

Original Article

Relationship of Microparticles to Progenitor Cells as a Measure of Vascular Health in a Diabetic Population

Anne M. Curtis,^{1,2} Lifeng Zhang,³ Elizabeth Medenilla,³ Ming Gui,²
 Patrick F. Wilkinson,¹ Erding Hu,² Jay Giri,³ Vijay Doraiswamy,³ Sampath Gunda,³
 Mark E. Burgert,² Jonni S Moore,³ Jay M. Edelberg,² and Emile R. Mohler, III^{3*}

¹Science Foundation Ireland (SFI), Wilton Park House, Wilton Place, Dublin 2, Ireland

²GlaxoSmithKline (GSK) 709 Swedeland Road, UW 2109, King of Prussia, Pennsylvania 19406

³Department of Medicine, Cardiovascular Division, Section of Vascular Medicine, University of Pennsylvania, School of Medicine, Philadelphia, Pennsylvania 19104

Objective: Quantitative measures are needed to identify diabetic patients at higher risk for CV events. Cell-derived microparticles (MPs) are submicron membrane vesicles released from activated cells that are indicative of cell damage. Progenitor cells (PCs) including proangiogenic cells (PACs), often termed endothelial progenitor cells (EPCs), are mediators of reparative capacity. We examined whether the relationship of MPs to PCs/PACs could be used as an improved and clinically feasible index of vascular pathology.

Methods and Results: Plasma samples were collected from patients with early-stage (ES, Diagnosis < 1 year) and long-term (LT, Diagnosis > 5 years,) Type 2 diabetes and compared with age related healthy subjects (H). PC and MP subtypes were measured by a combination of flow cytometry and ELISA-based methods. The ratio of procoagulant MPs/CD34⁺ PCs proved a valuable index to distinguish between subject groups ($P = 0.01$). This index of compromised vascular function was highest in the LT group despite intensive statin therapy and was more informative than a range of soluble protein biomarkers.

Conclusions: This is the first report of a relationship between MPs and PCs in Type 2 diabetes. This ratio may provide a quantitative and clinically feasible measurement of vascular dysfunction and cardiovascular risk in patients with diabetes. © 2010 Clinical Cytometry Society

Key terms: endothelial; progenitor; vascular; diabetes; microparticles

How to cite this article: Curtis AM, Zhang L, Medenilla E, Gui M, Wilkinson PF, Hu E, Giri Y, Doraiswamy V, Gunda S, Burgert ME, Moore JS, Edelberg JM, Mohler ER III. Relationship of microparticles to progenitor cells as a measure of vascular health in a diabetic population. *Cytometry Part B* 2010; 00B: 000–000.

Diabetes mellitus is associated with accelerated atherosclerosis, leading to a two- to threefold increase in the incidence of cardiovascular disease (1). Quantitative methods to distinguish cardiovascular disease risk remain elusive in the diabetic population. Current approaches rely on assessing endothelial function, including invasive, and noninvasive methods (2), and the association of circulating plasma levels of soluble proteins such as high sensitivity C reactive protein (CRP) (3). Many of these approaches are either complicated, operator dependant, or nonspecific (2). For example, the precise cellular source of soluble proteins is often unknown and can confound interpretation. Accurate, convenient and biologically informative markers

and associated methods are necessary to assist in the early detection and treatment of vascular complications in diabetic patients.

Grant sponsor: NIH National Heart Lung and Blood Institute; Grant number: K12 HL083772-01; Grant sponsor: GSK.

*Correspondence to: Emile R. Mohler, III, MD, Hospital of the University of Pennsylvania, 3400 Spruce Street, 4 Penn Tower, Philadelphia, PA 19104. E-mail: mohlere@uphs.upenn.edu

Received 13 January 2010; Revision 26 March 2010; Accepted 31 March 2010

Published online in Wiley InterScience (www.interscience.wiley.com).

DOI: 10.1002/cyto.b.20528

In this regard, cell-derived MPs and progenitor cells (PCs), defined as CD34 positive cells, and proangiogenic cells (PACs), also CD34 positive along with having endothelial surface markers, have emerged as surrogate markers for cellular damage and repair, respectively. Both have been associated independently with a range of vascular diseases including diabetes (4-7).

MPs are membrane encapsulated vesicles of $\sim 1 \mu\text{m}$ or less in diameter, derived from surface membranes under conditions of cellular activation or apoptosis (8). These submicron vesicles are released into circulation, carrying with them an array of surface markers, used to identify their cellular source. Exposed membrane phosphatidylserine (PS) and tissue factor, along with a plethora of other surface molecules and cytoplasmic components, including nuclear material (9), enable MPs to impact on a variety of biological functions, including coagulation, thrombosis, and angiogenesis (10). Direct receptor-ligand interactions (11) and fusion and delivery of intracellular contents (9,12) are mechanisms employed by MP's to target and impact host cells. Increases in endothelial MPs (EMPs) (13), platelet MPs (PMPs) (14), and monocyte MPs (MMPs) (15) have all been implicated in vascular pathology, including myocardial infarction. Circulating EMPs, generated from the surface of the endothelium have received considerable attention as a surrogate marker for endothelial dysfunction (4,16,17). Because of disease-specific changes and associated biological activity, these vesicles are considered active paracrine agents that aggravate further vascular dysfunction (18) and promote inflammation (19). To date, studies assessing MPs have relied on flow cytometry methods performed in specialized laboratories. The utility of a plate based method has not been tested in comparison to flow cytometry assay.

PACs (formerly given the designation of endothelial progenitor cells) play a significant role in vascular homeostasis, and in the development and resolution of cardiovascular pathologies. For example, the number and function of PACs are altered immediately after a myocardial ischemic event, and in individuals at high risk of pathologic cardiovascular events (7). In addition, MPs are lowered and PACs are increased after treatment with HMG CoA reductase inhibitors (statins) (20,21). A study of hypercholesterolemic patients with higher CD31⁺/CD42⁻ EMPs and lower PACs than normocholesterolemic subjects supports this hypothesis (22).

We hypothesize that the ratio of MPs to PCs and/or PACs provides an indicator of cellular damage, including the integrity of the endothelium and a loss of endothelial repair capacity that could be relevant in Type 2 diabetes. This ratio was directly associated with aortic pulse wave velocity (aPWV), providing a functional link between plasma cholesterol levels, MPs, PACs, endothelial injury, and arterial stiffness.

To test this hypothesis we evaluated the vascular health of two diabetic subpopulations using MPs and PCs/PACs as surrogate markers. As the risk of vascular events increases with the duration of diabetes (23), we

recruited individuals recently diagnosed and those diagnosed for five years or more with diabetes to provide separation of these two populations.

