Research Article

Colony Forming Unites-Endothelial Progenitor Cells (CFU-EPCs): A Surrogate Marker for Diabetic Retinopathy and High Cardiovascular Mortality Rate

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ABSTRACT

Purpose: Diabetic retinopathy is a risk factor for increased cardiovascular death. Our purpose was to find a significant difference in levels of endothelial progenitor cells (EPCs) in the peripheral blood of patients at different stages of diabetic retinopathy. Design: A prospective study. Colony forming units of endothelial progenitor cells (CFU-EPCs) in peripheral blood were counted. 40 subjects were enrolled (10 healthy (41 ± 8 y), 10 type 2 diabetes mellitus (T2DM) (64 ± 12 y) without retinopathy, 10 T2DM patients (62 ± 26 y) with non-proliferative retinopathy (NPDR), 10 T2DM patients (66 ± 9 y) with proliferative retinopathy (PDR)). The study was approevd by the ethics committee of the hospital and every subject signed a soncent form before enrollment. Methods: Growing CFU-EPCs was by the Hill's EPCs protocol. Blood was drawn early in the morning and was processed within 1 hour. Mononuclear cells were separated and cultured on fibronectin-coated plates with EndoCult medium (Stem Cell technologies, Vancouver BC Canada) for 5 days. CFU-EPCs were counted on day 5 (an average of 8 wells). Results: Healthy subjects had 36 ± 8 CFU-EPCs, patients without retinopathy had 13 ± 12 CFU-EPCs ($p<0.01$), patients with NPDR 22 ± 26 CFU-EPCs (p=NS), and 2 ± 2 CFU-EPCs in patients with PDR ($p<0.01$). A significant difference was found between patients with PDR and with NPDR ($p<0.05$). Conclusions: CFU-EPCs are inhibited in T2DM patients with DPR. Levels of CFU-EPCs may be used as a surrogate biologic marker for severity of diabetic retinopathy and for cumulative vascular-risk.

Keywords: Diabetic retinopathy, endothelial progenitor cells, blood

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INTRODUCTION

Proliferative diabetic retinopathy may occur in up to 50% of patients with type 1 Diabetes Mellitus (T1DM) [1] and in 10% of patients with T2DM [2] who have DM for 15 years. The prevalence of proliferative retinopathy is higher among patients with T2DM treated with insulin [3]. The Wisconsin Epidemiologic Study of Diabetic Retinopathy (WESDR) examined 960 younger onset and 1370 older inset patients with T1DM and T2DM for diabetic retinopathy, macular edema, visual acuity and cataract and these patients were followed for 16 years [4]. This prospective study [4] and other studies [5,6] have shown a 5 year survival rate in the young-onset subjects of 97% in those who had no or minimal non proliferative retinopathy, and 76% in those with proliferative retinopathy, and in the older-onset group 72% survival rate in those who did not have retinopathy or had minimal retinopathy, and 52% in those with proliferative retinopathy [4]. In order to understand the mechanism of diabetic proliferative retinopathy and to the high rate of cardiovascular mortality that follows, we examined previously levels of inflammation and angiogenesis in different stages of retinopathy [7]. Our novel finding at the time was that patients with proliferative diabetic retinopathy had low levels of inflammation and vascular endothelial growth factor (VEGF) compared to diabetic patients without retinopathy or with non-proliferative retinopathy. Levels of inflammatory markers (C reactive protein (CRP) and vascular cell adhesion molecule 1

{VCAM-1}) as well as vascular endothelial growth factor (VEGF) tended to increase in patients with T2DM, and increased even more with severity of the diabetic retinopathy; however, patients who already developed proliferative retinopathy (and were not treated with anti-VEGF injections) their levels of inflammatory markers and VEGF were inhibited to levels that were similar to the control group [7]. In order to explore this phenomena and to better understand the mechanism that leads eventually to higher cardiovascular death rate in patients with diabetic proliferative retinopathy we decided to study the ability of diabetic patients to grow colony forming units of endothelial progenitor cells (CFU-EPCs).

METHODS

Study subjects

We studied 40 subjects (half were women in each group) - 10 healthy controls (mean age 41 ± 8 years old), 10 T2DM patients with no retinopathy (mean age 64 ± 12 years old), 10 T2DM patients with non-proliferative retinopathy (mean age 62 ± 26 years old) and 10 T2DM patients with proliferative retinopathy who were not treated with anti VEGF injections (mean age 66 ± 9 years old).

