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Depletion of Circulating Progenitor Cells Precedes Overt Diabetes: A Substudy from the VA Enhanced Fitness Trial

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Abstract

Background—One theory of aging and disease development is that chronic injury (pathology) results in activation of regenerative processes and initial repair, with overt disease arising only after exhaustion of reparative capability leads to inadequate repair. While depletion of circulating progenitor cells (CPCs) has been noted in diabetes, the degree to which CPC depletion predates and is associated with propensity to develop overt disease is unclear.

Methods—The Enhanced Fitness trial enrolled overweight/obese (body mass index >25) sedentary patients with glucose intolerance but without overt diabetes. Baseline CPCs were measured in 129 patients based on the cell surface markers CD34, CD133, and aldehyde dehydrogenase (ALDH) activity. $HgbA_{1C}$, fasting insulin and glucose levels, and HOMA calculations were ascertained.

Results—Lower counts of early angiogenic CPCs identified as $CD34^+$, $CD34^+CD133^+$, and ALDH-bright ($ALDH^{br}$) cells were associated with impairments in glucose homeostasis as reflected by $HgbA_{1C}$, but not fasting insulin, glucose, or HOMA-IR. These associations remained when corrected for age and cardiovascular risk factors.

Conclusions/Interpretation—The numbers of $CD34^+$ and $ALDH^{br}$ CPCs were significantly lower in patients with impaired glucose tolerance. Depletion of reparative capacity as reflected by loss of CPCs may presage overt disease as exemplified in this pre-diabetes model.

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Introduction

One model of chronic disease development suggests that tissue injury leads to activation of reparative processes via endogenous progenitor cells. Chronic injury over time eventually results in an exhaustion of this reparative capacity, inadequate repair, and clinically evident disease.

One measure of progenitor cell mediated reparative capacity is the number of circulating progenitor cells (CPCs). CPCs are depleted with advancing age (1, 2) and in a number of chronic diseases (1, 3); however, the degree to which CPC depletion predates and contributes to progression to overt disease is unclear. One model of chronic disease development suggests that subclinical chronic injury mobilizes CPCs as a reparative mechanism, leading to eventual exhaustion of this reparative capacity, depletion of CPCs, and subsequent unrepaired injury leading to overt disease. If true, depletion of reparative processes might be manifest prior to the onset of clinically detectable disease (4) and be directly related to the degree of pathology; however, it has been difficult to find a model allowing such an assessment.

The Enhancing Fitness in Older Overweight Veterans with Impaired Glucose Tolerance (Enhanced Fitness) trial exclusively enrolled patients with impaired glucose tolerance but without overt diabetes mellitus and not on medications affecting glucose metabolism. This population offers a unique opportunity to study the relationship between CPC numbers and a continuous measure of preclinical disease.

METHODS

The design and outcomes of the Enhanced Fitness trial have been previously described (5, 6). Briefly, eligible patients had to be older (age 60 years), have impaired glucose tolerance as defined at the time of study design as a fasting glucose between 100–125 mg/dL, be free from a diagnosis of diabetes, have a hemoglobin A1C (HgbA1c) below 7%, not be on diabetes medications, and have a body mass index (BMI) between 25 and 45 kg/m^2. Blood chemistries, except for fasting insulin, were analyzed at the VA central laboratory by technicians not affiliated with the study. Comorbidity was assessed using the Older Americans Resources & Services (OARS) Comorbidity and Symptom Index by trained researchers following rigorous validated survey methods [6].

As part of a substudy after separate IRB approval was granted, 137 of the 138 eligible patients underwent analysis of CPCs at baseline based on expression of CD34, CD133, and CD133/CD34 as well as aldehyde dehydrogenase (ALDH) activity (2).

CPC Enumeration

Prior to assessments of physical function, blood was collected into citrated tubes and subjected to centrifugation (1800 g × 20 min). After plasma removal, the buffy coat was transferred to a 50 mL conical tube. Cells were washed, suspended, and recollected after brief (1800 g × 5 min) centrifugation. Cells were then counted and allocated for analysis based on cell surface marker expression or aldehyde dehydrogenase (ALDH) activity. All analyses were performed within 3 hours of sample collection.

Approximately 4 million mononuclear cells were analyzed for the relative content of cells with low orthogonal light scatter and high ALDH activity content (ALDH-bright [ALDH^{br}] cells) using Aldecount (Aldagen Inc., Durham, NC) per the manufacturer's instructions (1).