RESEARCH DESIGN AND METHODS

Patient Recruitment and Study Design

Patients diagnosed with Type 2 diabetes within the preceding 12 months were termed "Early Stage" (ES), and those diagnosed more than five years ago were termed "Long Term" (LT). Age related "Healthy" (H) subjects were recruited into the study on the basis of having no prior or current history of diabetic- or cardiovascular-related conditions and were not taking any type of CV-related medication including statins or medication for hyperlipidemia, hypertension or diabetes. Four (36%) of the ES group and 14 (67%) of the LT group were receiving statin medication, along with a range of other medications to treat diabetes, hypertension, and other conditions. Regarding family history of cardiovascular disease, 12 of the 18 (66%) subjects in the healthy group, 10 of the 11 (90%) in the early stage and every participant (100%) in the long-term group responded that a prior family history of cardiovascular disease existed. The average length of diabetes in the LT patients was 20 years. All study participants were non-smokers. Written informed consent was obtained from all study participants and study protocols were approved by the Institutional Review Board (IRB). Each subject donated ~ 50 mL of venous blood at ~ 8 am in the morning and all subjects had fasted the night beforehand. Blood was collected in heparinized (Baxter) syringes for cell and soluble protein analysis (30 mL), Sodium-Citrate for microparticles (10 mL) and EDTA for lipid analysis (10 mL). Demographic data, medical/medication history, physical examination, and vital signs were recorded for each subject.

Flow Cytometry for PCs/PACs

Less than 1 h post sample collection, white blood cells were isolated from 30 mL of blood using ammonium chloride lysis as previously described (5). Platelet counts were not determined. Cell staining, gating strategy, flow cytometric methods, and analysis were followed as described (5). Approximately 5E6 cells were stained with a 6-color antibody panel: FITC-anti-CD31 (PECAM) (Pharmingen), PE-anti-CD133 (Miltenyi Biotec.), PerCP-Cy5.5-anti-CD3,-CD19,-CD33 (Becton Dickinson), APC-H7 anti-CD45 (Becton Dickinson), PE-Cy7-anti-CD34 (Becton Dickinson), and APC-anti-VEGF-R2 (R&D Systems). Viability was assessed by propidium iodide exclusion. Using a Becton-Dickinson LSRII cytometer, 2E6 live events were processed for each sample and the six fluorescent markers along with light scatter allowed only viable, low to medium side scatter. Singlets that were CD3,19,33-negative were analyzed for PCs and PACs. Singlets were gated as the prominent cluster of cells identified from a plot of side scatter width versus forward scatter width to ensure that cell aggregates

Table 1
Cell or Particle Genotype Per Surface Marker by Flow Cytometry

Cell/Particle Type	Marker definition
Progenitor cell (PC)	CD133 ⁺ , CD34 ⁺ , CD133 ⁺ /34 ⁺
Proangiogenic cell (PAC)	CD133 ⁺ VEGF-R2 ⁺ , CD34 ⁺ VEGF-R2 ⁺ , CD133 ⁺ CD34 ⁺ VEGF-R2 ⁺
Endothelial microparticle (EMP)	CD144 ⁺
Platelet microparticle (PMP)	CD41 ⁺
Monocyte microparticles (MMP)	CD14 ⁺
Phosphatidylserine ⁺ (PS ⁺) MP	AnnV ⁺

were excluded from analysis. Fluorescence minus one (FMO) samples was used as negative controls. Cell populations (PCs and PACs) were quantified as a percentage of mononuclear cells calculated as number per ml blood. Analysis focused on subset definitions for PCs and PACs (Table 1). Data analysis was performed using FlowJo analytical software (Treestar, Ashland, OR).

MP Isolation

Platelet-poor plasma (PPP) was obtained from citrated blood within an hour after blood collection in order to isolate MPs. Whole blood was centrifuged at 1,500g for

15 min, supernatant collected, and PPP obtained by centrifugation at 13,500g for 5 min at room temperature. For each subject, PPP was aliquoted into separate tubes and stored at -80°C until subsequent use. All samples used were subjected to only one freeze-thaw cycle.

Flow Cytometry for MPs

For characterization and quantification of MPs, PPP was incubated with a mixture of Annexin-V (FITC), PE-CY5-CD41a, APC-CD14 (BD Biosciences) and PE-CD144 (R&D System) in 1X BD annexin-V binding buffer (10 mM HEPES, pH 7.4, 140 mM NaCl, and 2.5 mM CaCl_2) (BD Biosciences) for 30 min at RT in darkness, then 1X BD annexin-V binding buffer was added to make total volume of 1 mL/tube. The negative control was prepared as PPP stained with Annexin-V (FITC) and same amount of matched isotype control antibodies in calcium-free binding buffer. Using a BD Biosciences FACScanto cytometer, a P1 region ($<1\ \mu\text{m}$) on FSC-H and SSC-H scatter (log scale) was defined by calibrator beads (Fig. 1A) The number of MPs per μL was determined using the P1 region and also 6- μm microsphere beads (Bacteria Counting Kit, Invitrogen) to determine volume of sample (μL) analyzed (Fig. 1B). The number of MPs stained with each specific Ab and AnnexinV was analyzed and determined using FACSDiva software (BD Biosciences) and expressed as MPs/ μL . Characterization of

