Endothelial progenitor cell dysfunction in diabetes mellitus

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Chapter 6

Endothelial Progenitor Cell Dysfunction in Hyperglycemia originates in Myeloid Precursor Cells in the Bone Marrow

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Abstract

Bone marrow (BM)-derived endothelial progenitor cells (EPC) contribute to endothelial maintenance and repair. Risk factors for ischemic vascular disease such as diabetes mellitus not only affect the mature endothelium, but also lead to EPC dysfunction. To explore the impact of hyperglycemia on the differentiation of EPC from bone marrow progenitor cells, mice were maintained hyperglycemic for 6 weeks. Cultures generating EPC, macrophages (Mph) or dendritic cells (DC) from hyperglycemic BM yielded 40% fewer EPC and 50% more Mph compared to control bone marrow. These changes were directly related to the HbA1C levels of the donor mice. In contrast, BM-derived DC numbers were not affected by hyperglycemia. The composition of the BM was not altered; in particular the numbers of $CD31^{+}/Ly6C^{+}$ cells, which serve as common progenitors for EPC, Mph and DC were unaffected. In addition to their lower quantity, BM-derived EPC from hyperglycemic mice were less angiogenic and more pro-inflammatory in terms of endocytosis, T-cell activation and IL-12 production. HMGCoA reductase inhibition by statin supplementation of the culture medium counteracted these hyperglycemia-induced changes. Our data indicate that EPC dysfunction in diabetes is BM-derived, due to hyperglycemia-induced alteration of myeloid progenitor cell differentiation.

Introduction

Loss of endothelial integrity and an impaired capacity for ischemia-induced neovascularization leads to ischemic vascular disease in diabetes[1-3]. A recently identified risk factor for these vascular complications is dysfunction of bone marrow (BM)-derived endothelial progenitor cells (EPC). Under normal circumstances, EPC contribute to vascular homeostasis by replacing apoptotic or lost endothelial cells[4,5]. In addition, EPC can home to sites of denudation[6], ischemia or elevated shear stress to stimulate neovascularisation or arteriogenesis[7,8]. In patients with type 1[9] and 2 diabetes[10] and in diabetic animals[11-13], the number of circulating EPC is decreased and functional parameters such as adhesion, migration and the paracrine secretion of proangiogenic factors are impaired, likely as a consequence of hyperglycemia. Moreover, a recent study showed that the number of circulating EPC inversely correlates with the severity of peripheral vascular complications of patients with type 2 diabetes further supporting a role for EPC dysfunction in the pathogenesis of ischemic vascular disease[14]. Following these notions, EPC are increasingly recognized as a potential therapeutic target for the prevention of ischemic vascular disease. However, the molecular mechanisms underlying EPC dysfunction in diabetes are complex and may include reduced cell survival[11,15] due to an increased sensitivity to oxidative stress[16] or activation of a p53-dependent pathway that leads to cellular senescence[16,17].

Human EPC can be derived from either $CD34^+$, $CD34^-$ or $CD34^{low}$ cells and can be cultured from BM aspirates and peripheral blood $CD14^+$ mononuclear cell fractions[18-21]. This heterogeneity in precursor cells likely reflects the different stages of EPC as they mature from the early bone marrow-derived stem cells towards mature vascular endothelial cells.

Many studies have shown that cells of the myeloid lineage display high plasticity and that seemingly “lineage-committed” myeloid cells, given the appropriate conditions, can differentiate into cells of another lineage with different functional properties[22]. For example, in the presence of inflammatory cytokines, the normal differentiation of monocytes into macrophages (Mph) can be skewed to yield dendritic cells (DC)[23]. We recently demonstrated that angiogenic EPC develop from an immature, $CD31^+$/Ly-$6C^+$ myeloid progenitor fraction in mouse BM[24]. Upon stimulation by the proper differentiation factors, Mph and DC are also derived from this fraction[25,26], suggesting a common myeloid progenitor pool for EPC, Mph, granulocytes, monocytes and DC.

As diabetes is increasingly appreciated as a systemic pro-inflammatory state[27,28] that might affect cell-fate specification or developmental decisions, we here investigated the differentiation of EPC, Mph and DC from BM-derived myeloid progenitor cells of hyperglycemic and normoglycemic mice. Our results support the hypothesis that EPC dysfunction in diabetes is bone marrow-derived due to hyperglycemia-induced alterations in differentiation of myeloid progenitor cells.

