Time Course and Mechanisms of Circulating Progenitor Cell Reduction in the Natural History of Type 2 Diabetes Mellitus

ONLINE-ONLY APPENDIX

Corresponding author.
Dr. Gian Paolo Fadini, MD
Email: gianpaolofadini@hotmail.com; or gianpaolo.fadini@unipd.it

RESEARCH DESIGN AND METHODS

Patients. Between January 2004 and December 2007, we performed progenitor cell count in about 1200 individuals. For the present study, we reviewed our case records to select subjects for whom carbohydrate metabolism state and cardiovascular parameters were known. This allowed to identify 425 subjects, divided in: 205 with normal glucose tolerance (NGT), 40 with impaired fasting glucose (IFG), 43 with impaired glucose tolerance (IGT), 32 with new-onset type 2 diabetes, 64 with ≤10 years long-lasting diabetes, 20 with 10-19 years long-lasting diabetes, and 22 with ≥20 years long-lasting diabetes. NGT, IFG, IGT and new onset diabetes were defined on the basis of fasting and 2-hours post-OGTT plasma glucose. IFG was defined as fasting plasma glucose ≥100 mg/dl. IGT was defined as 2 hour post-challenge plasma glucose ≥140 mg/dl. DM was defined as either fasting plasma glucose ≥126 mg/dl, or 2-hours post-OGTT plasma glucose ≥200 mg/dl. None of the patients had acute onset diabetes with uncontrolled hyperglycemia. Known diabetes was categorized according to disease duration using available clinical records. For all subjects, the following data were collected: age, sex, body mass index, smoking habit (of one or more cigarettes per day), systolic and diastolic blood pressure, body mass index (BMI), total cholesterol, HDL-cholesterol, and triglycerides concentrations. fasting plasma glucose, glycated haemoglobin (HbA1c), and prevalent CVD. LDL cholesterol was calculated using Friedewald's formula. CVD was defined as any significant coronary artery disease (history of past myocardial infarction or stable angina, or evidence of hemodynamically relevant coronary atherosclerosis), or cerebrovascular disease (history of stroke or evidence of carotid atherosclerosis with stenosis ≥30%), or peripheral arterial disease (history of claudication, rest pain, revascularization or amputation, or evidence of significant leg artery atherosclerosis). Retinopathy (any stage) was defined by funduscopic examination according to the International Clinical Diabetic Retinopathy Disease Severity Scale (1), or as a history of past laser phototherapy. Chronic renal failure was defined as an estimated glomerular filtration rare <60 ml/min/m^2 according to the MDRD formula. Detailed information about neuropathy were not available, but this was not considered a major shortcoming, as there is no evidence that neuropathy is actually associated with progenitor cell alteration in diabetic patients (2). We also recorded data on medications, with a special focus on drugs that potentially modulate EPCs, such as statins, ACE-inhibitors and angiotensin-II receptor antagonists, and insulin (3). The following exclusion criteria were always applied before selecting patients for progenitor cell count: acute disease or infection, recent (within 3 months) surgical intervention or acute coronary syndrome, immunological disease or immune suppression.

All subjects had progenitor cell count determined. In a subset of 98 individuals (63 NGT, 16 IFG or IGT, 19 DM, see supplemental table III) we also measured plasma VEGF concentrations and, in a selected subset of 34 subjects either NGT (n=17) or diabetic (n=17), we analyzed progenitor cell apoptosis. Clinical characteristics of these subjects are reported in supplemental table IV. In addition, coupled peripheral blood and bone marrow samples were obtained from 20 patients (10 with and 10 without diabetes) undergoing cell therapies at the Department of Internal Medicine III, Wolfgang Goethe University, Frankfurt (Germany). Clinical characteristics of these subjects are reported in supplemental table V. The same exclusion criteria as described above were applied and this substudy was approved by the local Ethical Committee.

