Published in final edited form as:

Am J Cardiol. 2007 January 1; 99(1): 46–48.

Sex Differences in Circulating Endothelial Progenitor Cell Colony-Forming Capacity and Migratory Activity in Middle-Aged Adults

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Abstract

Middle-aged women have a lower prevalence and incidence of cardiovascular events than men. The mechanisms responsible for this sex-specific difference are unclear. Numerical and functional impairment of bone marrow-derived circulating endothelial progenitor cells (EPCs) is associated with increased cardio- and cerebrovascular morbidity and mortality. It is currently unknown whether there are sex-related differences in EPC number and function in middle-aged adults. We tested the hypothesis that EPCs isolated from middle-aged women demonstrate greater colony forming capacity and migratory activity compared with men of similar age. Peripheral blood samples were collected from 50 sedentary adults; 25 men (age: 59 ± 1 yr) and 25 women (58 ± 1 yr). Mononuclear cells were isolated, preplated for 2 days and nonadherent cells were further cultured for 7 days to determine EPC colony forming units. Migratory activity of EPCs was determined using a modified Boyden chamber. The number of EPC colony forming units was significantly higher ($\sim 150\%$) in samples collected from women (16 ± 3) compared with men (7 ± 1). In addition, EPC migration (relative fluorescent units) was $\sim 40\%$ greater in women (729 ± 74) versus men (530 ± 67). These results demonstrate that EPC colony forming capacity and migratory activity are higher in middle-aged women compared with men.

Keywords

endothelial progenitor cells; colony-forming units; migration

Cardiovascular disease is the most frequent cause of death in both men and women. However, between the ages of 45 and 65 years, the prevalence of coronary heart disease and the incidence of myocardial infarction and stroke is $\sim 50\%$ higher in men compared with women. The mechanisms responsible for this sex-related disparity are currently unclear. Interestingly, there are no apparent sex differences in the incidence and prevalence of traditional cardiovascular disease risk factors such as hypertension, obesity, tobacco use, hyperlipidemia, diabetes, or sedentary lifestyle in middle-aged adults¹. Clinical interest in bone marrow-derived circulating endothelial progenitor cells as a novel vascular risk factor has increased due to their importance in vascular repair and noted inverse relation with endothelial dysfunction as well as cardiovascular and cerebrovascular disease²⁻⁴. Indeed, reduced EPC number and migratory activity have been linked to atherosclerotic disease progression and cardiovascular events

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5-7. Currently, it is unknown whether there are sex-specific differences in numerical and functional characteristics of circulating EPCs. Accordingly, we tested the hypothesis that EPCs isolated from middle-aged women would demonstrate greater colony-forming capacity and migratory activity compared with men of similar age.

METHODS AND RESULTS

Peripheral blood samples were collected from 50 healthy, sedentary adults: 25 men and 25 women. All subjects were non-obese ($\text{BMI} \leq 30 \text{ kg/m}^2$), normotensive, nonsmokers, non-medicated and free of overt cardiovascular, metabolic and hematologic disease, as assessed by medical history, resting and exercise electrocardiograms, and fasting blood chemistries. The women were at least 1 year postmenopausal (range 1 to 20 years) and had never taken or had discontinued use of hormone replacement therapy at least 1 year before the start of the study. Framingham risk score was calculated as previously described⁸. All of the subjects provided written informed consent according to the guidelines of the University of Colorado at Boulder.