Materials and methods

Animals

Moderate to severe hyperglycemia was induced in 6 week old C57BL/6J and FVB/N mice (Harlan, Horst, The Netherlands) by intraperitoneal (IP) injections of streptozotocin (STZ, 80 mg/kg body weight; Sigma-Aldrich) in 0.05 mol/l Na-Citrate buffer pH 4.5, on two consecutive days. Blood glucose levels were examined by glucose-oxidase technique (OneTouch Ultra system, Lifescan) 1 week after STZ injection and mice with blood glucose levels below 20 mmol/l mice received two additional injections on two consecutive days. When necessary this procedure was repeated a third time. STZ treatment was stopped when blood glucose levels above 20 mmol/l were reached and glucose levels and total body weight were monitored weekly for a period of 6 weeks. Control mice were injected with buffer only at the onset of the experiments. Mice from the STZ treatment groups that showed no elevated blood glucose levels (below 15 mmol/l) were excluded from the study. All procedures involving animal handling were in accordance to national and institutional guidelines of the Erasmus MC, Rotterdam, The Netherlands.

Glycosylated hemoglobin

Glycosylated hemoglobin (HbA1C) levels were measured by High Performance Liquid Chromatography (HPLC) using a reverse-phase cation exchange column and detected by a dual wavelength colorimetric (415 and 500 nm) analyzer (ADAMS A1c HA8160; Arkray).

Isolation and differentiation of murine EPC, DC and Mph

BM-cell suspensions were prepared by flushing femora and tibiae with RPMI medium (Gibco) supplemented with 10% FCS (Gibco) and antibiotics (penicillin/streptomycin, Gibco). To culture EPC, total BM cell suspensions were plated at a density of 1.25 x 10^6

cells per cm^2 in 24-well plates (Nunc) coated with fibronectin (10 µg/ml, Sigma). Cells were cultured for 7 days in M199 medium supplemented with 20% FBS (Invitrogen), 0.05 mg/ml Bovine Pituitary Extract (Invitrogen), antibiotics, and 10 units/ml heparin (Leo Pharma BV).

For Mph and DC, 2 $x10^6$ BM cells were cultured in complete RPMI medium described above and plated on 10 cm Petri dishes (BD Biosciences). Medium was replaced every two days. For DC and Mph cultures, the medium was supplemented with 20 ng/ml recombinant murine GM-CSF (Biosource) and 10 ng/ml recombinant murine M-CSF (Peprotech), respectively. Atorvastatin (Pfizer) was dissolved in ethanol and, in indicated experiments, added to the medium to a final concentration of 0.1 µmol/l. Medium used in the control experiments contained ethanol at a similar final concentration ($5x10^{-5}$ %) as present in the cultures treated with atorvastatin.

Monoclonal antibodies, conjugates and flow-cytrometric analysis

Undiluted culture supernatant of the hybridoma F4/80 was directly used for staining. ER-MP12/CD31 was purified and biotinylated and ER-MP20/Ly-6C was conjugated to FITC[25,29]. Phycoerythrin-(PE-) labeled CD11c and biotinylated anti-MHC class II antibodies were purchased from BD Biosciences. Secondary FITC- or R-PE-labeled goat anti-rat IgG antibodies (mouse-absorbed; GαR-FITC or GαR-PE) were purchased from Caltag Laboratories. Biotinylated antibodies were detected with allophycocyanin-conjugated streptavidin (BD Biosciences). For EPC characterization, cells were stained with rhodamine-labeled Bandeiraea simplicifolia lectin (10µg/ml, BSL-1, Vector Labs) for 1 h. To measure the uptake of Dil-labeled acetylated LDL (acLDL, Molecular Probes), cells were incubated with acLDL (2.4 µg/ml) at 37°C for 2h. Flowcytometric analyses were done on a Calibur flowcytometer (BD Biosciences) using CellQuest software (BD Biosciences).

Cytokine detection

For cytokine measurements EPC were incubated with 50 ng/ml lipopolysaccharide (LPS, Sigma) overnight at 37°C. Culture supernatants were collected and cells were counted for normalization. IL-12p40 and IL-12p70 ELISA kits (R&D) were used according to the manufacturer's protocol.

MLR assay

Mixed leukocyte reactions (MLR) were done with allogeneic T cells from C57BL/6J or FVB/N splenocytes. Cells were incubated with antibodies recognizing CD11b, CD45 and MHCII and anti-rat IgG microbeads. Naive T cells were obtained by negative selection using a magnetic cell sorter. DC, Mph and EPC were irradiated sub-lethally. T cells (1.5 x 10^5 cells/well) were added to varying concentrations of stimulator cells depending on the desired stimulator-responder cell ratio. Proliferation of T cells was measured after 4 days by uptake of ^{3}H-thymidine (1 μCi/well, DuPont-NEN) and measured as counts per minute (cpm).

