Lipopolysaccharide-induced VEGF production and ambient oxidative stress in type 2 diabetes

Short Title: VEGF production and ethnicity in type 2 diabetes

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The authors have nothing to disclose.

Abstract

Context

Oxidative stress is implicated in the development of microvascular disease and is associated with an upregulation of vascular endothelial growth factor (VEGF) which is pathogenetically linked to microvascular complications of diabetes. Patients of African origin have an increased susceptibility to microvascular kidney disease compared with Caucasians, the reasons and the mechanisms that contributes to this vulnerability are unclear.

Objectives

Primary) Investigate whether there are ethnic differences in Lipopolysaccharide induced monocyte VEGF production in whole blood cell cultures. Secondary) whether stimulated VEGF production is related to prevailing oxidative stress assessed by plasma lipid hydroperoxides (LOOH) and α-Tocopherol.

Design and Setting

Cross sectional study at a secondary care centre in North London, UK, serving an inner-city community of 154,000 adults.

Patients

African-Caribbean and Caucasian patients with type 2 diabetes (n=52)

Results

Lipopolysaccharide induced production of VEGF in whole blood cultures (61.8[31.9] pg/mL to 78.4[36.0] pg/mL; $p<0.001$) that correlated positively with LOOH levels ($r=0.3$, $P=0.04$) and was significantly higher in African-Caribbean than Caucasian type 2 diabetes patients (404 [207.5] vs 268.8 [137.0] pg/mL $X10^9$/L monocytes; $P=0.018$). Plasma α-Tocopherol concentration was higher in Caucasian patients (40.3[18.3] vs 30.0[9.6] µmol/L; $p=0.04$) compared to African-Caribbeans.

Conclusions:

This study suggests that the redox environment influences VEGF production in response to proinflammatory stimuli in type 2 diabetes. The differential responsiveness by ethnic origin may be of relevance in the variations in susceptibility to the long-term microvascular complications.

Introduction

Diabetes mellitus affects more than 415 million individuals worldwide (1), the most common form is type 2 diabetes, that is characterized by persistent hyperglycaemia, the degree and the duration of which are well established as central in the development of vascular complications including diabetic kidney disease. This complication has a predilection for patients of African descent compared with Caucasian origin and is the leading cause of end stage renal disease (ESRD) (2). The incidence of ESRD related diabetes is four to six times higher in patients of African descent compared to Caucasians (3).

It is understood that hyperglycaemia gives rise to the accumulation of advanced glycation end product proteins and reactive oxygen species which, together with their deficient disposal causes a metabolic imbalance known as oxidative stress (4, 5). In diabetic conditions, lipid hydroperoxide levels and histological damage of increased oxidative stress is increased in the kidney of animal models and can be reduced by antioxidant therapy (6, 7). The mechanisms related to free radical exposure that gives rise to tissue damage involves induction of pro-inflammatory pathways and cytokine release (8, 9).

Reactive oxygen species upregulate vascular endothelial growth factor (VEGF) expression in various cell types, such as endothelial cells, smooth muscle cells, and macrophages (10, 11). Hohenstein et al (2006) reported increased VEGF expression by many different cell types in diabetic glomeruli compared to controls (12). VEGF increases the transcapillary leak of albumin

and therefore may contribute to microvascular disease. However, it is unknown if this mechanism is relevant to the enhanced risk of nephropathy seen in certain sub-groups of patients with diabetes.

Meta-analysis studies showed that VEGF genetic polymorphisms are associated with increased risk of diabetic nephropathy in Asian and Caucasian patients (13, 14). We have previously reported ethnic differences in VEGF +405 polymorphism in patients with diabetes which has been shown to influence circulating levels of the cytokine (15). However, a genome-wide analysis has not shown consistent relationships between VEGF polymorphisms and circulating protein in different populations suggesting that other non-heritable, modulating factors contribute to differences in circulating levels (16). We reported increased oxidative stress in African-Caribbean patients with type 2 diabetes compared to Caucasian patients as assessed by lipid peroxidation product, antioxidant nutrients and antioxidant enzyme activities (17-19). The reasons for this observation or the mechanisms that could account for these differences are unclear. Therefore, we investigated the relationship of markers of oxidative stress and VEGF production in patients with type 2 diabetes from different ethnic backgrounds.

