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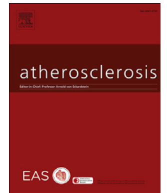

Reduced circulating stem cells associate with excess fasting and post-load NEFA exposure in healthy adults with normal glucose tolerance

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Background and aims: Reduced levels of circulating stem cells (CSCs) predict cardiovascular events and death, but the factors underlying variability of CSCs in healthy adults are mostly unknown. Previous studies detected associations of CSCs with glucose tolerance or insulin resistance, while the role of fatty acids has been overlooked. We herein aimed to describe in better detail the metabolic abnormalities associated with a reduced CSC level.

Methods: This was a cross-sectional study on 94 healthy male and female individuals with normal glucose tolerance, aged 18–65 years. All participants underwent an oral glucose tolerance test (OGTT) with blood samples collected at 0, 10, 20, 30, 60, 90 and 120 min. Mathematical models were applied to plasma glucose, insulin, *C*-peptide and non-esterified fatty acids (NEFA) concentrations. CSCs were defined as $CD34^+$ or $CD133^+$.

Results: Participants (mean ± SEM age 43.8 ± 0.7; 41% males) were divided according to CSC levels below (low) or above (high) the median value and metabolic parameters were compared. There was no significant baseline difference between groups except for higher concentrations of fasting NEFA in subjects with low CSCs. Upon OGTT, individuals with low CSCs had higher area under curve (AUC) of NEFA ($p < 0.001$) and no significant differences in glucose, insulin and *C*-peptide. Several insulin sensitivity and beta cell function indexes were not significantly different, except for a decrease in the disposition index (DI) in subjects with low CSCs. CSCs were associated with excess NEFA levels independently from age and DI.

Conclusions: We show for the first time that, in healthy adults with normal glucose tolerance, low CSCs are strongly associated with excess NEFA exposure. The pathophysiological consequence of this association needs to be interpreted in view of the prognostic role of CSCs. Future studies should explore whether excess NEFA and low CSCs and are causally interconnected.

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1. Introduction

In unstimulated conditions, hematopoietic stem cells circulate in the bloodstream at a very low frequency (~3/μl) [1]. While massive stem cell release occurs during acute inflammation or growth factor stimulation, a certain degree of variability can be observed within this physiologic range even in healthy individuals. On the other side, endocrine and metabolic diseases are associated with significant reductions in the levels of circulating stem cells (CSCs), as shown for diabetes and acromegaly [2,3]. The meaning of CSC variations within the normal range remained long obscure, but it has been now clarified that below-median CSC levels independently associate with a 2–3 fold increased risk of cardiovascular events and death [4,5]. Because of the vascular regenerative properties of $CD34^+$ CSCs [6] and of the subpopulation of $CD34^+KDR^+$ endothelial progenitor cells (EPCs) [7], pauperization of these cells in peripheral blood is believed to impair cardiovascular homeostasis and tissue repair. Moreover, shortage of CSCs and EPCs reflects pathologic changes ongoing in the bone marrow [2,8], which in turn can result in inflammation and impaired tissue homeostasis

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[9].

In diseased conditions, such as diabetes, several mechanisms can result in bone marrow damage and reduction of CSC levels [2], but the clinical correlates of CSC variations in healthy subjects are mostly unknown. In view of the strong negative prognostic implications of low CSC level, it is important to clarify which are the underlying pathophysiologic processes, in order to devise preventive and therapeutic approaches. We previously reported that glucose tolerance is negatively associated with CSC levels and that CSC reduction precedes development of overt diabetes [10]. However, the metabolic determinants of CSCs in individuals with normal glucose tolerance have never been explored. Notably, most previous studies interpreted CSC levels against glucose tolerance or insulin resistance, while the role of fatty acids has been overlooked, despite the fact that glucose and fatty acid metabolism are physiologically interconnected [11]. Interestingly, CSCs are sensitive to glucose toxicity [12], and are also affected by fatty acid metabolism [13]. We herein hypothesize that the CSC depletion observed in metabolic diseases may be attributable to derangements in fatty acid metabolism, which can in turn impair insulin section, induce insulin resistance and glucose intolerance [14]. This is supported by the evidence that fatty infiltration of the bone marrow, occurring with age and in human diabetes [12], acts locally as a negative regulation of CSC activity [15].

The present study was designed to describe in detail metabolic defects associated with changes in CSC levels in healthy glucose tolerant adults. To this end, we collected data on glucose, insulin, *C*-peptide and non-esterified fatty acids (NEFA) levels during the frequently sampled oral glucose tolerance tests (OGTT). This method allows deriving at the same time several indexes of insulin secretion, insulin sensitivity, and fatty acid metabolism, thereby being ideally suitable to dissect the major metabolic determinant of CSCs in healthy glucose tolerant individuals. Specifically, as fasting NEFA show wide variability [16], a dynamic test is more useful to study NEFA metabolism [17].