In vitro angiogenesis assay.

Conditioned media were obtained by replacing the medium of 6-day EPC cultures with serum-free EC basal medium-2 (Clonetics) supplemented with EGM-2 single aliquots (without vascular endothelial growth factor and basic fibroblast growth factor) and culturing the cells for an additional 20 h. EPC were counted and conditioned media were diluted to correct for cell numbers. After 14 h, tube formation by HUVECs was measured by staining the viable cells with Calcein-AM (5 μg/ml, Molecular Probes). HUVEC cultures were isolated as described[30] and used at passage 3 or less. For quantification, total tube area was determined using images obtained with an inverted fluorescence microscope and Scion Imaging software (Scion Corporation) and expressed in arbitrary units of tube length.

Endocytosis assay

Uptake of dextran-FITC was determined for 30 min, both at 4°C and 37°C. Subsequently, the cells were washed three times with PBS/BSA1% and total uptake of FITC label was measured by FACS analyses and expressed by the difference in geometric mean that resulted from subtracting the values obtained at 4°C from the values obtained at 37°C.

Statistical analysis

Differences between treatment groups were analyzed Student's *t* test. Results are expressed as mean ± SD. Linear regression analyses and Pearson correlation were used for comparison of the number of EPC/Mph and HbA1c. Probability values of $P < 0.05$ were considered statistically significant.

Results

Hyperglycemia induction in mice

In both mouse strains used (C57BL/6J and FVB/N), STZ treatment increased blood glucose levels up to 3.5 fold, associated with increased HbA1C levels (Table 1). Body weight was 25-30% reduced in hyperglycemic mice of both strains when compared to normoglycemic control animals. Total BM cell isolations from tibiae and femora yielded an equal number of cells in hyperglycemic and control C57BL/6J mice whereas in FVB/N mice the hyperglycemic state was associated with a minor 1.3 fold increase in the number of BM-derived cells.

	C57BL/6J Control	C57BL/6J STZ	FVB/N Control	FVB/N STZ
n	18	26	17	23
Blood glucose (mmol/l)	6.7 ± 2.0	24.1 ± 5.5 *	9.1 ± 1.8	27.8 ± 4.5 *
HbA1c %	3.9 ± 0.2	7.6 ± 0.7 *	4.1 ± 0.5	5.9 ± 0.5 *
Weight (gram)	28.5 ± 2.2	20.0 ± 1.2 *	30.5 ± 2.3	22.5 ± 2.9 *
Bone marrow cells $x10^8$	0.8 ± 0.3	0.8 ± 0.2	0.7 ± 0.2	0.9 ± 0.3

Table 1. Mouse characteristics.

Parameters were measured after 6 weeks of STZ-induced hyperglycemia or in littermate control mice matched for age and gender that were injected with buffer. Values represent mean ± SD, *: $P < 0.05$ v.s. buffer.

Hyperglycemia differentially alters the potential of BM cells to yield EPC, Mph and DC

To determine the effects of hyperglycemia on the potential of BM progenitors to generate EPC, total BM cells were harvested from hyperglycemic and control mice and equal numbers of cells were cultured for 7 days to differentiate into EPC. We previously provided evidence that EPC, Mph and DC may share a common myeloid progenitor pool in the BM[24]. Therefore, the potential of the BM cells to differentiate into $F480^+$ Mph and $CD11c^+$ DC, in M-CSF- and GM-CSF-stimulated cultures, respectively, was assessed in parallel to the EPC cultures. In EPC cultures from BM of hyperglycemic mice from both strains we observed on average a 40% reduction in the number of EPC, identified as cells positive for

CD31 and acLDL (Fig. 1A). In contrast, the number of Mph was approximately 50% increased. No differences were observed in the yield of DC in cultures from both strains of mice. Figure 1B shows that the number of EPC obtained inversely correlated with peripheral blood HbA1c levels of individual mice, indicating that the observed decrease in EPC numbers directly relates to the degree of hyperglycemia. In contrast, the number of Mph showed a positive correlation with the degree of hyperglycemia whereas the varying numbers of DC derived from the cultures showed no relationship with HbA1c levels. Taken