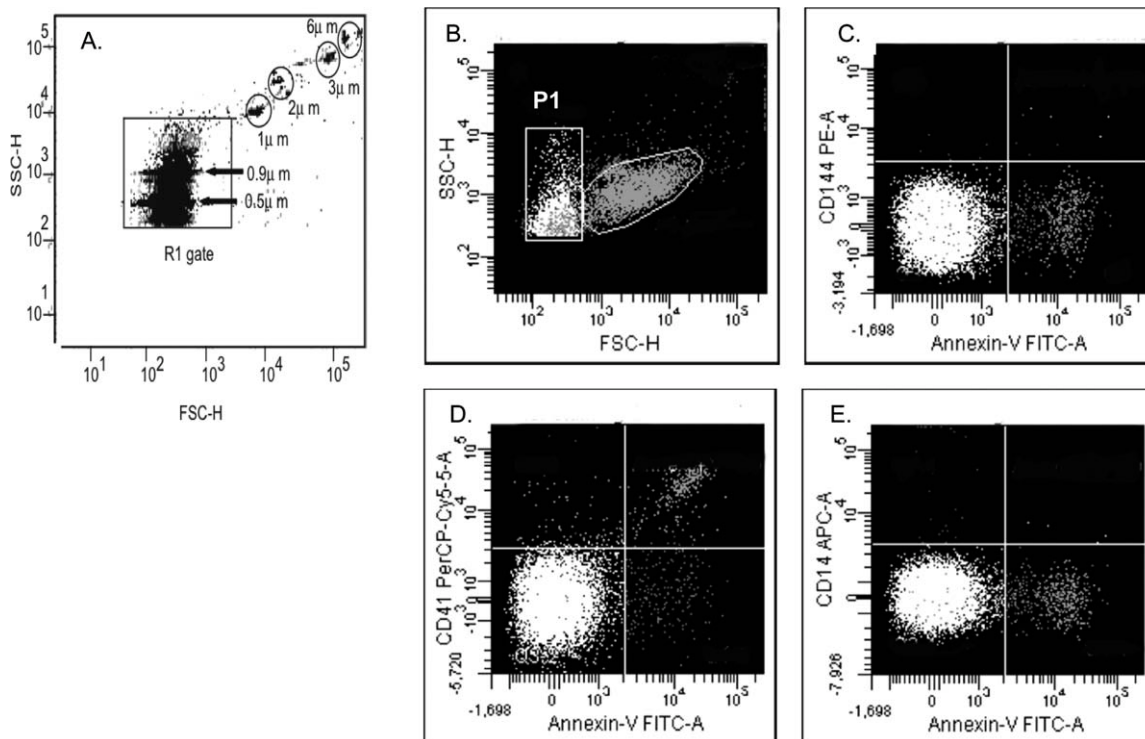


Fig. 1. Gating strategy for MP analysis. MPs were identified by first gating on the P1 region on the FSC/SSC plot defined by calibrator beads of less than $1\ \mu\text{m}$ (A and B). The origin of the microparticles was determined by coexpression of Annexin-V and CD144 for endothelial derived MPs (C), Annexin-V and CD41 for platelet-derived MPs (D), Annexin-V and CD14 for monocyte-derived MPs (E).

cellular origin of MPs by positive antibody staining is listed in Table 1.

MP Plate-Based Capture Assay

Concentrations of PS⁺ MPs were determined using the Zymuphen MP Activity Kit (Aniara-CAT#A521096). In this assay, MPs were captured from PPP onto insolubilized annexin-V and their PS content was measured by a functional prothrombinase assay. This offers an indirect measurement of total MP procoagulant activity via measurement of nM of phosphatidylserine on the outer membrane surface. MPs measured by this method are expressed in nM of phosphatidylserine equivalent.

Soluble Proteins

Soluble proteins were determined from citrated plasma by electro-chemiluminescent detection using commercially tested kits, as per the manufacturer's instructions. Meso-Scale Discovery multiplex kits, including the Vascular Injury II assay kit (CAT # K11136C-1) was used to measure SAA, CRP, VCAM1, and ICAM1. IL6, IL8, TNF α and IL1 β were measured using the Human Pro-Inflammatory Base Kit (K15025A-5). The Human Hypoxia Assay (CAT# K15122C-1) measured VEGF, IGFBF-1 and EPO. Plasminogen activator inhibitor (PAI-1) was detected with the Imubind kit from American Diagnostica. Stromal cell-derived factor 1 (SDF-1) was measured by R&D systems (CAT# DY350). IL1 β , SAA, IL6, and IL8 were below detection with these assays.

HbA1c

HbA1c was performed using the Primus boronate affinity HPLC method (Primus Corporation, Kansas City, MO) according to the manufacture's protocol.

Lipid Profile Analysis

Blood samples were collected in EDTA for lipid analysis. HDL was performed using an enzymatic in-vitro assay for the direct quantitative determination of human HDL-cholesterol on Roche automated clinical chemistry analyzers following by the manufacture's protocol. Triglyceride and cholesterol were performed using VITROS TRIG slides and VITROS chemistry products calibrator kit 2 on VITROS chemistry systems (VITROS 950 Chemistry System). LDL was calculated by the Friedewald Equation. (24).

Complete Blood Count (CBC) and White Blood Count (WBC) Differentiation Analysis

Blood cell analysis was performed using COULTER[®] LH 780 Hematology Analyzer (Beckman Coulter) following the manufacture's protocol.

Statistical Analysis

All univariate comparisons of the disease state groups used two-sided nonparametric tests or Chi-squared comparisons of proportions, which did not require Gaussian distributions. Most of the measured characteristics, especially the MP, PACs, and soluble protein levels had

strongly non-Gaussian distributions. The disease state group comparisons of the subject characteristics did not assume disease state related trends in the responses. These comparisons used the Kruskal-Wallis (KW) test (nonparametric ANOVA comparisons). The analyses of the PC, PAC, MP, and soluble protein levels assumed trends in the responses relative to the disease state. The assessment of the statistical significance of these trends used the Jonckheere-Terpstra (JT) test (nonparametric trend comparisons). The post-hoc comparisons of differences between individual disease state groups (e.g. ES versus LT) used the Wilcoxon two sample test.

The comparisons of the proportions of patients by gender applied the exact Chi-square test of the equality of proportions across the groups. This test compared the observed proportions in the disease state groups against the hypothesis that the proportions were the same for all the disease state groups. The other characteristic proportions (e.g. statin use) were criteria for exclusion from the H group, thus only the ES and LT groups were compared. The analysis of the MP and PAC relationships to the disease state after adjustment for specific covariates were based on logistical regression models with the covariates already included in the models.

On the basis of the assumption of a similar relationship of the disease state differences and the response variabilities, a power calculation was performed with data from Koga et al. (4) using CD144⁺ EMPs. The sample size of 11 in ES, 22 in LT, and 18 in H, had ~90% power in detecting the relative disease state differences (control versus Diabetes Mellitus) as noted in the aforementioned study.

RESULTS

Subject Design and Characteristics

On the basis of previous findings that diabetes is a risk factor for cardiovascular disease and duration with diabetes is a further additive component of that risk (23) we recruited ES and LT patients. Subject characteristics are presented in Table 2. A total of 41 subjects (mean age 57 years) were included. Age and gender did not differ between the three groups. As anticipated, the standard marker of blood glucose control, glycated hemoglobin, HbA_{1c}, was significantly altered between all diabetic and healthy individuals, but could not discriminate between ES and LT groups. The lower levels of LDL observed in the diabetic groups was most likely related to statin use (36% in ES, 67% in LT). To investigate this effect in the diabetic groups, we plotted LDL levels in each group separated by statin use, Figure 2A. LT diabetics receiving statins had reduced LDL levels compared to healthy individuals, indicating that without such therapy LDL levels in this cohort would have been substantially higher. The fact that LDL levels were lower in LT patients without statins versus with statins may be due the small sample size of the former ($n = 7$) compared with the latter ($n = 14$). HDL and Triglycerides

