ORIGINAL RESEARCH

Relationship between hemoglobin and CD40 ligand in prediabetes

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Abstract

Purpose: Hemoglobin (Hb) regulates the endothelial function by modulating the bio-availability of NO at the tissue level. A significant direct relationship is present between the Hb levels and endothelial functions in patients with Type 2 diabetes. Testing whether this association also exists in subjects with prediabetes is important because prediabetes is associated with an increased risk of cardiovascular disease and mortality. Therefore, we investigated the association of Hb both with the classical cardiac risk factors and the markers for endothelial dysfunction and inflammation, in subjects with impaired glucose tolerance (IGT).

Methods: We enrolled 69 normotensive, and cardiovascular events free subjects with IGT (M=40, age=45.50±6.8 yr). Plasma insulin, hsCRP, soluble CD40L, vonWillebrand factor, p-selectin levels were measured. The parameters given according to the higher and lower median Hb values of the subjects were compared.

Results: Subjects with the higher Hb levels exhibited lower HDL-C (46.68±10.8 mg/dl vs 51.5±8.9mg/dl; $P=0.04$) and higher systolic (122.57±6.2mmHg vs 116.17±7.4mmHg; $p<0.001$) and diastolic (79.14±3.73mmHg vs 75.58±6.1mmHg; $P=0.005$) blood pressures and sCD40L (7.9±3.8ng/ml vs 6.07±2.1 ng/ml; $P=0.02$) levels. Hb levels were correlated to the HDL cholesterol, sCD40L, systolic and diastolic blood pressures and waist circumference ($r=-0.28$, $P=0.02$; $r=0.29$, $P=0.02$;

$r=0.53$, $P<0.001$; $r=0.41$, $P=0.001$; $r=0.42$, $P<0.001$ respectively). According to the multiple logistic regression analysis, Hb was the determinant of sCD40L levels ($\beta=0.437$, $P<0.001$).

Conclusion: These results indicate that there may be a link with higher Hb values and cardiovascular risk factors in patients with IGT. Further investigation is warranted to understand the clinical implications of these findings in subjects with prediabetes.

Endothelial dysfunction, the impairment of regulatory function of the endothelium for vasodilatation, smooth muscle proliferation, and fibrinolysis, is pivotal in the pathogenesis of cardiovascular disease. Hemoglobin (Hb) is a well known carrier and buffer of nitric oxide (NO) which regulates endothelial function by modulating the bio-availability of NO at the tissue level.¹ Alterations in red cell mass may impair endothelial function and exert adverse cardiovascular events.² Full correction of anemia may do more harm than good in chronic kidney disease (CKD).^{3,4} This phenomenon has been attributed to the adverse effect of erythropoietin in patients with systemic illness and inflammation.⁵ However, our recent data showed a

direct association between Hb concentration and endothelial function in patients with chronic kidney disease (CKD stage 3-4), who are not under treatment with erythropoietin⁶, or in diabetic patients with proteinuria.⁷ Similar data have also been shown in patients with normal renal function with either type 2 diabetes⁸ or hypertension.⁹ Overall, these findings indicate that alteration of Hb concentration is relevant in the pathophysiology of impaired endothelial function.

Prediabetes is a state of increased risk of cardiovascular disease and mortality¹⁰ in which endothelial dysfunction (ED) is consistently present.^{11,12} The prevalence of prediabetes is about 30% in the adult population.¹³ This high frequency necessitates a meticulous search for the main contributors to the mechanism of ED in prediabetes. Due to the above data about the role of hemoglobin in the mechanism of ED, we designed a study in prediabetic patients to investigate the relationship between the hemoglobin level and the well established factors that take place in the mechanism of ED. We hypothesized that higher hemoglobin levels may correlate with markers of endothelial dysfunction and risk factors for cardiovascular disease in prediabetes.