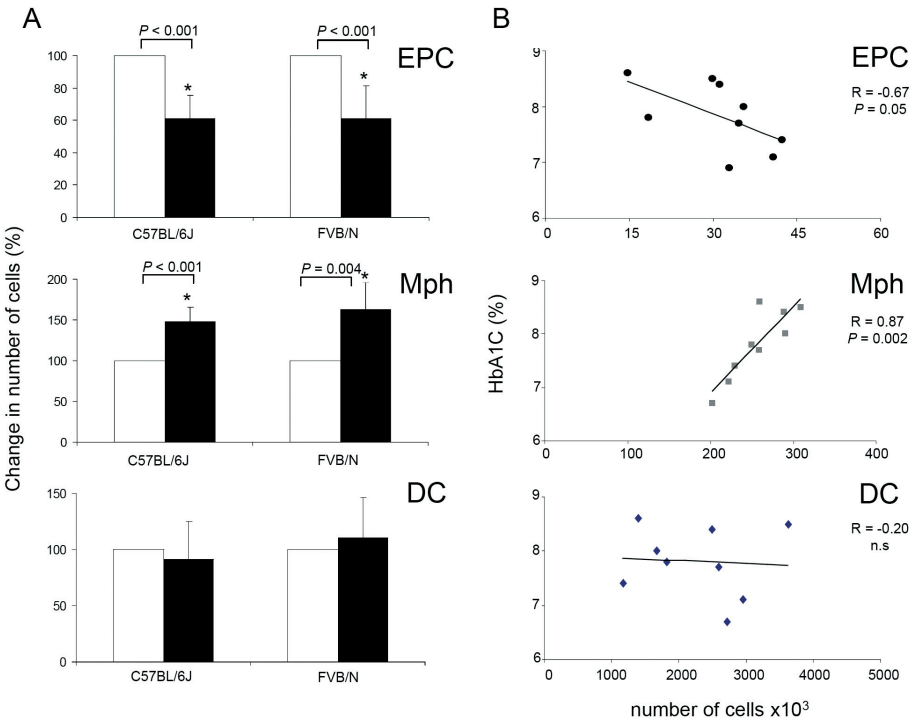
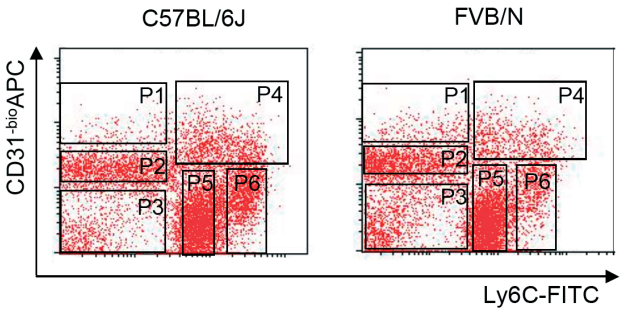


Figure 1. Hyperglycemia alters the differentiation potential of myeloid progenitors in BM.
(A) Relative change in the number of bone marrow-derived EPC, Mph and DC from STZ-treated mice (black bars) compared to control mice (white bars). Values for EPC, Mph and DC derived from BM from mice treated with buffer are set to 100%. Top panel: number of $CD31^{high}$ /Dil-acLDL-positive, attaching cells in EPC cultures (C57BL/6J; 4 experiments, n_{buffer} = 18 and n_{STZ} = 26 and FVB/N; 5 experiments, n_{buffer}= 17 and n_{STZ} = 23). Middle panel: the number of F4/80 positive cells in M-CSF-stimulated Mph cultures (C57BL/6J; 3 experiments, n_{buffer} = 13 and n_{STZ} = 18 and FVB/N; 3 experiments, n_{buffer} = 13 and n_{STZ} = 16). Bottom panel: number of CD11c positive cells in GM-CSF-stimulated dendritic cell cultures (C57BL/6J; 3 experiments, n_{buffer} = 13 and n_{STZ} = 18 and FVB/N; 3 experiments, n_{buffer} = 13 and n_{STZ} = 16). (B) Correlations between the number of cultured EPC (top panel), Mph (middle panel) and DC (bottom panel) and hyperglycemia as assessed by HbA1c. Representative experiment using BM from hyperglycemic C57BL/6J mice (n = 9).

together, hyperglycemia affects myeloid progenitor cells in BM and alters their potential to differentiate into different myeloid lineages.