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A tube containing diethylaminobenzaldehyde was used as a negative control. After 30 minutes at 37°C, the cells were centrifuged, placed on ice, and flow cytometry performed.

For cell surface marker assessments, isolated mononuclear cells were concentrated into Iscove's Modified Dulbecco's Medium containing 2% fetal calf serum (10^7 cells/mL). Non-specific antibody binding was inhibited using FcR reagent (Becton Dickinson, Franklin Lakes, NJ) (10 μl × 10 minutes), and cells incubated with CD133-phycoerythrin (Miltenyi Biotec, Auburn, CA), CD34-fluorescein isothiocyanate (Miltenyi Biotec), CD146-allophycocyanin (Becton Dickinson), and CD14-phycoerythrin-Cyanin7 (Becton Dickinson). Dead and dying cells were excluded using 7-amino-actinomycin D (Invitrogen, Carlsbad, CA) added just prior to flow cytometry. Flow cytometry was performed by trained technicians blinded to patient identity using an LSR CANTO flow cytometer (BD Biosciences, San Jose, CA) and analyzed using FlowJo software (Treestar, Costa Mesa, CA). Quality control measures were performed daily using BD Comp Beads (BD Biosciences).

Analytical Methods

Reported frequencies were expressed as percentages of the mononuclear cell population.

Associations were tested using non-parametric methods (Spearman correlations), given non-normal distribution of CPCs numbers. Statistical significance was declared at $p<0.05$. All analyses were performed using SAS version 9.3. (SAS Institute, Inc., Cary, NC). The research protocol was reviewed and approved annually by the Durham VA and the Duke University Medical Center Institutional Review Board.

RESULTS

The characteristics of patients undergoing CPC evaluation mirror those seen in the overall Enhanced Fitness cohort (5). Patients had a mean age of 66.6 years (range 60–81), were 67% white, and 98% male. Significant portions of patients reported findings of arthritis (51%), heart disease (27%), circulation problems (13%), and hypertension (70%). Mean values (standard deviation) of BMI were 31.5 kg/m^2 (3.8), fasting glucose 109.9 mg/dL (7.1), fasting insulin 10.3 μIU/mL (5.6), HgbA1C 5.9% (0.45), and Homeostasis Model of Assessment-Insulin Resistance (HOMA-IR) 1.42 (0.8).

The numbers of CPCs (assessed by ALDH activity or expression of CD34 and CD133) were significantly negatively associated with patients' $HgbA_{1C}$ levels (Table 1). In contrast, CPC numbers were not significantly associated with fasting insulin, fasting glucose or calculated HOMA-IR value (Table 1).

To assess if the association of CPCs with $HgbA_{1C}$ was independent of age and cardiovascular risk factors, we performed the analysis adjusted for age and the presence of hypertension, previous heart disease, as well as total and LDL cholesterol (Table 2). As indicated this did not substantively change the results.

DISCUSSION

We report that degree of glycemia, as reflected by $HgbA_{1C}$ level, is associated with lower levels of CPCs, even in prediabetic patients. We did not see similar associations with fasting insulin or glucose levels or with calculated HOMA-IR.

Our observations suggest that HgbA1C is associated with diminished CPC numbers even prior to the development of clinically overt disease. HgbA1C is well correlated with mean

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glucose concentrations over the lifespan of the red blood cell, as well as being a predictor of future cardiovascular events (7). In contrast, insulin levels (and the subsequently calculated HOMA-IR) are more subject to both assay and physiologic variability. It is perhaps surprising that a correlation was seen with $HgbA_{1C}$ but not fasting glucose levels; however, $HgbA_{1C}$ is reflective of overall glycemia and different physiologic mechanisms contribute to glucose levels in the fasting versus fed state. Although processes such as intestinal nutrient absorption, glycogenolysis, gluconeogenesis, and glucose disposal all contribute to circulating glucose levels, the primary determinant of fasting glycemia is hepatic glucose output (8). The many metabolic abnormalities that contribute to hyperglycemia and HgbA1c values are significantly more complex than can be estimated via glucose or insulin measurement, or HOMA-IR calculation. Our findings suggest that diminished CPCs are most closely related to the degree of overall glucose elevation, a key predictor of subsequent outcomes (7). It is interesting that Fadini and colleagues also reported that CPCs were not reduced in patients with elevated fasting glucose levels, but were in patients with post-prandial glucose elevation (3), consistent with an association between chronic glucose elevation (as reflected in $HgbA_{1C}$) and CPC depletion. These and our observations may be consistent with *in vitro* reports that document the effect of hyperglycemia on bone marrow or circulating progenitor cell function (9–11).

