Circulating Vascular Progenitor Cells in patients with Type 1 Diabetes and Microalbuminuria

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**Objective:** Patients with type 1 diabetes mellitus (T1DM) and microalbuminuria are at increased risk of cardiovascular disease (CVD). Abnormalities in vascular progenitor cells, which participate in vascular repair, may be implicated in this susceptibility.

**Research design and methods:** We studied the number and function of vascular progenitor cells in 22 T1DM patients with history of microalbuminuria (MA+) and 22 T1DM patients without history of microalbuminuria (MA-), of similar age, diabetes duration, glycaemic control, renal function, and no history of CVD.

**Results:** MA+ patients had lower circulating CD34+ and CD34+/CD133+ cells number compared to MA- (p<0.006). In *in vitro* functional assays, MA+ patients had significantly lower number of colony-forming units, and impaired VEGF-A mediated tube formation, when compared to MA-patients (p<0.01).

**Conclusions:** In T1DM with microalbuminuria, a marker of microvascular injury and a risk factor for CVD, circulating vascular progenitor cells number is reduced and function is impaired.
The number of circulating endothelial progenitor cells (EPCs) inversely relates to cardiovascular disease (CVD) (1-3); microalbuminuria is one of the earliest manifestations of diabetic nephropathy and a marker of CVD (4). A subset of patients with type-1 diabetes mellitus (T1DM) is susceptible to diabetic nephropathy, a condition characterised by a higher risk of cardiovascular morbidity and mortality (4,5). T1DM patients without complications have lower number of circulating progenitor cells (CPCs) as compared to healthy controls (6,7). To gain insights into the susceptibility to CVD in T1DM, we studied circulating vascular progenitor cells number and function in T1DM patients with and without microalbuminuria.

**RESEARCH DESIGN AND METHODS**

T1DM patients were recruited from Guy’s and St Thomas’ Hospital (London, UK). Patients with microalbuminuria (MA+) had a positive history of early morning urine albumin creatinine ratio (ACR) $\geq 3.5$ mg/mmol (in at least two out of three consecutive measures), were on anti-hypertensive therapy, and had evidence of diabetic retinopathy. The normoalbuminuric (MA-) T1DM patients were defined as patients with $\geq 20$ years diabetes duration, ACR consistently $<3.5$ mg/mmol, on no anti-hypertensive therapy. Exclusion criteria were: history of CVD, non-diabetic renal disease, and renal impairment defined as a serum creatinine $>130\mu$mol/l. The study was approved by the local Ethics Committee.

Blood pressure, measured in the dominant arm with the patient seated after a five-minute rest using an automated sphygmomanometer (Dinamap-8100T, GE-Medical, Slough, UK), was calculated from the mean of three consecutive measurements. Fasting plasma glucose, serum total cholesterol and creatinine were determined using a Cobas-Mira-Plus analyser (Roche-Diagnostics, Basel, Switzerland). Glycated haemoglobin ($\mathrm{HbA_1c}$) was measured by liquid chromatography (Primus-CLC330, Kansas City, USA).

CPCs were investigated as described (3). Leukocytes were studied from peripheral blood after red cells lysis with ammonium chloride buffer. At least 500 CD34$^+$/CD133$^+$ events were collected for each patient and showed more than $1 \times 10^6$ events in the lymphomonocytes gated area. Intra-assay coefficient of variation was $<8\%$. Data are presented as events/$10^6$ lymphomonocytes (Fig.1).

The cell colony forming units-Hill (CFU-Hill) assay was performed as described (2). For the tube formation assay early (7-days) and late (14-days culture) EPCs were studied in the presence and absence of Vascular Endothelial Growth Factor (VEGF)-A (3,8). Cells were characterized by immunofluorescence (supplement material, Fig.1 available at http://care.diabetesjournals.org).

Measurements and data analysis (SPSS-15) were performed blinded to group allocation. Not-normally and normally distributed variables were compared by Kruskall-Wallis and Student’s t-test (2-sided) respectively; when more than two groups were compared, ANOVA (LSD post-hoc test) was used. $p \leq 0.05$ was considered statistically significant.

**RESULTS**

Twenty-two MA+(17M/5F) and 22MA-(13M/9F) subjects of Caucasian origin were studied ($p=0.23$ for gender between groups). There were no differences between the two groups (MA+ vs. MA-) in age, mean$\pm$SD, $50.3\pm11.9$ vs. $49.8\pm6.6$ yrs, diabetes duration $35.2\pm10.2$ vs. $30.9\pm7.7$ yrs, BMI $25.2\pm3.4$ vs.
27.9±6.3 kg/m², HbA1c 8.1±1.6 vs. 8.3±1.3 %, total cholesterol 4.8±0.8 vs. 4.6±0.6 mmol/l, estimated glomerular filtration rate (eGFR-MDRD formula) 79.3±23.6 vs. 86.0±27.8 ml/min, systolic 127.5±15.7 vs. 125.3±9.5 and diastolic blood pressure 74.7±8 vs. 72.1±7.5 mmHg. All patients had evidence of diabetic retinopathy and eGFR>50ml/min.