Methods

We studied 52 patients with type 2 diabetes who were part of the Prospective Evaluation of Early Nephropathy and its Treatment (PREVENT) study. Patients were considered to be of African-Caribbean (AC) origin if both parents were native to either African or Caribbean countries. Caucasian (CA) white patients were native of Western European or Mediterranean countries.

Individuals with a history of cardiovascular disease defined as having a clinical record of ischaemic heart disease (angina, myocardial infarction, coronary artery revascularization and or heart failure), peripheral vascular disease (intermittent claudication or peripheral artery revascularization) or cerebrovascular disease (transient ischaemic episodes or stroke), a history of malignancy or any other life threatening illness, current pregnancy, clinical proteinuria (albumin:creatinine ratio [ACR] >30 mg/mmol) or inter-current illness were excluded. Microalbuminuria was diagnosed if ACR was ≥ 3 and < 30mg/mmol in at least 2 of 3 sterile, early morning urine samples. Therapeutic regimens for hypertension and glucose lowering, and smoking history (as either current/ex-smoker or non-smoker) were recorded. The study was approved by the ethics committee of the Whittington Hospital Trust and all patients provided written, informed consent.

Patients were studied in the post-prandial state after 12 hour fast. Body mass index (BMI) was calculated from weight in kg divided by height in m^2. Sitting blood pressure was measured after 10 minutes rest using a validated automated machine (OMRON 705HEM CP; OMRON Healthcare, West Sussex, U.K.) using an appropriate cuff size. Venous blood was taken from an

antecubital vein. Glycosylated haemoglobin A1c (HbA1c) was measured by a high-performance liquid chromatography system (Menarini 8140; Menarini Diagnostics, Wokingham, U.K.). Total cholesterol and total triglycerides were estimated using enzymatic methods (Boehringer-Mannheim, Mannheim, Germany). Low density lipoprotein-cholesterol was calculated using the formula 3/4 (Total cholesterol - HDL-cholesterol) mmol/l described by de Cordova (20). Urinary albumin and creatinine were measured by immunoturbidimetry (Cobas Fara, Roche Diagnostics, Lewes, UK) and the Jaffe rate reaction methods, respectively.

Plasma lipid hydroperoxide (LOOH) concentrations (range in non-diabetic subjects: 0.22-6.22 µmol/L) was measured by ferrous oxidation-xylenol orange (FOX-2) assay in conjunction with triphenylphosphine method (21). The inter- and intra-assay coefficients of variation (CV) of the FOX-2 assay are <5 and <6%, respectively. Plasma α-tocopherol concentrations was measured by HPLC as previously described (17) and corrected for lipid profile with inter- and intra-assay coefficients of variation of 3%. Total monocyte and platelet counts were measured in whole venous blood (Advia 120, Bayer, Basingstoke, UK).

Cell culture

To measure cytokine production, whole blood cell cultures were incubated in triplicates with or without lipopolysaccharide (LPS) (25mg/mL) to activate monocytes (22). The inter- and intra-assay CVs for VEGF are 6 and 8% respectively. Concentration of the main circulating 165 amino acid VEGF-A isoform in culture supernatants was determined using an enzyme-linked

immunosorbent assay (ELISA) kit, according to the manufacturer's protocol (R&D Systems Ltd, Abingdon, UK).

Statistics

Analyses were performed using Stata 14.2 (Stata Corp, Texas, USA). Continuous variables were compared using parametric or non-parametric tests according to their distribution. Categorical variables were compared using the Chi-squared or Fishers exact tests. Variables with skewed distribution were log transformed before analyses. At an alpha of 0.05, the study had 98% power to detect a 16 pg/ml increase in LPS-stimulated VEGF. The multivariate model was based upon inputting those variables that were significantly different between the groups and/or of biological relevance to VEGF release. All tests were 2-tailed and a p value <0.05 was accepted as being statistically significant.

Results

The African-Caribbean and Caucasian groups had similar chronological age, body mass index, systolic and diastolic blood pressure, fasting plasma glucose, glycated haemoglobin and cholesterols, and prevalence of retinopathy and microalbuminuria. There were more males in the African- Caribbean group and they tended to have a longer duration of diabetes in comparison to the Caucasian cohort. Whilst the latter were more likely to have a positive smoking history, higher triglyceride concentrations, monocyte and platelet counts (Table 1). There were no statistically

significant differences in the proportions of patients in the African-Caribbean and Caucasian groups that were prescribed oral hypoglycaemic agents (Metformin and/or Sulphonylureas) or Insulin (48 vs 53 or 36 vs 25%;p=0.713) for blood glucose management, angiotensin converting enzyme inhibitors or angiotensin 2 receptor antagonists to lower blood pressure (44 vs 50%;p=0.896) or HMG Co-A reductase inhibitors to lower cholesterol (43 vs 56%;p=1.00).

