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Endothelial cell inhibitory autoantibodies are associated with laser photocoagulation in adults from the Veterans Affairs Diabetes Trial

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Abstract

Basic fibroblast growth factor (bFGF) is a potent endothelial cell mitogen that does not normally circulate, but increases in microalbuminuric adult type 2 diabetes mellitus. Earlier work indicated an unexpected association between low levels of plasma bFGF immunoreactivity and the subsequent 4-year need for laser treatment in 172 patients from the Veterans Affairs Diabetes Trial (mean: age, 59 years; diabetes duration, 11 years; baseline hemoglobin A_{1c}, 9.5%). In the present study, we tested for an association between endothelial cell inhibitory autoantibodies in plasma and the need for laser treatment. Inhibitory activity in endothelial cells from the immunoglobulin G fractions of plasma was significantly associated ($P = .002$) with low plasma bFGF immunoreactivity. There was a significant association ($P = .003$) between endothelial cell inhibitory autoantibodies in baseline plasma and the time to occurrence of first laser treatment after 4 years of study treatment. After adjusting for other risk factors, endothelial cell inhibitory activity greater than 90% vs less than or equal to 90% (hazard ratio, 0.2; $P = .003$) and low-density lipoprotein cholesterol concentration (hazard ratio, 0.98; $P = .02$) were each significant predictors of the time to first postrandomization laser occurrence. These results suggest that circulating autoantibodies inhibitory in endothelial cells may contribute to the need for laser treatment in adult men with advanced type 2 diabetes mellitus. Among the possible risk factors evaluated, baseline insulin use was the only variable significantly inversely ($P = .02$) associated with the baseline occurrence of inhibitory endothelial cell autoantibodies. It could not be determined whether insulin use may decrease the occurrence of endothelial cell inhibitory autoantibodies in advancing adult type 2 diabetes mellitus.
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1. Introduction

Diabetic retinopathy is a leading cause of new blindness in adults in the United States [1]. There is growing evidence that factors besides hyperglycemia also contribute to the risk of progression of diabetic retinopathy [2] or the need for laser treatment [3]. Macular edema is the leading cause of visual impairment in type 2 diabetes mellitus [4]. A particularly strong association between albuminuria and macular edema in type 2 diabetes mellitus [5] suggests that diffuse vascular injury [6] may contribute to visual impairment in type 2 diabetes mellitus.

Basic fibroblast growth factor (bFGF) is a potent angiogenesis factor that does not normally circulate [7,8]. Yet plasma bFGF levels were increased in association with micro- or macroalbuminuria in adult type 2 diabetes mellitus [9]. We found an unexpected significant association between low levels of plasma bFGF immunoreactivity and the need for laser treatment in adults with type 2 diabetes mellitus from the Veterans Affairs Diabetes Trial [10] (VADT). In the present study, we tested for an association between plasma autoantibodies to endothelial cells (ECs) and the need for laser treatment in a subset of 162 subjects from the VADT.

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We now report a significant association between inhibitory EC autoantibodies in plasma and the need for laser treatment in adults with advanced type 2 diabetes mellitus. To our knowledge, this is the first report that spontaneously occurring, circulating autoantibodies inhibitory in ECs may contribute to the need for laser treatment in adults with advanced type 2 diabetes mellitus.

2. Subjects and methods

2.1. Study subjects

Informed consent for the Investigational Review Board–approved substudy was obtained from 172 diabetic subjects at 5 outpatient sites who had consented to participate in the main VADT. Blood drawing was performed at each site in the morning in subjects who had fasted overnight. EDTA plasma was aliquoted and shipped frozen (dry ice) to a central laboratory (Maveric, Boston Veterans Affairs Medical Center, Boston, MA) where it was inventoried and stored at −80°C for 1 to 2 years. Archived, coded frozen EDTA plasma from consecutively enrolled patients was shipped to the laboratory of Dr Zimering (VA New Jersey Health Care System, Lyons, NJ) where bFGF immunoreactivity (bFGF-IR) and bioassays were performed. All other assays were performed in the Central Laboratory of the VADT (Tufts University, Boston, MA).

All subjects were older than 40 years old. Ninety-seven percent of patients were men. Baseline clinical characteristics in the subject group were previously reported [10] and are shown later in Table 4.

2.2. Medications

All patients were taking antidiabetic medications at baseline including oral agents and/or insulin. Patients randomized to the standard or intensive glycemic treatment group were treated for at least 5 years (and some up to 7.5 years) with the same classes of medications including insulin and the thiazolidinedione (TZD) rosiglitazone.

