Published in final edited form as:

Atherosclerosis. 2010 July ; 211(1): 297–302. doi:10.1016/j.atherosclerosis.2010.01.036.

Decreased Number and Impaired Functionality of Endothelial Progenitor Cells in Subjects with Metabolic Syndrome: Implications for Increased Cardiovascular Risk

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Abstract

Objective—Metabolic syndrome (MetS) is characterized by low-grade inflammation and confers an increased risk for cardiovascular disease. Endothelial progenitor cells (EPCs) are a measure of vascular health and are decreased in patients with various risk factors for cardiovascular disease (CVD). There is a paucity of data examining the EPC status especially in terms of their functionality in MetS subjects without diabetes or cardiovascular disease. We aimed to enumerate and functionally characterize EPCs in subjects with MetS in comparison to healthy controls.

Methods—The study was performed at the University of California Davis Medical Center. Healthy controls (n=31) and MetS (n=46) subjects were included in the study. EPCs were enumerated in fasting blood by KDR/CD34 dual positivity. Functionality was assessed by the colony forming units (CFU) assay, migration and tubule formation.

Results—Subjects with MetS had significantly decreased number of EPCs compared to control subjects. Furthermore, EPCs from MetS subjects depicted significantly impaired clonogenic capacity i.e., decreased colony forming units, and impaired capacity to incorporate into tubular structures suggesting functional impairment of EPCs from MetS subjects.

Conclusions—We make the novel observation that MetS subjects without diabetes or CVD have decreased EPC number and impaired functionality as compared to control subjects. These findings could contribute to the increased CV risk in this population.

Keywords

CD34+KDR+ cells; EPC functions; CRP; Metabolic Syndrome

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None of the authors report any conflicts of interest.

Introduction

Metabolic syndrome (MetS) is a cluster of dysglycemia, dyslipidemia, obesity and hypertension that has reached epidemic proportions in industrialized countries [1]. Patients with Metabolic Syndrome have an increased risk for cardiovascular disease (CVD)[2,3].

Endothelial progenitor cells (EPCs) are a subtype of bone marrow–derived progenitor cells expressing surface antigens of both hematopoietic stem cells and endothelial cells: they are involved in adult neovasculogenesis and maintenance of vascular integrity. An altered status of circulating EPCs represents a marker of endothelial dysfunction and vascular health [4–6]. It has been reported that persons at risk for coronary artery disease (CAD) have decreased number of circulating EPCs [7]. Also, lower number of EPCs has been reported in a wide range of conditions including chronic kidney disease, rheumatoid arthritis (RA), diabetes, obesity and CAD [7–14]. Increasing age is also strongly associated with decreased EPCs number. Furthermore, functional properties of EPC may be of equal or probably greater importance than quantitative alterations [15]. Importantly, various features of MetS have been reported to individually correlate with decreased number and functionality of EPCs [16,17]. Although a few investigators have described EPC number or progenitor cell (PC) number in subjects with MetS having either diabetes [18,19], CAD [20] and obesity (males only) [21], however, no data is available on functionality of EPCs in drug-naïve MetS subjects especially in the absence of diabetes and CAD [21]. Furthermore, various pharmacological agents, such as statins, angiotensin receptor blockers (ARBs), PPAR gamma agonists increase EPC number [8,22, 23] and thus were an exclusion criterion.

We hypothesized that EPC might be numerically and/or functionally impaired in drug naïve MetS subjects (both males and females), which may account for the increased CV risk in this subject population. We quantitated the number and functional capacity of EPCs obtained from the MetS subjects, not on statins, angiotensin receptor blockers (ARBs) or PPAR gamma agonists, and compared them with the matched control subjects.

Subjects and Methods

All subjects were recruited from Sacramento county through fliers and advertisements in the newspaper. The subjects (age 21–70 yrs) with the MetS (n=46) and healthy controls (n=31) were studied. Metabolic syndrome was defined using the criteria of the National Cholesterol Education Program Adult Treatment Panel III was used [24]. Briefly, the subject classified as MetS must have at least three risk factors to sustain the diagnosis, including either central obesity, hypertension, dyslipidemia (low HDL, high triglycerides) and or hypertension on anti-hypertensive medications. The control subject needed to have ≤ 2 features of MetS and not be on blood pressure (BP) medications. Other exclusion criteria for controls were: a fasting plasma glucose (>100 mg/dl) and, a triglycerides (TG >200 mg/dl).

