Circulating Endothelial Progenitors Cells as A Marker for Vascular Complications in Diabetic Patients

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Abstract

Introduction: Knowing that the degree of hyperglycaemia correlates directly with the development of vascular morbidities and its role as a key of pathogenic factor in the development of endothelial dysfunction. Therefore a common approach for the prevention and treatment of diabetic complications relies on the understanding of the endothelial dysfunction pathophysiology.

Aim of the Work: This study aimed to focus on the mechanism of Endothelial Progenitor cells (EPCs) reduction and dysfunction associated with diabetes discussing their role in virtually all diabetic complications.

Materials and Methods: Twenty nine diabetic patients and ten normal healthy controls were included in the study after giving informed consent. Diabetic patients were (8) males and (21) females with mean age of (49.7 ± 13.9) 9 cases (31%) were with macrovascular complications, 13 cases (45%) with microvascular complications and 7 cases (24%) were with no vascular complications 16 cases (65%) were with (type I) diabetes and 13 cases (44%) were with type (II) diabetes mellitus. 11 cases (37%) were on oral hypoglycaemic medications and (18) cases (62%) were on insulin treatment. The mean duration of diabetes mellitus was (11.5 ± 6.5) years (ranged from 1 year 25 ys duration) type II diabetes was defined according to American Diabetes association criteria (the expert committee on the diagnosis and classification of diabetes mellitus, 1997). All subjects were also subjected to routine laboratory investigations, fasting and post prandial blood glucose, glcosylated Hb (HbA1C) expression of endothelial marker CD133 which was determined by flow cytometric analysis of whole blood sample and culture of endothelial progenitor cells.

Results: In the current study it was found that CD133 was significantly lower in all diabetic patient groups than control group \( p=0.000 \). Regarding HbA1C it was not correlated with CD133 \( p=0.1 \) and number of colonies in culture it was higher in macro and microvascular complication groups than control group \( p=0.04 \) and 0.000 respectively while there is no significant difference between nonvascular complication group and the control group \( p=0.06 \).

Conclusions: This study conclude that circulating EPCS CD133 can be used as a marker for vascular complications in all diabetic patient and have a prominent role in pathogenesis of all diabetic complications groups (macro, micro, and no vascular complication groups) moreover HbA1C must be used as an independent marker of diabetes, and number of colonies in culture can be used as an indicator of activity of circulating endothelial cells progenitor as diabetic hyperglycaemia is one of the stress condition.

Key Words: Circulating endothelial progenitor cells – Vascular complications – Diabetic patients.

Introduction

THE incidence of diabetes is increasing exponentially. 10% of the population over 20 years of age have diabetes (type 1 + type 2) whereas another 26% have pre-diabetes (defined as impaired fasting glucose levels), many of whom are unaware of their diagnosis [1]. Vascular diseases associated with diabetes contribute significantly to the morbidity and mortality of this chronic disease. In fact, 73% of adults with diabetes are hypertensive and >65% dies from cardiovascular disease or stroke [2].

A critical component of vascular health is the efficient repair of vascular endothelium and the ability to form new blood vessels. EPCs are circulating immature cells that contribute to vascular homeostasis and compensatory angiogenesis. EPCs may participate in both of these processes. Numerous studies demonstrate an indirect correlation between circulating EPCs numbers and vascular disease risk as in individuals with coronary heart disease. Diabetic EPCs display functional impairment such as reduced proliferation, adhesion, migration and incorporation into tubular structures. Hyperglycaemia, associated with endothelial dysfunction and reduced new blood vessels growth, is a primary cause of vascular complications in diabetes [3].

EPCs were discovered in 1997 as circulating cells with the ability to differentiate into mature
endothelium and take part in neoangiogenesis [4]. EPCs share markers of hematopoietic (CD34 and CD133) and endothelial (KDR, CD31, and VWF) lineages [5]. EPCs are derived from bone marrow and can be mobilized to peripheral circulation in response to many stimuli such as tissue ischaemia [6], through the release of growth factors and cytokines, which mobilizes EPCs, which once in the peripheral circulation specifically home on the ischaemic sites to stimulate compensatory angiogenesis [7]. Moreover, EPCs constitute a circulating pool of cells able to form a cellular patch at sites of endothelial injury, thus contributing directly to homeostasis and repair of endothelial layer; these observations suggest that EPCs have a major role in cardiovascular biology, in fact, the extent of the circulating EPC pool is now considered a mirror of cardiovascular health. Virtually all risk factors for atherosclerosis have been associated with decrease and/or dysfunction of circulating EPCs [8]. While an expanded EPC pool is associated with a decreased cardiovascular mortality [9].