2. Patients and methods

2.1. Enrolment and characterization of study participants

Individuals were enrolled from a screening program performed at the Division of Metabolic Diseases of the University Hospital of Padova. The study was approved by the local Ethical Committee and conducted according to the Principles of the Declaration of Helsinki. All participants provided written informed consent. Male and female adults aged 18–65 years could be enrolled provided they were free from the following exclusion criteria: any abnormalities in glucose tolerance, as defined by ADA glucose criteria [18], including IFG (impaired fasting glucose, 100–125 mg/dl), IGT (impaired glucose tolerance, 2 h glucose 140–199) or diabetes, morbid obesity (BMI >40 kg/m^2), any acute disease or infection, chronic inflammatory disease, recent surgery or cardiovascular event, special diets (e.g. vegan, gluten-free, or very poor in carbohydrates), use of medications that could affect metabolism, pregnancy or lactation, or inability to provide informed consent. Individuals accessed the outpatient clinic at 8:00 a.m. for baseline examination and metabolic tests. We recorded the following data: age, sex, height, weight, BMI, waist circumference, systolic and diastolic blood pressure, heart rate, smoking habit, alcohol consumption, systolic and diastolic blood pressure and heart rate. A fasting blood sample was obtained for the determination of total cholesterol, HDL cholesterol, triglycerides and concentrations of markers of inflammation and endothelial activation.

2.2. OGTT and model parameters

All participants received a standard 75 g oral glucose tolerance test (OGTT): blood samples were collected at −5, −1, 10, 20, 30, 60, 90, and 120 min from a cannulated antecubital vein for the determination of plasma glucose (PG), plasma immunoreactive insulin (IRI), immunoreactive *C*-peptide (IRCP) and non-esterified fatty acids (NEFA). Mathematical models were used to compute indexes of insulin resistance: Sensitivity Index (SI) [19]; Oral Glucose Insulin Sensitivity (OGIS) [20]; Matsuda index ISI_{comp} [21]; Stumvoll index (or estimated metabolic clearance rate, MCR_{est}) [22]. Derived indexes of beta cell function were: global beta cell function (Φ_{tot}) and disposition index (DI) [19]; the insulinogenic index, IGI and the c-peptide based IGI or $\Delta CP_{30}/\Delta G_{30}$ [23]. The NEFA minimal model was used to compute parameters of NEFA kinetics, as previously described [24]. This specific method combines a mathematical approach with genetic algorithms to derive parameters of NEFA kinetics and to get insight into their relationship with glucose and insulin metabolism during an OGTT. Gradient-free genetic algorithms outperform other model search strategies in problems involving high dimensional, noisy and multimodal functions, like integrated metabolic regulation. The model was previously validated to describe abnormal NEFA kinetics in women with former gestational diabetes [24].

2.3. Quantification of markers of inflammation and endothelial activation

Commercially available ELISA kits were used to determine the concentrations of ICAM-1, VCAM-1, TNF-α, IL-6, thrombomodulin, and high sensitive CRP (hsCRP). All were from R&D Systems.

2.4. Flow cytometry

Circulating CPCs and EPCs were quantified by flow cytometry on whole blood samples, as previously described [25]. The gating strategy is illustrated in Fig. 1. Briefly, after red blood cell lysis, cells were stained with anti-CD45, anti-CD34 (Becton Dickinson), CD133 (Miltenyi Biotec), and KDR (R&D System) monoclonal antibodies. After gating $CD34^+$ or $CD133^+$ cells in the $CD45^{dim/neg}$ mononuclear cell population, cells were scored for dual or triple expression of KDR. Usually, generic circulating stem/progenitor cells have been defined as $CD34^+$, $CD133^+$ or $CD34^+CD133^+$ cells, whereas EPC are usually defined as $CD34^+KDR^+$, $CD133^+KDR^+$ and $CD34^+CD133^+KDR^+$ cells [26]. At least 5–10 × 10^6 events were acquired and cell count was always expressed per 10^6 events. Baseline progenitor cell levels were quantified by the same two trained operators using the same method and materials throughout the study. Reproducibility of this method has been reported previously, with CV ranging from 6.3% for $CD34^+$ cells to 15–16% for $CD34^+KDR^+$ cells [27]. The availability of up to 6 cell phenotypes defined by expression of the 3 markers generates an inflation of type I error during statistical analysis and the biological differences among generic and endothelial progenitor cells are mostly unknown. Therefore, in this study we elected one single total measure for circulating stem cells (CSCs) and one for endothelial progenitor cells (EPCs). CSCs were defined as cells expressing CD34 or CD133 and calculated as: $CD34^+/10^6 + CD133^+/10^6 - CD34^+CD133^+/10^6$. EPCs were defined as $CD34^+$ or $CD133^+$ cells expressing KDR and calculated as $CD34^+KDR^+/10^6 + CD133^+KDR^+/10^6 - CD34^+CD133^+KDR^+/10^6$.

2.5. Statistical analysis

Normality was checked using the Kolmogorov-Smirnov test and non-normal variables were log transformed before analysis.

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