EPC colony-forming capacity was determined as previously described^{4,9}. Briefly, peripheral-blood mononuclear cells were isolated by Ficoll density-gradient centrifugation (Histopaque 1077, Sigma), washed, resuspended in growth medium (Medium 199, Gibco), supplemented with 20% fetal-calf serum, penicillin (100 U/mL), and streptomycin (100 mg/mL), and plated on 6-well plates coated with human fibronectin (BD Biosciences) for 48 hours. Thereafter, non-adherent cells were collected, and 5×10^5 cells were seeded onto 24-well fibronectin-coated plates (BD Biosciences). Growth medium was changed every 3 days, and the colony-forming units (CFUs) were counted in 4 random wells by two independent investigators blinded to sample identification, on day 7. Only CFUs consisting of multiple thin, flat cells emanating from a central cluster of rounded cells were counted. Endothelial cell lineage was confirmed in selected samples by fluorescent-activated cell sorting analysis utilizing antibodies recognizing VEGFR-2, CD34 and CD133.

To determine migration, non-adherent cells (4×10^5) were resuspended in serum-free culture medium (Medium 199, Gibco) and loaded in the upper chamber of a 24-well modified Boyden chamber coated with fibronectin (FluoroBlok, BD Biosciences). The upper chamber was placed in the lower chamber containing culture medium supplemented with vascular endothelial growth factor (2 ng/mL) and incubated for 22 hours at 5% CO_2 , at 37°C. Cells were then labeled with calcein AM (Molecular Probes) and the fluorescence of the migrated cells was determined in triplicate with the mean relative fluorescent units presented. Plasma concentrations of oxidized low-density lipoprotein, C-reactive protein, tumor necrosis factor- α , and asymmetric dimethylarginine were determined by enzyme immunoassay.

Group differences were determined by analysis of variance. Relations between variables of interest were assessed by means of Pearson's correlation coefficient and linear regression analysis. Data are reported as mean $\pm$ SEM. Statistical significance was set at $P < 0.05$.

Selected subject characteristics are presented in the table. Although none of the subjects were obese, the men demonstrated significantly higher body mass and waist circumference, whereas the women had higher body fat percentage. Aside from HDL-cholesterol, there were no other sex-related differences in metabolic variables or inflammatory cytokines. There were no differences in Framingham risk score between the men and the women, however, the estimated 10-year coronary heart disease risk was significantly higher in men compared with the women. We observed no relation between risk score or estimated risk with EPC colony number or migration.

The capacity of EPCs to form colonies was markedly higher ($\sim 120\%$; $p < 0.05$) in women (16 ± 3 CFUs) compared with men (7 ± 1 CFUs) (Figure). There was also a significant sex-related difference in EPC migration. EPC migratory activity was $\sim 40\%$ higher in samples from women compared with men (729 ± 74 vs. 530 ± 67 relative fluorescent units) (Figure). Within in each group no significant univariate correlations were observed between EPC colony formation or migration and any anthropometric, metabolic, or inflammatory variables.

DISCUSSION

The primary new finding of the present study is that EPC colony forming capacity and migratory activity are markedly higher in middle-aged women compared to men. Numerical and functional differences in circulating EPCs may contribute to the sex-related disparity reported in endothelial function¹⁰ and cardiovascular events¹ in this segment of the population.

Circulating EPCs have generated interest as a novel biological marker of endothelial dysfunction and a prognostic indicator of cardiovascular morbidity and mortality^{4,7,11,12}. EPC number (assessed either by colony-forming units or flow cytometry) and migratory activity have been shown to be significantly diminished in coronary and cerebrovascular disease patients compared with healthy age-matched controls^{5,11,13}. In fact, two recent clinical studies reported that reduced levels of circulating EPCs independently predict atherosclerotic disease progression and death from cardiovascular causes in patients with established coronary artery disease. Moreover, after adjusting for disease activity and risk factors, low numbers of circulating EPCs were associated with a four-fold increased risk of a future cardiovascular event^{7,11}. In a seminal study, Hill et al.⁴ observed a strong correlation between the number of EPC colony-forming units and both flow-mediated brachial artery reactivity and overall Framingham risk score in middle-aged men. Importantly, the authors reported that EPC colony formation was a stronger predictor of endothelial vasodilator function than conventional risk factors; suggesting that a potential mechanism for endothelial dysfunction may be a lack of EPCs to maintain and repair the endothelial monolayer¹². This postulate is supported by the observation that EPC-mediated vascular repair is associated with normalization of endothelial function at the site of injury¹⁴. We have previously shown, in a similar population of middle-aged adults, that endothelial fibrinolytic capacity is greater in women than men¹⁰; it is tempting to speculate that this difference may be due, at least in part, to EPC bioavailability and bioactivity.