2.3. Laser photocoagulation

Information regarding laser photocoagulation for retinopathy was obtained from questionnaires administered at the baseline and each annual visit. Baseline determination of EC bioactivity in the protein A eluate from plasma or bFGF-IR (at Veterans Affairs New Jersey Health Care System) was masked to the information about laser photocoagulation occurrence.

The risk factors associated with time to first laser treatment were modeled in 147 subjects in whom postbaseline data about laser occurrence were available between the second and sixth postbaseline annual visits. Laser events occurring during the first year of study follow-up were disregarded to minimize the effect on the time to first laser occurrence of preexisting retinal lesions.

2.4. Baseline fundus photographs

Baseline fundus photographs were obtained in all patients. The photographs were evaluated at the Central Fundus Photography Reading Center, University of Wisconsin, Madison, WI. The frequencies of no retinopathy, microaneurysms, and mild nonproliferative, severe nonproliferative, and proliferative retinopathy were 29%, 18%, 29%, 17%, and 7%, respectively. Macular edema was present in 16 (10.3%) of 156 patients in whom it could be assessed from photographs.

2.5. Laboratory and clinical measures

Standard laboratory and clinical measures were determined as previously described [11]. Urinary albumin-creatinine ratio was calculated as albumin concentration/creatinine concentration × 100. Low-density lipoprotein (LDL) cholesterol was calculated using the Friedewald equation on all samples with plasma triglyceride concentration less than 400 mg/dL. Blood pressure (BP) was recorded in the seated position after 5-minute rest. Three consecutive readings were obtained, and the median value of the 3 consecutive determinations was used for analysis.

2.6. Plasma samples

Archived, coded EDTA plasma samples were kept frozen (−70°C) for up to 4 years before assay of protein A eluate fractions for bioactivity in ECs. Bioactivity in protein A eluate fractions from sera was previously shown to be stable for 5 years or longer at −20°C [12]. Endothelial cell inhibitory activity in the protein A eluate fractions from plasma was stable after storage at 0°C to 4°C for 6 months or longer.

2.7. Basic fibroblast growth factor assays: cut point for "low" vs "high" bFGF-IR

Basic fibroblast growth factor immunoreactivity in plasma was determined using a sensitive specific 2-site enzyme-linked immunoassay (R&D Systems, Minneapolis, MN) as previously described [13]. We dichotomized this measurement at the value of 4.5 pg/mL, the previously reported upper limit in normal adult men [14].

2.8. Cell culture and growth assays

Bovine pulmonary artery ECs (Clonetics, San Diego, CA) were maintained at 37°C in 5% CO_2/95% air in EC growth medium (Clonetics) plus 10% fetal calf serum. Bovine pulmonary artery cells were passaged continuously and used between passages 4 and 10.

2.9. Colorimetric estimation of cell number

Endothelial cell proliferation assays were carried out as previously reported [12]. Confluent cells were trypsinized and plated at 10^3 to 10^4 cells per well in Medium 199 plus 10% fetal calf serum in 96-well plates. After 1 or 2 days of

incubation for cells to reach 60% to 80% confluency, test fractions (1:50 dilution of protein A eluates of plasma) were added to wells in quadruplicate. After 2 days of incubation in the presence of test fractions, wells were washed with phosphate-buffered saline and processed for the colorimetric estimation of number of cells, that is, cell-associated acid phosphatase activity, as previously described [12]. There was a linear relationship between EC number and optical density at 410 nm as previously described [12]. Growth-promoting activity is expressed as a percentage of the number for cells grown in the absence of test protein A eluate fractions in a control well. *Significant inhibitory activity* (≤90%) is defined as that occurring outside the reference range for control, that is, unexposed cells. Each point represents the mean of quadruplicate determinations. The intra- and interassay coefficients of variation were 4% and 7% at 1:50 dilution of protein A–eluted fractions from plasma of 3 diabetic subjects (n = 3 assays in each patient).

2.10. Protein A affinity chromatography

Protein A affinity chromatography was carried out as previously described [12]. Four-tenths–milliliter aliquots of plasma were adjusted to pH 8.0 by adding 0.8 mL 100 mmol/L Tris (pH 8). After syringe filtration to clarify samples, 1 mL was applied to a 1-mL column of packed protein A beads (Pierce Chemical, Rockford, IL) equilibrated in 100 mmol/L Tris (pH 8.0). The column was washed and eluted as previously described [12]. The eluate fractions containing nearly all the recovered protein were pH neutralized and stored at 0°C to 4°C. Inhibitory activity in protein A eluate fractions was unchanged, appearing in the retentate fraction after dialysis (10 mmol/L phosphate, pH 7.4) and ultrafiltration on a 10-kd MW cutoff membrane (Centricon-10; Millipore, Bedford, MA). All fractions were sterile filtered (Millipore, 0.2 μm) before assay for growth-promoting activity.

