance of these observations remains to be determined, but smoking may be considered as an accelerating factor for nephropathy. Previous studies showed that 0.4 pmol/L VEGF increases intracellular free-calcium concentrations,[9] and half-maximal mitogenesis can be induced at 2 to 3 pmol/L.[10] Since plasma IR-VEGF reached these levels in some of our diabetic patients after smoking, a smoking-induced increase in plasma VEGF may lead to a worsening of vascular complications, especially in the kidney.

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