Hyperglycemia has no major quantitative impact on the subpopulation composition of myeloid progenitors of the BM

To evaluate if the observed altered number of bone marrow-derived EPC and Mph from hyperglycemic mice was due to quantitative shifts in myeloid progenitor fractions of the BM, total BM was immunostained using ER-MP12 (anti-mouse CD31) and ER-MP20 (anti-mouse Ly-6C) and analyzed by flowcytometry. Using this combination of markers[25], 6 distinct subpopulations of BM cells can be identified, each with varying degrees of lineage commitment and progenitor potential (Figure 2). The populations consist of: (P1) 70% blast



	C57BL/6J Change %	FVB/N Change %
$CD31^{high}/Ly6C^{low}$ (P1)	112 ± 34	104 ± 62
$CD31^{med}/Ly6C^{low}$ (P2)	58 ± 10*	57 ± 30*
$CD31^{low}/Ly6C^{low}$ (P3)	108 ± 18	104 ± 16
$CD31^{high}/Ly6C^{high}$ (P4)	128 ± 54	121 ± 56
$CD31^{low}/Ly6C^{med}$ (P5)	148 ± 63	104 ± 35
$CD31^{low}/Ly6C^{high}$ (P6)	112 ± 95	129 ± 98

Figure 2. Hyperglycemia has no major quantitative impact on the myeloid progenitor fractions of the BM.

BM of the control and hyperglycemic mice was harvested and immediately stained with anti-CD31 and anti-Ly-6C. Using this combination of markers, 6 distinct populations can be identified (P1-P6). For each subpopulation, the average increase or decrease in BM cell fractions from hyperglycemic mice is depicted as the percentage change relative to nondiabetic mice. In the top panel representative scatter plots from hyperglycemic BM are shown for each strain. For both strains the average percentage and standard deviations were calculated from 3 experiments (C57BL/6J: $n_{buffer} = 15$ and $n_{STZ} = 18$ and FVB/N; $n_{buffer} = 13$ and $n_{STZ} = 14$, $*P < 0.001$)

cells and 25% lymphoid cells; (P2) lymphoid cells; (P3) erythroid cells; (P4) myeloid progenitors and plasmacytoid cells; (P5) granulocytes; (P6) 75% monocytes and 20% monocyte progenitors[25]. As previously shown, Mph and DC can originate from $CD31^{high}$/$Ly6C^{low}$(P1), $CD31^{high}$/$Ly6C^{high}$(P4) and $CD31^{low}$/$Ly6C^{high}$(P6) fractions[25,26]. However, short term cultured EPC are mainly derived from the $CD31^{high}$/$Ly6C^{high}$ fraction[24]. When comparing the BM myeloid cell subpopulations from hyperglycemic and control mice, we found no significant quantitative differences in the three fractions from which EPC, Mph and DC can be derived in short term cultures (Figure 2). The only subpopulation that was altered was the $CD31^{med}$/$Ly6C^{low}$ lymphoid and HSC fraction (P2) that was 40% decreased in the BM of hyperglycemic mice of both strains. In line with the observation that the total cell number that can be harvested from the BM of these mice is not, or only marginally changed (Table 1), we conclude that hyperglycemia does not affect the quantitative composition of the myeloid progenitor subpopulations in the BM, but rather alters their differentiation potential.

BM-derived EPC from hyperglycemic mice display a pro-inflammatory phenotype

To investigate whether functional properties of EPC cultured from hyperglycemic BM are altered in comparison to those from control BM, we compared their angiogenic and pro-inflammatory potentials (Table 2). We first determined the angiogenic properties of EPC-conditioned medium in an *in vitro* angiogenesis model. When HUVEC were seeded on matrigel and maintained for 14 h in conditioned medium of EPC derived from control C57BL/6J and FVB/N mice, tube formation was markedly stimulated when compared to non-conditioned medium (non-conditioned media 0.37 ± 0.13 A.U. of tube formation (n = 3) $P = 0.04$ and $P < 0.001$ respectively). Conditioned media of EPC from FVB/N mice induced more capillary formation than EPC-conditioned media of C57BL/6J mice ($P = 0.01$). In contrast, conditioned media of EPC from hyperglycemic mice supported tube formation by only 43% (C57BL/6J, $P = 0.03$) and 35 % (FVB/N, $P = 0.02$) compared to the control values (Table 2). Next we examined to what extent the different EPC display pro-inflammatory properties that are typical for myeloid antigen-presenting cells like the ability to endocytose, the capacity to activate T cell proliferation and the expression of pro-inflammatory cytokines. Table 2 shows that EPC from hyperglycemic mice displayed a trend towards a higher capacity to endocytose large dextran molecules, when compared to EPC from normoglycemic mice. C57BL/6J EPC show a higher capacity to endocytose when compared to FVB/N mice ($P < 0.001$). To assess whether the capacity of Mph derived

