ORIGINAL ARTICLE

The Association of Serum Vascular Endothelial Growth Factor and Ferritin in Diabetic Microvascular Disease

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Abstract

Background: Vascular endothelial growth factor (VEGF) is involved in the pathogenesis of diabetic microvascular disease. Most diabetes patients have higher serum levels of ferritin that may participate in diabetic vascular complications through high oxidative stress induced by iron. However, the mechanistic link between ferritin and VEGF is obscure. The study investigated the association of VEGF and ferritin in patients with diabetic microvascular disease.

Patients and Methods: Sixty patients with type 2 diabetes mellitus (T2DM) and 26 healthy individuals were selected in this study. Serum ferritin, VEGF, hematological parameters, and clinical data were assessed in this cohort. The Spearman rank method was used to evaluate the associations among them.

Results: Serum levels of VEGF and ferritin were significantly higher in diabetes patients compared with the controls; levels of both were elevated with development of the disease. There were positive correlations between VEGF and glucose levels and between VEGF and ferritin in diabetes groups, especially in patients with diabetic retinopathy. Positive correlations were also found between VEGF level and the parameters of age, hemoglobin, and albumin in patients with diabetes hypertension.

Conclusions: Our data suggest that high ferritin levels in T2DM are closely related to the development of diabetic vascular complications through interaction with VEGF.

Introduction

TYPE 2 DIABETES MELLITUS (T2DM) is responsible for the increasing prevalence of associated vascular complications leading to considerable morbidity and mortality, and it has become a priority to characterize their pathophysiological mechanisms to develop novel, rational therapeutic strategies.^{1,2} Two major abnormalities of T2DM are the failure of β -cell function and the deterioration of systemic insulin sensitivity. Studies suggest that the decreased β -cell mass and the dysfunction of islets are the main reasons for β -cell failure in T2DM. The pancreatic islets are highly vascular tissues, and their dysfunctions of vascular structure are associated with deterioration of insulin secretion.³ The formation of vascular structure depends on the expression of vascular endothelial growth factor (VEGF) in β -cells. VEGF, the most potent pro-angiogenic growth factor, plays a key role in angiogenesis. VEGF is also regarded as an important factor for normal β -cell function.⁴ Clinically, diabetes is associated with failure of various organs, especially the blood vessels. T2DM can lead to many complications over time. These complications, such as nephropathy, retinopathy, neuropathy, impaired wound healing, and accelerated atherosclerosis, are implicated in a large number of cellular and subcellular changes in vessels.⁵

Studies have found that VEGF is involved in the pathogenesis of diabetic complications.^{6–8} Hyperglycemia-induced diacylglycerol, a lipid molecule, activates protein kinase C in the vascular tissues, and the latter in turn promotes VEGF signaling, resulting in diabetic microvascular complications.⁹ Other factors, including hypoxia, gender, smoking, elevated levels of blood lipids, inflammatory status, and activated stress axes, may affect the synthesis and secretion of VEGF; among them, the major physiological stimulus for VEGF expression is the cellular hypoxia.¹⁰

Adaptive responses of cells to hypoxia are mediated by hypoxia inducible factor-1 (HIF-1). HIF-1 (especially HIF-1 α) is a primary regulator of VEGF expression.¹¹ HIF-1 α is also required for normal β -cell function, and HIF-1 α dysregulation may contribute to the pathogenesis of T2DM. Iron is a transition metal that can catalyze the conversion of poorly active free radicals into highly active free radicals. The latter attacks cell membranes, lipids, proteins, and DNA, causing tissue damage, and may play an important role in the pathogenesis of diabetes. The physiological roles of iron are numerous, and new activities have recently been uncovered, including the regulation of the levels of HIF-1.¹² Once iron enters the cell, it is rapidly taken up by cytoplasmic ferritin.¹³ Ferritin is a multimeric iron-storage protein consisting of 24 subunits of two types: heavy and light.

The ratio of these two chains is tissue specific and controls iron storage and availability. Alteration in the ferritin heavy/light ratio is due to changes in the availability of iron in a labile pool. This causes significant changes in the uptake and storage of iron, the rate of ferritin synthesis and degradation, and the secretion of VEGF.¹⁴ Therefore, ferritin has a vast capacity to sequester and store large amounts of iron, in a nontoxic form, and has been reported to protect cardiomyocytes against iron-mediated doxorubicin toxicity.¹⁵ As the main form of iron stored *in vivo*, ferritin could reflect body iron status in healthy individuals. Epidemiological studies have reported that a positive correlation exists between iron stores and the risk of T2DM.^{16,17} Elevated levels of iron stores may induce diabetes through oxidative damage to pancreatic β -cells, impair hepatic insulin extraction by the liver, and interfere with insulin's ability to suppress hepatic glucose production. In addition, iron overload may contribute initially to insulin resistance and subsequently to decreased insulin secretion and ultimately result in diabetes.¹⁸ This is confirmed by the evidence of reduced hyperinsulinemia in the presence of iron depletion.¹⁹ At present, the relationship of a high level of serum ferritin and hyperglycemia has become a hot topic in the investigation of T2DM.²⁰