No one of the patients or the volunteers had renal impairment or cardiovascular disease, coronary artery disease or heart failure from any etiology. All patients were treated with HMG-CoA reductase inhibitors (statins) and with angiotensin converting enzyme inhibitors (ACE inhibitors).

No one of the patients or the healthy volunteers had documented coronary artery disease or known clinical atherosclerosis. T2DM patients were recruited from the ophthalmology outpatient clinic and had to sign a consent form before enrolment to the study. Subjects were excluded from the study if they had known or symptomatic cardiovascular disease or had any chronic condition such as cancer, acute of chronic infection, autoimmune or inflammatory conditions.

All enrolled subjects underwent a detailed assessment of cardiovascular risk after signing an informed consent document approved by the institutional review board of the Baruch Padeh Poria Medical Center. All subjects continued with their regular glucose control medications as well as statins.

GROWTH OF COLONY FORMING UNITS OF ENDOTHELIAL PROGENITOR CELLS (CFU-EPCs)

The investigator who performed the laboratory experiments was blinded to the patients' clinical data. Venous blood samples were drawn from an antecubital vein into ethylene diamine tetra acetic acid-containing tubes. Forty milliliters of blood were processed; peripheral blood mononuclear cells were isolated by Ficoll density-gradient centrifugation, washed twice in phosphate-buffered saline with 5% fetal bovine serum, and re-suspended in media (EndoCult basal media with supplements; Stem Cell Technologies, Vancouver BC Canada, for endothelial progenitor cell colony-forming assay). Cells were plated on human fibronectin-coated plates (BIOCOAT; Becton Dickinson Labware Bedford Mass) at a density of 5×10^6 cells/well and incubated at 37°C in humidified 5% CO_2. After 48 hours the non-adherent cells were re-plated onto fibronectin-coated 24-well plates at a density of 1×10^6 cells/well. After 5 days, colony forming units (defined as a central core of rounded cells surrounded by elongated, spindle-shaped cells) were counted manually in 8 wells of a 24-well plate. The average number of colony forming units per well is represented.

A colony of endothelial progenitor cells consisted of multiple thin, flat spindle-like cells emanating from a central cluster of rounded oval cells. A central cluster alone without associated emerging cells, the "sunflower" image, was not counted as a colony. Colonies were counted manually in a minimum of eight wells by observers who were unaware of the subjects' clinical profiles.

STATISTICAL ANALYSIS

Data are expressed as means ± SD. A one-way ANOVA with Tukey's HSD test was utilized to compare the number of CFU-EPCs between groups of patients and between patients and healthy controls.

RESULTS

Formation of Cfu-Epcs and severity of retinopathy

Peripheral blood mononuclear cells formed distinct colonies on fibronectin coated plates. A CFU-EPC was defined as a "sunflower" with oval cells in the middle and elongated spindle

like cells sprouting from the core to the periphery, and the image could be described as a "sunflower". Most of the CFU-EPCs were located on the margins of the culture plate and tended to be organized in groups (**Figure 1**). We assessed whether the level of circulating endothelial progenitor cells correlated with the presence or absence of T2DM and the severity of the diabetic retinopathy (**Figure 2**). Compared to the healthy controls the numbers of CFU-EPCs were significantly reduced in T2DM patients without retinopathy (36 ± 8 vs. 13 ± 12, p<0.01) and in patients with proliferative retinopathy (36 ± 8 *vs.* 2 ± 2, p<0.01). However, no significant change was observed comparing the number of CFU-EPCs between the controls and patients with T2DM with non-proliferative retinopathy (36 ± 8 *vs.* 22 ± 26, p=NS), implying that the number of CFU-EPCs increased in this group of patients from the low numbers of CFU-EPCs observed in patients without retinopathy, and as the disease progressed to proliferative retinopathy CFU-EPCs were inhibited (**Table 1**).