The study of EPC biology consists two related aspects: quantitative evaluation of the EPC pool and functional assessment. Circulating EPCs can be quantified directly in ex-vivo using flow cytometry which is considered a gold standard for this purpose [10]. Typical surface antigens to identify EPCs are CD34, CD133 and KDR.

Functional characteristics are explored in vitro using standardized protocols culture [11].

- Proliferation refers to the ability of EPCs to expand and form colonies in culture.
- Adhesion is a further step required for both re-endothelialization and angiogenesis. It is assessed as the ability to adhere to a monolayer of mature endothelium in culture.
- Migration of EPCs through the extracellular matrix is crucial for the growth of new vessels and is generally assessed in vitro as the ability to invade the lower side of a boyden-like chamber.
- Spatially organize to form vascular structures. This property can be assessed in vitro as a tube formation assay in which EPCs are seeded with human umbilical vein endothelial cells on a gel of extracellular matrix proteins.
- Fadini et al., [12] states that, there is at least two different types of EPCs, they could be classified in ex-vivo cultured system according to their time dependent appearance.

1- Early adherent EPCs appear 4 days after plating, they appear as elongated with spindle shape.

2- Late EPCs appear twenty-one days after plating exhibiting cobblestone like morphology both of them share some endothelial phenotype but show different morphology, proliferation rate, survival feature and functions in neovascularization [13].

Knowing that the degree of hyperglycaemia correlates directly with the development of vascular morbidities and it is a key of pathogenic factor in the development of endothelial dysfunction. This study aimed to focus on the mechanism of EPCs reduction and dysfunction associated with diabetes discussing their role in virtually all diabetic complications.

**Patients and Methods**

Twenty nine diabetic patients and ten normal healthy controls were included in the study after giving informed consent diabetic patients they were (8 males) males and (21) females with mean age of (49.7±13.9) 7 cases (27%) were with macrovascular complications, 13 cases (65%) with microvascular complications and 7 cases (24%) were with no vascular complications 16 cases (55%) were with (type I) diabetes and 13 cases (44%) were with type (II) diabetes mellitus 11 cases (37%) were on oral hypoglycaemic medications and (18) cases (62%) were on insulin treatment. The mean duration of diabetes mellitus was (11.5±6.5) years. Their ages ranged from (1 year to 25 ys duration) type II diabetes was defined according to American Diabetes association criteria (the expert committee of the diagnosis and classification of diabetes mellitus, 1997).

Screening oral glucose tolerance tests are performed as part of routine care in all normal control. All controls had a normal screening 50 g oral glucose challenge, defined as 1-h glucose <140, and have normal blood pressure.

All diabetic patients were under treatment (Diet control, oral hypoglycaemic drugs, ± or insulin treatment). Non of these patients had received drugs influencing vascular integrity.

Exclusion criteria for all patients and controls were trauma, surgery or acute organ ischaemia within the preceding 3 months, severe liver disease, renal failure, cancer, chronic inflammatory diseases and fever or acute inflammatory or infections conditions and any disease affecting glucose
metabolism i.e., polycystic ovarian syndrome, Cushing syndrome) or taking medications known to affect glucose metabolism (i.e., glucocorticoids) were excluded from the study.

All subjects were subjected to history taking, detailed clinical examination. All subjects were also subjected to routine laboratory investigations, fasting and post prandial blood glucose, glycosylated Hb (HbA1C) expression of endothelial marker CD 133 which was determined by flow cytometric analysis of whole blood sample and culture of endothelial progenitor cells.

All diabetic vascular complications were classified into macro, micro or without vascular complications according to the presence or absence of vasculopathy. These complications were investigated by ECG and cardiac enzymes magnetic resonance imaging direct and indirect retinal examination abdominal ultrasonography arterial and venous Doppler examinations.