For both groups, other exclusion criteria were: diabetes, clinical atherosclerosis (CAD, PAD, CVD etc), smoking, hypo- or hyperthyroidism, malabsorption, anticoagulant therapy, steroid therapy, anti-inflammatory drugs, statin & other hypolipidemic therapy, hypoglycemic agents, ARBs, TG >400 mg/dl (for MetS subjects), oral contraceptives, use of antioxidant supplements in the past 6 months; pregnancy, abnormal complete blood count and alcohol consumption >1 oz/day; consumption of N-3 PUFA, postmenopausal women on estrogen replacement therapy, wounds, retinopathy, recent surgery, inflammatory or malignant disease which may influence EPC kinetics, CRP >10 mg/l, chronic high intensity exercisers (exercise >100 min per week).

Informed consent was obtained from participants in the study, which was approved by the Institutional Review Board at University of California Davis. After history and physical examination, fasting blood was obtained. A complete blood count, plasma lipid and lipoprotein

profile, urea nitrogen, creatinine, aspartate aminotransferase, alanine aminotransferase, glucose and TSH were assayed by standard laboratory techniques in the Clinical Pathology Laboratory. Insulin levels were assayed by ELISA (Linco Biosystems) and HOMA-IR was calculated from glucose and insulin levels as described previously [19]. After subjects were screened, if they fit the selection criteria, they were requested to come to the UC Davis CCRC for a fasting blood draw.

Enumeration of Peripheral blood EPCs by Flow Cytometry

One ml of whole blood (heparinized) was used to quantitate the number of EPCs by the analysis for the expression of surface antigens by flow cytometry. In brief, 100 μ L of peripheral blood was incubated initially with Fc-gamma receptor blocking agent followed by separate incubation with a phycoerythrin-conjugated antibody against human KDR and PE-Cy5-conjugated antibody against human CD34, and both antibodies together. Control stainings were performed with isotype-matched and species-specific antibodies. Incubation was followed by lysis with BD lysing solution, washing and fixation in 1% paraformaldehyde. Acquisition was then performed on a Becton Dickinson FACS Array flow cytometer (FACScan) and included 100,000 events per sample. Cells positive for both CD34 and KDR were characterized as EPCs. Also, the number of progenitor cells (CD34+ only) was quantitated. These analyses were performed for all the subjects (C, n=30 and Met S, n=46) in duplicate. Intra-assay coefficient of variation was performed in 5 different samples performed 10 times and was 11%.

Cell Culture Enrichment of EPCs

EPC isolation and culture were performed as described [12,19,25]. Briefly, mononuclear cells (MNCs) were isolated from 20 ml of fasting heparinized blood by Ficoll Hypaque centrifugation. MNCs (4×10^6 cells in 3 ml medium) were plated on fibronectin-coated 6-well dishes (Becton Dickinson Biosciences) and cultivated in growth medium 199 (Gibco) containing 20% fetal calf serum plus 100 ng/ml vascular endothelial growth factor (VEGF) and penicillin (100 U/mL)/streptomycin (100 μ g/mL, Gibco) for 48 hours. Nonadherent cells were then recollected, centrifuged, resuspended in fresh medium and 10^6 cells/well in 1 ml medium were replated onto fibronectin-coated, 24-wells plates and cultured in triplicate for 7 days in growth medium that was changed every 3 days.

EPC characterization

On the 7th day, a subset (n=7/group from matched pairs of C and MetS subjects whose EPCs were cultivated and processed together) of adherent cells underwent cytochemical analysis after incubation with DiI-LDL and FITC Ulex-lectin as described [10]. After staining, the cells were viewed under inverted fluorescent microscope.

To further confirm endothelial cell phenotype of EPC, cells from 24 well plates were removed from surface with PBS-EDTA and were surface stained for KDR and CD31.

Functional Assays—The cells growing in 24 well plates were used for the following assays.