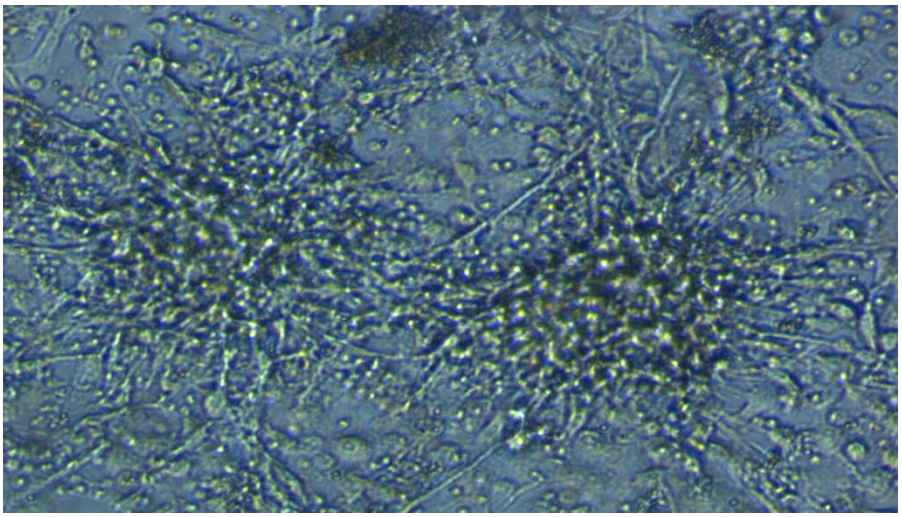

Figure 1: 2 colony forming units of endothelial progenitor cells (CFU-EPC) are demonstrated. You can see the "sunflower" image with oval cells in the middle and elongated spindle-like cells sprouting from the core to the periphery. Most of the CFU-EPCs were located on the margins of the culture plate and tended to be organized in groups.

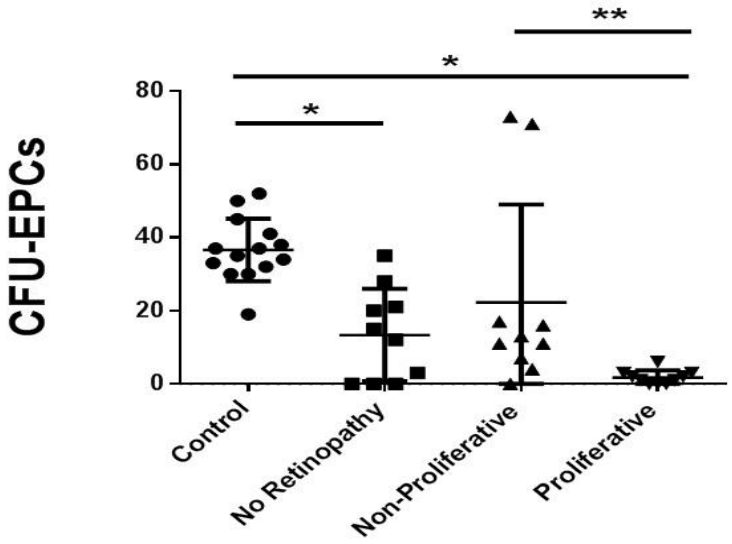

Figure 2: Colony forming unit-endothelial progenitor cells (CFU-EPCs) in patients with type 2 diabetes mellitus (T2DM) and healthy controls. T2DM patient populations with and without retinopathy and controls have significantly different numbers of CFU-EPCs (P<0.001, ANOVA). Specifically, T22DM patients with no retinopathy and with proliferative

retinopathy have less CFU-EPCs compared to controls (*P<0.01, ANOVA). In addition, T2DM patients with non-proliferative retinopathy have more colonies than patients with proliferative retinopathy (P<0.05).**

Table 1: Levels of CFU-EPCs in different stages of diabetic retinopathy.

	Healthy	**T2DM No Retinopathy**	**T2DM Mild Retinopathy**	**T2DM Proliferative Retinopathy**
CFU-EPCs	36 ± 8	13 ± 12	22 ± 26	2 ±2
*p-value	0.001	0.292	0.05	
**P-value		0.001	0.186	0.00001
Age (Y old)	41 ± 8	64 ± 12	62 ± 26	66 ± 9
*p-value	0.0009	0.617	0.491	
**p-value		0.0009	0.0003	0.00001

CFU-EPCs=Colony forming units of endothelial progenitor cells (an average count of 8 wells).
*p-value=Comparing between groups.
**p-value=Comparing between each group and the healthy volunteers group.

Mean age of the patients was 64 ± 8 years old in the proliferative retinopathy group and 62 ± 26 years old in the non-proliferative retinopathy group (p=0.491), and 64 ± 12 years old in the no-retinopathy DM group (p=0.617). However, the mean age of the healthy volunteers was 41 ± 8 years old, with a significant difference from the patients' groups (p=0.0009 compared with the no-retinopathy group, p=0.0003 compared with the non-proliferative group, and p=0.00001 compared with the proliferative retinopathy group).