**Methods:**

Blood samples were collected in 2 tubes for each patient and control: one containing EDTA for:

- CD133 expression which was determined by flow cytometric analysis (MACs Cat number 130-080-801):
  1. $10^7$ nucleated cells was suspended per 80 $\mu$L of buffer.
  2. 20 $\mu$L of FCR Blocking reagent was added.
  3. 10 $\mu$L of CD 133/1 (AC 133) antibody was added.
  4. Then mixed well and refrigerated for 10 minutes in the dark (4-8ºC).
  5. The cells were washed by adding 1-2ml of buffer per $10^7$ cells and centrifuged at 300xg for 10 minutes. The supernatant was aspirated completely.
  6. Cell pellet was resuspended in a suitable amount of buffer for analysis by flow cytometry or fluorescence microscopy.
  7. Cells were counted using EP ICS-Elite flow cytometer.
  8. 10,000 event was counted/sample [14].

- Glycosylated HbA1C using the spectrophotometric method for determination of HbA1C in blood by Ion exchange method in tube (Far diagnostics code 3655, 3658).

The other tube contain Na heparine for:

- Culture of endothelial progenitor cells. Stem cell technologies Cat number 05900 # 100ml 05950 # 500 ml

**Culture of endothelial progenitor cells in peripheral blood:**

Using the components of Endo Cult Liquid Medium Kit Basal Medium and supplements have been pre-screened and performance tested in an established in vitro colony assay [15].

**Day 0 day to 5 CEU-Hill Colony assay**

**Day 0:**

- Peripheral blood was collected using sodium heparin as an anticoagulant to avoid clotting or clumping of the sample.
- $10^7$ mononuclear cells was prepared per sample for each experiment.
- Mononuclear cell suspension was prepared by light density separation using Ficoll-paque in plus catalog no. # 07907/07957).
- 2ml/well of Endo Cult Liquid Medium was added to a 6 well-fibronectin-coated plate from Bio Sciences Discovery Lab ware, BD Catalog # 354402, 6 well plates, 5/pack.

**Day 2:**

After 2 days harvest the non-adherent cells containing the cFu Hill colony forming cells and culture for an additional three days to allow formation of cFu-Hill colonies.

**Day 5:**

- The number of cFu-Hill colonies was counted per well for each sample. cFu-Hill colonies was defined as a central core of round cells with radiating elongated spindle like cells at the periphery.
- Colonies were fixed and stained.

**Statistical analysis:**

Data were statistically described in terms of range, mean ± standard deviation (±SD), median, frequencies (number of cases) and percentages when appropriate. Comparison of quantitative variables between the study groups was done using Mann Whitney U test for independent samples when comparing 2 groups and Kruskal Wallis analysis of variance (ANOVA) test when comparing more than 2 groups. Correlation between various variables was done using Spearman rank correlation equation for non normal variables. A probability value ($p$-value) less than 0.05 was considered statistically significant. All statistical calculations were done using computer programs Microsoft Excel 2003 (Microsoft Corporation, NY, USA) and SPSS (Statistical Package for the Social Science; SPSS Inc., Chicago, IL, USA) version 15 for Microsoft Windows.
Results

We studied 29 patients (31%) with macrovascular complications, 13 (45%) with microvascular complications and 7 (24%) with no vascular complications. Of diabetic patients 16 (55%) were type I diabetes and 13 (44%) were type II diabetes Fig. (1).

18 (62%) were on insulin treatment and 11 (37%) were on oral hypoglycaemic drugs and the study also included 10 normal healthy control subjects with age and sex matched group.

Demographic and metabolic characteristics of patients and control subjects are shown in Table (1).

By comparing diabetic patients as a whole with the control group it was found that:

- CD133, were significantly lower in diabetic patients than control group ($p=0.000$) while HbA1C and No of colonies were significantly higher in diabetic patients than control group ($p=0.000$ and $p=0.001$ respectively) (Table 2 & Fig. 2).

- Comparing diabetic group with no vascular complications with control group, it was found that CD133 was significantly lower than control group ($p=0.000$) while HBA1C and No of colonies were significantly higher then control group ($p=0.003$ and $p=0.000$ respectively) (Table 4 & Fig. 2).

- By comparing diabetic group with no vascular complications with the control group, it was found that CD133 was significantly lower than control group, $p=0.001$ while HbA1C was significantly higher than control group $p=0.001$ and there is no significant difference regarding no of colonies between these two group ($p=0.06$) (Table 5 & Fig. 2).

- It was found, that there is no correlation between CD133 and the age, duration of disease, HbA1C and number of colonies in culture $p=0.2, 0.6, 0.1, 0.9$ respectively (Table 6 and Figs. 3-6).

Table (1): Demographic and clinical parameters in diabetic patients.

