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ARTICLE

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Circulating endothelial cells are elevated in patients with type 2 diabetes mellitus independently of HbA_1c

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Abstract *Aims/hypothesis:* Patients with diabetes mellitus are well known to be at high risk for vascular disease. Circulating endothelial cells (CECs) have been reported to be an ex vivo indicator of vascular injury. We investigated the presence of CECs in the peripheral blood of 25 patients with diabetes mellitus and in nine non-diabetic control donors. *Methods:* Endothelial cells were isolated from peripheral blood with anti-CD-146–coated immunomagnetic Dynabeads, and were stained with acridine orange dye and counted by fluorescence microscopy. The cells were also stained for von Willebrand factor and *Ulex europaeus* lectin 1. *Results:* Patients with diabetes mellitus had an elevated number of CECs (mean 69±30 cells/ml, range 35–126) compared with healthy controls (mean 10±5 cells/ml, range 3–18) ($p<0.001$). The increase in CECs did not correlate with the levels of HbA_1c. Circulating endothelial cell numbers were elevated regardless of glucose levels, suggesting that, even with control of glucose levels, there is increased endothelial cell sloughing. *Conclusions:* Our study suggests that the higher number of CECs in patients with type 2 diabetes may reflect ongoing vascular injury that is not directly dependent on glucose control.

Keywords Circulating endothelial cells · Diabetes mellitus · Hyperglycaemia · Vascular injury

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Abbreviations CECs: Circulating endothelial cells · FcR: Fc receptor · ROS: Reactive oxygen species · UEA-1: *Ulex europaeus* lectin 1

Introduction

Diabetes mellitus has become increasingly prevalent over the past several decades owing to the advancing age of the population, a substantially increased prevalence of obesity, and decreased physical activity, all of which have been attributed to a western lifestyle. Diabetes mellitus is associated with an increased risk of cardiovascular disease, including atherosclerosis. The clinical severity of vascular complications in diabetes has in part been attributed to endothelial injury and dysfunction [1]. Exposure of endothelial cells to elevated glucose levels causes glucose oxidation, resulting in the generation of excess reactive oxygen species (ROS) in endothelial cells. A reduction in antioxidant reserves has been attributed to endothelial cell dysfunction in diabetes, even in patients with well-controlled glucose levels [2–4]. Hyperglycaemia-mediated local formation of ROS is considered to be the major factor contributing to endothelial dysfunction, including abnormalities in cell cycling [4] and delayed replication [5, 6], and these abnormalities can be reversed by exogenous antioxidant agents [7, 8] as well as an increased expression of antioxidant enzymes [9]. Hyperglycaemia has been demonstrated to stimulate the induction of apoptosis in endothelial cells by a mechanism that involves the generation of ROS and superoxide anion formation [10]. Moreover, high glucose conditions have been shown to facilitate the susceptibility of various serum proteins to oxidation, which contributes to the inhibition of endothelial cell proliferation [11].

In normal individuals, the number of circulating endothelial cells (CECs) is low. The number is markedly increased in conditions associated with a high degree of endothelial cell injury, such as myocardial infarction [12], sickle cell crisis [13], thrombotic thrombocytopenic purpura [14], Behçet's disease [15], active systemic lupus

erythematosus [16], active cytomegalovirus [17], anti-neutrophil cytoplasmic antibody-associated small vessel vasculitis [18] and *Rickettsia conorii* infection [19]. Although there is no real proof that CECs correlate with the extent of endothelial damage, it is clear that several pathological situations associated with endothelial damage are also associated with high levels of CECs.

We have previously shown that human microvascular endothelial cells, when exposed to high glucose concentrations, manifest an increase in apoptosis [20]. We therefore hypothesised that hyperglycaemia-mediated oxidative stress results in endothelial cell injury and that this may be reflected by a concurrent increase in CECs. Elevation of CECs has been reported to be an ex vivo indicator of vascular injury, and their number seems to vary according to the extent of endothelial injury [21, 22]. We examined the levels of CECs in subjects with type 2 diabetes mellitus and in normal subjects. We further attempted to correlate these findings with the levels of HbA_1c).