Colony-forming Unit Assay

The ability to clonally expand and create colonies in an endothelial-specific medium is a key functional feature of EPCs. The CFU assay [7] was performed in all the subjects. CFUs, characterized by a central cluster surrounded by emerging cells were counted at day 7 in 24 well plates in a minimum of 3 wells per subject (C, n=25, MetS, n=43) and the average count was recorded.

EPC Migration Assay

EPC migratory function, which is essential for angiogenesis, was examined using a modified Boyden chamber technique in 16 Control subjects and 36 MetS subjects. A 24-well Transwell apparatus (Chemicon) with 8- μ m pores was used. The chamber was immersed in a 24-well plate, which was filled with growth factor-free 199 media or medium with 100 ng/ml of human VEGF (R and D systems). EPCs ($\sim 4 \times 10^4$) were stained with CFDA (10 μ M final conc) and placed in the inserts in duplicate along with one blank well. Also, one well of the 24 well plate was used for recording total number of cells added. The cells in this well were lysed by adding 1% Tris-Triton at the start of incubation itself. After incubation for 12 hours, the membrane was washed briefly with PBS and the upper side of membrane was wiped gently with a cotton ball. To the lower chamber was added Tris-Triton to lyse the cells which had migrated to the bottom of inserts. Migration was measured as MFI using spectrofluorometer. The results are expressed in MFI units as % migration of the cells with respect to total number of cells used.

Matrigel Tube Formation Assay

A Matrigel tube formation assay was performed to assess the ability of EPC to incorporate into endothelial cell vascular structures, which is believed to be important in new vessel formation. Growth factor reduced matrigel (Becton Dickinson) was layered into 48 well plates for $\frac{1}{2}$ hr at 37°C. EPCs were stained with DiI (5 μ M conc; Molecular Probes) to distinguish them from HAEC. DiI-labeled EPC (1×10^4) and HAEC (4×10^4) were mixed and plated together followed by incubation at 37°C overnight with complete culture medium. Incorporated cells were counted from 4 random microscope fields per each subject (C, n=15, MetS, n=15).

Statistical Analysis

Data are expressed as mean $\pm$ SD or, for skewed variables, as median and interquartile range. Log transformations were applied to skewed data prior to parametric analyses. We were not able to get complete data sets for EPC functionality on all subjects due to the inadequacy of the EPCs obtained from each subject. Comparisons between the control and metabolic syndrome groups were made with two-sample t-tests. Spearman's rank correlation coefficients were computed to assess the association between metabolic risk factors and EPC numbers and their functionality. Multiple regression models were constructed to evaluate predictors of EPC dependent variables. Independent variables assessed included age, BMI, waist circumference, blood pressure, plasma glucose, triglycerides, HOMA-IR, LDL-C, HDL-C, and CRP. Data were analyzed using SAS version 9.1.3 (SAS Institute, Cary, NC, USA).

Results

Table 1 shows the salient characteristics of the study subjects. There were no significant differences in age and gender between the 2 groups. MetS subjects had significantly higher body mass index (BMI), waist circumference, blood pressure (both systolic and diastolic) as well as fasting plasma glucose than those in the control group. Total cholesterol, triglycerides LDL and HDL, insulin and HOMA-IR were also significantly different in MetS subjects than controls. The MetS subjects were on hypertensive medications including beta blockers, ACE inhibitors, Ca channel blockers and diuretics (27%). Furthermore, MetS had significantly higher levels of CRP, a down stream marker of inflammation depicting higher systemic inflammation than control subjects.

We quantitated the number of CD34+ only and CD34+KDR+ cells (EPCs) in the peripheral blood of all the subjects in duplicate using flow cytometry. In addition to decreased CD34 (progenitor cells) counts in MetS subjects compared to controls [Control 12.7 (11,21) mfi vs. MetS 8.2 (6.4,12), $p < 0.001$], CD34+KDR+ EPCs were significantly ($p < 0.001$) reduced by 34% in subjects with MetS compared with their age-matched healthy volunteers (Fig 1).

Representative scatterplots are depicted in Supplementary Fig 1. Regression analysis revealed that CRP, triglycerides, age, and plasma glucose were the strongest predictors of reduced circulating CD34+KDR+ cells (adjusted R-squared=0.147, model p=0.01) (Table 2).