DISCUSSION

Our study has demonstrated that T2DM patients with proliferative retinopathy have the lowest levels of CFU-EPCs compared with other groups of patients with diabetes mellitus. Our present study shows clearly that overall diabetic patients have an impaired ability to grow in culture CFU-EPCs from the peripheral blood. However, when DM is advancing and the retinopathy is getting worse towards non-proliferative retinopathy there is an enhancement of CFU-EPCs' growth that goes altogether with our previous findings of higher CRP, VCAM-1 and VEGF levels in that stage [7], that stimulated and encouraged angiogenesis and the development of the vascular chaos that is found in diabetic patients with stage 4 proliferative retinopathy. However, at the stage of proliferative retinopathy there is a down regulation and inhibition of EPCs in the peripheral blood with inhibition of their function and their ability to grow colonies and form CFU-EPCs. It could be a mechanism of exhaustion or a protective mechanism following the inhibition of markers of inflammation and angiogenesis [7] that have been described before at that particular stage of retinopathy. The paradigm today is that elevated levels of markers of inflammation accompany diabetic retinopathy, providing a link between inflammation and the development of microvascular complications of diabetes [8,9]. However, patients with diabetic retinopathy have not been studied in the same way that we have studied them – we measured markers of inflammation and VEGF in the peripheral blood in every stage of retinopathy and our findings have demonstrated that in the most severe form of diabetic retinopathy there is a phase of inhibition of vascular growth factors and inflammatory proteins [7].

The Wisconsin Epidemiologic Study of Diabetic Retinopathy (WESDR) had demonstrated epidemiologically that proliferative retinopathy was associated with all-cause mortality and ischemic cardiovascular disease mortality in all age groups [4]. The findings of WESDR were consistent with other reports, like the study published by Davies et al. [5]. that followed

T2DM patients for 13 years and found that patients who were diagnosed before age 50 had a 5 years survival of 56% for those with proliferative retinopathy compared with 99% for those with no or minimal retinopathy at baseline. Similarly a retrospective study reported a 55% 5 year-survival rate for T2DM patients with proliferative retinopathy compared to 92% for those with minimal retinopathy or 92% for those without retinopathy [6]. Another study from England has demonstrated that after correcting for age, gender and systemic factors retinopathy severity was associated with an increased 6 year all-cause mortality with a relative risk of 3.4 [10]. An American cohort study of Mexican Americans that followed patients for 8 years found once more that retinopathy severity was associated with mortality [11]. More than that, data from the WESDR showed that severity of proliferative retinopathy was strongly associated with stroke mortality in patients older than 50 years old [4]. These data was consistent with earlier findings that of age adjusted relative risk of proliferative retinopathy of 2.9 for incidental stroke in older-onset persons treated with insulin, and a relative risk of 6.0 for older-onset persons not taking insulin [12].

Endothelial damage represents a balance between magnitude of injury and ability to repair. Reduced number and impaired function of EPCs including proliferation, adhesion, and attachment have been described in patients with T2DM [13] and at early stage in atherosclerosis [14]. However, when the atherosclerotic plaque is activated EPCs level increase. Previous studies [15,16] have demonstrated that endothelial progenitor cells increased significantly in patients with acute myocardial infarction a few days after the acute vascular event. Fadini et al. have suggested that EPC level in diabetic patients may have a biphasic trend during the different stages of atherosclerosis development [17] Reduced number and impaired function of EPCs have been demonstrated in diabetic patients with insulin resistance [13] and at early stages of atherosclerosis [14]. When plaque complications occur, EPCs level increase, like in patients with myocardial infarction, unstable angina pectoris, and other vascular events/injury [18].

Our methodology of looking separately on diabetic patients at different stages of diabetic retinopathy enabled us to find a possible mechanism that may explain the grave outcome of patients with proliferative retinopathy and may be used as a bio-assay and a biomarker to estimate severity and risk assessment of patients with diabetes mellitus.

The nature and size of our study do not permit to determine whether low levels of CFU-EPCs could accurately predict subsequent cardiovascular events, however, a possible mechanistic explanation has been suggested. Establishing a definitive cause and effect relation requires studies in which the levels of endothelial progenitor cells are experimentally manipulated and the biologic or therapeutic effects assessed. Rather, we believe that our data suggest that circulating endothelial progenitor cells have a role in vascular homeostasis, and we can speculate (and suggest to continue this study with larger populations) that CFU-EPCs grown from the peripheral blood could be used as a biomarker for diabetic retinopathy and microangiopathy severity and may explain the epidemiological data that have shown a deleterious outcome for patients with proliferative retinopathy [9].

It could be also the key for future interventions, and patients with diabetes mellitus with impaired ability to grow endothelial progenitor colonies from the peripheral blood may need stem cells' transplantation to improve survival and prevent cardiovascular (including cerebral) events and death.

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