Progenitor cell count. Peripheral blood and bone marrow progenitor cells were counted by flow cytometry using antibodies against the stem antigen CD34 and the endothelial antigen KDR. To saturate sites for unspecific binding, before staining with the specific monoclonal antibody, cells were treated with foetal calf serum for 10 min, and then washed with buffer containing phosphate-buffered saline and 0.5% bovine albumin. Then cells were incubated with a fluoroisothiocyanin (FITC)-conjugated anti-human CD34 monoclonal antibody (Becton Dickinson) and with a phycoerythrin-conjugated anti-human KDR monoclonal antibody (R&D Systems). Control isotypes IgG1 and IgG2a Abs were obtained from Becton Dickinson. CD34+ cells were identified in the mononuclear cell fraction as cells with a high specific fluorescence intensity and a low side scatter, in comparison with the negative controls. Dual expression of KDR was then assessed in gated CD34+ cells. In all analyses, 5×10^5 events were acquired, scored using a FACS Calibur Analyser (Becton Dickinson), and processed using the Macintosh CELLQuest software program (Becton Dickinson). Cell count was always expressed per 10^6 events. Reproducibility of CD34+ cell count was good, with a coefficient of variation (CV) in random samples of less than 5%. In a subset of 34 individuals, cells were also stained with a FITC-conjugated Annexin V antibody (BD Pharmingen), which recognizes early apoptotic cells that externalize phosphatidilserine. CD34+ cell apoptosis was assessed as the percentage expression of Annexin V on CD34+ cells. Dead cells were excluded from the analysis.

Venous plasma VEGF concentrations. In a subset of 98 patients, VEGF was measured in venous plasma using a commercially available ELISA kit (R&D Systems) according to manufacturer's instructions.

Statistical analyses. Data are expressed as Mean±SD, unless otherwise specified. Progenitor cell count is always expressed as cell count per 10^6 cytometric events. Normal distribution of progenitor cell counts was verified with the Kolmogorov-Smirnov test. Comparison between two or more groups was performed using Student's t test or ANOVA (with Bonferroni correction), respectively. The Chi square was used for categorical variables. Correlation between two continuous variables was assessed with Pearson's r coefficient. To derive an estimate of progenitor cell variation that was independent of possible confounders, we used non-standardized coefficients from multiple linear regression analyses, in which each category of patients was entered as a dichotomous variable (0 or 1) compared to NGT. To adjust data, we first used a full model (model 1) including age, sex, smoking habit, BMI, systolic and diastolic blood pressure, HDL and LDL cholesterol, triglyceride concentrations, fasting plasma glucose, HbA1c, presence/absence of cardiovascular disease, chronic renal failure and retinopathy, and all medications (all variables listed in table 1, except for total cholesterol). However, given the high number of variables to be controlled for, the uncertainty of the estimate (standard error of rough regression coefficient) was very large. Therefore, to select a limited number of unrelated highly significant variables, we applied a stepwise regression approach, and repeated the analyses controlling only for this parsimonious set of variables (model 2). SPSS ver. 13.0 was used and statistical significance was accepted at $p<0.05$.

REFERENCES

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Supplemental Table I. Variation of circulating CD34+ cell counts in different categories of patients, as compared with NGT. Observed values are reported as absolute variation and % of variation versus NGT patients. Adjusted values are derived from non-standardized B coefficients of multiple linear regression analyses to control for variables included in the model (see Statistical methods), and % of variation is computed versus NGT.

	Observed values			**Adjusted values (model 1)**				**Adjusted values (model 2)**			
	Absolute variation	% variation	p	B	SE	p	% variation	B	SE	p	% variation
IFG	-4.8	-1.1	0.889	-6.8	52.1	0.32	-1.5	-4.8	36.8	0.89	-1.1
IGT	-96.9	-21.7	0.003	-72.1	35.2	0.05	-16.1	-83.2	32.4	0.01	-18.6
DM new	-182.2	-40.8	<0.001	-151.0	62.9	0.007	-33.8	-141.5	54.4	0.01	-31.7
DM < 10 y	-133.1	-29.8	<0.001	-96.8	58.9	0.23	-21.7	-60.5	46.7	0.20	-13.5
DM 10-20 y	-140.9	-31.6	0.001	-131.7	147.0	0.96	-29.5	-93.5	48.3	0.054	-20.1
DM > 20 y	-213.7	-47.9	<0.001	-175.6	144.7	0.89	-39.3	-155.7	87.1	0.07	-34.9

Supplemental Table II. Variation of circulating CD34+KDR+ cell counts in different categories of patients, as compared with NGT. Observed values are reported as absolute variation and % of variation versus NGT patients. Adjusted values are derived from non-standardized B coefficients of multiple linear regression analyses to control for variables included in the model (see Statistical methods), and % of variation is computed versus NGT. Model 2 is adjusted for variables selected by a stepwise multiple logistic procedure (IFG: LDL cholesterol; IGT: sex and LDL; DM new: age and LDL; 0-10 year: LDL, plasma glucose, OHA use; 10-20 years: LDL; >20 years: age and LDL).