Lipopolysaccharide significantly increased VEGF concentrations from 61.8[31.9] pg/mL to 78.4[36.0] pg/mL; p<0.001. Plasma LOOH and LPS stimulated VEGF release corrected for monocyte count was significantly higher in African-Caribbean patients than Caucasian patients (Figure 1). Plasma LOOH correlated with VEGF concentration (rho=0.3; p=0.04). Plasma α-Tocopherol concentration was higher in a subset of a group (n=19) of the Caucasian patients (40.3[18.3] vs 30.0[9.6] µmol/L; p=0.04) compared with group of African-Caribbean patients (n=15). In multivariate analysis, current and previous history of smoking, female gender, Caucasian ethnicity (with marginal significance) and age all had negative β coefficients. In this model, plasma LOOH remained the only statistically significant independent predictor (Table 2).

Discussion

Our study has found that in patients with type 2 diabetes mellitus, the production of VEGF from LPS stimulated whole blood cell cultures is higher and proportional to biochemical evidence of greater exposure to oxidative stress in patients of African-Caribbean compared with Caucasian origin. These findings are consistent with *in vitro* studies showing the induction of VEGF by LPS

in monocytes and its upregulation by superoxide radical generating systems in a time and dose-dependent manner (23).

A circulating, cellular source of VEGF most notably, appears to have an important role in the reparation of ischaemic tissues. In animal models of myocardial ischaemia, restoration of blood flow and preservation of function is associated with VEGF protein production and VEGF receptor gene expression (24, 25). Studies in humans with myocardial infarction have shown that circulating VEGF is elevated and the VEGF gene upregulated during the acute phase of injury in both arterial smooth muscle cells and infiltrating macrophages (26, 27). Furthermore, after acute cerebral infarction elevation of circulating VEGF occurs in relation to the size of the lesion and the associated leucocytes (28). Leucocytes, which can be less populous in people of African origin, have the same relationship with low-grade inflammation and cardio-metabolic risk seen in other ethnic groups with higher counts (29). However, the differences in VEGF response we observed suggests that monocyte function may be modified by the higher levels of glucose-induced oxidative stress that occurs in the patients of African-Caribbean origin.

Monocyte-derived, VEGF plays a key role in chronic vascular disease of significance to vasculopathy in diabetes (30). Increasing evidence implicates increased tissue production of VEGF in the development of diabetic retinopathy. In this context, increased expression of VEGF in the retina and raised levels in the aqueous occur in relation to hypoxia resulting in deleterious angiogenesis (31-33). Circulating VEGF may be a marker of future renal disease in patients with diabetes (34). The vascular permeability enhancing effects of VEGF may play a role in the rise in

urinary albumin excretion. Albuminuria in turn mediates release of other proinflammatory cytokines (35). In the evolution of diabetic nephropathy, monocytic infiltration is a feature of the development of tubulo-interstitial lesions. Therefore, oxidative stress induced VEGF could participate in the cascade of albuminuria, upregulation of chemoattractant molecules, increased monocyte attraction and trafficking of proinflammatory molecules and fibrogenic cytokines such as transforming growth factor β1 within the kidney. In a streptozotocin murine model of diabetes, increased podocyte VEGF signalling has been shown to significantly worsen the characteristic histological features of nephropathy (36). Lee at al reported that in cultured murine podocytes, glucose-dependent increases in oxidative stress and VEGF could be completely ameliorated by different antioxidants (37). Moreover, it has been reported that the renal changes associated with the db/db model of diabetes could be abrogated by neutralising anti-VEGF antibody (38). In addition, VEGF receptor tyrosine kinase inhibitor (SU5416) reduced albuminuria in type 2 diabetes db/db mouse model (39), supporting the involvement and interplay of increased oxidative stress with VEGF in the pathogenesis of diabetic nephropathy. An association between high circulating levels of VEGF and the oxidative effects of ferritin suggests that both have a role in the development of complications in patients with diabetes (40). Also, a recently described association between advanced chronic kidney disease and VEGF implies that it may also have a role in renal disease progression (41).