Decreased number of EPCs in the MetS group compared with control group was also evident when analyzed for Ulex-lectin and DiI-LDL staining from cells growing on 24 well plates on 7th day after culture (Supplementary Fig 2). These cells also stained positive for the endothelial lineage markers, KDR and PECAM-1.

Significantly fewer EPC CFUs were found from MNCs culture of MetS subjects compared with normal controls (48% decrease) at day 7 p<0.001 (Fig 2a). These CFUs stained positive for Di-LDL and FITC- Ulex lectin consistent with endothelial lineage of cells. Moreover, regression analysis revealed that CRP was a strong predictor (p=0.01) of impaired clonogenic capacity, followed by adiposity, HDL, and BP variables, (adjusted R-squared=0.18, model p=0.005) (Table 2).

The migratory function of EPC in response to VEGF, which is believed to be important during neovascularization, was evaluated using a modified Boyden chamber. The migration capacity of EPCs from MetS, whilst lower, was not significantly different from control subjects (Fig 2b).

EPC incorporation into the tubular networks formed by HAEC was evaluated in culture using Matrigel, which is used to evaluate vasculogenic capacity. The data presented is representative of 15 paired subjects in C and MetS groups for whom the EPC isolation and matrigel assay was performed on the same day and time since these assays are time dependent and results vary largely on culture conditions [18,19,25]. Significantly fewer EPCs of MetS subjects were incorporated into tubules compared with the EPCs from normal controls (Fig 2c). Multiple regression analysis identified CRP as a significant predictor (p=0.04) when adjusted for age and HDL-C (adjusted R-squared=0.203, model p=0.03) (Table 2).

Discussion

Several recent reports indicate that the number of circulating EPCs is an independent indicator of overall cardiovascular health. Common conditions predisposing to atherosclerosis, such as hypercholesterolemia, hypertension, diabetes, and smoking are associated with endothelial dysfunction. There is a decrease in the number of EPCs in chronic inflammatory states and in individuals with cardiovascular risk factors [7,10,21]. Furthermore, subjects with MetS have been shown to have endothelial dysfunction. Also, individual features of MetS have been reported to contribute to decreased EPC number. A few investigators [18–21] have analyzed progenitor cell number and EPCs number in subjects with MetS and reported decreased cell numbers; however, no data exist in literature about the functionality of EPCs in MetS [21]. Also, it is important to emphasize that MetS subjects studied earlier were on variety of medications, including oral hypoglycemics, ARBs, statins etc, and these medications have been reported to affect EPC number and functionality [4,22,23]. Overall, there has been a paucity of data on EPCs in drug naïve MetS subjects, free of diabetes, hypercholesterolemia and/or CAD which prompted the current study. Our patients were not on statins, ARBs and PPAR agonists, since these drugs interfere with EPC number and functionality. In this study, we demonstrate that the number of circulating PCs and EPCs is decreased in MetS subjects. Circulating functional EPCs are thought to derive from immature undifferentiated progenitor cells. There are several controversies in the literature regarding the phenotypic characterization of the EPCs. Fadini et al have shown that CD34+KDR+ cell number is reduced in early and late atherosclerotic disease and correlate negatively with endothelial function [25]. A sound rationale to choose the CD34+KDR+ combination is that this is the only putative EPC