Table 2
Patient Characteristics

	H (18)	ES (11)	LT (22)	P
Age (y)	57 ± 14	51 ± 13	64 ± 13	NS
Gender (% males)	61%	64%	81%	NS
Office systolic BP (mmHg)	125 ± 11	133 ± 14	139 ± 13 [†]	*
Office diastolic BP (mmHg)	78 ± 5	80 ± 11	76 ± 12	NS
Total cholesterol (mg/dL)	185 ± 34	168 ± 43	156 ± 34 [†]	**
LDL (mg/dL)	109 ± 32	84 ± 23 [†]	80 ± 31 [†]	**
HDL (mg/dL)	55 ± 11	44 ± 13	51 ± 19	NS
TG (mg/dL)	107 ± 61	169 ± 129	126 ± 103	NS
HgbA1c	5.3 ± 0.3	7.2 ± 1.7 [‡]	7.1 ± 1.5 [†]	**
WBC	5.3 ± 1	6.2 ± 2	5.8 ± 1	NS
Monocyte Count (n/μL)	432 ± 148	531 ± 255	483 ± 118	NS
Lymphocyte Count (n/μL)	1557 ± 522	2074 ± 920	1660 ± 634	NS
RBC (million/μL)	4.48 ± 0.45	4.55 ± 0.36	3.99 ± 0.54 ^{,#}	**
Hemoglobin (g/dL)	13.92 ± 1.58	13.46 ± 1.83	12.22 ± 1.45	**
Hypertension	0	7 (64%) [‡]	15 (68%) [†]	NA
Hypercholesterolemia	0	8 (72%) [‡]	14 (63%) [†]	NA
Myocardial infarction/angina	0	1 (9%)	2 (9%)	NA
Stroke	0	0	1 (4%)	NA
Peripheral artery disease	0	1 (9%)	3 (14%)	NA
Statin (% use)	0	4 (36%)	14 (67%)	NA

Data are means ± SD.

* $P < 0.01$, ** $P < 0.0001$, NS, nonsignificant; NA, not analyzed. Post-hoc comparison significance levels (not adjusted for multiple comparisons). [†] $P < 0.01$ LT vs. H, [‡] $P < 0.01$ ES vs. H, ^{||} $P < 0.05$ LT vs. H, [†] $P < 0.05$ ES vs. H, [#] $P < 0.05$ LT vs. ES.

KW was used to test significance between all three groups for measured values. For measured data, post-hoc analyses between patient groups used the two sample Wilcoxon test. Comparisons for gender data used the two-sided exact Pearson Chi Square test for comparisons of the three groups and for the post-hoc analyses. The characteristics with zero healthy patients had that as a group requirement, thus comparisons to that group were not analyzed.

did not differ between the three groups. Although LDL levels were controlled in the LT group, systolic blood pressure was higher ($P < 0.01$) in LT versus H groups. Importantly, as our studies investigated the role of certain cell-derived markers (particularly monocyte MPs), the monocyte and lymphocyte counts were statistically unchanged among the three groups. Red blood cell count was not altered between ES and H groups but decreased significantly in the LT group. Similarly, hemoglobin levels were significantly lower in the LT versus H group (Table 2).

Measurement and Characterization of Progenitor Cells and Microparticles

Assessment of PCs, PACs, and total MPs along with a range of cell-specific MPs was performed. A general relationship was observed in which PC and PAC levels decreased, and MPs and most MP subtypes increased with either onset or disease duration (Table 3). The exceptions to the above was that PCs increased from H to the ES state, but all fell below H levels in the LT disease state, however this was not statistically significant ($P < 0.05$). PACs were detected at significantly lower frequencies than PCs and some significant changes were observed. All three PACs were approximately equivalent between H and ES disease state, but CD133VEGF-R2 dropped significantly from ES to LT subjects. Significant reductions in triple positive PACs, CD133⁺CD34⁺ VEGF-R2⁺ were only observed between H and LT groups. It is interesting to note that the number of individuals within

each group with undetectable levels of triple positive PACs (CD133⁺ CD34⁺ CDVEGF-R2⁺) by flow cytometry also rose dramatically with onset of disease, (33% in H, 63% in ES, and 68% in LT).

For cell-derived MPs, levels of circulating EMPs and PMPs were altered significantly between H and LT ($P = 0.03$). CD14⁺ monocyte derived MPs were neither affected by disease state or duration (Table 3). Changes in AnnV⁺ MPs measured by flow cytometry and nM of PS⁺ MPs measured by the plate based assay, were statistically equivalent ($P = 0.02$). However, the flow cytometry method could detect differences with onset of disease, H vs. ES, whereas the plate-based method detected changes with disease duration, ES and LT. The differing resolution between the two assays maybe due to their separate read-outs; cytometry (AnnV⁺ MPs) counts the number of PS⁺ MPs whereas the plate captures PS⁺ MPs and quantifies using a prothrombinase assay. Nonetheless, both assays detected differences between the H and LT. These results demonstrate the possibility of employing a more feasible plate based assay to measure MPs in clinical studies than flow cytometry.

A covariate adjustment was also performed to assess if the observed group differences remained significant after the covariates, age, gender, hypertension, and statin use were included in the regression model. As hypertension and statin use were exclusion criteria within the H group, only age and gender could be used in those comparisons. With adjustment for age and gender, AnnV⁺ MPs was still significant ($P = 0.02$) between H and ES as

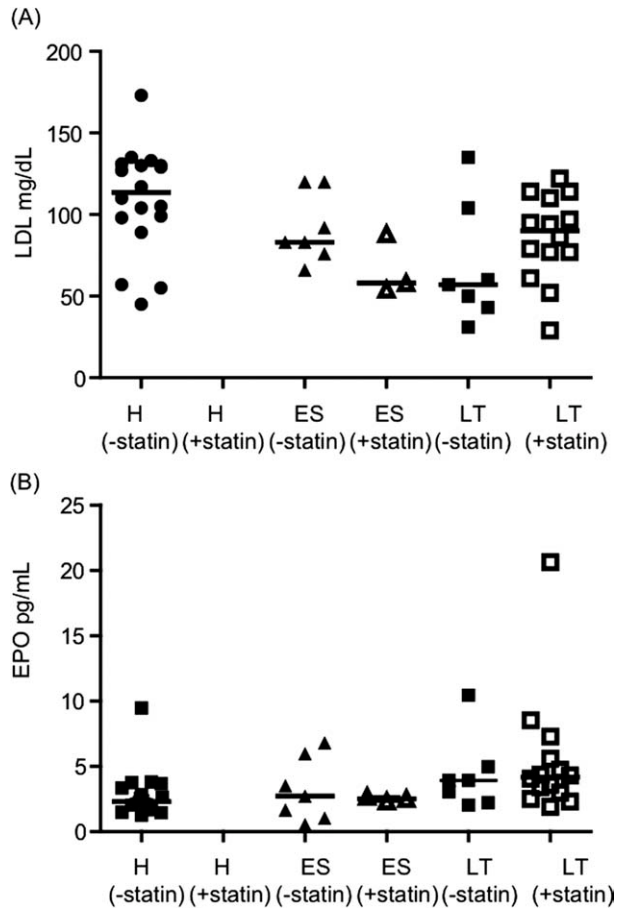


Fig. 2. Scatter plots (with median lines) showing low density lipoprotein levels (panel A) and EPO level (panel B) compared to statin use. LDL, low density lipoprotein cholesterol; EPO, erythropoietin; H, healthy; ES, early stage diabetes; LT, long-term diabetes.

were EMPs ($P = 0.03$) and PMPs ($P = 0.01$) between H and LT. AnnV⁺ MPs were borderline significant ($P = 0.053$) between H and LT. For comparisons between ES and LT, adjusting for age, gender, hypertension, and sta-

tin use, no other covariate improves the model after age is included.