<table>
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<th>Sex</th>
<th>Duration of disease ys</th>
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<th>Type of diabetes</th>
<th>Treatment</th>
<th>CD133</th>
<th>HbA1C</th>
<th>Culture</th>
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Table (2): Comparing CD133, HbA1C, No of colonies in culture between diabetic patients group as a whole and the control group.

<table>
<thead>
<tr>
<th></th>
<th>Cases (n=29)</th>
<th>Control (n=10)</th>
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<td>CD133</td>
<td>0.404±0.60</td>
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<td>HbA1C</td>
<td>9.32±2.50</td>
<td>5.100±1.66</td>
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<td>No of colonies in culture</td>
<td>9.07±3.37</td>
<td>4.50±1.26</td>
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Table (3): Comparing CD133, HbA1C, No of colonies in culture between macrovascular complication group and the control.

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<td>HbA1C</td>
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<tr>
<td>No of colonies in culture</td>
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<td>4.50±1.26</td>
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Table (4): Comparing CD133, HbA1C, No of colonies in culture between microvascular complications group and the control.

<table>
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<td>HbA1C</td>
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<td>No of colonies in culture</td>
<td>10.60±2.74</td>
<td>4.50±1.26</td>
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Table (5): Comparing CD133, HbA1C, No of colonies in culture between diabetic patients (no vascular complication group) and the control group.

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<td>No of colonies in culture</td>
<td>7.29±3.25</td>
<td>4.50±1.26</td>
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Table (6): Correlation between CD133 and age, duration of disease as HbA1C no of colonies.

<table>
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<th></th>
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<td>HbA1C</td>
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<tr>
<td>No of colonies in culture</td>
<td>0.008</td>
<td>0.9</td>
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Fig. (1): Subgrouping of diabetic patients according to vascular complications.

Fig. (2): CD133 in the 3 diabetic groups and the control group.

Fig. (3): Correlation between CD133 and age among all diabetic groups.

Fig. (4): Correlation between CD133 and disease duration among all diabetic groups.
Discussion

The release of EPCs from bone marrow in response to mobilizing stimuli depends on complex interactions in the local marrow microenvironment. Tissue ischaemia is considered the strongest stimulus for EPC mobilization, through the activation of hypoxia-sensing systems, such as hypoxia inducible factor (HIF-1).

HIF-1 is a heterodimeric transcription factor composed of α and β subunits. While β is constitutively expressed, α expression is regulated by cellular oxygen concentration.

Other, vascular endothelial growth factor (VEGF), stromal derived factor (SDF-1) and erythropoietin allow EPCs to undergo transendothelial migration and to pass into the peripheral blood by means of attenuating stromal cell-stem cell interaction by rearranging extracellular matrix [16]. It has been shown that the expression of angiogenic factors VEG-F and HIF-1 α are reduced in heart of diabetic patients during acute coronary syndromes [17].

Oxidative stress plays a crucial role in the pathogenesis of diabetic complications. As stress induced apoptosis may be one mechanism of EPC reduction in diabetes as EPCs might decrease because of increase apoptosis and that EPCs are stress sensitive as estrogen and physical exercise increase cultured EPCs by inhibiting apoptosis, while C-reactive protein and systemic hypoxia down regulate EPCs by enhancing apoptosis [18].

Expression of endothelial nitric oxide synthase (eNOS) is known to be essential for the survival, migration and angiogenesis of either EPCs or endothelial cells. (NO) derived from eNOS has been identified as promoting the mobilization of EPCs from the bone marrow through nitrosylation and elevated vascular endothelial growth factor (VEGF) [18].

NOS is considered a protective enzyme not only for its role in NO synthesis, but also because its inhibition is associated with the production of ROS through NADPH consuming enzyme as inflammatory cytokines (tumour necrosis factor-a and IL-6 can directly activate NADPH oxidase [19].

It has been suggested that high glucose level may impair eNOS expression resulting in reduced NO production which is associated with reduced proliferation and an increase in apoptosis of endothelial cells and potentially contributes to the development of endothelial dysfunction and atherosclerosis in diabetes [20].

It has been shown that Reactive Oxygen Species (ROS) may play a key role in high glucose induce apoptosis of mature vascular endothelial cells [21].

Excessive generation of ROS may contribute to endothelial dysfunction while low levels of ROS are essential and participate in intracellular signaling pathways [22]. In diabetic patients increased production of ROS could contribute to the onset of development of diabetic vascular complication [23].