The "iron hypothesis" was initially presented as an explanation for the sex difference in prevalence of cardiovascular disease; the proposal is that modest levels of stored iron, far less than a conventional iron overload, may promote cardiovascular disease, whereas sustained iron depletion can protect against it.²¹ That a chronic iron level reduction in response to frequent blood donation is associated with improved vascular function in the brachial artery²² suggested that iron plays a role in proliferation of vascular smooth muscle cells.²³ Iron is able to generate powerful reactive oxygen species (ROS) that can activate angiogenesis through HIF-1 α ,²⁴ which is the main stimulator of VEGF production.

Both VEGF and ferritin are involved in the development of T2DM; however, there are scarce data to show the relationship between them. Recently, a study showed that ferritin was involved in regulating angiogenesis by binding to the anti-angiogenic domain of high-molecular-weight kininogen. Ferritin antagonized high-molecular-weight kininogen's effects that lead to increased blood vessel growth.²⁵ Serum ferritin levels may be elevated in order to function as an angiogenic modulator, working to increase new blood vessel growth.²⁶ On the other hand, cellular hypoxia, the major physiological stimulus for VEGF expression, could regulate cellular iron uptake through negative feedback of the hypoxic conditions.²⁷ Thus, common regulating signals may have existed between ferritin and VEGF, both of which are related to angiogenesis of T2DM. Diabetes is a well-recognized modifiable cardiovascular risk factor associated with vascular endothelial dysfunction,²⁸ and most diabetes patients may have iron overload.

At present, there are few clinical data to elucidate the association between ferritin/VEGF and the pathogenesis of diabetic microangiopathy. In order to explore it, in this study T2DM patients with diabetic microangiopathies were selected, and their serum levels of VEGF and ferritin as well as other parameters were assessed.

Patients and Methods

This study was approved by the Ethics Committee of Shantou University Medical College and conducted from

May 2010 to January 2011. Eighty-six individuals were selected for this study, including 17 cases of diabetic retinopathy (DR), 25 cases of diabetic nephropathy (DN), 18 cases of diabetic hypertension (DH), and 26 age-matched healthy controls. All patients and the controls gave written informed consents to the project's aims. All patients met the following standards during follow-up: (1) glycosylated hemoglobin (Hb) A1c (HbA1c) level of $\geq 6.5\%$ (according to the National Glycohemoglobin Standardization Program certification and standardized with the Diabetes Control and Complications Trial criteria), (2) plasma glucose level of ≥ 7.8 mmol/L after a 8-h fast, (3) an oral glucose tolerance test performed on two or more different days yielding a fasting plasma glucose level of ≥ 7.8 mmol/L, 1-h postprandial glucose level of ≥ 11.1 mmol/L, and 2-h postprandial glucose level of ≥ 11.1 mmol/L, (4) accompanied by symptoms of a hyperglycemia or hyperglycemia crisis (a random plasma glucose level of ≥ 11.1 mmol/L), and (5) standards 1–3 were to be confirmed again when there were no longer symptoms of hyperglycemia.

DR was diagnosed when the individual met one or more of the following criteria: (1) a history of diabetes, (2) impaired vision accompanied by retinal hemorrhage and beaded vein presented in the fundus photograph, (3) fundus fluorescein angiography displaying hard or soft exudates and/or microangioma, and (4) exclusion of retinopathy caused by some other etiology. The degree of disease severity is classified according to Wilkinson et al.,²⁹ based on publications by the Early Treatment Diabetic Retinopathy Study and the Wisconsin Epidemiologic Study of Diabetic Retinopathy, which includes the following stages: (1) no apparent retinopathy, (2) mild nonproliferative DR (microaneurysms only), (3) moderate nonproliferative DR, (4) severe nonproliferative DR with any of the following syndromes of (a) more than 20 intraretinal hemorrhages in each of four quadrants, (b) definite venous beading in two or more quadrants, or (c) prominent intraretinal microvascular anomalies in one or more quadrants, and (5) proliferative DR (PDR) with one or more of the following—(a) neovascularization and/or (b) vitreous or preretinal hemorrhage.^{29,30} Of the five stages, the first three stages belong to the low-risk group, whereas the fourth and fifth stages belong to the high-risk group. Therefore, we divided the DR patients into two groups: the mild-to-moderate group (the low-risk group) and the severe group (nonproliferative retinopathy and proliferative retinopathy).