Methods

Patients and controls Following protocol approval by the human subject review boards of New York Medical College and the Westchester Medical Center, peripheral blood samples were collected from diabetic patients (n=25) and non-diabetic volunteer control subjects (n=9). All subjects gave informed consent. Inclusion criteria for the diabetic group were previous diagnosis of type 2 diabetes, objective documentation of chronic hyperglycaemia with HbA_1c of 6% or greater at the time of diagnosis, 18 years of age or above, and any gender or race. Control group inclusion criteria were good general health, no significant past medical history, 18 years of age or above, documented normal fasting blood glucose, and any gender or race. Exclusion criteria for both groups included uncontrolled hypertension (BP >140/90), LDL greater than 130 mg/dl, active inflammatory/infectious process, history of atherosclerosis (i.e. coronary artery disease, peripheral vascular disease or carotid artery disease), history of stroke, creatinine greater than 2 mg/dl, malignancy and smoking. Additional exclusion criteria for the control group included glucose intolerance (i.e. fasting blood sugar above 110 mg/dl) and past medical history of diabetes.

Isolation and measurement of CECs To identify endothelial cells, we used two different methods and compared the results. In the first method, we used immunomagnetic beads to detect endothelial cells. Monodispersed magnetisable particles (Dynabeads, CELLection Pan Mouse IgG kit) were obtained from Dynal (Lake Success, NY, USA). Dynabeads are 45-μm-diameter polystyrene beads coated with affinity-purified pan anti-mouse immunoglobulin G_1 covalently bound to the surface. The beads were washed according to the manufacturer's instructions using a strong magnet (MPC6; Dynal) to remove sodium azide. Typically, 100 μl of bead suspension is then non-covalently coated with 10 μg/ml CD-146 antibody (Novus Biologicals, Littleton, CO, USA). CD-146 is expressed almost exclusively in mature endothelial cells with the exception of some tumour cell lines [23, 24].

The second method used *Ulex europaeus* 1 (UEA-1), which is a lectin that specifically binds to endothelial cells. In this method, 1 ml of CD-146-extracted cell suspension was incubated with 10 μl *Ulex* (1:1,000) for 2 h at room temperature. An aliquot of this suspension was placed on a slide, and endothelial cells were visualised by fluorescence microscopy.

Blood samples were obtained as follows. Peripheral blood (15 ml) was obtained with a 21-G butterfly needle and the first 7.5 ml was discarded. It has been previously demonstrated that these measures are sufficient to avoid any influence of venipuncture or arterial vs venous sampling, and that diurnal variations can be excluded [24]. Of the remaining sample, 2 ml of blood was diluted with 2 ml of phosphate-buffered saline, 0.1% bovine serum albumin sodium azide, and 20 μl Fc receptor (FcR) blocking agent (Miltenyi, Gladbach, Germany), and incubated with 100 μl anti-CD146-coated beads (1.4×10^8 coated beads/ml) for 30 min at 4 °C in a Dynal mixer at 50 rpm. Cells bound to anti-CD146-coupled beads were separated from blood in a

Table 1 Patient characteristics

	Diabetic subjects	Control subjects
n	25	10
Age, mean (range)	54.0 (39–79)	46.6 (31–69)
Male	8 (32%)	4 (40%)
Female	17 (68%)	6 (60%)
HbA_1c	7.78% (5.1–11.9%)	–
Additional cardiac risk factors (per subject)	1.9 (1–4)	0.4 (0–1)
Medications		
Metformin	11	0
Insulin	9	0
Sulphonylureas	4	0
Glitazones	6	0
ACE inhibitors	15	1
Beta blockers	8	0
Thiazides	2	0
Statins	11	0
Fibrates	1	0
AT_1 blockers	3	0
Digoxin	0	1
Furosemide	2	0
Nitrates	2	0
Aspirin	2	0
Clopidogrel	1	0
Proton pump inhibitors	0	2
Antibiotics	4	0
Warfarin	0	1
Levothyroxine	1	1

