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RESEARCH LETTER

Putative endothelial progenitor cells predict long-term mortality in type-2 diabetes

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Introduction

Putative endothelial progenitor cells (pEPCs) may be identified by the expression of combinations of cell surface antigenic markers, including haematopoietic stem cell markers CD34, CD117, CD133 and/or the endothelial cell marker kinase insert domain receptor (KDR), in different combinations [1, 2]. However, since the discovery of "putative" endothelial progenitor cells over 20 years ago by Asahara [3], debate is still ongoing with regard to the precise nomenclature and best method used to detect and measure this population of cells [1, 2].

Ten years ago, we demonstrated that patients with type 2 diabetes have significantly lower levels of a range of pEPCs compared to healthy controls [4]. This generalised reduction of pEPCs has also been observed by others [5, 6]. Furthermore, levels of circulating progenitor cells have been shown to negatively correlate with the presence of diabetic complications [4, 5], as well as predicting microvascular and cardiovascular outcomes in type 2 diabetic patients [4, 7, 8]. However, to date, no study has specifically examined whether baseline pEPC levels are associated with long-term (>10 years) mortality in type 2 diabetes patients.

Patients and methods

Blood samples were collected from patients referred to the Diabetes Unit at the University Hospital of Siena, Italy, from May 2006 to August 2007. Diagnosis of type 2 diabetes was the only criterion for inclusion [9], whereas exclusion criteria included diagnosis of type 1 diabetes and presence of any of the following self-reported medical conditions: auto-immune diseases, current or prior cancer, infection and any other unrelated disease. Peripheral blood mononuclear cells (PBMCs) were collected from 98 patients with type 2 diabetes and analysed by flow cytometry for cells expressing a range of pEPC cell-surface markers. pEPCs were estimated from total (lymphocyte and monocyte) PBMC population. Patient clinical characteristics and methodology for quantification of pEPCs have previously been described in detail [4].

In the present study, we have extended the follow-up of this same cohort to examine the association between a panel of pEPCs (CD34/CD31, CD34/CD117, CD34/CD133, CD34/KDR, CD117/KDR and CD34/CD133/KDR) and all cause mortality up to 10 years after baseline examination. Information on time and cause of death was collected during routine visits and retrospectively examined from electronic patient records or by phone call. Ethics committee approval and written informed consent were obtained from all participants.

Statistical analysis

Statistical analysis was performed using MedCalc software (Mariakerke, Belgium). Data are expressed as mean ±

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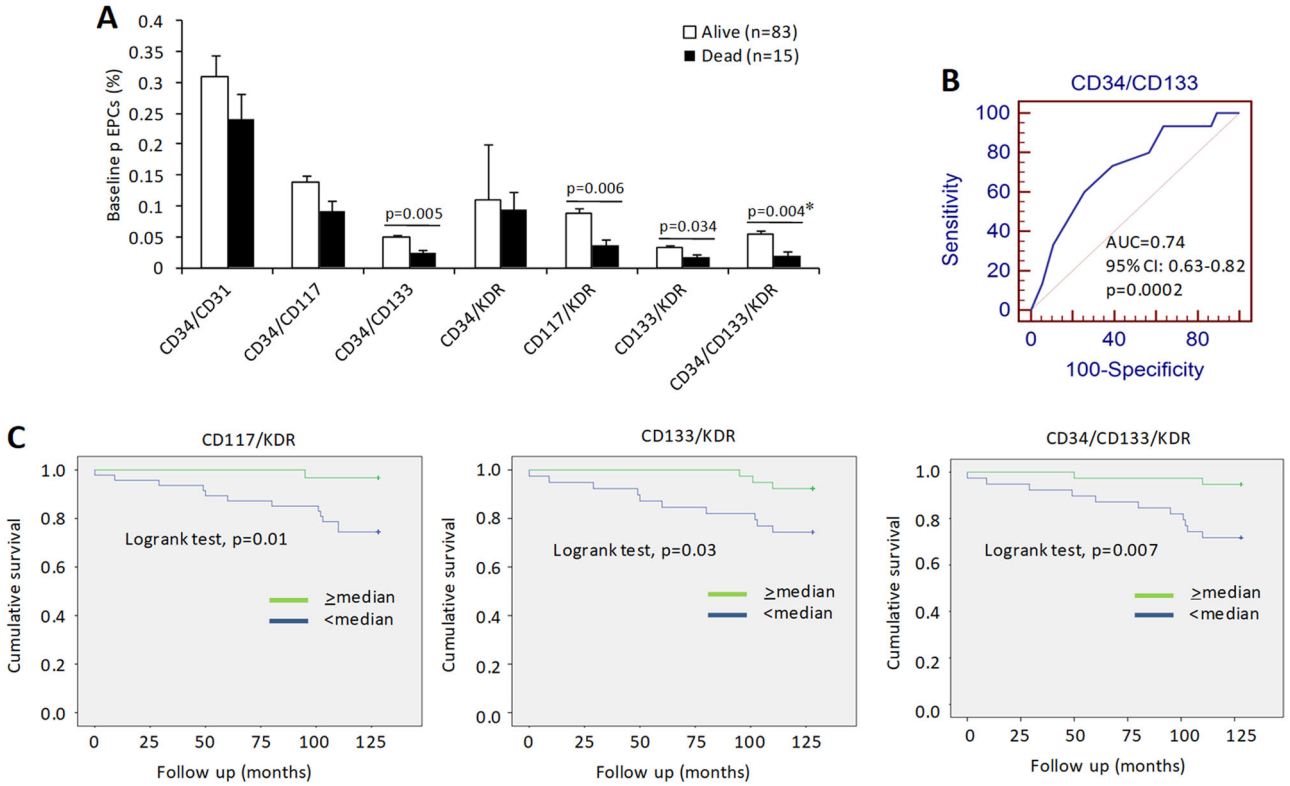