DN was defined by increased urinary albumin excretion (UAE) in the absence of other renal diseases combined with a long history of diabetes, the presence of proteinuria at a rate of >0.5 g/24 h, microalbuminuria (UAE >20 μ g/min and ≤ 199 μ g/min), and macroalbuminuria (UAE ≥ 200 μ g/min), which appeared more than two times in 6 months, and accompanied by abnormal renal function that was represented by an abnormal serum creatinine (CRE) level, calculated CRE clearance, or glomerular filtration rate. The classification of disease severity in our study was according to the staging criteria of Mogensen et al.,³¹ which includes the following stages: stage 1, characterized by kidney hypertrophy, increased glomerular filtration rate (>150 mL/min), and normal UAE rate (<20 μ g/min); stage 2, no obvious clinical manifestations, UAE rate is normal (<20 μ g/mL), and glomerular filtration rate is normal or elevated slightly; stage 3, characterized by persistent microalbuminuria, UAE rate of 20–200 μ g/mL, and the microalbuminuria is abnormal

(30–300 mg/24 h); stage 4, overt DN, characterized by persistent proteinuria (>500 mg/24 h) and UAE rate of >200 μ g/mL; and stage 5, end-stage renal failure with uremia due to DN, persistent proteinuria, and glomerular filtration rate of <15 mL/min. According to the urine protein excretion, all DN patients in our study were in stages 2–5. We then divided the patients into two groups: the mild-to-moderate group (stages 2–3, with a urinary protein excretion rate of <200 μ g/min) and the severe group (stages 4–5, with a urinary protein excretion rate of $\geq$ 200 μ g/min).

DH was defined in the diabetes patients such that systolic blood pressure (SBP) was consistently >140 mm Hg, diastolic blood pressure (DBP) was consistently >90 mm Hg, or both after measurement three times on different days without use of any antihypertensive drug. The diagnostic criteria of hypertension were formulated by the World Health Organization/International Society of Hypertension in 1999³²: stage 1, SBP ranging from 140 to 159 mm Hg and/or DBP ranging from 90 to 99 mm Hg; stage 2, SBP ranging from 160 to 179 mm Hg and/or DBP ranging from 100 to 109 mm Hg; and stage 3, SBP $\geq$ 180 mm Hg and/or DBP $\geq$ 110 mm Hg. All our patients with DH were in stages 1–2; therefore we divided the DH patients in two groups according to the criteria of mild-to-moderate hypertension (stage 1) and severe hypertension (stage 2).

To study the association of ferritin and VEGF in diabetes patients, we redivided the 60 patients into three groups according to serum levels of ferritin: Group A, ferritin levels <300 ng/mL; Group B, ferritin levels 300–500 ng/mL; and Group C, ferritin levels >500 ng/mL. T2DM patients who had malignancies and hepatic disease were excluded, as radiotherapy and/or chemotherapy might alter the patients' metabolism. None of the subjects had acute inflammation or received a blood transfusion prior to the start of the study.

Collection and evaluation of samples

Blood samples were obtained by using two types of tubes: one contained EDTA, whereas the other was without any anticoagulant. Blood in the EDTA tube was analyzed by an automatic cell counter (model Coulter[®] LH 750 hematological analyzer; Beckman Coulter, Fullerton, CA) for the determination of complete blood count, including red blood cells, Hb, mean corpuscular volume, white blood cells, and HbA1c. Blood without anticoagulant was kept for 2 h at room temperature for biochemical detections including liver function (aspartate aminotransferase, alanine aminotransferase, γ -glutamyl transferase, and albumin) and renal function (blood urea nitrogen and CRE) by an automatic biochemical analyzer (model LX20; Beckman Coulter). Serum levels of VEGF, interleukin (IL)-6, erythropoietin, and ferritin were measured by the enzyme-linked immunosorbent assay method (Cusabio Biotech, Newark, NJ).

Statistical analyses

Data were given as mean $\pm$ SD values based on the normal distribution or as median (25th–75th percentile) values when data were non-normally distributed. Statistical differences among groups were assessed by one-way analysis of variance of post hoc multiple comparisons: least significant difference (normal distribution data) or nonparametric statistics. The Kruskal–Wallis H, Mann–Whitney U, and Wilcoxon rank tests

were used to assess non-normal distribution data. The associations among serum levels of VEGF, ferritin, IL-6, and erythropoietin were tested with the Spearman rank correlation. Bivariate correlations were used to evaluate the factors that regulated VEGF production in each group. SPSS for Windows version 17.0 software was used for statistical analyses (SPSS Inc., Chicago, IL). Values of $P < 0.05$ were considered to be statistically significant.