Evaluating the MP/PC Ratio

The measurement of double and triple positive PACs and of cell derived MPs, by flow cytometry, is both technically challenging and expensive. We investigated the value of measuring single positive PCs by flow cytometry and PS⁺ MPs by the plate based assay. CD34⁺ PCs displayed borderline significant reductions from H to ES ($P = 0.06$) whilst the measurement of procoagulant MPs by the plate-based assay displayed a significant upward trend with ($P = 0.02$) Figure 3.

We assessed whether the ratio of MP/PC offered additional information over the investigation of PCs/PACs or MPs alone. Interestingly, the ratio of PS⁺ MPs/CD34⁺ PC proved a valuable index to distinguish the subject groups ($P = 0.01$) (Fig. 3) and this change was more significant than any of the other single PC, PAC, or MP subtypes analyzed (Table 3). We also compared PCs, PACs, and MPs to a range of soluble protein markers. The MP/PC ratio was more predictive than a number of often utilized soluble proteins including CRP, ICAM1, and VCAM1 (Table 4). These soluble proteins were quantified via multiplex assays, a cost effective and efficient method often employed in clinical studies. TNF α , VEGF, IGFBF-1, SDF-1, and PAI-1 although detectable were not significant (data not shown).

Of interest, EPO concentration was higher in the LT group ($P = 0.004$), (Table 4) and was not due to exogenous recombinant human EPO (rhEPO) or linked to statin use (Fig. 2B). Those within the LT group had significantly depressed red blood cell (RBC) count and hemoglobin and were therefore approaching an anemic state in comparison to both Healthy and ES (Table 2).

DISCUSSION

To our knowledge, this is the first study showing PACs decrease and MP increase with both onset and duration of Type 2 diabetes. Additionally, we provide

Table 3
Cell and Cell Derived Biomarkers

Marker type	H	ES	LT	P
CD133 ⁺ PC	954 (712–157)	1052 (538–1277)	639 (448–1243)	0.08
CD34 ⁺ PC	2676 (1763–4644)	3432 (2153–4125)	1818 (1341–3268)	0.06
CD133 ⁺ CD34 ⁺ PC	778 (618–1251)	868 (407–1228)	605 (356–1208)	0.15
CD133 ⁺ VEGF-R2 ⁺ PAC	8.6 (0–20)	18.3 (0–21)	0 (0–9) [#]	0.04*
CD34 ⁺ VEGF-R2 ⁺ PAC	15.3 (9–26)	7.9 (6–40)	8.0 (0–18) [#]	0.03*
CD133 ⁺ CD34 ⁺ VEGF-R2 ⁺ PAC	6.9 (0–12)	0 (0–20)	0 (0–7)	0.04*
CD144 ⁺ MPs	204 (93–411)	453 (187–616)	571 (207–892)	0.03*
CD41 ⁺ MPs	300 (155–583)	693 (239–894)	367 (258–939)	0.03*
CD14 ⁺ MPs	120 (68–347)	340 (178–469)	230 (77–488)	0.15
AnnV ⁺ MPs	355 (240–760)	761 (449–996) [†]	682 (330–1044)	0.02*
nM of PS ⁺ MPs	0.31 (0.21–0.36)	0.27 (0.16–0.40)	0.45 (0.28–0.56) ^{, #}	0.02*

Data are medians (25%–75% interquartile range). P(A)C values correspond to Cells/mL, MP values correspond to MPs/ μ L except PS⁺ MPs which corresponds to nM of PS equivalent by plate-based assay. H, healthy; ES, early stage diabetes; LT, long-term diabetes. * $P < 0.05$. Post-hoc comparison significance levels (not adjusted for multiple comparisons). ^{||} $P < 0.05$ LT vs. H, [†] $P < 0.05$ ES vs. H, [#] $P < 0.05$ LT vs. ES.

^P calculated by JT test to test for differences between the three groups. Post-hoc analyses between patient groups used the two sample Wilcoxon test.

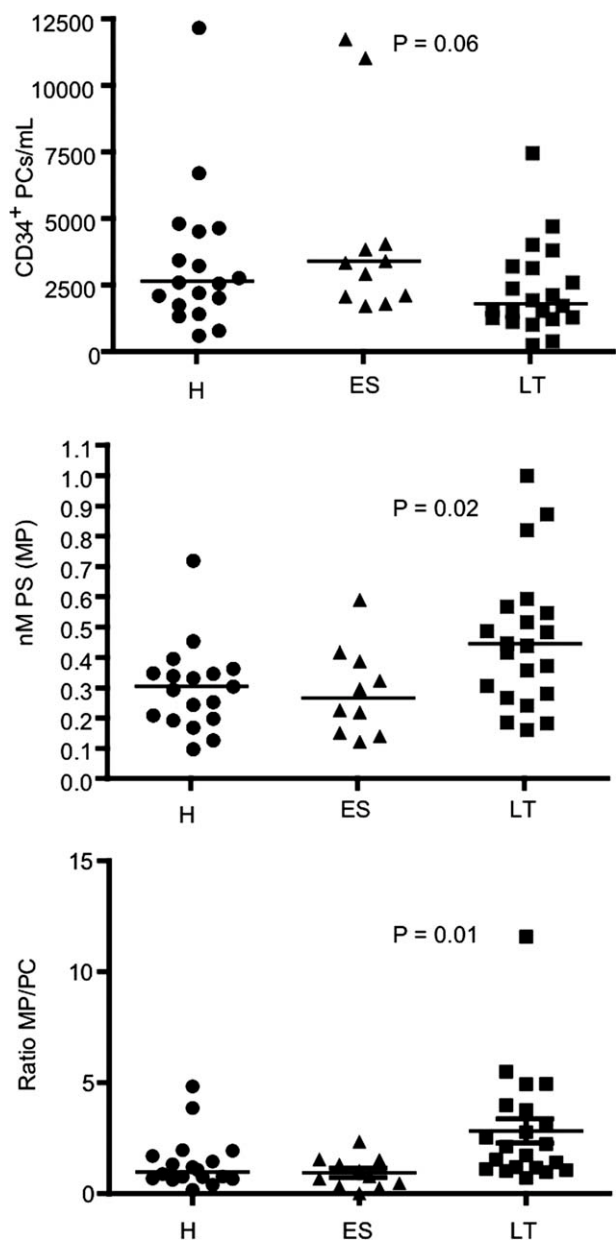


FIG. 3. Scatter plots (with median lines) and significance of CD34⁺ PCs, nM of PS⁺ MPs by plate-based assay and ratio of nM of PS⁺ MPs/CD34⁺ PCs. *P* calculated by KW. H, healthy; ES, early stage diabetes; LT, long-term diabetes; MP, microparticle; PC, progenitor cell.