Fig. 1 Baseline levels of pEPCs predict long-term mortality in type-2 diabetes. **a** Levels of different pEPC populations in alive and dead patients. **b** ROC analysis was used to assess the ability of pEPC levels to discriminate patients who died and those alive. **c** Kaplan–Meier curves for high (≥median) and low (<median) pEPC combinations in type 2 diabetes patients over 10-year follow up. Data expressed as mean ± SEM. *Statistical significance after Bonferroni correction. AUC area under curve, pEPC putative endothelial progenitor cell

standard error of the mean or number and percentage. Comparison between two groups was performed using the Student's *t*-test for continuous variables or Mann–Whitney test for non-parametric variables. The Bonferroni procedure was used to correct for multiple testing and inflation of type I error. Categorical variables were compared using Fisher's exact test. Given the relatively low number of deaths ($n = 15$), the need to control for confounding factors and the general rule to include into a multiple Cox model a number of variables ranging from 5 to 9 for each case experiencing the event of interest [10], the relationship between predictor variables (including pEPCs) and mortality was assessed by bivariate Cox-proportional hazard regression models (i.e. models including 1 variable for every 7–8 cases of death). The Kaplan–Meier estimation method was computed to assess the probability of survival in patients with "high" and "low" pEPC levels and compared statistically using log rank test. High and low levels were based on median values, as previously described [8]. Receiver operating characteristic curve (ROC) analysis was used to assess the ability of pEPC levels to discriminate patients who died and those alive. A *p*-value of <0.05 was considered statistically significant.

Results

From the 98 patients examined from 2006–2007, 15 (15.3%) died over the 10-year follow-up period. Cause of death was cardiovascular/vascular related in 12/15 (80%) patients: myocardial infarction ($n = 4$), heart failure ($n = 3$), and 1 from carotid artery occlusion, critical limb ischemia, cerebral atherosclerosis, cerebral infarction and stroke, whereas 3 patients died from cancer (2 from pancreatic cancer and 1 from cerebral neoplasia). As expected, patients who died were older (68.5 ± 2.5 vs. 59.4 ± 1.1, $p = 0.001$), had increased prevalence of cardiovascular disease (66.7 vs. 24.1%, $p = 0.011$) and increased number of risk factors [4] (6.9 ± 0.63 vs. 5.2 ± 0.2, $p = 0.005$), than patients still alive (ESM Table 1). Baseline pEPC levels (expressing CD34/CD133, CD117/KDR, CD133/KDR, and CD34/CD133/KDR) were significantly lower (approximately 50% reduction) in patients who died (ESM Table 1, Fig. 1a). Considering all predictor variables, Cox-proportional hazard regression analyses revealed that albuminuria, advanced age, the presence of CVD were associated with a 1.01 (95% CI: 1–1.02, $p = 0.032$), 1.08 (95% CI: 1.02–1.1, $p = 0.009$) and 4.1-fold (95% CI: 1.5–11.4, $p = 0.007$)