2.11. Protein determinations

Protein concentrations were determined by a bicinchoninic acid protein assay kit (Pierce Chemical).

2.12. Statistics

Cox proportional hazards regression analysis was used to model time to first postbaseline laser treatment as a function of possible baseline risk factors. Those possible risk factors were a set of clinical risk variables that based upon published literature [15,16] (age, diabetes duration, antibody group ≤90% vs >90%, history of hypertension, LDL cholesterol concentration, baseline hemoglobin A_{1c} [HbA_{1c}]) are known or likely to be associated with retinopathy or laser treatment. Backward elimination was used to determine those variables that contributed significantly ($P \leq .05$) to the model. With this procedure, we found that the excluded clinical variables (age, history of hypertension, baseline HbA_{1c}, baseline insulin, angiotensin-converting enzyme [ACE] inhibitor, angiotensin receptor blocker [ARB] use, glycemic treatment arm) all had *P* values greater than .20. Low plasma bFGF-IR was an excluded variable that had a *P* value of .12.

3. Results

3.1. Association between inhibitory activity in ECs from protein A eluates and low plasma bFGF-IR

In the current study, we compared bioactivity in ECs from a 1:50 dilution of the protein A eluate fractions of plasma to baseline plasma bFGF-IR in available samples from 162 of the 172 subjects. There was a highly significant association between inhibitory activity in ECs from the protein A eluates of plasma and low plasma bFGF-IR ($P = .002$, Table 1). Fifty-two (32%) of 162 subjects had inhibitory bioactivity in ECs from the protein A eluate fractions of plasma (Table 1).

3.2. Protein A–eluted activity in ECs and laser treatment

The proportion of subjects unaffected by postbaseline laser differed with respect to the presence or absence of plasma autoantibodies inhibitory in ECs (Fig. 1). The separation for antibody groups was apparent after 24 months of follow-up and remained relatively constant between 24 and 48 months of treatment in the VADT. For example, after 36 months of study treatment, 21% vs 5% of subjects with or without plasma EC inhibitory autoantibodies, respectively ($P = .003$), had at least 1 postbaseline laser event. Extending the analysis for up to 5 or to 6 years of study follow-up, a significant difference ($P = .02$) between the antibody groups regarding time to first laser occurrence was still evident, in the direction of increased laser occurrence for the presence of inhibitory EC autoantibodies in plasma.

3.3. Effect of clinical risk factors on the need for laser treatment

The best fit model of risk factors associated with the time to first laser treatment during 4 years of follow-up included the following variables as significant predictors: EC autoantibodies greater than 90% vs less than or equal to 90% (hazard ratio [HR], 0.20; $P = .003$) and LDL cholesterol concentration (HR, 0.98; $P = .02$) (Table 2). The results were unchanged after adjusting for diabetes treatment group or

Table 1
Association between inhibitory bioactivity in ECs from the protein A eluates of plasma and low baseline bFGF immunoreactivity

Antibody[a]	bFGF-IR (pg/mL)		
	Low (0-4.4)	High (≥4.5)	*P* value
≤90%	36 (69%)	16 (31%)	.002[b]
>90%	48 (44%)	62 (56%)	

[a] A 1:50 dilution of protein A eluate of plasma was assayed for change in EC number as described in "Subjects and methods." Results are number (percentage) of subjects.

[b] *P* value from χ^2 test.

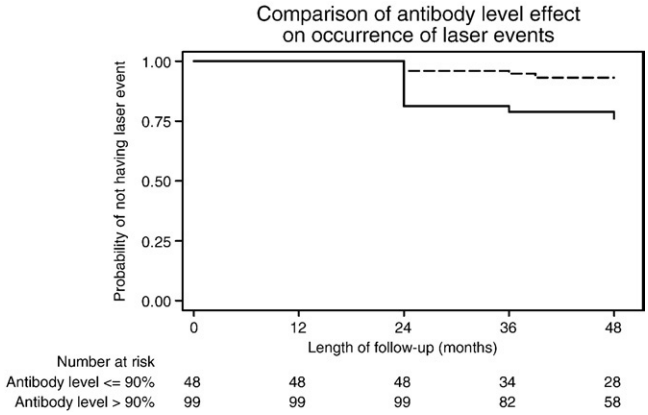

Fig. 1. Comparison of antibody level effect on occurrence of laser events. The difference in time to occurrence of first laser for antibody groups was statistically significant, $P = .003$. Dashed line indicates group with antibody level greater than 90%.

