Endothelial Progenitor Cells and the Diabetic Paradox

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An unexplained paradox puzzles diabetologists: diabetic patients must face both poor vessel growth in ischemic heart and limbs and increased angiogenesis in retinal complications (1,2).

Endothelial progenitor cells (EPCs) are marrow-derived cells involved in adult neovascularization and endothelial homeostasis (3,4). It has been postulated that low EPCs in peripheral blood may have a role in cardiovascular disease, and we have demonstrated that EPCs are reduced in macrovascular diabetes complications (5,6). On the other hand, an excess of EPCs may be involved in pathologic neoangiogenesis of cancer and proliferative retinopathy (7,8). Therefore, diabetes complications may be associated with both decreased and increased EPCs. Recently, novel therapeutic approaches have been directed to enrich the EPC pool in ischemic diseases and to block EPC function in proliferative diseases (9,10). These approaches in diabetic subjects require cautious evaluation of the implications carried by the paradox and new studies to unravel its causes (11,12). This study was carried out to investigate the contemporaneous effects of retinal and peripheral vascular complications on circulating progenitor cells.

RESEARCH DESIGN AND METHODS

— Ethics committee approval was obtained, and after giving informed consent, 60 type 2 diabetic patients were prospectively included. Patients were characterized in terms of peripheral arterial disease (PAD) and diabetic retinopathy (DR) as the most representative complications at the two extremities of the diabetic paradox. PAD was diagnosed by minimal criteria (including history of claudication or rest pain, pulse examination, ankle-brachial indexes, and ultrasonography) and eventually confirmed by angiography. Diabetic retinopathy (severe nonproliferative or proliferative) was defined by a dilated and comprehensive eye examination and acquisition of high-quality stereoscopic photographs by an ophthalmologist, eventually confirmed by fluorangiography. Patients were divided into the following groups: DR^-PAD^- ($n = 15$), DR^-PAD^+ ($n = 30$), DR^+PAD^- ($n = 5$), and DR^+PAD^+ ($n = 10$).

Peripheral blood progenitor cells were analyzed for the expression of surface antigens with direct two-color flow cytometry using fluorescein isothiocyanate–conjugated anti-human CD34 mAb (Becton Dickinson) and phycoethrin-conjugated anti-human kinase insert domain receptor mAb (R&D Systems). The frequencies of positive cells were determined by a two-dimensional side-scatter fluorescence dot-plot analysis of the samples using a FACScan analyzer and the Macintosh CELLQuest software (Becton Dickinson).

For culture of EPCs, mononuclear cells were separated from peripheral blood of five DR^-PAD^+ and five DR^+PAD^- subjects using Ficoll (Sigma) and plated on fibronectin-coated dishes. Cells were grown in supplemented endothelial growth medium (Clonetics) for 15 days. Attaching cells rapidly assumed an endothelial-like shape and, after 6 days of culture, proliferated in clusters with a core of rounded cells and radiating spindle-shaped cells. Clusters were counted on days 6, 9, 12, and 15 in randomly selected microscopic fields by two independent operators blind to the patient status. It is currently agreed that this culture model allows positive selection of true EPCs that should represent the only survived cell population. To confirm the endothelial phenotype, cells were stained with DiI-Acetylated LDL (Molecular Probes) and fluorescein isothiocyanate–conjugated Ulex-Lectin (Sigma-Aldrich).

Data are expressed as means ± SE and flow cytometry results as number of cells per 10^6 events. Comparison between two groups was performed by Student's t test, and the χ^2 test was used for dichotomous variables. Statistical significance was accepted at $P \leq 0.05$.