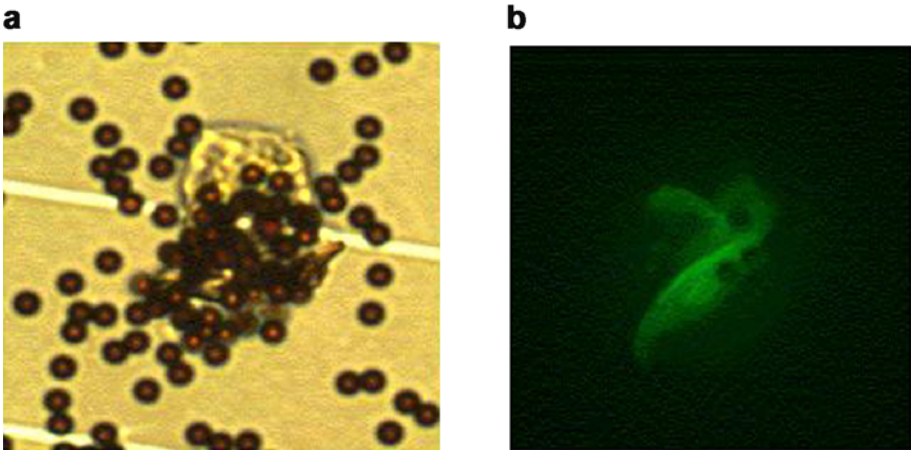

Fig. 1 Morphology of CECs with Dynabeads attached in normal or diabetic blood. Typical CECs captured by Dynabeads coated with anti-CD146 under light microscope (**a**), or fluorescent microscope (**b**). CECs were stained with acridine orange, isolated by Dynabeads, and stained with UEA-1 lectin.

Dynal magnet, washed (three washings using PBS–BSA 0.1% and repetitive mixing for 5 min in the Dynal mixer at 4 °C), dissolved in 100 μl buffer, mixed with acridine, and counted in a Nageotte chamber (Brand, Wertheim, Germany). To serve as positive controls, various concentrations of fresh HUVECs were diluted in blood from healthy volunteers. Recovery was in the range of 89–93% with excellent preservation of cell morphology.

The following steps were taken to prevent non-specific binding of leucocytes to Dynabeads. The cell suspension was flushed vigorously through a 20-μl pipette tip, and citrate was added to the buffer with an FcR blocking agent. Side-by-side assays were performed with M-450 Dynabeads coated with human antibodies against mouse IgG, but without anti-endothelial antibody to check for non-specific binding to Dynabeads. Cells were identified on the basis of morphology and adherence to beads alone, so that concurrent immunocytochemistry was not necessary. Analysis was done using an inverted and upright epi-fluorescent Olympus IX 81 microscope with motorised stage and a Nageotte haemocytometer for quantitation.

Endothelial cells are larger than blood cells, are about 20–50 μm in diameter, and have a well-delineated round or oval cell shape. Our criteria required that endothelial cells had at least ten beads attached. Cells with less than ten beads were counted only if they had a well-preserved and recognisable morphology (i.e. clear nucleus with a well-delimited cytoplasm and a size that corresponds to endothelial cells) and if the concentration of beads over the endothelial cells was more than the surrounding field. These cells were also stained by anti-von Willebrand factor VIII antibody [25]. For aggregates, the number of cells was deduced from the number of nuclei or from the number of spherical rosetted features discriminated in the aggregate. Numeration of endothelial cells was confirmed by counting performed in parallel to cells stained with UEA-1 and acridine.

Statistical analysis Statistical significance between the experimental groups was determined by ANOVA analysis; $p<0.05$ was considered statistically significant. The data are presented as means ± SE.

Table 2 Recovery of HUVECs added to healthy donor blood

Number of cells added	Number of cells recovered	Percentage recovery
0 (Control blood)	3±2	100
100 HUVECs	89±8	89
200 HUVECs	186±12	93
400 HUVECs	362±17	90

Endothelial cells were detected using immunobeads as described in text

Results

We examined the effect of diabetes mellitus on endothelial cell sloughing in 25 diabetic patients and nine non-diabetic