relative risk of death respectively, whereas a 0.01% higher baseline level of pEPCs expressing CD34/CD133 was associated with a 30% lower rate of mortality (HR: 0.7, 95% CI: 0.5–0.9, $p < 0.01$) (ESM Table 2). Similarly, pEPCs expressing CD34/CD133/KDR also emerged as being significantly associated with mortality (ESM Table 3). ROC analysis revealed a significant predictive power of levels of pEPCs expressing CD34/CD133 in predicting mortality (AUC = 0.74, 95% CI: 0.63–0.82, $p = 0.0002$). Stratifying pEPC populations by "high" and "low" levels, revealed that cumulative survival was significantly greater in patients having "high" levels of the following pEPC combinations: CD177/KDR, CD133/KDR and CD34/CD133/KDR (Fig. 1c).

Discussion

This study responds to our initial hypothesis, as to whether baseline pEPC levels may be associated with long-term mortality in type 2 diabetes patients. Having previously identified a panel of five antigenic marker combinations (CD34/CD31, CD34/CD117, CD34/CD133, CD34/KDR, and CD34/CD133/KDR) known to be present on both haematopoietic cells and pEPCs that are significantly lower in type 2 diabetes patients and negatively correlated with disease severity [4], we next retrospectively compared baseline levels of these pEPC combinations (in addition to CD117/KDR and CD133/KDR combinations) in patients who died compared to those still alive, in addition to examining the association between risk factors and mortality. Our findings demonstrate that although classical risk factors such as albuminuria [11], advanced age [12] and the presence of CVD [13] at baseline are strong predictors of death, low levels of pEPCs can independently predict increased mortality risk over a period of 10 years in patients with type 2 diabetes.

In a recent meta-analysis of longitudinal studies (including 4155 patients monitored for approximately 2 years), Rigato and colleagues observed that lower levels of circulating progenitor cells expressing CD34+ and CD34+CD133+ were associated with an approximately 2-fold increased risk of cardiovascular events and death [7]. Meta-regression of this same study did not detect a correlation between the prevalence of diabetes and hazard ratios [7]. This meta-analysis study was also limited by heterogeneity, in terms of setting, patient population, underlying clinical condition, outcome definition, duration of follow-up, and progenitor cell phenotype. A Japanese study has also recently shown an association between CD34+ cells and the presence of cardiovascular events over a 4.6-year follow-up [14]. Similarly, Fadini demonstrated that lower levels of progenitor cells (CD34+ and CD34/CD133+) predict the occurrence of cardiovascular events, but not death, in type 2 diabetes patients with high cardiovascular risk followed over a 6-year period [8]. Although levels of CD34+ cells (similar to other single marker expression such as CD117 and CD133) were not lower in patients who died, baseline levels of pEPCs expressing CD34/CD133 emerged in our analysis as being significantly lower in patients who died, as well as strong predictors of mortality, although this population did not emerge as being statistically significant following KM survival analysis ($p = 0.09$), likely due to the small number of events. Furthermore, our cohort included patients with relatively similar clinical background who did not present with high cardiovascular risk at baseline. In this respect, the patient profile may represent the real-life setting to a greater extent, where less stringent exclusion criteria were implemented, therefore less prone to potential problems of selection bias. There are some limitations of the present study that need to be acknowledged. First, the number of events analysed by flow cytometry was less than desired. To address this, investigators followed a strict protocol and routinely examined the reproducibility of flow cytometry analysis in order to reduce variation. Second, the small number of patients who died precluded the possibility of including all variables in a multivariate Cox regression model. However, the follow up period of 10 years, combined with a wide panel of pEPCs are unique strengths of this study, offering important insights into the potential use of specific pEPC combinations as surrogate markers for mortality risk in type 2 diabetes. Implementation of recent consensus on the nomenclature of EPCs [2] in studies to evaluate the long-term monitoring pEPCs/EPCs as potential predictors of cardiovascular-related mortality are needed.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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