	C57BL/6J Control (n)	C57BL/6J STZ (n)	*P* value	FVB/N Control (n)	FVB/N STZ (n)	*P* value
Angiogenesis length tubes (A.U.)	0.47 ± 0.17 (8)	0.27 ± 0.12 (8)	0.03 *	0.77 ± 0.19 (12)	0.50 ± 0.20 (12)	0.02*
Endocytosis EPC Δ geo mean	63.6 ± 18.0 (9)	78.8 ± 21.2 (15)	0.12	20.7± 17.9 (8)	42.3 ± 15.2 (8)	0.09
Endocytosis Mph Δ geo mean	47.0 ± 8.6 (3)	62.7 ± 8.0 (3)	0.13	78.3 ± 7.6 (3)	68.8 ± 11.4 (3)	0.28
MLR ^{3}H-thymidine incorporation (%)	100 (9)	176 ± 71 (15)	0.15	100 (6)	139 ± 27 (8)	0.08
IL-12p40 concentration (pg/ml)	26.9 ± 13.2 (8)	64.4 ± 19.3 (8)	0.001*	29.3 ± 13.5 (8)	56.6 ± 24.3 (8)	0.02*
IL-12p70 concentration (pg/ml)	78.4 ± 28.1 (8)	155 ± 52.5 (8)	0.006*	62.7 ± 44.3 (8)	262 ± 63.1 (8)	< 0.001*

Table 2. Angiogenic and pro-inflammatory properties.
Functional analyses of EPC and Mph cultured for 7 days from control and hyperglycemic BM. Listed are the capacity of EPC-conditioned media to stimulate *in vitro* formation of tubular structures by HUVEC (n = 8, each group), the capacity of EPC and Mph to endocytose large dextran molecules labeled with FITC as measured by FACS analyses and expressed as the delta geo mean (Δ), the capacity of EPC to stimulate T cell proliferation as assessed by allogeneic MLR (determined by measuring ^{3}H-thymidine incorporation and expressed in percentage compared to EPC from controls), and the expression of the pro-inflammatory cytokine IL-12p40 and IL-12 p70 production by EPC after LPS stimulation (n = 8 per group).

from hyperglycemic mice to endocytose was altered, Mph were cultured from hyperglycemic and control mice. Mph derived from hyperglycemic BM from C57BL6/J mice showed a trend towards a higher capacity to take up large dextran molecules, while FVB/N-derived Mph showed a small but not significant decrease when derived from hyperglycemic BM.

When the EPC fractions were tested for their capacity to stimulate T-cell proliferation in mixed lymphocyte reaction assays (MLR), again for both strains we observed a trend towards a higher inflammatory capacity of the EPC from hyperglycemic BM. This was supported by our findings on the ability of the cells to produce IL-12 upon LPS stimulation IL-12p70, the active form of the pro-inflammatory IL-12, can regulate T cell-mediated immune responses by promoting Th1 development and we have previously shown that it is the predominant IL-12 subtype produced by EPC[24]. As shown in table 2, IL-12p70 levels were elevated in the conditioned medium of EPC from hyperglycemic mice, ranging from 2.3 fold for C57BL/6J mice ($P = 0.006$) up to 4 fold for FVB/N mice ($P < 0.001$). The IL-12p40 subunit was also about 2 fold higher in supernatants from EPC derived from hyperglycemic mice as compared to the supernatants of control EPC (C57BL/6J mice ($P = 0.001$), FVB/N mice ($P = 0.02$). Because elevated inflammatory properties were observed under hyperglycemic conditions, BM-derived EPC were further phenotypically

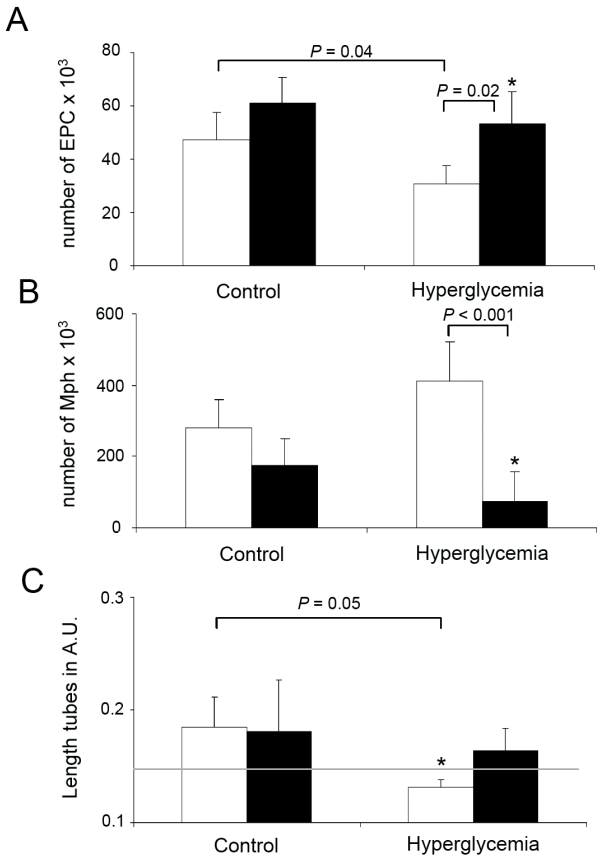


Figure 3. Atorvastatin reverses hyperglycemia-induced changes in progenitor commitment and EPC function.