In our study, it would appear that oxidative stress that determined the VEGF response to the inflammatory stimulus may be a proxy for ethnic origin. **Exposure to hyperglycaemia though is a possible explanation of the differences in redox status between the groups (42). Duration of diabetes was significantly longer in univariate analysis in the African-Caribbean group**

which however, failed to reach statistical significance as independent predictor in multivariate analysis. Dietary factors could be relevant and it is notable that a survey from the United States suggests that 40% of minority ethnic groups with diabetes have a deficient micronutrient intake including vitamin E (43). A limitation of our study was that we did not collect dietary details from our cohort so we were not able to determine whether the differences in oxidative stress between the groups were related to the intake of vitamin E. In summary, we show that a variation in VEGF production by activated, pro-inflammatory cells is related to ambient oxidative stress. Infiltrating monocytes contribute to renal disease and these findings may have relevance to differing susceptibility to ESRD. Further clinical studies are required to examine the role of circulating monocyte VEGF production in the renal complications of diabetes.

Author Contributions.

- Designed research: KAE
- Performed research: KAE, KZ, JNZ
- Data analysis: KAE
- Manuscript preparation, writing and editing: KAE, KZ, JNZ

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Legend

Table 1. Demographic, clinical, biochemical and haematological characteristics of African-Caribbean and Caucasian patients with type 2 diabetes

Table 2. Multivariate regression analysis with LPS-stimulated VEGF release corrected for monocyte as the dependent variable

Figure 1. Fasting mean (SEM), plasma lipid hydroperoxide (LOOH) in open bars, and vascular endothelial growth factor (VEGF) in solid bars, after stimulation with lipopolysaccharide corrected for monocyte count in whole blood cell cultures from patients of African- Caribbean (AC) and Caucasian (CA) origin with type 2 diabetes

Demographic, clinical biochemical and haematological parameters	African-Caribbean (n=22)	Caucasian (n=30)	p
Age (years)	63.0 ± 6.4	59.0 ± 10.4	0.12
Duration of diabetes	13.0 ± 9.5	8.3 ± 6.4	**0.04**
BMI (Kg/m^2)	28.8 ± 2.8	29.8 ± 5.6	0.47
Systolic blood pressure(mmHg)	158.9 ± 17.8	153.8 ± 25.8	0.44
Diastolic blood pressure (mmHg)	90.9 ± 8.2	87.4 ± 11.9	0.56
Gender (Male/Female) %	57/43	32/68	0.08
Smoking History (%)			
Current	9	11	
Previous	27	49	**0.005**
Never	64	40	
Microalbuminuria (%)	40	36	0.79
Total Cholesterol (mmol/L)	5.3 ± 0.8	5.4 ± 0.8	0.51
LDL-cholesterol (mmol/L)	2.2 ± 0.59	2.6 ± 0.91	0.06
HDL-cholesterol (mmol/L)	1.61 ± 0.48	1.35 ± 0.56	0.07
Triglycerides (mmol/L)	1.3 ± 0.5	1.8 ± 0.9	**0.03**
Fasting plasma glucose (mmol/l)	9.2 ± 3.8	10.9 ± 4.6	0.18

HbA1c (%)	8.3 ± 0.9	7.8 ± 1.8	0.21
Platelet count ($x10^9$/L)	196.9 ± 57.9	236.8 ± 74.5	0.07
Monocyte count ($x10^9$/L)	0.21 ± 0.1	0.36 ± 0.17	**0.001**

[1] Data expressed as Mean ± SD

[1] **Table 1**. Demographic, clinical, biochemical and haematological characteristics of African-Caribbean and Caucasian patients with type 2 diabetes patients

Variable	β-coefficient	t	P value	95% CI
Log_{10} LOOH	167.23	3.67	<0.001	75.6 to 258.9
Gender	-7.12	-0.45	0.66	-39.3 to 25.0
Current Smoker	-25.62	-0.98	0.33	-78.2 to 27.0
Previous Smoker	-33.63	-1.96	0.06	-68.1 to 0.8
Ethnicity	-24.36	-1.44	0.16	-58.4 to 9.6
Duration Diabetes	-1.43	-1.84	0.07	-3.0 to 0.1
Log_{10} triglyceride	-0.17	-0.31	0.76	-1.3 to 0.9

[1]

[1] **Table 2.** Multivariate regression analysis with increase in VEGF release corrected for monocyte count as the dependent variable

Figure 1

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