evidence for the value of assessing the ratio of MPs to PCs (nM of PS⁺ MPs/CD34⁺ PCs), a measurement that is clinically feasible and more informative than some standard protein markers. PCs and MPs are not byproducts of cardiovascular disease but active components of the disease, and therefore reflect specific disease pathways. For instance, MPs are not only markers of cellular damage but also active agents in promoting endothelial dysfunction (18) and coagulation (10). A reduction in PCs and PACs indicates a loss of vascular reparative ability (6). In demonstrating that levels of both PCs/PACs and MPs correlate with duration of diabetic disease, our results also

provide mechanistic insights into the stepwise etiology of diabetes and its contribution to vascular pathology.

The presence of coronary artery disease (CAD) is often asymptomatic in individuals with diabetes (25). Although recent noninvasive studies indicate that CAD can be detected in significant numbers of these individuals, a routine screening approach has not been shown to be clinically useful or cost effective (26). Biomarkers, and particularly cell-derived biomarkers, may prove to be more predictive of cardiovascular events. The search for cell biomarkers of patients at risk for vascular complications is promising but has not become a part of clinical practice because a rapid, easy to perform cell-based assay has not been validated.

A developing and exciting biomarker strategy is the measurement of MPs and assessment of circulating progenitor cells (5,27). MPs are shed by cells as a consequence of cellular injury resulting from activation or death (apoptosis), thus elevation of plasma MPs may be a surrogate marker for vascular cell damage and EMPs are considered markers for endothelial dysfunction (4,16,17). Elevated MPs are found in coronary artery disease or thrombotic microangiopathies, supporting this hypothesis. Further, some evidence exists that the levels of circulating hematopoietic and progenitor cells are reflective of vascular health (27).

In a prospective observational study of diabetic patients referred for angiography, elevated EMP levels, measured using flow cytometry, were predictive for the presence of coronary artery lesions, odds ratio 3.5 (1.8-6.9) (4). Further, it was a more significant independent risk factor than duration of diabetes, lipid levels, or the presence of hypertension. Interestingly, elevated endothelial MPs (CD144⁺) identified a subpopulation of diabetic patients without typical angina symptoms who had atherosclerotic disease on coronary angiography. They also found that endothelial MP levels inversely correlated with coronary blood flow in response to acetylcholine stimulation of endothelium-dependent vasodilatation (4). Similarly, in another study, EMP levels were 2.5 times higher in the presence of high-risk coronary lesions compared with low risk lesions (28). A recent prospective study demonstrated that EMPs in patients at high risk of cardiovascular disease (by virtue of established risk factors) were independent predictors of future events (17).

The etiology of high levels of MPs and low levels of PC and PACs in patients with diabetes is unclear. Microparticles are constitutively released from the surface of cells but can be triggered by cell activation or apoptosis (29). We hypothesize that the high oxidative stress induced by the diabetic state with enhanced reactive oxygen species (ROS) generation and lipid peroxidation promotes MP release. Endothelial nitric oxide synthase (eNOS) decoupling, leading to a switch from the NO producing enzyme to superoxide production may participate in the production of MP (30,31). In fact, endothelial MPs themselves promote endothelial dysfunction through reduction in NO (13) and stimulation of

Table 4
Soluble Protein Analysis

	H	ES	LT	P
EPO (pg/mL)	2.8 ± 1.8	3.0 ± 2	5.1 ± 4.2 [†]	0.004**
CRP (pg/mL)	13.8 ± 13	77 ± 90 [¶]	42 ± 83	0.109
ICAM1 (pg/mL)	1.4 ± 0.5	2.7 ± 1.8 [¶]	1.6 ± 0.1 [#]	0.382
VCAM1 (pg/mL)	2.43 ± 0.9	3.58 ± 2.01	3.22 ± 1.31	0.071

***P* < 0.01 H, healthy; ES, early stage diabetes; LT, long-term diabetes. Post-hoc comparison significance levels (not adjusted for multiple comparisons). [†]*P* < 0.01 LT vs. H, [¶]*P* < 0.05 ES vs. H, [#]*P* < 0.05 LT vs. ES.

P calculated by JT test to test for differences between the three groups. Post-hoc analyses between patient groups used the two sample Wilcoxon test.

superoxide from the endothelium. MPs also contain the NAD(P)H oxidase subunit p22^{phox} (32). This intriguing observation suggests that delivery of NAD(P)H subunits to the endothelium by MPs may enhance superoxide production producing a constant cycle of MP production and ROS generation. The administration of an angiotensin II receptor blocker to patients with Type 2 diabetes decreased levels of monocyte and endothelial derived MPs, but not in hypertensive patients without diabetes (33). The administration of statins also reduces MP number in patients with diabetes (20,34).

The diabetic state impairs PC mobilization from the bone marrow and PACs in this disease state display functional impairment, such as reduced proliferation, adhesion, migration, and incorporation, into tubular structures (35,36). The pathological mechanisms underlying reduced circulating PACs in diabetes include bone marrow dysfunction and shortened survival in peripheral blood (6). Indeed, disease-associated MPs may themselves impact negatively on PACs. In-vitro studies indicate that MPs from hypercholesterolemic patients caused a significant PAC loss over MPs harvested from normocholesterolemic controls (22).

Circulating levels of PACs are stimulated by endogenous factors such as proangiogenic cytokines and erythropoietin (37,38). In this study, patients with diabetes had higher levels of erythropoietin than nondiabetics despite lower levels of PACs and hemoglobin. This observation may be due to impaired response of the bone marrow to erythropoietin, driven by enhanced ROS and inflammatory cytokines associated with the diabetic condition. Another study also found increases in EPO levels in microalbuminuric Type 2 diabetes, that was associated with anemic status and also reduced hemoglobin (39). Furthermore, a pathological alteration in the phosphatidylinositol (PI) 3-kinase/protein kinase-B and endothelial nitric oxide synthase eNOS pathways seen in diabetics may be responsible in part to reduced levels of EPCs (40). Again, higher or pathological MPs seen in diabetic patients may play a causative role in reducing eNOS (16).