Number of EPC (A) and Mph (B) cultured from BM of control and hyperglycemic mice (n = 6 per group). (C) Tube formation in arbitrary units (AU) in response to medium conditioned by EPC from control and hyperglycemic mice (n = 6 per group) cultured in the absence (white bars) or presence of atorvastatine (black bars).

characterized by flow cytometry analyses. However, when EPC from hyperglycemic and control mice were stained with either anti-F480 or anti-CD11c antibodies, to reveal a more Mph- or DC-like phenotype, no significant differences could be observed (data not shown). Taken together, EPC derived from hyperglycemic mice have a reduced angiogenic capacity but are more pro-inflammatory as they secrete more IL-12 and they show a trend for an elevated capability to endocytose exogenous material and to stimulate naïve T cells.

Statin treatment in vitro restores affected progenitor commitment and EPC function of hyperglycemic mice

HMG-CoA reductase inhibitors have been shown to be potent anti-inflammatory agents[31]. Moreover, they elevate EPC numbers *in vitro*[32] and *in vivo*[33] and interfere with the differentiation of monocytes towards Mph[34,35]. Therefore, in separate experiments, we explored the effect of atorvastatin on the hyperglycemia-induced effects on BM-derived EPC and Mph. BM cells of hyper- and normoglycemic C57BL/6J mice were cultured for 7 days to obtain EPC and Mph. As expected, lower numbers of EPC were derived from BM of hyperglycemic mice ($P = 0.04$). When atorvastatin (0.1 μmol/l) was present in EPC cultures from hyperglycemic mouse BM, the number of EPC was normalized to levels obtained from control mice (Figure 3A). Conversely, the number of Mph generated in M-CSF stimulated cultures of both hyperglycemic and control BM was markedly reduced in the presence of atorvastatin ($P < 0.001$; Figure 3B).

To evaluate the effect of atorvastatin on EPC function, an *in vitro* angiogenesis assay was performed with conditioned medium of EPC cultured in the presence of atorvastatin. As shown in figure 3C, we again observed significant reduction in tube formation when HUVEC were subjected to conditioned media of EPC derived from hyperglycemic mice ($P = 0.05$). This loss in paracrine angiogenic capacity was attenuated when the EPC from hyperglycemic mice were cultured in the presence of atorvastatin.

Discussion

Diabetes can be envisioned as a disease characterized by a chronic systemic inflammatory state disturbing the function of multiple vital body systems, in particular the vasculature[27,28]. We hypothesized that hampered differentiation of EPC from myeloid progenitor cells in the BM might contribute significantly to the suboptimal endothelial repair under hyperglycemic conditions. Indeed, we observed a marked 40% reduction in the number of EPC that could be cultured from BM of mice that were made hyperglycemic by streptozotocin treatment. Similar to what we previously observed for the number of EPC that could be cultured from PB-MNC from patients with type 1 diabetes[9], the observed reduction was inversely correlated with HbA1C levels suggesting a causal role for hyperglycemia. In addition, EPC from hyperglycemic murine BM displayed impaired angiogenic activity. Again, EPC from patients with type 1 diabetes are likewise impaired in