RESULTS

— Mean population age was 68.1 ± 1.2 years (range 43–86). No significant differences were present between groups as for age, duration of diabetes, HbA_{1c}, BMI, prevalence of hypertension, smoke, and diabetic nephropathy (incipient and overt), although DR^+ tended to have higher albumin excretion rate than DR^- patients (75.7 ± 22.2 vs. 33.9 ± 8.1; $P = 0.24$). DR^+ patients showed lower $CD34^+$ cells than DR^- patients (202.7 ± 18.4 vs. 301.9 ± 20.8; $P < 0.01$), while there was no difference in $CD34^+KDR^+$ cells (61.4 ± 5.4 vs. 64.9 ± 10.6; $P = 0.48$). Consequently, patients with DR had higher $CD34^+/CD34^+KDR^+$ percent ratio (34.8 ± 6.3 vs. 22.7 ± 2.2; $P = 0.02$). $CD34^+$ cells were reduced only in DR^+ compared with DR^- patients, while $CD34^+KDR^+$ cells were reduced only in PAD^+ patients. The independent negative associations of $CD34^+$ cells with DR and of $CD34^+KDR^+$ cells with PAD were confirmed in multivariate analyses including age, sex, BMI, hypertension, smoking, and HbA_{1c}. DR^+PAD^- patients had the

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Abbreviations: DR, diabetic retinopathy; EPC, endothelial progenitor cell; PAD, peripheral arterial disease.

A table elsewhere in this issue shows conventional and Système International (SI) units and conversion factors for many substances.