	Observed values			**Adjusted values (model 1)**				**Adjusted values (model 2)**			
	Absolute variation	% variation	P	B	SE	p	% variation	B	SE	p	% variation
IFG	-2.3	-3.2	0.797	-8.6	13.7	0.534	-12.0	-3.6	9.7	0.711	-5.0
IGT	+1.4	2.0	0.863	+6.3	12.2	0.603	+8.9	+3.6	11.1	0.745	+5.1
DM new	-27.4	-38.4	0.002	-17.1	22.2	0.442	-23.9	-11.7	12.7	0.358	-16.4
DM < 10 y	-4.8	-6.8	0.491	+19.6	14.6	0.181	+27.4	-14.9	18.9	0.430	-20.9
DM 10-20 y	-26.7	-37.4	0.015	-55.6	39.8	0.164	-77.8	-16.8	11.8	0.158	-23.5
DM > 20 y	-20.7	-29.0	0.046	+3.35	35.4	0.925	+4.7	-10.8	12.1	0.370	-15.2

Supplemental table III. Clinical characteristics of patients assayed for plasma VEGF concentrations. Data expressed as mean ± standard error or as percentage. * significantly different among groups.

Characteristic	NGT (n=63)	Prediabetic (n=16)	Diabetic (n=19)
Age (years)	52.7±2.0	58.6±2.0	65.8±2.1*
Male sex (%)	79	75	100
Smoking (%)	41	31	16
Hypertension (%)	37	56	95*
Dyslipidemia (%)	47	80	89*
Nephropathy (%)	8	13	32*
CVD (%)	59	81	89*
Statins (%)	36	75	79*
Anti-platelet agents (%)	51	81	89*
ACE-I / ARBs (%)	49	94	84*
Beta-blockers (%)	58	81	88
Other anti-hypertensives (%)	29	6	26
Insulin (%)	0	0	31*
Oral antidiabetic drugs (%)	0	0	31*

Supplemental table IV. Clinical characteristics of patients assayed for CD34+ cell apoptosis. Data expressed as mean ± standard error or as percentage. * significantly different versus non diabetic patients.

Characteristic	NGT (n=17)	Diabetic (n=17)
Age (years)	44.7±1.9	63.9±1.9*
Male sex (%)	100.0	70.6
Smoking (%)	11.8	5.9
Hypertension (%)	17.6	35.2
Dyslipidemia (%)	41.1	35.2
Nephropathy (%)	0.0	11.8
Retinopathy (%)	0.0	11.8
CVD (%)	0.0	29.4*
Statins (%)	0.0	64.7*
Anti-platelet agents (%)	0.0	58.8*
ACE-I / ARBs (%)	23.5	76.5*
Other anti-hypertensives (%)	0.0	64.7*
Insulin (%)	0.0	35.3*
Oral antidiabetic drugs (%)	0.0	76.5*

Supplemental table V. Clinical characteristics of patients assayed for coupled bone marrow and peripheral blood progenitor cell counts. Data expressed as mean ± standard error or as percentage. * significantly different versus non diabetic patients.

Characteristic	**Non diabetic (n=10)**	**Diabetic (n=10)**
Age (years)	65.8±2.5	66.4±3.6
Male sex (%)	80	90
Smoking (%)	20	10
Hypertension (%)	50	100*
Dyslipidemia (%)	80	90
Nephropathy (%)	0	30
CVD (%)	80	80
Statins (%)	70	70
Anti-platelet agents (%)	70	90
ACE-I / ARBs (%)	80	90
Beta-blockers (%)	80	80
Other anti-hypertensives (%)	0	30
Insulin (%)	0	40*
Oral antidiabetic drugs (%)	0	40*

Supplemental Figure I. Observed and adjusted variation of circulating CD34+KDR+ cells in patients grouped according to carbohydrate metabolism or diabetes duration, as appropriate. The mean value of NGT patients was taken to represent the zero point. Bars indicate 95% confidence intervals of means (observed values) and estimates (adjusted values). * observed values significantly different versus NGT. † adjusted values significantly different versus NGT. Model 1 (A) and model 2 (B) refers to the strategy used to control for confounders (see statistical methods).