Results

General clinical data in the diabetes groups and the controls

Data were obtained from 86 (35 men, 51 women) participants. General characteristics of the three diabetes groups and the controls are summarized in Table 1. Glucose levels in the all diabetes groups were significantly higher than in the controls ($P = 0.014$ for DR, $P < 0.001$ for DN, and $P < 0.001$ for DH, respectively). The same was also shown in HbA1c ($P < 0.001$ for all). The body mass index values were higher in the diabetes groups compared with the controls ($P < 0.001$ for all), but no difference was found among the three diabetes groups ($P > 0.05$ for all). SBP was significantly higher in the diabetes groups than the controls ($P = 0.007$ in DR, $P = 0.010$ in DN, and $P < 0.001$ in DH, respectively). In particular, SBP in the DH group was higher than in the DR and DN groups, but no difference was found between the DN and DR groups ($P > 0.05$ for both). No differences were found in DBP between the diabetes groups and the controls as well as among the diabetes groups ($P > 0.05$ for all). CRE and blood urea nitrogen levels were elevated significantly in the DN group compared with the other two diabetes groups ($P < 0.001$ for both), and no differences existed among the DR group, the DH group, and the control groups ($P > 0.05$ for all). Although white blood cell counts in the diabetes patients were significantly higher than in the controls ($P < 0.05$ for all), no difference existed among the diabetes groups. In addition, no difference in high-sensitivity C-reactive protein (hsCRP) levels was found between the diabetes patients and the controls, indicating that the diabetes patients in our study had no acute-phase response. Hb concentrations decreased in the DR ($P = 0.019$), DN ($P < 0.001$), and DH ($P = 0.001$) groups compared with the controls, and the Hb level in the DN group was significant lower than those of the DR ($P = 0.005$) and DH ($P = 0.012$) groups. No difference in erythropoietin levels was found among the groups, as well as in the mean corpuscular volume value ($P > 0.05$). Albumin levels were significantly lower in the diabetes groups than in the controls ($P < 0.001$), and the albumin level in the DN group was lower than in the DR group ($P < 0.001$), but no difference was found between the DN and DH groups ($P > 0.05$). Serum IL-6 levels were elevated significantly in the diabetes groups ($P < 0.001$ for all groups) compared with the controls, but no difference was found among the three diabetes groups.

Levels of ferritin and VEGF in the diabetes groups and the controls

Serum ferritin concentrations were higher in the diabetes groups than in the controls (DR, $P < 0.001$; DN, $P < 0.001$; DH, $P = 0.049$). A significant difference was found between the DR

TABLE 1. CLINICAL AND LABORATORY DATA IN THE DIABETES GROUPS AND THE CONTROLS

	DR	DN	DH	Control
n (M/F)	17 (7/10)	25 (10/15)	18 (6/12)	26 (12/14)
Age (years)	63.00 (59.00–72.00) ^a	64.00 (55.00–70.50) ^a	60.50 (53.25–67.00)	57.00 (53.00–61.75)
BMI (kg/m ²)	27.09 (24.79–28.24) ^b	27.56 (24.66–29.03) ^b	26.53 (24.76–28.10) ^b	22.37 (20.58–23.87)
SBP (mm Hg)	144.00 (128.00–161.50) ^b	145.00 (123.00–178.50) ^b	150.00 (142.50–166.50) ^b	128.00 (120.00–136.25)
DBP (mm Hg)	85.12 ± 14.29	83.76 ± 12.19	85.94 ± 11.41	80.35 ± 8.15
AST (U/L)	20.00 (19.00–25.00) ^{ce}	18.00 (14.50–21.50) ^{af}	26.50 (20.75–36.50)	23.00 (18.75–32.25)
ALT (U/L)	26.00 (14.00–30.00)	16.00 (10.05–28.50) ^{be}	25.00 (19.50–38.25)	25.50 (21.50–37.50)
GGT (U/L)	23.00 (18.50–43.00)	26.00 (17.50–48.50)	36.50 (20.25–49.50)	26.00 (18.50–33.50)
CRE (U/L)	84.00 (71.00–100.50) ^d	236.00 (116.00–689.00) ^{bf}	71.50 (54.25–109.50)	67.50 (58.00–89.75)
BUN (U/L)	4.52 (3.90–6.10) ^d	10.70 (6.10–18.95) ^{bf}	4.90 (3.38–6.80)	4.15 (3.48–5.35)
GLU (mmol/L)	7.64 (4.87–10.12) ^a	7.53 (5.97–10.57) ^b	8.63 (6.97–10.67) ^b	5.35 (4.99–5.62)
HbA1c (%)	6.47 (5.63–8.67) ^b	6.68 (6.12–8.48) ^b	6.96 (5.96–8.64) ^b	4.62 (4.14–5.03)
WBC (×10 ⁹ /L)	8.40 (6.55–9.45) ^b	8.10 (6.10–13.59) ^b	7.70 (6.75–10.05) ^a	6.53 (5.70–7.26)
Hb (g/L)	125.00 (102.00–134.50) ^{ad}	97.00 (78.00–111.00) ^{be}	118.50 (102.00–130.75) ^b	135.50 (127.50–145.25)
EPO (IU/mL)	22.86 (20.56–33.02)	25.36 (19.50–30.75)	23.68 (16.90–30.65)	23.36 (18.32–29.18)
MCV (fL)	89.80 (84.75–91.70)	89.30 (85.40–91.05)	84.80 (74.95–90.23)	87.90 (86.13–89.93)
ALB (g/L)	36.90 (34.35–40.05) ^{bd}	31.60 (28.65–34.40) ^b	35.35 (31.70–38.33) ^b	42.60 (40.83–45.73)
hsCRP (pg/mL)	12.75 (11.08–14.71)	12.84 (9.69–14.56)	11.56 (9.51–16.91)	12.12 (10.34–17.10)
IL-6 (pg/mL)	213.05 (152.49–302.38) ^b	194.50 (80.26–287.22) ^b	245.66 (131.28–302.00) ^b	39.93 (16.38–76.07)
Ferritin (ng/mL)	438.61 ± 135.82 ^{be}	394.50 ± 164.13 ^b	302.36 ± 194.00 ^a	205.83 ± 134.19
VEGF (pg/mL)	366.02 (313.03–592.03) ^{bf}	319.23 (201.26–450.41) ^b	269.41 (208.49–395.06) ^a	211.36 (146.79–290.49)