Methods

Subjects and Controls

The local ethics committee of Gulhane Medical School approved the study protocol and subjects gave informed consent. Subjects for the study were selected from patients who were referred for routine annual screening to the Gulhane Outpatient Clinics of Internal Medicine and whose current medical status necessitated oral glucose tolerance testing (overweight with positive family history of diabetes, blood glucose > 100mg/dl, history of gestational diabetes). Sixty-nine patients with impaired glucose tolerance (IGT) (male=40, age=45.95±6.8yr) were eligible and gave consent to be enrolled. Part of the data from these subjects was published previously.¹⁴⁻¹⁶ The exclusion cri-

teria included a history of any metabolic or inflammatory disorder, dyslipidemia [low-density lipoprotein (LDL)-cholesterol > 130 mg/dl], hypertension (systolic blood pressure >140mmHg and diastolic blood pressure > 90 mmHg), obesity [body mass index (BMI) > 30 kg/m²], clinical evidence of cardiovascular disease (history of acute myocardial infarction, coronary angioplasty, or bypass surgery, symptoms of angina, dyspnea, or claudication, electrocardiographic abnormality) as well as rheumatic disease, renal, hepatic, or thyroid dysfunction, concomitant medications (including herbals), regular alcohol, or drug consumption, smoking, or pregnancy. All subjects underwent detailed physical examination.

Anthropometric measurements

Weight and height were measured without shoes and in light clothing and BMI was calculated as weight divided by squared height (kg/m²). Systolic and diastolic blood pressures were measured using a manual mercury sphygmomanometer. Two readings were obtained five minutes apart in the sitting position, and the mean of the two was recorded as the blood pressure.

Laboratory analyses

Glucose tolerance status

All participants underwent standard oral glucose tolerance test (OGTT) according to the American Diabetes Association criteria.¹⁷ OGTT was performed after 10–12 h of overnight fasting by ingesting 75 g of oral glucose load over 2-min, and obtaining blood samples at baseline and 2 h after the glucose load for plasma glucose measurement. Subjects were instructed not to restrict carbohydrate intake in the week before the test. No patient had any acute infection or stress during or before the procedure. Normal glucose tolerance was defined as fasting and 2-hour plasma glucose of < 100 mg/dl and < 140 mg/dl, respectively.

TABLE 1. Characteristics of the prediabetic patients according to the median hemoglobin levels.

	Total group (n=69)	Below median Hb (n=34)	Above median Hb (n=35)	P
Age (yr)	45.50±6.8	46.02±7.1	45.00±6.6	0.53 [†]
Sex (M/F)	40/29	8/26	32/3	<0.001*
BMI (kg/m ²)	27.97±3.0	28.08±3.1	27.97±3.1	0.8 [†]
Waist(cm)	93.37±13.1	91.85±8.4	94.86±16.5	0.35 [†]
SBP (mmHg)	119.42±7.5	116.17±7.4	122.57±6.2	<0.001[†]
DBP (mmHg)	77.39±5.3	75.58±6.1	79.14±3.73	0.005[†]
FPG (mg/dl)	111.07±7.0	110.61±6.9	111.51±7.3	0.60 [†]
Total C (mg/dl)	215.0±45.8	221.73±51.2	208.45±39.5	0.23 [†]
HDL- C (mg/dl)	49.05±10.2	51.5±8.9	46.68±10.8	0.04[†]
LDL-C (mg/dl)	129.02±36.3	132.12±39.2	126.02±33.4	0.44 [†]
TG (mg/dl)	175.04±103.4	164.61±91.2	185.17±114.3	0.41 [†]
Insulin (mg/dl)	13.5±7.2	13.34±8.2	13.80±6.1	0.79 [†]
hsCRP (mg/dl)	2.64±1.8	3.05±1.9	2.3±1.6	0.06 [†]
p-Selectin (ng/ml)	429.22±223.5	384.64±188	472.54±246	0.10 [†]
vWF (IU/dl)	0.72±0.3	0.73±0.3	0.71±0.3	0.83 [†]
sCD40L (ng/ml)	7.0±3.2	6.07±2.1	7.9±3.8	0.02[†]
Uric acid (mg/dl)	5.48±1.2	5.1±1.1	5.75±1.2	0.05 [†]
Hemoglobin (g/dl)	14.47±1.3	13.40±0.8	15.50±0.7	<0.001[†]

Data are expressed as mean value ± SD. Independent sample *t* test[†], Chi-square test*.