phenotype that has demonstrated repeatedly and convincingly to be an independent predictor of cardiovascular outcomes [25–28]. In a 10-month follow-up study, Schmidt-Lucke et al [27] showed that the level of CD34+KDR+ cells independently predicted cardiovascular events and progression of atherosclerosis in a mixed population of healthy subjects and cardiovascular patients. In a larger study, Werner et al. [28] have reported that CD34+KDR+ cell count predicted cardiovascular events and cardiovascular death during a 12-month follow-up in 519 patients with CAD. Overall, it has been reported that these EPCs i.e., CD34+KDR+ cells, which are CD45- represent a subpopulation of circulating EPCs [29] and due to their functional properties, may represent the “true” EPCs. Whilst the major focus of this study was to examine EPC functionality in MetS, our findings with respect to decreased progenitor cell numbers accord with the larger study by Fadini et al [19] and contrasts with the smaller study in 18 obese males with Metabolic Syndrome [20]. Thus, it appears that both progenitor cells and EPCs are decreased in MetS. Our data of decreased number of EPCs in MetS is consistent with those reported by Fadini et al in diabetics [18,19] and Westerweel et al [20] in obese males. However, it needs to be emphasized that none of our subjects were diabetic and the majority with MetS were females (77%). On the other hand, Satoh et al [30] have reported increased EPCs in MetS subjects; however, the majority of their patients were studied after an acute coronary event. Despite this increase, the EPCs manifested decreased telomere length and increased oxidative DNA damage, suggesting a paradoxically dysfunctional population of EPCs. Importantly, our study population of MetS did not include patients with diabetes or CVD. One person had an LDL-C > 160 mg/dL, exclusion of the subject data from analyses did not change any of the results, thus these results hold true for MetS subjects without hypercholesterolemia also. Therefore, we extend further and underscore the decreased PC and EPC number in MetS previously reported in MetS patients with diabetes and obese males. Furthermore, using CD34+KDR+ as a dependent variable, age, plasma glucose, triglycerides, and CRP were found to be variables predictive of EPC (CD34+KDR+) measurements.

The EPC characterization for its endothelial lineage in the current study was done for multiple markers such as DiI uptake and concomitant Ulex-lectin. The endothelial lineage was further documented by the expression of KDR and CD31. Endothelial dysfunction (ED) is an early step in the pathogenesis of atherosclerosis. Importantly, CRP which is not only a cardiovascular risk marker but also a mediator in atherogenesis has been earlier reported by our group to cause ED mediated by uncoupling of eNOS in HAECs [31]. CRP treatment of EPCs in vitro resulted in altered eNOS activity [32]. There is growing evidence that bone marrow-derived EPC participates in endothelial repair. This process can be divided into 3 stages: mobilization from bone marrow, homing into the sites of vascular injury, and incorporation into the endothelium of the injured or newly formed blood vessels [28]. Importantly, not only the extent of the EPC pool is an indicator of vascular health, but also normal functions of EPC are required for adequate homeostasis [28]. The novelty of our study is the functional characterization (CFUs, migration and tubulogenesis) of EPCs in MetS subjects which has not been reported earlier in comparison to controls.

We report a highly significant ($p < 0.001$) decrease in the number of CFUs in MetS compared to controls. Multivariate analysis revealed that BMI, HDL-C, and CRP were independent predictors for the reduction in the number of EPCs colonies. In support of our findings, earlier George et al [33] have reported a negative correlation between CRP levels and circulating EPC numbers (defined as CFU assay) in patients with unstable angina.

Current data suggest that EPC may be incorporated into damaged endothelium and may work in concert with existing endothelial cells to form blood vessels rather than forming entirely new vessels de novo [19,26]. Our results reveal that the incorporation of EPCs into EC was reduced in MetS subjects compared to controls which suggest that EPC incorporation into damaged endothelium or neovascularization foci may be impaired in MetS condition. These

phenomena could contribute to the deterioration in the repair of damaged endothelium or angiogenesis in MetS.

Interestingly, individual risk factors seem to differentially affect the number and functional capacity of EPCs. These data suggest that different mechanisms contribute to the impairment in functional activity compared with the reduced levels of circulating EPCs. Overall, CRP emerged as a major predictor in most of the parameters analyzed in the current study. Our study is in line with and extends the role of CRP not only as an inflammatory biomarker but also biomediator in the disease process. Our data implicate CRP in dysregulation of EPCs and based on multivariate analysis, this observation is significant for calling attention to a possible effect of CRP in contributing to atherosclerosis in MetS. This effect may result from impaired ability for endothelial repair and regeneration due to reduced EPCs number. Verma's group [32] has shown experimentally that CRP attenuates EPC survival, function and differentiation by induction of ROS. Future studies will examine mechanisms by which increased CRP levels may be linked to EPC dysfunction.