Data are mean ± SD values or median (25th–75th percentile) values as indicated.

^a*P* < 0.05, ^b*P* < 0.01 when diabetes groups (diabetic retinopathy [DR], diabetic nephropathy [DN], and diabetes hypertension [DH]) were compared with control subjects.

^c*P* < 0.05, ^d*P* < 0.01 when the DR group was compared with the DN group.

^e*P* < 0.05, ^f*P* < 0.01 when the DR and DN groups were compared with the DH group.

ALB, albumin; ALT, alanine aminotransferase; AST, aspartate aminotransferase; BMI, body mass index; BUN, urea nitrogen; CRE, creatinine; DBP, diastolic blood pressure; EPO, erythropoietin; GGT, γ -glutamyl transferase; GLU, glucose; Hb, hemoglobin; HbA1c, glycosylated hemoglobin A1c; hsCRP, high-sensitivity C-reactive protein; IL-6, interleukin 6; M/F, males/females; MCV, mean corpuscular volume; SBP, systolic blood pressure; VEGF, vascular endothelial growth factor; WBC, white blood cell.

and DH groups (*P* = 0.012), but no marked difference was found between the DR and DN groups (*P* = 0.375), as well as between the DN and DH groups (*P* = 0.062) (Table 1).

Serum VEGF levels were elevated significantly in the diabetes groups compared with the controls (*P* < 0.001 for all). The VEGF level in the DR group was significantly higher than that in the DH group (*P* = 0.013), but there was no difference between the DR and DN groups (*P* = 0.130), as well as between the DN and DH groups (*P* = 0.221) (Table 1).

The influencing factors on ferritin and VEGF expression in the diabetes groups and the controls

To analyze the association that existed between ferritin and VEGF in T2DM, the correlation between ferritin and VEGF was studied, as well as the associations among ferritin/VEGF with other parameters in the diabetes patients and the controls. To minimize the statistical error caused by small samples, we put all diabetes patients into one group. Spearman rank correlations were used to evaluate these associations in this study. The levels of VEGF in the combined diabetes group (DR, DN, and DH) and the controls are shown in Figures 1–3. In the combined diabetes groups, a significant positive correlation was found between VEGF and ferritin (*R* = 0.298, *P* = 0.021), as well as VEGF and glucose (*R* = 0.232, *P* = 0.031). Specifically, there is a positive correlation between VEGF and ferritin in the DR group (*R* = 0.536, *P* = 0.027) (Figs. 1a and b and 3a). In the DH group, a positive correlation between VEGF and age (*R* = 0.500, *P* = 0.034) and negative correlations between VEGF and albumin and between VEGF and Hb (*R* = −0.543, *P* = 0.020 and

R = −0.616, *P* = 0.006) were found (Fig. 3b–d). No relationship was found between VEGF and ferritin in the controls (*R* = 0.170, *P* = 0.407). A positive correlation was found between ferritin and age in the combined diabetes group (*R* = 0.313, *P* = 0.015) (Fig. 1c) but not in the controls (*R* = −0.252, *P* = 0.215). No correlation was found between ferritin and IL-6 in the diabetes group (*R* = 0.018, *P* = 0.894), but significant positive associations between ferritin and IL-6 (*R* = 0.595, *P* = 0.001) and between ferritin and Hb were found (*R* = 0.477, *P* = 0.014) in the control group (Fig. 2b and c).