Limitations of the Study

A limitation in our study is the lack of a functional measurement of cardiovascular disease (such as angiography or pulse-wave velocity) to test the predictive value of our markers. This was beyond the scope of this study.

Our conclusions are based on the assumption that vascular risk increases with duration of diabetic disease as reported in other studies (4,17,22,23).

Further limitations are that the surface markers for PACs are controversial and may not reflect the ability to proliferate or to be incorporated into vessels in vivo. We did however use multiple cells as per our previously validated PAC assay that allows for assessment of many cell combinations (5). The issue of specificity of surface markers for MP subtype identification and the ability to accurately measure MPs by current flow cytometry methods also poses concern. However, our absolute numbers of CD144⁺ endothelial MPs are in line with those published by other groups (16,41). Although EMPs number less than PMPs in our study, the proportion of EMPs to PMPs is slightly higher than that measured in other studies (42).

CONCLUSIONS

The main findings obtained from the experimental study are as follows. First, we found significant alterations in both PCs/PACs and cell derived MPs in Type 2 diabetes and also with duration of disease. Second, we illustrate the potential value of using the ratio of MPs/PCs, which encompasses two biologically relevant markers that impact functionally on disease progression. Third, we show that this ratio may be more informative than many individual standard protein biomarkers commonly used to stratify individuals at heightened cardiovascular risk. From a clinical standpoint, the results from our investigation indicate that a single platform high throughput, multiplexed flow cytometry assay for hematopoietic progenitors and plate-based assay for MPs is a feasible and cost effective method to identify those individuals at highest risk for cardiovascular events.

ACKNOWLEDGMENTS

This study was supported in part by an unrestricted grant from GSK. Dr. Mohler's salary is partially funded via NIH National Heart Lung and Blood Institute grant K12 HL083772-01.

LITERATURE CITED

1. Kannel WB, McGee DL. Diabetes and cardiovascular disease. The Framingham study. *JAMA* 1979;241:2035-2038.
2. Kuvin JT, Patel AR, Sliney KA, Pandian NG, Rand WM, Udelson JE, Karas RH. Peripheral vascular endothelial function testing as a non-invasive indicator of coronary artery disease. *J Am Coll Cardiol* 2001;38:1843-1849.