100% of the MA+ patients were on angiotensin converting enzyme inhibitors (ACE-I) as compared to 0% in MA- (p=0.005). Twenty MA+ patients were on statins as compared to 5MA- (p=0.05); 5MA+ and 4MA- were smokers.

MA+ patients had significantly lower number of CD34+ and CD34+/CD133+ cells when compared MA-; CD34+/KDR+, CD34+/CD133+/KDR+, and CD133+/KDR+ cells number was similar between groups (Fig.1A,B).

The cell colony forming units-Hill (CFU-Hill) assay was conducted in 10MA+(6M/4F) and 8MA-(5M/3F) consecutive patients from the population described comparable for all characteristics. Colony formation was lower in MA+ (MA+ median[interquartile range], 43[35-56] vs. MA- 93[52-103], p=0.01).

Tube formation experiments were conducted in 9MA+(5M/4F) and 7MA-(4M/3F) T1DM consecutive patients as above. In experiments with early EPCs we did not observe differences between groups. In experiments with late EPCs, we observed a VEGF-A mediated increase in tube surface-area only in MA- patients; MA+ patients had a significant lower tube number than MA- (Fig.1C,D).

CONCLUSIONS

We demonstrated a lower number of circulating CD34+ and CD34+/CD133+ cells and CFU-Hill in T1DM/MA+ compared to T1DM/MA- patients. VEGF-A-mediated in vitro tube formation was observed only in cells derived from MA- patients, suggesting impaired vascular repair processes in MA+.

Microalbuminuria is a strong predictor for CVD in long standing T1DM (5), and in our study microalbuminuria associates with low number of CPCs, a recognised marker of CVD.

ACE-I increase the number and function of vascular progenitor cells (9), despite a more prevalent use of these medications in the MA+ group, we still observe a significant lower number of CD34+, CD34+/CD133+, and impaired tube formation in MA+ patients. Conversely the effect of statins on progenitor cells has been controversial (9,10) and this may represent a confounder in our study.

The number of CD34+/KDR+ and CD34+/CD133+ cells is reduced in hypertensive patients (11); normalization of blood pressure with renin-angiotensin system inhibitors is paralleled by normalisation of these cells (11). This suggests that in our T1DM/MA+ population (100% on ACE-I) the observed reduction in CD34+ and CD34+/CD133+ cells is independent of blood pressure. Further we found no correlation between systolic or diastolic blood pressure and CD34+ and CD34+/CD133+ within the MA+ or MA- groups.

Our observations are in line with the described inverse relationship between CD34+ cells and progression of nephropathy in T2DM (12). Indeed significant renal impairment affects progenitor cells number and function (13), however both our groups had relatively preserved and comparable renal function and we did not observe a correlation between progenitor cells number and eGFR.

All our patients had retinopathy; however its severity was not measured. The observation that microalbuminuric diabetic patients have more severe retinopathy (14), a condition paralleled by higher CPCs (15), could have underestimated the observed differences.

In conclusion, in a relatively ‘protected’ population of T1DM patients, with or without...
microalbuminuria, circulating vascular progenitor cells may be a mean by which individuals respond to the vascular risk linked with diabetes and improve their long-term vascular health, while others, unable to respond to insults, are at higher risk for renal and vascular diseases.

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Figure legend

Figure 1: Circulating vascular progenitor cell (A-B) and tube formation assay (C-D) in T1DM patients with and without microalbuminuria.
Circulating CD34+ and CD34+/CD133+ progenitor cells number was lower in MA+ (□) vs. MA- (□)(*p<0.006)(A). No difference was seen in CD34+/CD133+/KDR+, CD34+/KDR+ and CD133+/KDR+ cells (B)(n=22 for MA+; n=22 for MA-, data is presented as median and interquartile range).
In experiments conducted with late endothelial progenitor cultured cells (C-D), surface area occupied by complete tube per field was similar in MA+ and MA- in vehicle treated cells (□). VEGF-A (■) increased tube surface formation only in MA- (MA-/vehicle vs. MA-/VEGF-A, *p=0.016; MA+/VEGF-A vs. MA-/VEGF-A, **p=0.003).
Tube number was similar between vehicle (□) and VEGF-A (■) treated conditions within MA+ and MA- groups. MA+ patients had a significant lower tube number than MA- both in vehicle and VEGF-A treated cells (MA+/vehicle vs. MA-/vehicle, #p=0.02; MA+/VEGF-A vs. MA-/VEGF-A, **p=0.01)(D). All experiments were conducted in triplicate and the average obtained for each patient was used for statistical analysis (n=9 for MA+; n=7 for MA-, data are presented as mean±SD).
REFERENCES

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