In the combined diabetes group, a positive correlation between IL-6 and hsCRP (*R* = 0.264, *P* = 0.042) and a negative correlation between IL-6 and blood urea nitrogen were found (*R* = −0.279, *P* = 0.031) (Fig. 1d and e). No correlation was found between ferritin level and those of the parameters erythropoietin, glucose, HbA1c, CRE, blood urea nitrogen, and hsCRP in the diabetes group as well as in the controls.

Effect of ferritin on VEGF expression in the diabetes patients and the controls

To study whether there is a dose–response relationship between ferritin and VEGF, the 60 diabetes patients were redivided into three groups according to ferritin level (Group A, ferritin levels < 300 ng/mL; Group B, ferritin levels 300–500 ng/mL; and Group C, ferritin levels > 500 ng/mL) (Table 2). As ferritin is also an acute-phase protein, some inflammatory markers were evaluated in our study to exclude the possibility that the elevated ferritin level were due to acute inflammation. The white blood cell count in Group A was lower than in Groups B and C

FIG. 1. (a–e) Correlations among vascular endothelial growth factor (VEGF), ferritin, glucose (GLU), age, interleukin-6 (IL-6), high-sensitivity C-reactive protein (hsCRP), and blood urea nitrogen (BUN) in type 2 diabetes mellitus patients.

($P=0.014$ and $P=0.013$, respectively), and no difference was found between Groups B and C ($P=0.269$). In addition, expression of the inflammatory cytokine IL-6 was not different among the three groups ($P>0.05$ for all). Although the hsCRP level in Group B was higher than in Group A ($P=0.024$), no difference was found between Groups A and C or between Groups B and C ($P>0.05$ for both). Mean corpuscular volume values in Groups A and B were lower than in Group C ($P=0.020$ and $P=0.041$, respectively). Specifically, VEGF levels in Groups

A and B were lower than in Group C ($P=0.009$ and $P=0.035$, respectively). There was a trend of increasing VEGF level with elevated ferritin level in the diabetes groups (Fig. 1a).

Levels of VEGF and ferritin in patients with different disease severity of DR, DN, and DH

According to the classification standards of clinical disease severity, we divided the DR patients into two groups: the

FIG. 2. (a–c) Correlations among vascular endothelial growth factor (VEGF), erythropoietin (EPO), ferritin, interleukin-6 (IL-6), and hemoglobin (Hb) in the control group.

mild-to-moderate group and the severe group. VEGF values in the severe group were significant higher than those of the mild-to-moderate and the control groups ($P=0.002$ and $P<0.001$, respectively), and there was no difference between the mild-to-moderate group and the control group ($P=0.093$). The data suggested that elevated VEGF levels were associated with disease severity in patients with DR. Ferritin levels in the severe group were significant higher than in the controls ($P<0.001$), but no difference was found between the mild-to-moderate group and the severe group or between the mild-to-moderate group and the controls ($P=0.206$ and $P=0.207$, respectively) (Fig. 4a).

According to the urinary protein levels, the patients with DN were divided into two groups: the mild-to-moderate group and the severe group. Levels of both VEGF and ferritin in the severe group were significant higher than those in the

mild-to-moderate group and the controls ($P=0.014$ and $P<0.001$ for VEGF and $P=0.024$ and $P<0.001$ for ferritin, respectively), but no differences were found between the mild-to-moderate group and the controls ($P=0.214$ for VEGF and $P=0.109$ for ferritin) (Fig. 4b). Our data suggested that levels of VEGF and ferritin in patients with DN were closely related to their disease severity.

The patients with DH were divided into two groups: the mild-to-moderate group and the severe group. The levels of VEGF in the severe and the mild-to-moderate groups were significant higher than in the controls ($P=0.021$ and $P=0.046$, respectively), but no differences was found between the severe and the mild-to-moderate groups ($P=0.976$). There was no significant difference in ferritin levels among the three groups ($P>0.05$ for all) (Fig. 4c). The results indicated that the levels of VEGF were more valuable in reflecting the disease

FIG. 3. (a–d) Correlations among vascular endothelial growth factor (VEGF), ferritin, age, albumin (ALB), and hemoglobin (Hb) in the diabetic retinopathy (DR) and diabetes hypertension (DH) subgroups.

severity in patients with DH compared with the levels of ferritin.

Discussion and Conclusions

In this study, serum ferritin levels were significant higher in the diabetes patients than in the controls, which is consistent with the finding of a recent study that increased serum ferritin level and body iron stores had been recognized as a feature of T2DM.³³ The possible mechanism may due to the up-regulation of transferrin receptor, glucose, and insulin-like growth factor 2, which are colocalized in the cell membrane, and the presence of insulin could result in the simultaneous translocation of all three proteins. Therefore, insulin-mediated glucose transportation may lead to increasing expression of transferrin receptor on the cell surface, resulting in increased

uptake of extracellular iron. On the other aspect, iron overload, either in the circulation or in the specific tissues, may raise the risk of T2DM through contributing insulin resistance.³⁴ Low molecular iron catalyzes the Fenton reaction that produces oxygen radicals to cause the increase of ROS, and the soluble iron accelerates the generation of ROS from H_2O_2 ; it is therefore toxic to cells. In this study, our finding of significant higher levels of ferritin in the diabetes groups suggests that abundant ferritin may induce increasing amounts of ROS. A recent proposal of ROS-mediated vascular damage induced by hyperglycemia in diabetes is appealing. Iron could promote oxidative stress; strong pro-oxidant effects of iron associated with increased levels of oxidative stress were thought to play a role in diabetic vascular complications.³⁵