BMI: Body Mass index, SBP: systolic blood pressure, DBP: Diastolic blood pressure, FPG: fasting blood glucose, TG: triglycerides, hsCRP: high sensitive C reactive protein, vWF: vonWillebrand factor, sCD40L: soluble CD40 ligand.

Fasting plasma glucose < 126 mg/dl and 2-hour plasma glucose of 140–200 mg/dl were accepted as impaired glucose tolerance (IGT).

Biochemical parameters

Blood samples were collected between 08:00 and 08:30 A.M. after 12 h fasting. The tubes were promptly centrifuged, and the plasma was separated and stored at -80 °C. All plasma samples were run in the same assay. Glucose, total cholesterol, high density lipoprotein (HDL)-cholesterol and triglyceride (TG) levels were measured by the enzymatic colorimetric method with Olympus AU 600 auto analyzer using reagents from Olympus Diagnostics, (GmbH, Hamburg, Germany). Low density lipoprotein (LDL)-cholesterol was calculated by Friedewald's formula.¹⁸ Serum basal insulin level was determined in duplicate by the coated tube method (DPC-USA). vWF was measured using a commercially available enzyme-

linked immunosorbent assay kit (Technoclone, Surrey UK). All assays were performed in duplicate. Human Soluble CD40 Ligand (sCD40L) was measured by ELISA (Quantikine, R & D Systems Inc, Minneapolis, USA) [sensitivity: (minimum detectable concentration) = 0.095 ng/ml, intraCV: 4.0% and inter CV: 6.8%]. Serum hsCRP was determined by turbidimetric fixed rate method¹⁹ by an automated analyzer (Olympus AU-2700, Mishima, Japan). sP-selectin was measured using a commercially available enzyme-linked immunosorbent assay kit (Human sP-selectin, BMS 219 / 3, Bender MedSystems GmbH, Vienna, Austria). Both tests were run in duplicate.

Statistical analyses

The distribution characteristics of the variables were evaluated by Kolmogorov-Smirnov Test and then Levene's test was used to determine the homogeneity of variance. Differences between the parameters were measured by Chi-Square and independent samples T test where necessary. The association between

TABLE 2. Multiple regression models to reveal the contribution of Hb on the parameters of the prediabetic patients.

	HDL Cholesterol (β , P)	Systolic BP (β , P)	sCD40L (β , P)
Hemoglobin	NS	NS	0.437(<0.001)
Age	NS	NS	NS
Sex	0.129 (0.003)	0.437(<0.001)	NS
BMI	NS	NS	NS
SBP	NS	NS	NS
DBP	NS	0.491(<0.001)	NS
p-selectin	NS	NS	0.371(<0.001)
Total Cholesterol	NS	NS	NS

the parameters was investigated by Spearman Correlation test. All values were expressed as mean $\pm$ SD. $P < 0.05$ was accepted as statistically significant.

Results

Demographic and clinical characteristics of the subjects categorized on the basis of median Hb levels are reported in Table 1. Subjects with Hb levels in the upper median did not differ from those of the lower median as for age, BMI, waist circumference, serum glucose, insulin, total cholesterol, LDL cholesterol, triglycerides, uric acid, hs CRP, p-selectin and vWF levels. However, subjects with the higher Hb levels exhibited lower HDL Cholesterol and higher systolic and diastolic blood pressures and sCD40L levels. Also the number of women was higher in the group who had less than median Hb levels. Hemoglobin was correlated with the HDL cholesterol, sCD40L, systolic and diastolic blood pressures and waist circumference ($r = -0.28$, $P = 0.02$; $r = 0.29$, $P = 0.02$; $r = 0.53$, $P < 0.001$; $r = 0.41$, $P = 0.001$; $r = 0.42$, $P < 0.001$ respectively).

Multivariate analysis

To analyze the independent contribution of hemoglobin, we constructed a series of multiple regression models (table-2). Gender was an independent risk fac-

FIGURE 1. Association between the sCD40L and the Hemoglobin levels of the patients with Prediabetes.

tor for HDL cholesterol, waist circumference and systolic and diastolic blood pressures. However, only the Hb and the p-selectin levels were independent determinants of the sCD40L levels (table-2) (figure-1). No additional interactions were detected among the remaining risk factors.