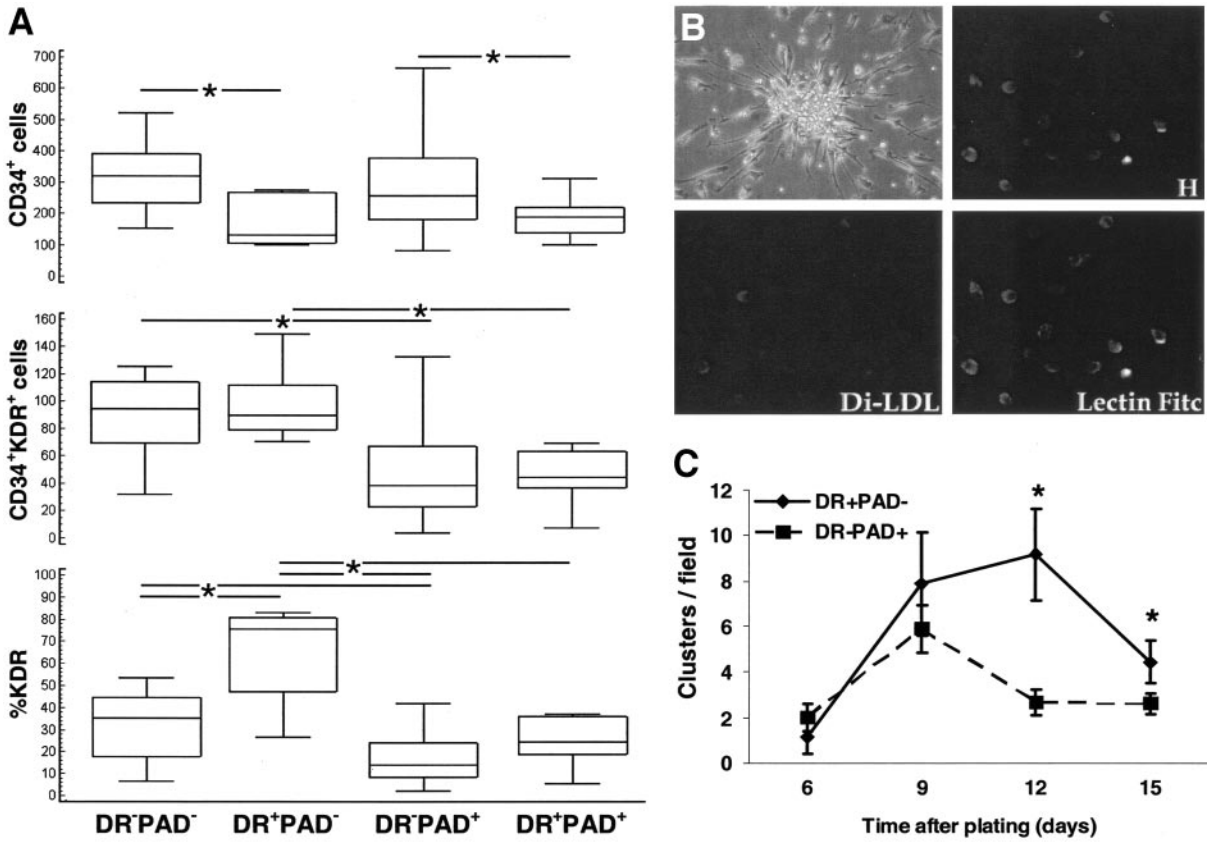

Figure 1—*A: Box-and-whisker plot showing the levels of CD34$^+$ and CD34$^+$KDR$^+$ cells and their ratio in diabetic patients divided into four groups according to the presence of DR and/or PAD. The* central box *represents the values from the lower to upper quartile (25–75 percentile), and the* middle line *represents the median. A line extends from the minimum to the maximum value, excluding values extending 1.5 times or more the interquartile range from the lower and upper quartile. Horizontal lines indicate which groups were compared. B: A cellular cluster of spindle-shaped cells, representing proliferation of cultured EPCs, and cells double stained with DiI-AcLDL (red) and fluorescein isothiocyanate–conjugated–Ulex lectin (green), indicating endothelial differentiation of progenitor cells from peripheral blood. Nuclei are stained in blue. C: Enumeration of cell clusters at different time points revealed that the clonogenic expansion capacity of cultured EPCs during the 15-day culture period is significantly superior in DR patients compared with PAD patients.* *P $<$ *0.05.*

highest percentage of KDR expression on CD34$^+$ cells (64.1 ± 9.5%), suggesting increased endothelial differentiation. On the opposite, the lowest percent ratio was seen in DR$^-$PAD$^+$ patients (17.3 ± 2.2%) (Fig. 1*A*).

To confirm the enhanced endothelial differentiation of circulating progenitors in DR$^+$ versus PAD$^+$ patients, EPC culture assay was performed, showing that the clonogenic expansion capacity was better in DR$^+$PAD$^-$ patients than in DR$^-$PAD$^+$ patients. Moreover, cells obtained 15 days after plating, which were confirmed to exhibit an endothelial phenotype and are believed to represent true EPCs, were significantly more abundant from DR$^+$PAD$^-$ patients than from DR$^-$PAD$^+$ patients (Fig. 1*B* and *C*).

CONCLUSIONS — We show that CD34$^+$ cells and CD34$^+$KDR$^+$ cells are differentially altered in the presence of DR and PAD. These two common diabetes complications exhibit a very different behavior in terms of angiogenic response to ischemia, and this contrast has been termed "diabetic paradox." Many growth factors have been proposed to have a role in this phenomenon (13–15), all of which are potent stimuli for progenitor cell mobilization and homing to ischemic tissues (16). Indeed, recent data indicate that marrow-derived cells are involved in retinal neovascularization and that DR$^+$ patients have increased levels of circulating progenitors (8,17). Conversely, macrovascular complications are characterized by reduced angiogenesis and exhausted EPC levels (6).

Our findings suggest that enhanced endothelial differentiation of circulating progenitors characterize DR, as shown by the high CD34$^+$KDR$^+$ proportion and the enhanced efficiency of EPC culture in contrast with the poor endothelial differentiation of PAD patients.

It has been proposed that growth factor alterations are confined to the retina and analysis of peripheral blood is not sensitive enough (18), but this seems untrue for progenitor cells, as their characterization from peripheral blood correlates with the clinical pattern. However, due to the small number of subjects in the DR$^+$PAD$^-$ group and the preliminary nature of the present study, future research is needed to confirm this hypothesis.

In summary, we report that two subpopulations of circulating progenitor cells are differentially regulated in DR and PAD, which locate at the two extremities of the diabetic paradox. Increased endothelial differentiation in DR is unmasked with this novel approach.

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