Iron is also important in regulation of VEGF production through its effects on HIF-1. HIF-1 is the most potential

TABLE 2. CLINICAL AND LABORATORY DATA IN DIABETES PATIENTS WITH DIFFERENT LEVELS OF FERRITIN

	Ferritin group		
	Group A (<300 ng/mL)	Group B (300–500 ng/mL)	Group C (>500 ng/mL)
n (M/F)	18 (7/11)	24 (8/16)	18 (8/10)
Age (years)	59.50 (53.25–67.25)	62.00 (55.25–69.25)	65.50 (61.75–70.25)
AST (U/L)	21.00 (16.75–26.25)	20.00 (15.50–27.75)	20.00 (19.00–34.00)
ALT (U/L)	23.50 (14.75–31.75)	21.50 (12.25–30.00)	24.50 (15.50–31.25)
GGT (U/L)	22.50 (15.75–47.50)	26.50 (19.50–42.50)	28.50 (18.75–53.50)
CRE (U/L)	83.50 (60.25–393.75)	108.00 (69.25–225.50)	108.50 (73.00–692.00)
BUN (U/L)	6.10 (3.55–10.05)	5.10 (4.21–9.60)	6.15 (3.85–22.38)
GLU (mmol/L)	7.63 (6.06–9.31)	6.70 (5.00–10.93)	8.09 (7.39–16.56)
HbA1c (%)	6.54 (6.06–8.41)	7.38 (5.51–8.31)	6.77 (6.11–10.19)
WBC ($\times 10^9$)	7.10 (5.45–8.83) ^d	8.25 (7.33–10.88)	9.50 (6.60–15.25) ^a
Hb (g/L)	107.72 $\pm$ 20.3	115.58 $\pm$ 26.86	102.00 $\pm$ 27.92
IL-6 (pg/mL)	214.22 $\pm$ 92.65	205.28 $\pm$ 111.18	215.14 $\pm$ 128.72
VEGF (pg/mL)	288.12 (200.09–371.31)	303.45 (251.85–399.42)	454.06 (307.07–606.50) ^{bc}
EPO (IU/mL)	22.20 (18.67–29.41)	22.96 (18.69–36.24)	25.70 (22.25–31.14)
MCV (fL)	84.90 (79.93–90.90)	87.65 (82.78–89.80)	90.00 (86.90–93.68) ^{ac}
ALB (g/L)	34.81 $\pm$ 4.26	33.17 $\pm$ 5.77	33.26 $\pm$ 5.02
hsCRP (pg/mL)	11.15 (8.30–13.91) ^d	14.65 (11.10–20.39)	12.75 (11.20–13.88)

Data are mean $\pm$ SD values or median (25th–75th percentile) values as indicated.

^a $P < 0.05$, ^b $P < 0.01$ when Group C was compared with Group A.

^c $P < 0.05$ when Group C was compared with Group B.

^d $P < 0.05$ when Group A was compared with Group B.

ALB, albumin; ALT, alanine aminotransferase; AST, aspartate aminotransferase; BUN, urea nitrogen; CRE, creatinine; EPO, erythropoietin; GGT, γ -glutamyl transferase; GLU, glucose; Hb, hemoglobin; HbA1c, glycosylated hemoglobin A1c; hsCRP, high-sensitivity C-reactive protein; IL-6, interleukin 6; M/F, males/females; MCV, mean corpuscular volume; VEGF, vascular endothelial growth factor; WBC, white blood cell.

stimulator of VEGF production that regulates VEGF synthesis at the transcriptional level.¹⁴ Oxygen tension has been shown to be a key regulator of VEGF gene expression both in vitro and in vivo.³⁶ It is well known that the diabetes subjects may experience macro- and microangiopathy, with the latter leading to DR, DN, and diabetic neuropathy. Angiogenesis is an essential biological process in the progression of vascular complications in diabetes. The VEGF family and its receptors have been shown to be the fundamental regulators of the cell signaling of angiogenesis. In this study, angiogenesis in diabetes patients was investigated by measuring their serum levels of VEGF. Our results showed that significantly higher levels of VEGF are found in the diabetes patients compared with the controls, suggesting that the elevated VEGF level in the diabetes patients may contribute to their vascular complications. This was also supported by our data that the serum VEGF level in patients with DR (the most typical microvascular complication in T2DM) was the highest among all the diabetes groups, suggesting that aberrant expression of VEGF may play the crucial role in diabetic microangiopathy. In addition, our data showed that the VEGF levels in both the severe DR and DN groups were significantly higher than those in the mild-to-moderate and the control groups, indicating that VEGF is an important factor in the development of both PDR and diabetic macular edema.³⁷ VEGF alters the retinal capillary permeability by increasing the phosphorylation of proteins involved with tight junctions.³⁸ In DN, experimental models show that overproduction of VEGF is a pathogenic condition for podocytopathy³⁹; by activating the VEGF receptor 2 on endothelial cells, VEGF increases capillary permeability. Although we could not find a direct association between VEGF and albuminuria in DN patients, both