baseline ACE inhibitor, ARB use, or insulin treatment. The same variables—EC autoantibodies greater than 90% vs less than or equal to 90% (HR, 0.28; $P = .008$), duration of diabetes (HR, 1.06; $P = .017$), and LDL cholesterol concentration (HR, 0.98; $P = .009$) (Table 2)—also were significantly associated with time to first laser after up to 5 years of study treatment. No first laser events occurred in the year after 5 years of follow-up.

3.4. Relation of EC inhibitory autoantibodies to baseline risk factors

When comparing the 2 plasma EC autoantibody groups, there was no significant difference in mean values of the variables patient age, BMI, waist-hip ratio, baseline glycosylated hemoglobin, diabetes duration, systolic BP, urine albumin-creatinine ratio, or plasma LDL cholesterol concentration (Table 3). There was a significant ($P = .02$) inverse association between plasma EC inhibitory autoantibodies and baseline insulin use and a marginal ($P = .07$) inverse association between plasma EC inhibitory autoantibodies and baseline fibrate use (Table 4). There was no association between plasma EC inhibitory autoantibodies and any other baseline categorical risk factor including race/ethnicity, history of hypertension, macrovascular disease prevalence, baseline use of TZDs, antihypertensive medications, or current smoking status (Table 4).

Table 2
Cox proportional hazard regression: time to first laser treatment

4 y postbaseline			
Variable	HR	95% CI	*P* value
Antibody group (>90% vs ≤90%)	0.20	0.07-0.58	.003
LDL cholesterol	0.98	0.96-1.0	.02
5 y postbaseline			
Antibody group (>90% vs ≤90%)	0.28	0.17-0.72	.008
Diabetes duration	1.06	1.01-1.11	.017
LDL cholesterol	0.98	0.99-0.96	.009

CI indicates confidence interval.

Table 3
Associations between inhibitory antibody activity (≤90%) and continuous baseline risk factors in 162 patients

Risk factor	Antibody ≤90%	Antibody >90%	*P* value[a]
Age (y)	59.5 ± 7.9	59.3 ± 8.6	.85
HbA_{1c} (%)	9.5 ± 1.4	9.4 ± 1.5	.74
Duration of diabetes (y)	10.4 ± 7.8	11.8 ± 8.2	.30
Urinary ACR (mg/g)	177.8 ± 632.5	104.9 ± 266.0	.43
LDL cholesterol (mg/dL)	105.3 ± 29.4	102.0 ± 34.2	.56
Systolic BP (mm Hg)	130.6 ± 16.0	130.0 ± 18.3	.84
BMI (kg/m^2)	31.0 ± 3.0	31.6 ± 4.6	.39
Waist-hip ratio	0.997 ± 0.073	1.006 ± 0.070	.45

Results are mean ± SD. ACR indicates albumin-creatinine ratio.
[a] *P* values from *t* test.

4. Discussion

The present data suggest a novel association between EC inhibitory autoantibodies and the need for laser treatment in

Table 4
Associations between inhibitory antibody activity (≤90%) and baseline categorical risk factors in 162 patients

Variable	Antibody ≤90%	Antibody >90%	*P* value[a]
Demographics			
Male	100	96.4	.16
Hispanic	19.2	14.6	.45
Non-Hispanic white	57.7	69.1	.15
African American	21.2	15.5	.37
Current smoker	17.3	17.3	1.00
Baseline medications			
β-Blocker	9.6	11.8	.68
ACE inhibitor	71.2	67.3	.62
ARB	9.6	3.6	.12
Calcium channel antagonist	21.2	19.1	.76
Thiazide diuretic	11.5	20.0	.18
Statin	59.6	66.4	.40
Fibrate	11.5	23.6	.07
TZD	21.2	18.2	.65
Insulin	32.7	52.7	.02
Sulfonylurea	71.2	61.8	.25
Metformin	80.8	75.5	.45
Thyroid hormone	3.9	7.3	.40
History			
Hypertension	67.3	75.2	.29
MI	9.6	16.2	.26
Coronary revascularization	19.2	20.0	.91
Any macrovascular event (MI, CABG, angina, stroke, PVD)	32.7	43.8	.18
Albuminuria (urinary ACR)			
Macro, ≥300 mg/g	9.6	8.7	.96
Micro, 30-299 mg/g	26.9	28.9	
Normo, <30 mg/g	63.5	62.5	