angiogenicity[9]. Our findings confirm that EPC-mediated neovascularization may be impaired in diabetes due to a reduction in number and angiogenic capacity of the circulating EPC, caused by their defective development from myeloid BM progenitors. Indeed, others have shown that when progenitor cells derived from a hyperglycemic background are transplanted into mice they fail to augment ischemia-induced neovascularization[12,13] and have decreased capacity for re-endothelialization following arterial injury[36]. However, our results also demonstrate that EPC derived from hyperglycemic BM display a pro-inflammatory phenotype as their capacities to endocytose, to activate T-cells and to produce IL-12 are increased. By mRNA expression profiling we recently demonstrated that human EPC from patients with type 1 diabetes over express numerous pro-inflammatory genes known to be associated with hyperglycemia and oxidative stress, including osteopontin, plasminogen activator type 1, lectin-like oxidized LDL receptor, thrombomodulin and type IV collagen[37]. Together these data suggest that when EPC from a diabetic background home to sites of ischemia or vascular injury, the pro-inflammatory nature of these cells may contribute to an adverse (immune) response that may be pro-atherogenic or contribute to the formation of neointima. EPC dysfunction was previously proposed to explain the pro-atherogenic nature of transplanted BM-MNC from apolipoprotein E-knockout mice[38] and may limit the direct use of autologous progenitor cell transplantation for therapeutic angiogenesis in patients with ischemic vascular disease[39,40]. Surprisingly, the reduction in the number of EPC obtained from hyperglycemic BM was associated with a concomitant increase in the ability of these BM cells to generate Mph while the number of DC that could be obtained upon appropriate stimulation was not changed. We have previously shown that Mph and DC can be derived from common myeloid progenitor cell subsets in murine BM[25,26], and recent findings by Fogg *et al*. confirm this notion as they indicate that Mph and DC share a common BM progenitor[41]. Here, we observed that the frequency of the different myeloid progenitor subsets in BM was not changed by hyperglycemia. Therefore, we propose that myeloid lineage differentiation potential of the BM progenitors was skewed as a consequence of the hyperglycemic state. Our observations suggest that inflammatory cytokines or elevated redox signaling may directly regulate the differentiation of various myeloid lineages from progenitor cells. NF-κB is a major transcription factor responsive to cytokines and reactive oxidant species. Indeed, recent studies indicate that NF- κB, acting via IRF4, directly influences DC vs. macrophage differentiation[42].

Recently we demonstrated that EPC, Mph and DC show a significant phenotypic overlap and that a major source of EPC from the BM is the $CD31^{high}/Ly6C^{high}$ fraction that predominantly contains myeloid progenitor cells that can also generate Mph and DC[24]. This myeloid origin of EPC is consistent with the observation that $CD34^{low}CD14^{+}$ cells in peripheral blood are a major source of human EPC[21]. Assuming that the EPC, the Mph and the DC all originate from a common myeloid progenitor, we speculate that the pro-inflammatory milieu in diabetes not only skews the differentiation of the myeloid progenitor cell towards the Mph but that it does so at the cost of the generation of EPC. Several recent studies support this concept of heterogeneity of myeloid cells in response to environmental stimuli. In mice, diet-induced obesity was shown to induce a phenotypic switch of the adipose tissue macrophages from an anti-inflammatory towards a pro-inflammatory state[43]. Another study showed that a subfraction of circulating $Ly6C^{high}$ monocytes was selectively and progressively expanded under hypercholesterolemic conditions. This subfraction, compared to $Ly6C^{low}$ monocytes, displayed a highly pro-inflammatory and atherogenic phenotype[44]. As pointed out before, short-term cultured BM-derived EPC are mainly derived from a myeloid cell fraction with high levels of Ly6C. As EPC display a higher pro-inflammatory phenotype under hyperglycemic conditions, their possible role in progression of atherogenesis in this metabolically altered environment needs to be further explored. Our data imply that hyperglycemia-dependent alterations on cell-fate specification are not restricted to embryonic development[45] but may also affect the differentiation of adult progenitor cells. The ultimate proof of this concept may involve the use of techniques that monitor clonal expansion of the myeloid progenitor cells and HSC-like cells and are the subject of current investigations.

HMG-CoA reductase inhibitors, statins, have been shown to increase the number of circulating EPC both in animal models[32,33] as well as in patients with stable coronary artery disease[46]. Also, statins generate potent anti-inflammatory actions[31] and can improve properties of dysfunctional EPC populations *in vitro*[4,33,46]. In addition, statins appear to modulate the *in vitro* differentiation of monocytes to Mph[35]. Therefore, we assessed whether the addition of atorvastatin, *in vitro*, could reverse diabetes-associated changes in the EPC and Mph cultures. Indeed, we observed that atorvastatin restored EPC differentiation to control levels, and also strongly decreased the elevated number of Mph generated in the BM cultures from hyperglycemic mice. Also the angiogenic capacity of the EPC derived from hyperglycemic BM was partially recovered when cells were cultured in the presence of atorvastatin.

In conclusion, our study shows that EPC dysfunction in diabetes stems from the BM due to hyperglycemia-induced alteration in differentiation of myeloid progenitor cells. The inflammatory nature of EPC in hyperglycemia may not only impair EPC function but potentially also contributes to premature atherosclerosis when these cells incorporate into the vessel walls. Our observation that statins can, at least in part, counteract these effects may not only provide a helpful tool to elucidate the molecular mechanism underlying EPC dysfunction but may also contribute to the beneficial effect of statin therapy on the circulating levels of EPC in patients.

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