While others have reported decreased CPCs in patients with diabetes and in some cases pre-diabetes, these reports have compared mean CPCs levels between cohorts of patients. Unlike these previous reports, our findings are the first to demonstrate a direct association between the degree of glucose intolerance as measured by a continuous measure and the degree of CPC depletion (3, 12). While we cannot definitively determine that chronic hyperglycemia or the metabolic derangements that accompany this condition result in CPC depletion in individuals, the association observed here, coupled with the observation that CPCs are depleted in diabetic patients (3), suggests that loss of reparative capacity precedes overt disease, consistent with our model (4). Interestingly, $HgbA_{1C}$ may be reflective of early insulin release and as such a measure of beta-cell aging (13). The relationship with CPC depletion suggests that beta cell depletion may be reflective of an inability of reparative capacity to replenish beta-cell numbers or function.

It is worth comment that while most of the literature suggests that diabetes is associated with reduced numbers or lower functioning CPCs, some reports have associated the presence of diabetic retinopathy with increased levels of at least some CPC subtypes (14, 15). In particular, the early CPCs analogous to the CPCs identified in our work appeared lower in diabetics without retinopathy, but were unchanged in those with retinopathy (14). These findings differed when progenitor cells were defined based on different cell surface expression patterns. Whether these findings are due to differences in CPC measurements and CPC populations under study, or represent fundamental differences in CPC biology, and how these CPC populations contribute to clinical manifestation of disease, warrants further study.

Interventions that halt or impede the loss or reparative function or enhance progenitor cell mediated-repair may delay or abrogate the appearance of clinical disease. In this regard, diabetic treatment strategies that may improve CPCs function are of particular interest (16). Further studies investigating the interaction between improvements in CPCs reparative capacity and effects on cardiovascular outcomes would be of particular interest.

Limitations

This study is limited in that it is a cross-sectional assessment of CPCs and $HgbA_{1C}$ in a select group of male patients with limited physical fitness; however, we believe this is also a study strength. The entry criteria allowed for careful screening for patients with early signs

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of altered glucose metabolism but without frank diabetes, and who were not on medical therapy that might impact glucose levels. This study design allowed us to query the relationship between modest degrees of hyperglycemia and CPCs in a tightly controlled setting. We were not able to assess the impact of hyperglycemia over time, and the study patient selection criteria do not allow us to extend our observations to include those with frank diabetes.

We relied on self-reported measures for such potential modifiers as the presence of hypertension, cardiac disease, and did not capture use of medications that may impact CPC numbers such as statins. Nonetheless, statin use would be expected to be higher in patients with diagnosed diabetes thereby reducing our ability to find an association between CPCs and measures such as $HgbA_{1C}$.

CONCLUSIONS

Even in patients without overt diabetes, chronic hyperglycemia is associated with a reduction in the numbers of CPCs, supporting a model in which disease may be preceded by a period of chronic injury leading to depletion of reparative capacity. Additional study of the role of CPCs depletion in this and other chronic disease states is warranted.

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Table 1

Association of CPCs and Measures of Glucose Metabolism

		CD34$^+$	**CD133$^+$**	**CD133$^+$CD34$^+$**	**ALDH^{bright}**
HgbA$_{1C}$	r	−0.20	−0.19	−0.20	−0.22
	p-value	0.02	0.02	0.03	0.01
Fasting insulin	r	−0.08	−0.02	−0.02	−0.03
	p-value	NS	NS	NS	NS
HOMA-IR	r	−0.08	−0.02	−0.03	−0.03
	p-value	NS	NS	NS	NS
Fasting glucose	r	−0.05	−0.02	−0.08	−0.08
	p-value	NS	NS	NS	NS

ALDH=aldehyde dehydrogenase; HOMA-IR=Homeostasis Model Assessment-Insulin Resistance.

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Table 2

Association of CPCs and Hemoglobin A1C (Multivariate*)

		$CD34^+$	$CD133^+$	$CD133^+CD34^+$	$ALDH^{bright}$
$HgbA_{1C}$	r	−0.19	−0.17	−0.19	−0.23
	p-value	0.04	0.06	0.04	0.01

*Corrected for age, presence of hypertension, history of cardiovascular disease, and total and LDL cholesterol. ALDH=aldehyde dehydrogenase; HgbA1C=hemoglobin A1C.