Results are percentage of patients. MI indicates myocardial infarction; CABG, coronary artery bypass graft; PVD, peripheral vascular disease.
[a] *P* values from χ^2 test.

patients with long-standing type 2 diabetes mellitus. The increased rate of laser treatment persisted for up to 5 years after initiation of study treatment despite the known strong influence of duration of diabetes. An earlier report of a significant association between low baseline plasma bFGF-IR level and the interim (4 years) need for laser treatment in a baseline subset of 172 subjects from the VADT [10] may be accounted for by a significant association between low baseline bFGF and EC inhibitory autoantibodies in plasma.

Endothelial cell binding autoantibodies were reported in type 1 diabetes mellitus in association with proliferative retinopathy [17]. Additional studies, however, failed to confirm an association between EC binding autoantibodies and either retinopathy or microvascular disease complications [18,19]. Our data are the first to suggest that immunoglobulin G autoantibodies in plasma from adults with type 2 diabetes mellitus inhibit ECs. This may be consistent with a report that immunoglobulin G autoantibodies from a subset of lupus patients with nephropathy induced apoptosis in ECs [20].

How autoantibodies arise in typical obese, adult, type 2 diabetes mellitus patients is not clear. All patients enrolled in the VADT were prescreened and were required to have evidence of detectable fasting C-peptide level. It is therefore unlikely that our subject group included many patients with either a systemic autoimmune condition or an autoimmune-mediated insulin deficiency. The prevalence of EC autoantibodies (32%) in our study group of older adult type 2 diabetes mellitus patients was in close agreement with that reported in younger type 1 diabetes mellitus subjects of similar duration [19], suggesting that factors related to long-standing diabetes per se predispose to the development of autoantibodies.

One possibility is that vascular injury results in the ectopic expression of self-antigens that are normally sequestered. Heparan sulfate proteoglycan (HSPG) is a low-affinity receptor for bFGF [21] that is abundant on ECs. Heparan sulfate proteoglycan is also a known target for autoimmunity [22, 23]. Heparanase degrades HSPG components of the subendothelial basement membrane [24], contributing to the loss of heparan sulfate proteoglycan thought to underlie generalized vascular injury in diabetes [25]. Release of HSPG or HSPG-bFGF complexes into the circulation through the action of heparanase [26] could provide a mechanism for the association found here between EC autoantibodies and low plasma bFGF-IR (Table 1).

The associations between baseline medication use and a lower frequency of baseline EC autoantibodies demonstrated here (Table 4) lend support to a possible mechanism involving factors that regulate heparanase expression. First, the significant association between baseline insulin use and a lower baseline prevalence of inhibitory EC autoantibodies is consistent with a report that insulin inhibits EC heparanase expression [27]. Second, the suggestion of an inverse association between baseline fibrate use and EC autoantibodies is consistent with the possibility that fibrates such as fenofibrate may reduce the need for laser treatment (eg, the Fenofibrate Intervention and Event Lowering in Diabetes [FIELD] study [3], a large clinical trial recently conducted in adults with type 2 diabetes mellitus) through an effect on inflammatory cytokines that can also modulate EC heparanase expression [28].

Our study is small, and the results only reflect the experience of men with long-standing diabetes. Still, the present data provide evidence for a novel pathogenetic mechanism contributing to the need for laser treatment. Our heterogeneous study group included 60% non-Hispanic white patients who had a higher baseline prevalence of cardiovascular disease (and significantly lower mean baseline LDL cholesterol concentration) compared with African American and Hispanic subjects. The significant inverse association between LDL cholesterol concentration and the need for laser treatment in our study group (Table 2) may indicate confounding by factors associated with non-Hispanic white race.

Recurrent macular edema requiring repeated laser treatments can contribute to impaired vision in patients with type 2 diabetes mellitus. Proliferative diabetic retinopathy may develop later in some patients with type 2 diabetes mellitus patients and is thought to be mediated by the effects of another potent, heparin-binding [29] angiogenesis factor, vascular endothelial cell growth factor [30,31]. It is possible that EC autoantibodies modulate the bioavailability of more than one kind of potent growth factor, for example, bFGF and vascular endothelial cell growth factor, capable of acting synergistically [32] to promote angiogenesis. In such cases, neovascularization may result through enhanced availability of angiogenic growth factors released after decreases in the affinity of EC autoantibodies [33] for circulating HSPG.

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