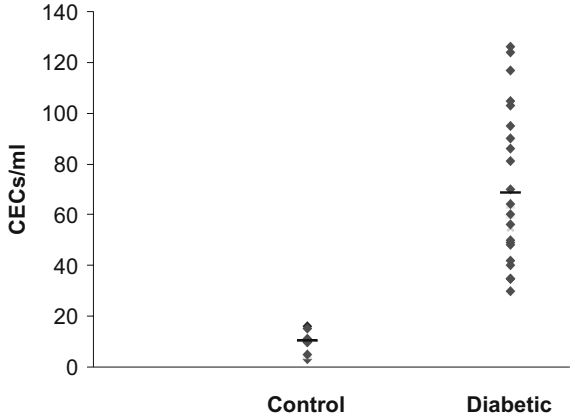

Fig. 2 The numbers of CECs in control or in diabetic donors were measured as described in "Methods." The number of CECs increased significantly in diabetic donors ($p<0.001$) relative to controls. CECs were isolated by magnetic beads with CD-146 antibody from normal donors or patients with diabetes. Control experiments using human umbilical vein endothelial cells are described in Table 2.

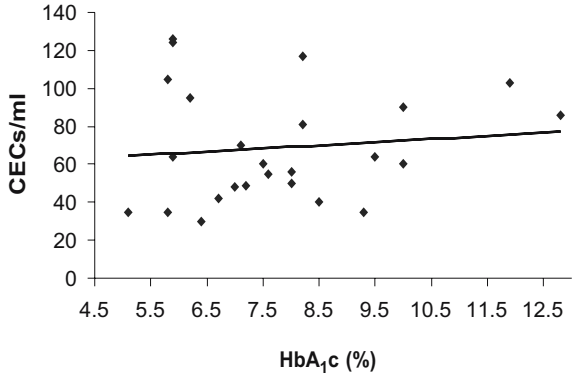

Fig. 3 Relationship between HbA_1c and CECs/ml in patients with diabetes, with linear regression. No correlation is observed (R^2=0.0117).

control subjects. Subject characteristics are summarised in Table 1. Glucose levels in diabetic patients studied were between 150 and 320 mg/dl. Diabetic patients were on a variety of medications, with the majority on ACE inhibitors and statins in addition to oral hypoglycaemic agents and/or insulin.

We have used a reproducible method for isolating CECs using immunomagnetic beads coated with CD-146 (specific antibodies for human endothelial cells) or by using UEA-1, a fluorescent lectin that specifically binds to endothelial cells. Parallel counting using both methods was identical. Figure 1 depicts endothelial cells isolated by immunomagnetic beads and stained with acridine (panel a) and endothelial cells in whole blood identified by *Ulex* staining (panel b). Both methods gave the same results. When blood from healthy controls was mixed with a known number of HUVECs and diluted with buffer and FcR blocking agent, the recovery of endothelial cells was between 89% and 93% (Table 2), confirming the efficiency of the standardised methods used.

As seen in Fig. 2, patients with diabetes had a significantly elevated number of CECs (mean 69±30 cells/ml, range 35–126) compared with healthy controls (mean 10±5 cells/ml, range 3–18) (p<0.001). Regression analysis demonstrated no significant difference between levels of

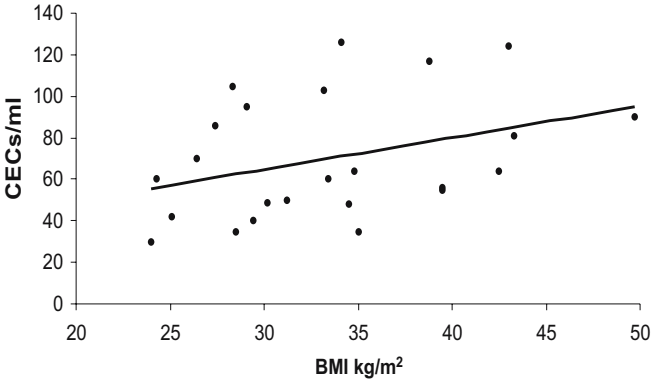

Fig. 4 CECs/ml plotted against BMI (kg/m^2) in patients with diabetes, with linear regression. A non-significant trend towards association is noted (R^2=0.1269, p=0.09).