Humpert et al. [24] have shown that insulin therapy achieve favorable clinical outcomes associated with glycaemic control during acute ischaemic syndromes and this is partly dependent on stimulation of EPCs-meditated neovascularization in the ischaemic myocardium, thus reducing residual ischaemia [24,25].

Diabetic bone marrow is less responsive to exogenous EPCs-mobilizing agents [26,27,28].

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Fig. (5): Correlation between CD133 and HbA1C among all diabetic groups.

Fig. (6): Correlation between CD 133 and number of colonies in culture among all diabetic groups.
In this study, we compared the circulating CD133 levels in macrovascular, microvascular and no vascular complications with control cases. The results showed statistically significant decrease in CD133 in all diabetic groups p-values was respectively (p=0.008, 0.000, 0.001).

This study was in accordance with Gigan et al., [29] which demonstrate decrease EPC level in diabetic patients and also shows that PVD (Peripheral Vascular Diseases) was associated with an extensively lower number of EPC as they demonstrate that CPCS and EPCS from diabetic patients were reduced by 33% and 40% respectively compared with healthy subjects (p<0.001) an inverse correlation was found between the number of EPCS and the values of fasting glucose \( r=0.49 \) \( p<0.006 \) and they state that peripheral vascular disease was associated with 47% reduction in EPCS (p<0.0001) patients with ischaemic foot lesions had the lowest levels of both EPCS and CPCS (p=0.02) they conclude that diabetic patients demonstrates decrease EPC levels and that PVD is associated with an extensively low number of EPCS. Depletion of EPCS in diabetic patients may be involved in the pathogenesis of peripheral vascular complications as a marker of severity for diabetic vasculopathy.

Also, this study was in agreement with Gian et al., [30] who demonstrated that EPC decrease is related to PAD severity and EPC function is altered in diabetic subjects with PAD (Peripheral Artery Diseases), strengthening the pathogenetic role of EPC dysregulation in diabetic vasculopathy. Diabetic patients with PAD displayed a significant 53% reduction in circulating EPCS versus non PAD patients and EPC levels were negatively correlated with the degree of carotid stenosis and the stage of leg claudication, moreover the clonogenic and adhesion capacity of cultured EPCS were significantly lower in diabetic patients with PAD versus other patients they concluded ther. EPC count may be considered a novel biological marker of peripheral atherosclerosis in diabetes.

Also this study, was in accordance with Sinson et al., [31] who demonstrated that circulating progenitor endothelial cells, endothelial progenitor cells in type-I DM with and without diabetic retinopathy they found that EPCS were reduced in mild non proliferative diabetic retinopathy 114±66 (p<0.001) and moderate-severe non proliferation diabetic retinopathy 77±40 (0.04) compared with control (244±115), while EPCS were unchanged in mild moderate proliferative diabetic retinopathy (248±155) compared with controls.

Regarding glycosylated haemoglobin in this study no correlation was found between CD133 level "circulating EPC" and HbA1C (p=0.1). This denotes that the cause of CD133 is not related to the hyperglycaemia but rather to the initial pathology of diabetes mellitus, so it must be used as an independent marker of diabetes.

Although diabetic patients showed decrease CD133 than control group, the number of colonies in culture was high then control group in macrovascular and microvascular complication groups (p=0.04, p=0.000 respectively), while diabetic patients with no vascular complication group showed no significant difference than control group (p=0.06).

This can be explained by increase activity of circulating EPCs in diabetes mellitus than in normal control group as diabetic hyperglycaemia is one of the stress condition.

Conclusion:

Bone marrow is a reservoir of immature cells that once in the blood stream, participate in regeneration and repair of many tissues thanks to their extreme plasticity. Reduction or dysfunction of EPCs may have a prominent role in the pathogenesis of all diabetic complications.

Reduction in circulating EPCs in diabetic patients may recognize at least three pathophysiological explanations:

• Impaired bone marrow mobilization.
• Defective proliferation.
• Enhanced apoptosis.

This study showed that circulating EPCS "CD133" can be used as a marker for vascular complications in all diabetic patient and have a prominent role in pathogenesis of all diabetic complications groups (macro, micro, and no vascular complication groups), moreover HbA1C must be used as an independent marker of diabetes and number of colonies in culture can be used as an indicator of activity of circulating endothelial cells progenitor as diabetic hyperglycaemia is one of the stress condition.

References

Circulating Endothelial Progenitors Cells as A Marker