3. Ridker PM, Stampfer MJ, Rifai N. Novel risk factors for systemic atherosclerosis: A comparison of C-reactive protein, fibrinogen, homocysteine, lipoprotein(a), and standard cholesterol screening as predictors of peripheral arterial disease. *JAMA* 2001;285:2481-2485.
4. Koga H, Sugiyama S, Kugiyama K, Watanabe K, Fukushima H, Tanaka T, Sakamoto T, Yoshimura M, Jinnouchi H, Ogawa H. Elevated levels of VE-cadherin-positive endothelial microparticles in patients with type 2 diabetes mellitus and coronary artery disease. *J Am Coll Cardiol* 2005;45:1622-1630.
5. Shaffer RG, Greene S, Arshi A, Supple G, Bantly A, Moore JS, Mohler ER III. Flow cytometric measurement of circulating endothelial cells: The effect of age and peripheral arterial disease on baseline levels of mature and progenitor populations. *Cytometry B Clin Cytom* 2006;70B:56-62.
6. Mohler ER III, Shi Y, Moore J, Bantly A, Hamamdzc D, Yoder M, Rader DJ, Putt M, Zhang L, Parmacek M, Wilensky RL. Diabetes reduces bone marrow and circulating porcine endothelial progenitor cells, an effect ameliorated by atorvastatin and independent of cholesterol. *Cytometry A* 2009;75A:75-82.
7. Möbius-Winkler S, Höllriegel R, Schuler G, Adams V. Endothelial progenitor cells: Implications for cardiovascular disease. *Cytometry A* 2009;75A:25-37.
8. Martinez MC, Tesse A, Zobairi F, Andriantsitohaina R. Shed membrane microparticles from circulating and vascular cells in regulating vascular function. *Am J Physiol Heart Circ Physiol* 2005;288:H1004-H1009.
9. Deregibus MC, Cantaluppi V, Calogero R, Lo Iacono M, Tetta C, Biancone L, Bruno S, Bussolati B, Camussi G. Endothelial progenitor cell derived microvesicles activate an angiogenic program in endothelial cells by a horizontal transfer of mRNA. *Blood* 2007;110:2440-2448.
10. Morel O, Toti F, Hugel B, Bakouboula B, Camoin-Jau L, Dignat-George F, Freyssinet JM. Procoagulant microparticles: Disrupting the vascular homeostasis equation? *Arterioscler Thromb Vasc Biol* 2006;26:2594-2604.
11. Falati S, Liu Q, Gross P, Merrill-Skoloff G, Chou J, Vandendries E, Celi A, Croce K, Furie BC, Furie B. Accumulation of tissue factor into developing thrombi in vivo is dependent upon microparticle P-selectin glycoprotein ligand 1 and platelet P-selectin. *J Exp Med* 2003;197:1585-1598.
12. Barry OP, Pratico D, Lawson JA, FitzGerald GA. Transcellular activation of platelets and endothelial cells by bioactive lipids in platelet microparticles. *J Clin Invest* 1997;99:2118-2127.
13. Boulanger CM, Scoazec A, Ebrahimiyan T, Henry P, Mathieu E, Tedgui A, Mallat Z. Circulating microparticles from patients with myocardial infarction cause endothelial dysfunction. *Circulation* 2001;104:2649-2652.
14. Morel O, Ohlmann P, Epailly E, Bakouboula B, Zobairi F, Jesel L, Meyer N, Chenard MP, Freyssinet JM, Bareiss P, Mazzucotelli JP, Toti F. Endothelial cell activation contributes to the release of procoagulant microparticles during acute cardiac allograft rejection. *J Heart Lung Transplant* 2008;27:38-45.
15. Matsumoto N, Nomura S, Kamihata H, Kimura Y, Iwasaka T. Increased level of oxidized LDL-dependent monocyte-derived microparticles in acute coronary syndrome. *Thromb Haemost* 2004;91:146-154.
16. Amabile N, Guerin AP, Leroyer A, Mallat Z, Nguyen C, Boudaert J, London GM, Tedgui A, Boulanger CM. Circulating endothelial microparticles are associated with vascular dysfunction in patients with end-stage renal failure. *J Am Soc Nephrol* 2005;16:3381-3388.
17. Nozaki T, Sugiyama S, Koga H, Sugamura K, Ohba K, Matsuzawa Y, Sumida H, Matsui K, Jinnouchi H, Ogawa H. Significance of a multiple biomarkers strategy including endothelial dysfunction to improve risk stratification for cardiovascular events in patients at high risk for coronary heart disease. *J Am Coll Cardiol* 2009;54:601-608.
18. Boulanger CM, Amabile N, Tedgui A. Circulating microparticles: A potential prognostic marker for atherosclerotic vascular disease. *Hypertension* 2006;48:180-186.
19. Curtis AM, Wilkinson PF, Gui M, Gales TL, Hu E, Edelberg JM. p38 mitogen-activated protein kinase targets the production of proinflammatory endothelial microparticles. *J Thromb Haemost* 2009;7:701-709.
20. Nomura S, Shouzu A, Omoto S, Nishikawa M, Iwasaka T. Effects of losartan and simvastatin on monocyte-derived microparticles in hypertensive patients with and without type 2 diabetes mellitus. *Clin Appl Thromb Hemost* 2004;10:133-141.
21. Pirro M, Bagaglia F, Paoletti L, Razzi R, Mannarino MR. Hypercholesterolemia-associated endothelial progenitor cell dysfunction. *Ther Adv Cardiovasc Dis* 2008;2:329-339.
22. Pirro M, Schillaci G, Paltriccina R, Bagaglia F, Menecali C, Mannarino MR, Capanni M, Velardi A, Mannarino E. Increased ratio of CD31+/CD42- microparticles to endothelial progenitors as a novel marker of atherosclerosis in hypercholesterolemia. *Arterioscler Thromb Vasc Biol* 2006;26:2530-2535.
23. Spijkerman AM, Dekker JM, Nijpels G, Jager A, Kostense PJ, van Hinsbergh VW, Bouter LM, Heine RJ, Stehouwer CD. Impact of diabetes duration and cardiovascular risk factors on mortality in type 2 diabetes: The Hoorn Study. *Eur J Clin Invest* 2002;32:924-930.
24. Friedewald WT, Levy RI, Frederickson DS. Estimation of the concentration of low-density lipoprotein cholesterol in plasma without the use of the preparative ultracentrifuge. *Clin Chem* 1972;18:499-502.
25. Haffner SM, Lehto S, Ronnema T, Pyörälä K, Laakso M. Mortality from coronary heart disease in subjects with type 2 diabetes and in nondiabetic subjects with and without prior myocardial infarction. *N Engl J Med* 1998;339:229-234.
26. Young LH, Wackers FJ, Chyun DA, Davey JA, Barrett EJ, Taillefer R, Heller GV, Iskandrian AE, Wittlin SD, Filipchuk N, Ratner RE, Inzucchi SE, DIAD Investigators. Cardiac outcomes after screening for asymptomatic coronary artery disease in patients with type 2 diabetes: The DIAD study: A randomized controlled trial. *JAMA* 2009;301:1547-1555.
27. Leroyer AS, Tedgui A, Boulanger CM. Role of microparticles in atherothrombosis. *J Intern Med* 2008;263:528-537.
28. Bernal-Mizrachi L, Jy W, Fierro C, Macdonough R, Velazques HA, Purow J, Jimenez JJ, Horstman LL, Ferreira A, de Marchena E, Ahn YS. Endothelial microparticles correlate with high-risk angiographic lesions in acute coronary syndromes. *Int J Cardiol* 2004;97:439-446.
29. Nomura S, Ozaki Y, Ikeda Y. Function and role of microparticles in various clinical settings. *Thromb Res* 2008;123:8-23.
30. Wang JM, Huang YJ, Wang Y, Xu MG, Wang LC, Wang SM, Tao J. Increased circulating CD31+/CD42- microparticles are associated with impaired systemic artery elasticity in healthy subjects. *Am J Hypertens* 2007;20:957-964.
31. Wang JM, Wang Y, Huang JY, Yang Z, Chen L, Wang LC, Tang AL, Lou ZF, Tao J. C-Reactive protein-induced endothelial microparticle generation in HUVECs is related to BH4-dependent NO formation. *J Vasc Res* 2007;44:241-248.
32. Brodsky SV, Zhang F, Nasjletti A, Goligorsky MS. Endothelium-derived microparticles impair endothelial function in vitro. *Am J Physiol Heart Circ Physiol* 2004;286:H1910-H1915.
33. Nomura S, Shouzu A, Omoto S, Nishikawa M, Fukuhara S, Iwasaka T. Effect of valsartan on monocyte/endothelial cell activation markers and adiponectin in hypertensive patients with type 2 diabetes mellitus. *Thromb Res* 2006;117:385-392.
34. Sommeijer DW, Joop K, Leyte A, Reitsma PH, ten Cate H. Pravastatin reduces fibrinogen receptor gpIIb on platelet-derived microparticles in patients with type 2 diabetes. *J Thromb Haemost* 2005;3:1168-1171.
35. Hill JM, Zalos G, Halcox JP, Schenke WH, Waclawiw MA, Quyyumi AA, Finkel T. Circulating endothelial progenitor cells, vascular function, and cardiovascular risk. *N Engl J Med* 2003;348:593-600.
36. Fadini GP, Sartore S, Agostini C, Avogaro A. Significance of endothelial progenitor cells in subjects with diabetes. *Diabetes Care* 2007;30:1305-1313.
37. Heeschen C, Aicher A, Lehmann R, Fichtlscherer S, Vasa M, Urbich C, Mildner-Rihm C, Martin H, Zeiher AM, Dimmeler S. Erythropoietin is a potent physiologic stimulus for endothelial progenitor cell mobilization. *Blood* 2003;102:1340-1346.
38. Bahlmann FH, de GK, Spandau JM, Landry AL, Hertel B, Duckert T, Boehm SM, Menne J, Haller H, Fliser D. Erythropoietin regulates endothelial progenitor cells. *Blood* 2004;103:921-926.
39. Bruno CM, Sciacca C, Bertino G, Cilio D, Pellicano R, Marchese AE, Politi G, Chinnici L. Circulating erythropoietin in microalbuminuric type 2 diabetic patients with normal renal function: A pilot study. *J Diabetes Complications* 2006;20:376-379.
40. Dimmeler S, Aicher A, Vasa M, Mildner-Rihm C, Adler K, Tiemann M, Rutten H, Fichtlscherer S, Martin H, Zeiher AM. HMG-CoA reductase inhibitors (statins) increase endothelial progenitor cells via the PI 3-kinase/Akt pathway. *J Clin Invest* 2001;108:391-397.
41. Boulanger CM, Amabile N, Guerin AP, Pannier B, Leroyer AS, Mallat Z, Tedgui A, London GM. In vivo shear stress determines circulating levels of endothelial microparticles in end-stage renal disease. *Hypertension* 2007;49:902-908.
42. Leroyer AS, Isobe H, Leseche G, Castier Y, Wassef M, Mallat Z, Binder BR, Tedgui A, Boulanger CM. Cellular origins and thrombotic activity of microparticles isolated from human atherosclerotic plaques. *J Am Coll Cardiol* 2007;49:772-777.