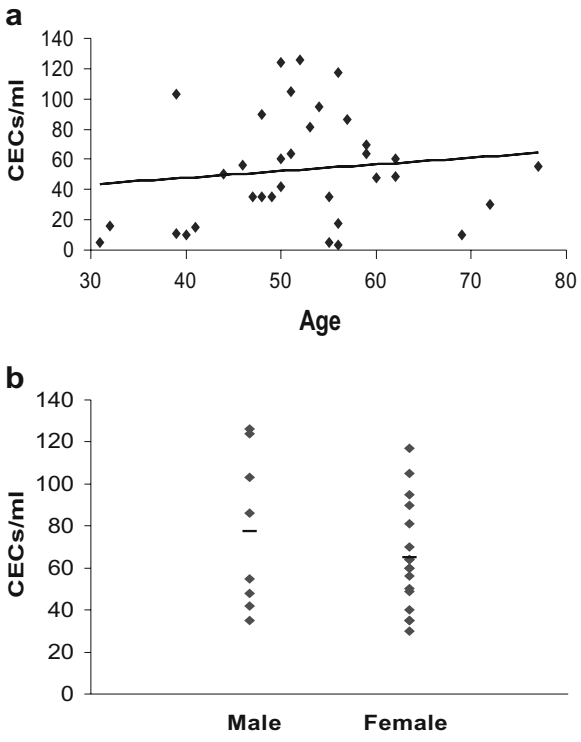

Fig. 5 **a** CECs/ml plotted against age in all subjects, with linear regression. No correlation is observed (R^2=0.015). **b** CECs/ml plotted against sex in patients with diabetes (8 males, 17 females). No correlation is observed.

HbA_1c and the number of CECs (Fig. 3). There was, however, a trend towards higher numbers of CECs in patients with a higher BMI (Fig. 4). As seen in Fig. 5a, there is no correlation between the number of CECs and age. Similarly, plotting the number of CECs against gender also demonstrated no significant association (Fig. 5b).

Discussion

The current study demonstrates a clear increase in CECs in patients with type 2 diabetes mellitus compared with normal control subjects. At this time, we are unable to ascertain what proportion of these cells are mechanically disrupted or apoptotic vascular endothelial cells and what proportion represent matured progenitor cells capable of expressing CD146 antibody. Prior work has demonstrated that stem-cell-derived circulating endothelial progenitor cells are decreased in patients with abnormal flow-mediated brachial vasodilatation and in patients with high Framingham risk scores [26]. Similarly, a decrease in the number of circulating endothelial progenitor cells has been reported in patients with risk factors for coronary artery disease [27]. Both of these studies suggest that the majority of cells documented in type 2 diabetics are products of endothelial cell sloughing rather than representing an increase in progenitor cells. Future studies aimed at determining the origin of CECs in type 2 diabetics by means of flow cytometry may serve to confirm this.

Several possible mechanisms may result in an increase in endothelial cell sloughing. Hyperglycaemia has been observed to increase superoxide anion levels in endothelial cells [28]. Superoxide anion exerts a vasoconstrictive effect through the conversion of nitric oxide to peroxynitrite, thereby consuming the endogenous vasodilator in the vasculature [28, 29]. Glucose exposure results in an increase in O_2^-, an increase in activation of cyclo-oxygenase-2 and an increase in the levels of angiotensin II [3, 4, 6, 30].

Angiotensin II has been shown to increase endothelial cell death, an effect that is reversed by increased haem oxygenase-1 gene expression [20, 31]. Inhibition of haem oxygenase activity has been shown to exacerbate both the inflammatory response in the arterial wall and to enhance endothelial cell death and the expression of adhesion molecules in animal models of atherosclerosis [32]. Therefore, a hyperglycaemia-mediated increase in angiotensin II can be a possible mechanism by which glucose increases endothelial cell sloughing, either directly or via glycated oxidants. It has been suggested that this kind of mechanism plays a key role in the development of atherosclerosis [33].

What is intriguing is that the extent of the increase in CECs in our study does not appear to directly correlate with the level of HbA_1c despite its occurrence in patients who present with hyperglycaemia. We noted a trend towards an increase in the number of CECs in patients with higher BMI; however, the small sample size precludes the assignment of statistical significance to this observation. A recent report documenting a disturbed antioxidant defence mechanism associated with insulin resistance may support a hypothesis that insulin resistance rather than hyperglycaemia per se is primarily responsible for the increase in number of CECs in type 2 diabetics [30]. This is supported by a previous observation that endothelium-dependent vasodilatation is impaired in normal volunteers subjected to more than 4 h of euglycaemic hyperinsulinaemia, an effect that is reversed by vitamin C [34]. Future studies in patients with type 1 diabetes mellitus as well as correlation of the number CECs with insulin levels in a larger cohort of patients with type 2 diabetes mellitus would serve to further address this hypothesis.