the VEGF and albuminuria levels were significant higher in patients with DN than in the controls. In addition, the VEGF level was elevated with the development of disease severity. Our data demonstrated that elevated VEGF levels are closely related with disease development in the diabetes patients.

Based on a study suggesting that there are potentially important relationships between intracellular iron metabolism and VEGF,¹⁴ it is predicted that iron chelation could suppress several angiogenic mediators, including VEGF, fibroblast growth factor-1, IL-1, IL-6, IL-8, and nuclear factor- κ B.⁴⁰ In the study of the long-term vascular effects of diabetes, streptozotocin-induced diabetes was found to be corrected by treatment with deferoxamine, suggesting that iron may participate in the development of vascular complications of diabetes.⁴¹ Iron bound by vascular elastin and collagen could focus on oxidative damage at the vascular wall.⁴² To further examine whether an elevated level of serum VEGF was associated with ferritin, we redivided the 60 diabetes patients into three groups by their ferritin levels: lower than normal, normal, and higher than normal ranges. The findings of our study showed that the high levels of ferritin co-occur with the high levels of VEGF, and a strong positive correlation was found between serum ferritin and VEGF in diabetes patients. In addition, our data also showed that the ferritin levels were elevated with the degree of disease severity in patients with DR and DN. A similar trend was also found in VEGF in the DR, DN, and DH groups. The increasing trend of VEGF and ferritin levels was kept from the mild-to-moderate stages to the severe stage in all diabetes groups. Our data suggest that the elevated level of ferritin, together with that of VEGF, may be involved in the development of vascular complications in diabetes.

(a) DR: Levels of VEGF and ferritin in groups severe, mild-to-moderate, and the control.
 (b) DN: Levels of VEGF and ferritin in groups severe, mild-to-moderate, and the control.
 (c) DH: Levels of VEGF and ferritin in groups severe, mild-to-moderate, and the control.

FIG. 4. Levels of vascular epithelial growth factor (VEGF) and ferritin in diabetes subgroups with mild-to-moderate or severe disease versus the control group: (a) diabetic retinopathy (DR), (b) diabetic nephropathy (DN), and (c) diabetic hypertension (DH). CI, confidence interval.

It is intriguing that positive correlations between ferritin and VEGF concentrations and between VEGF and glucose concentrations both were found in our cohort, suggesting an interplay may exist among hyperglycemia, ferritin, and VEGF. In the podocyte, chronic hyperglycemia and a low level of endothelial nitric oxide induced excess production of VEGF, and the latter drove the development and the progression of DN. The abnormal cross-talk between VEGF and nitric oxide pathways is fueled by the diabetic milieu, resulting in increased oxidative stress.⁴³ In this study, IL-6 levels in the DR, DN, and DH groups were significantly higher than in the controls, indicating that pro-inflammatory cytokines were involved in the progression of vascular complications in

diabetes. Inflammatory activity leads to an increase in vascular permeability, thickening of the glomerular basement membrane, endothelial cell apoptosis, and chronic inflammation.⁴⁴ We also found significant positive correlations existed between VEGF and age and between VEGF and Hb in the DH group, suggesting that VEGF expression may be regulated by multiple factors in diabetic microangiopathy.

In conclusion, our data showed a close association among hyperglycemia and levels of ferritin and VEGF, which may interweave with microvascular disease in diabetes. The elevated levels of ferritin and VEGF may contribute to the pathogenesis of T2DM microangiopathy. At present, testing for serum ferritin is routine in laboratory monitoring panels of

T2DM; given the close relationship between ferritin and VEGF, these biochemical markers would be helpful to predict the possibility of developing vascular complications during the disease progression and treatment. The limitations of the study are the absence of neuropathy and that the numbers of patients in this study are not big enough to show statistical significance. A large population should be needed to minimize statistical variance. In addition, we only analyzed the serum levels of VEGF and ferritin in patients with T2DM; although our data showed the close relationship between them, more experiments would be required to investigate the molecular mechanisms between VEGF and ferritin at the cellular level.

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Author Disclosure Statement

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