The mechanisms responsible for the sex-related difference in EPC clonogenic potential and migratory activity are unclear. It is important to emphasize that although a number of pathological, pharmacological and physiological factors have been reported to influence EPC mobilization and function; all of the adults in the present study were free of overt disease, non-medicated, non-smokers and sedentary. In addition, all of the women were at least 1-year postmenopausal and not taking estrogen replacement therapy, thus reducing the potential confounding effect of estrogen-mediated EPC mobilization. Increased levels of oxidative stress, inflammatory cytokines and asymmetric dimethylarginine, an endogenous inhibitor of endothelial nitric oxide synthase, have been linked to diminished mobilization, differentiation and function of EPCs¹⁵⁻¹⁸. However, we observed no sex-related differences in these factors in our study population. It is possible that the observed disparity in colony formation and migration may represent a primary phenotypic difference between men and women.

ACKNOWLEDGEMENTS

We would like to thank all of the subjects who participated in the study as well as Yoli Casas, Kimber Westbrook and Jared Greiner for their technical assistance. This study was supported by National Institutes of Health awards HL68030, HL077450, HL076434, and MO1 #RR00051 as well as American Heart Association award 0555678Z.

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Figure.
EPC colony forming units (A) and migration (B) in middle-aged men and women. Values are mean $\pm$ SEM. *P<0.05 vs men.

Table

Selected subject characteristics.

VARIABLE	MEN(N=25)	WOMEN(N=25)
Age (years)	59 ± 1	58 ± 1
Years postmenopausal (years)	-----	8 ± 1
Weight (kg)	82 ± 2	68 ± 2 *
Body fat (%)	26 ± 1	39 ± 1 *
Body mass index (kg/m ²)	26 ± 1	26 ± 1
Waist Circumference (cm)	92 ± 2	81 ± 2 *
Systolic blood pressure (mmHg)	120 ± 2	118 ± 2
Diastolic blood pressure (mmHg)	78 ± 1	74 ± 2
Total cholesterol (mmol/L)	5.2± 0.1	5.3 ± 0.1
(mg/dL)	202±6	206 ±6
High-density lipoprotein cholesterol (mmol/L)	1.3 ± 0.1	1.6 ± 0.1 *
(mg/dL)	51 ± 2	62 ± 3 *
Low-density lipoprotein cholesterol (mmol/L)	3.4± 0.1	3.2±0.2
(mg/dL)	130±5	125±6
Triglycerides (mmol/L)	1.2±0.1	1.1±0.1
(mg/dL)	100±9	107±11
Glucose (mmol/L)	5.1±0.1	5.0±0.1
(mg/dL)	93±2	90±1
Insulin (pmol/L)	28.8±2.1	33.6±3.2
(μU/mL)	4.8±0.4	5.6±0.5
Homeostasis model assessment of insulin resistance	1.1 ± 0.1	1.3 ± 0.1
Oxidized low-density lipoprotein (U/L)	51 ± 3	50 ± 3
C-reactive protein (mg/L)	2.9 ± 0.9	4.0 ± 1.4
Tumor necrosis factor-alpha (pg/mL)	2.1 ± 0.5	1.4 ± 0.1
Asymmetric dimethylarginine (μmol/L)	0.56 ± 0.03	0.50 ± 0.04
Framingham risk score	5 ± 0.3	5 ± 0.6 *
Estimated 10 year coronary heart disease risk (%)	8.6 ± 0.5	5.4 ± 0.6 *

Values are mean ± SEM.

* P<0.05 vs. men.