In summary, we have demonstrated that there is an increase in the number of CECs in patients with diabetes mellitus as compared with normal controls. This increase may be a contributing factor in the vascular pathology of the disease. Although related to the presence of elevated glucose levels, the extent of endothelial cell sloughing does not appear to be related to the severity of the hyperglycaemia. The increased number of CECs in type 2 diabetics provides direct evidence of endothelial injury and may be useful in the exploration of the vascular effects of diabetes mellitus. Future studies aimed at precisely distinguishing cell origin, identifying the mechanism by which they are generated, and associating their presence or absence with clinical endpoints may help to further define the vascular pathophysiology of patients with diabetes.

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References

1. Abraham NG, McClung J, Lafaro R, Wolin M (2003) Heme oxygenase overexpression attenuates glucose-mediated elevation of 8-epi-isoprostane F2α: linking heme to oxidative stress. Circulation 194[Suppl IV]:IV-265
2. Baynes JW (1991) Role of oxidative stress in development of complications in diabetes. Diabetes 40:405–412
3. Karpen CW, Cataland S, O'Dorisio TM, Panganamala RV (1985) Production of 12-hydroxyeicosatetraenoic acid and vitamin E status in platelets from type I human diabetic subjects. Diabetes 34:526–531
4. Zou MH, Shi C, Cohen RA (2002) High glucose via peroxynitrite causes tyrosine nitration and inactivation of prostacyclin synthase that is associated with thromboxane/prostaglandin H2 receptor-mediated apoptosis and adhesion molecular expression in cultured human aortic endothelial cells. Diabetes 51:198–203
5. Baumgartner-Parzer SM, Wagner L, Pettermann M, Grillari J, Gessl A, Waldhausl W (1995) High-glucose-triggered apoptosis in cultured endothelial cells. Diabetes 44:1323–1327
6. Lorenzi M, Nordberg JA, Toledo S (1987) High glucose prolongs cell-cycle traversal of cultured human endothelial cells. Diabetes 36:1261–1267
7. Curcio F, Ceriello A (1992) Decreased cultured endothelial cell proliferation in high glucose medium is reversed by antioxidants: new insights on the pathophysiological mechanisms of diabetic vascular complications. In Vitro Cell Dev Biol 28A:787–790
8. Lorenzi M, Cagliero E, Toledo S (1985) Glucose toxicity for human endothelial cells in culture delayed replication, disturbed cell cycle, and accelerated death. Diabetes 34:621–627
9. Ceriello A, Dello RP, Amstad P, Cerutti P (1996) High glucose induces antioxidant enzymes in human endothelial cells in culture: evidence linking hyperglycaemia and oxidative stress. Diabetes 45:471–477
10. Du X, Stocklauser-Farber K, Rosen P (1999) Generation of reactive oxygen intermediates, activation of NF-kappaB, and induction of apoptosis in human endothelial cells by glucose: role of nitric oxide synthase? Free Radic Biol Med 27:752–763
11. Nakao-Hayashi J, Ito H, Kawashima S (1992) An oxidative mechanism is involved in high glucose-induced serum protein modification causing inhibition of endothelial cell proliferation. Atherosclerosis 97:89–95
12. Mutin M, Canavy I, Blann A, Bory M, Sampol J, Dignat-George F (1999) Direct evidence of endothelial injury in acute myocardial infarction and unstable angina by demonstration of circulating endothelial cells. Blood 93:2951–2958
13. Solovey A, Lin Y, Browne P, Choong S, Wayner E, Hebbel RP (1997) Circulating activated endothelial cells in sickle cell anaemia. N Engl J Med 337:1584–1590
14. Romani DW, Fijnheer R, Brinkman HJ, Kersting S, Hene RJ, van Mourik JA (2003) Endothelial cell activation in thrombotic thrombocytopenic purpura (TTP): a prospective analysis. Br J Haematol 123:522–527
15. Camoin-Jau L, Kone-Paut I, Chabrol B, Sampol J, Dignat-George F (2000) Circulating endothelial cells in Behçet's disease with cerebral thrombophlebitis. Thromb Haemost 83:631–632
16. Clancy R, Marder G, Martin V, Belmont HM, Abramson SB, Buyon J (2001) Circulating activated endothelial cells in systemic lupus erythematosus: further evidence for diffuse vasculopathy. Arthritis Rheum 44:1203–1208