Discussion

The results show that prediabetic subjects with higher Hb levels have elevated blood pressures, lower HDL cholesterol levels and higher sCD40L levels. Moreover, the Hb level was modestly correlated to the components of the metabolic syndrome, HDL cholesterol, arterial blood pressure and waist circumference. These findings imply that the rise in Hb values seems to take part in the elevation of both the conventional and the emerging risk factors in prediabetic patients.

Hb regulates endothelial functions by the take up and transport of NO^{20,21}. The reaction between NO

and Hb yields several compounds one of which is a highly potent vasodilatory molecule, S-nitrosylated Hb. When the PO₂ is low, Hb modulates vasodilation by releasing NO to peripheral tissues.^{1,2} Glycosylation of Hb hinders NO release in diabetes and shifts the equilibrium among NO species towards irreversible compounds which cannot release NO.²² The association between high Hb and endothelial dysfunction has been reported in several chronic disorders including diabetes mellitus and hypertension.^{8,9} We have recently shown this relationship in nondiabetic CKD 3-4 patients⁶ and in patients with diabetic proteinuria.⁷ There is no data about the role of Hb in endothelial function in prediabetic patients. Prediabetes is highly prevalent and many of these subjects are living with undiagnosed high cardiovascular risk.¹³ Therefore, focusing on this population is important to provide further information on the previously reported inverse link between Hb and endothelial function. Our study population, composed of untreated, uncomplicated, overweight (but not frankly obese) people with prediabetes, provided the opportunity to investigate this relationship without being confounded by other cardiovascular risk factors.

In this study we also searched for the relation between Hb and sCD40L, vWF or p-selectin levels, the emerging risk factors for endothelial dysfunction. CD40/CD40L signaling amplifies the inflammatory activity in the endothelium²³ and contributes to progression of atherosclerosis.²⁴ sCD40L is a predictor for cardiovascular events in patients with high-risk atherosclerotic lesions²⁵ and is elevated in prediabetes and diabetes mellitus.^{14,26,27} vWF, a glycoprotein mediating platelet adhesion and aggregation²⁸ is increased in different states of endothelial damage.²⁹ The role of vWF as a marker for endothelial dysfunction was reported in patients with vascular disorders and in prediabetes.^{30,31} P-selectin is a cell adhesion molecule used as a plasma predictor of adverse cardiovascular events³² reported to increase in patients with obesity, insulin resistance or prediabetes.^{33,34} In our previous studies in prediabetic

patients, free of vascular complications, other metabolic disorders or drugs, we found higher sCD40L levels¹⁴ while we have not observed alterations of vWF and p-selectin levels.^{15,16} Overall, it can be inferred that sCD40L may be a more important contributor to the link between the Hb and the endothelial function in prediabetes.

There are some limitations of this study. The small number of patients and the cross-sectional nature of the study may prevent causal interpretations. Therefore, it cannot be excluded *a priori* that the factors responsible for endothelial dysfunction in these patients may either stimulate erythropoiesis or increase Hb. This seems unlikely, as the endothelial NO-synthase null mouse, a transgenic model with pronounced endothelial dysfunction, does not develop hemoconcentration.³⁵ Another point to mention is that sCD40L may only be a surrogate for endothelial function and direct measurement such as flow mediated dilatation with brachial ultrasound may present clearer associations with Hb levels. The uneven sex distribution between the groups may be another limitation. According to the multiple logistic regression analysis, sex was also the determinant of HDL cholesterol and systolic blood pressure. However, sex had no impact on the sCD40L in the regression analysis, while Hb was an independent determinant of sCD40L.

In conclusion, the present cross-sectional study in prediabetes shows that patients with higher Hb concentrations have higher sCD40L levels, higher systolic and diastolic blood pressures, and lower HDL cholesterol levels. Overall, these data support the previous *in vivo* studies showing that higher Hb was associated with the impaired endothelial function. It is important to emphasize that the biological interplay between NO released by the endothelium and the red blood cell has not been studied in detail. Therefore, further studies are warranted to clarify the clinical importance of these results.

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