In conclusion, our study demonstrates for the first time decreased number of EPCs along with impaired functionality in both male and female subjects with MetS without diabetes, hypercholesterolemia or manifest cardiovascular disease when compared to matched controls and clearly fills a hiatus with respect to EPC functionality in MetS. Also, these subjects were non-smokers and drug naïve except for anti-hypertensive medications. These findings may explain in part, the increased CV risk in MetS population. Future studies will focus on mechanistic insights and intervention strategies to ameliorate altered EPC status in this high risk population.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

The study was supported by ADA grant (IJ) and NIH K24 (AT00596, IJ) and UL1 RR024146 from the National Center for Research Resources (NCRR), a component of the National Institutes of Health (NIH), and to CTSC. Authors acknowledge assistance of Long Wang, PhD in assisting with subject recruitment and Manpreet Kaur for editorial assistance.

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Fig 1. Enumeration of EPCs by FACS. CD34/KDR dual positive cells in all the subjects were assessed as described in Methods. * p<0.001 compared to control subjects.

Figure 2a

Figure 2b

Figure 2c

Fig 2.

Fig 2a: CFU in control and MetS subjects. Number of CFUs were enumerated as described in Methods * $p<0.001$ compared to control EPCs

Fig 2b: Migration assay in control and MetS subjects. A Boyden chamber assay was used with growth factor-free medium or VEGF (100 ng/ml) supplemented medium.

Fig 2c: Vasculogenic capacity of EPCs (DiI-labeled) as measured by their incorporation into tubules formed by HAEC on matrigel. Fluorescence and phase contrast images are shown in the figure. Significantly fewer EPCs from MetS subjects were incorporated into the tubules formed by HAEC than those of the normal controls. EPCs incorporation in the MetS group was reduced by 33%. * $P<0.05$.

Table 1

Anthropometric, Clinical, and Biochemical Characteristics of the Subjects

	Control (n=31)	MetS (n=46)	P value
Age (yrs)	49 ± 11	54 ± 11	0.08
Females/Males	25/6	36/10	0.99
Waist circumference (cm)	94 ± 15	110±14	0.0001
Weight (kg)	85±22	102±19	0.0005
BMI (kg/m ²)	30±6	36±6	0.0002
Systolic BP(mmHg)	121 ± 12	130± 13	0.001
Diastolic BP (mmHg)	74 ± 8	82 ± 10	0.0003
Fasting Glucose (mg/dl)	90 ± 7	101± 10	0.0001
Total Cholesterol (mg/dl)	185 ± 33	199 ± 27	0.048
HDL-Cholesterol (mg/dl)	65 ± 13	41 ± 12	0.0001
LDL-Cholesterol (mg/dl)	114 ± 27	129± 20	0.0001
Triglycerides (mg/dl)	75 (62,95)	134 (106,172)	0.0001
Fasting Insulin (uIU/mL)	8.6 ± 4.9	16.6 ± 9.9	0.0001
HOMA-IR	1.7 (0.9,2.8)	1.1 (2.3,5.8)	0.0001
hsCRP (mg/L)	1.3 (0.5,3)	3.5(1.6,5.7)	0.005

Data are summarized as mean ± SD except for hsCRP, HOMA and triglycerides which are presented as median (25th percentile, 75th percentile)

Table 2

Summary of multiple regression models

Dependent variable CD 34+KDR+ [†]			
Variable	Parameter Estimate, β	Standard Error	p-value
Intercept	3.93	0.90	0.002
Age	0.01	0.007	0.07
Glucose	-0.017	0.009	0.05
Triglycerides [†]	-0.36	0.21	0.07
CRP [†]	-1.46	0.74	0.05
Interaction (triglycerides x CRP)	0.3	0.16	0.05
Dependent variable EPC CFU [†]			
Variable	Parameter Estimate, β	Standard Error	p-value
Intercept	1.96	1.69	0.25
BMI	0.04	0.02	0.03
Glucose	-0.02	0.01	0.09
HDL	0.02	0.009	0.02
DBP	-0.02	0.01	0.07
Triglycerides	0.41	0.28	0.14
CRP [†]	-0.29	0.11	0.01
Dependent variable Tubules			
Variable	Parameter Estimate, β	Standard Error	p-value
Intercept	7.71	9.31	0.42
Age	0.23	0.19	0.24
HDL	0.14	0.10	0.19
CRP [†]	-3.16	1.50	0.04

[†] log_e transformed prior to regression analysis