17. Percivalle E, Revello MG, Vago L, Morini F, Gerna G (1993) Circulating endothelial giant cells permissive for human cytomegalovirus (HCMV) are detected in disseminated HCMV infections with organ involvement. J Clin Invest 92:663–670
18. Woywodt A, Streiber F, de Groot K, Regelsberger H, Haller H, Haubitz M (2003) Circulating endothelial cells as markers for ANCA-associated small-vessel vasculitis. Lancet 361:206–210
19. George F, Brouqui P, Boffa MC et al (1993) Demonstration of *Rickettsia conorii*-induced endothelial injury in vivo by measuring circulating endothelial cells, thrombomodulin, and von Willebrand factor in patients with Mediterranean spotted fever. Blood 82:2109–2116
20. Abraham NG, Kushida T, McClung J et al (2003) Heme oxygenase-1 attenuates glucose mediated cell growth arrest and apoptosis in human microvessel endothelial cells. Circ Res 93:131–139
21. Dignat-George F, Sampol J (2000) Circulating endothelial cells in vascular disorders: new insights into an old concept. Eur J Haematol 65:215–220
22. Segal MS, Bihorac A, Koc M (2002) Circulating endothelial cells: tea leaves for renal disease. Am J Physiol Renal Physiol 283:F11–F19
23. Bardin N, George F, Mutin M et al (1996) S-endo 1, a pan-endothelial monoclonal antibody recognizing a novel human endothelial antigen. Tissue Antigens 48:531–539
24. Woywodt A, Schroeder M, Guinner W et al (2003) Elevated numbers of circulating endothelial cells in renal transplant recipients. Transplantation 76:1–4
25. Challah M, Nadaud S, Philippe M et al (1997) Circulating and cellular markers of endothelial dysfunction with aging in rats. Am J Physiol 273:H1941–H1948
26. Hill JM, Zalos G, Halcox JPJ et al (2003) Circulating endothelial progenitor cells, vascular function, and cardiovascular risk. N Engl J Med 348:593–600
27. Vasa M, Fichtlscherer S, Aicher A et al (2001) Number and migratory activity of circulating endothelial progenitor cells inversely correlate with risk factors for coronary artery disease. Circ Res 89:E1–E7
28. Katusic ZS (1996) Superoxide anion and endothelial regulation of arterial tone. Free Radic Biol Med 20:443–448
29. Griendling KK, Sorescu D, Ushio-Fukai M (2000) NAD(P)H oxidase: role in cardiovascular biology and disease. Circ Res 86:494–501
30. Bruce CR, Carey AL, Hawley JA, Febbraio MA (2003) Intramuscular heat shock protein 72 and heme oxygenase-1 mRNA are reduced in patients with type 2 diabetes: evidence that insulin resistance is associated with a disturbed antioxidant defense mechanism. Diabetes 52:2338–2345
31. LiVolti G, Seta F, Schwartzman ML, Nasjletti A, Abraham NG (2003) Heme oxygenase attenuates angiotensin II-mediated increase in cyclooxygenase-2 activity in human femoral endothelial cells. Hypertension 41:715–719
32. Abraham NG, Rezzani R, Rodella L et al (2004) Overexpression of human heme oxygenase-1 attenuates endothelial cell sloughing in experimental diabetes. Am J Physiol Heart Circ Physiol 287:H2468-H2477
33. Dimmeler S, Hermann C, Zeiher AM (1998) Apoptosis of endothelial cells contribution to the pathophysiology of atherosclerosis? Eur Cytokine Netw 9:697–698
34. Arcaro G, Cretti A, Balzano S et al (2002) Insulin causes endothelial dysfunction in humans: sites and mechanisms. Circulation 105:576–582