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Circulating Endothelial Progenitor Cells Are Increased in Patients with Classic Kaposi's Sarcoma

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TO THE EDITOR

Cancer is a disease largely dependent on neoangiogenesis. Accumulating evidence indicates that tumor angiogenesis is supported by the mobilization and incorporation of endothelial progenitor cells (EPCs), highly proliferative elements derived from the bone marrow (Rafii *et al.*, 2002). EPCs have been detected at increased frequency in the circulation of patients with different types of cancer, in some cases even correlated with tumor volume, so that they have been proposed to possibly represent a diagnostic and prognostic tool to be used as a surrogate marker in clinical studies (Bertolini *et al.*, 2006). To our knowledge, circulating EPCs have never been quantified in patients with Kaposi's sarcoma (KS), an angioproliferative malignancy in which the typical spindle-shaped tumor cells share many markers with vascular endothelial cells and are thought to be of endothelial origin (Dupin and Grange, 2006).

Circulating EPCs can be defined by the expression of cell-surface antigens. Although a unique consensus on the optimal markers to be used is still lacking, current literature supports that $CD34^+KDR^+$ is still the best antigenic combination to define EPCs (Fadini *et al.*, 2007), as only the level of $CD34^+KDR^+$ cells has been shown repeatedly and convincingly to be an independent predictor of cardiovascular events (Werner *et al.*, 2005; Schmidt-Lucke *et al.*, 2005). In a previous study, we reported that $CD34^+$ cells, which contain EPCs, are increased in patients with KS (Della Bella *et al.*, 2006). In this study, by flow cytometry we analyzed the frequency of EPCs, either identified as $CD34^+KDR^+$ cells or as $CD34^+KDR^+CD133^+$ cells, in the peripheral blood of patients with classic KS (cKS) compared with matched healthy controls. The selection of patients affected by the classic variant of the disease was aimed to avoid the confounding effects of HIV co-infection or immunosuppressive therapy that are present in the other clinical variants of KS. All patients had histologically confirmed diagnosis of KS, were positive for anti-human herpesvirus-8 (HHV8) antibodies, and negative for HIV. Staging was performed in accordance with our classification (Brambilla *et al.*, 2003). Circulating EPCs were measured at a single time point on fresh peripheral blood samples; staging at this time is summarized in Table 1. Ethical approval was obtained from the local Institutional Review Committee, and signed informed consent was obtained from all participants. The study was conduced according to the Declaration of Helsinki Principles. Heparinized whole-blood samples (100 µl) were incubated with biotin-conjugated anti-human kinase insert domain receptor (KDR) (Sigma-Aldrich, St Louis, MO), phycoerythrin-conjugated anti-human CD133 (Miltenyi-Biotec, GmbH, Bergisch Gladbach, Germany), and phycoerythrin-Cy5-conjugated anti-human CD34 (Beckman-Coulter Immunotech, Marseille, France) mAbs. KDR was

Abbreviations: cKS, classic Kaposi's sarcoma; EPC, endothelial progenitor cell; HHV-8, human herpesvirus-8; KDR, kinase insert domain receptor; KS, Kaposi's sarcoma

Table 1. Clinical characteristics of patients

Characteristic	Healthy controls (*n*=27)	cKS patients (*n*=29)	Intralesional therapy[1]
Age (years)[2]	75.6 ± 2.9	72.8 ± 2.3	
Sex, no. (%)			
Female	8 (29.6)	6 (20.7)	
Male	19 (70.4)	23 (79.3)	
KS stage[3], no. (%)			
I (maculo-nodular)			
A (slow)		5 (17.2)	2
B (rapid)		10 (34.5)	5
II (infiltrative)			
A (slow)		2 (6.9)	2
B (rapid)		6 (20.7)	4
III (florid)			
A (slow)		1 (3.5)	1
Bc (rapid with complications)		3 (10.3)	3
IV (disseminated)			
Bc (rapid with complications)		2 (6.9)	

cKS, classic Kaposi's sarcoma; KS, Kaposi's sarcoma; yr, years.
[1]Intralesional therapy consisted of vincristine, usual dose 0.1 mg per site. All patients received compressive device (elastic stockings). Patients in systemic therapy, either chemotherapy or IFN-α, were excluded.
[2]Mean ± SE.
[3]A, slow evolution; B, rapid evolution; rapid denotes an increase in the total number of nodules/plaques or in the total area of plaques in the 3 months following the last examination; c, complications; objective complications include ulcerations, bleeding, lymphedema, and lymphorrea; subjective complications include pain, functional grip, and ambulatory impotence (Brambilla *et al.*, 2003).

revealed using FITC-conjugated streptavidin (Sigma-Aldrich, St Louis, MO). Mononuclear cells were gated on forward *versus* side scatter plot to exclude granulocytes, dead cells, and debris. Cells were then sequentially gated on the basis of CD34, KDR, and CD133 expression (Figure 1a). Estimates of the absolute numbers of cells were calculated from the proportion of cells recorded by flow cytometry in the mononuclear gate multiplied by absolute mononuclear cell count measured using a standard hemacytometer. In few experiments, we included CD45 staining and confirmed, according to Duda *et al.* (2007) and Bertolini *et al.* (2006), that $CD34^{bright}$ cells in our analyses are $CD45^{dim}$ (not shown).

As shown in Figure 1b, the number of circulating $CD34^+$ cells was significantly higher in cKS patients than in controls ($P<0.001$), thus confirming our previous observation (Della Bella *et al.*, 2006). Also the number of EPCs, identified as either $CD34^+KDR^+$ or $CD34^+KDR^+CD133^+$ cells, resulted significantly higher in cKS ($P=0.012$ and $P=0.022$, respectively). Intriguingly, the increase of $CD34^+$ cells and $CD34^+KDR^+$ cells was significantly more pronounced in cKS patients with slowly evolving (all stages A) than rapidly evolving (all stages B) disease ($P=0.029$ and $P=0.002$, respectively) (Figure 1c). This finding may be explained with a localization of EPCs within the lesions during the active phases of the disease, as it may be suggested by the recent observation that the number of intralesional $CD34^+$ cells increases during the progression of KS from patch to nodular (Pyakurel *et al.*, 2006). No correlations between $CD34^+$ or $CD34^+KDR^+$ cells and clinical stage (I, II, III, IV), presence of complications, or local therapy were observed. Similar results were obtained when data were expressed as percentage of cells in the mononuclear cell population rather than absolute count. Identification of EPCs as $CD34^+KDR^+CD133^+$ did not allow us to point out differences between KS patients with different evolution pattern (Figure 1c), thus supporting the opinion that $CD34^+KDR^+$ may be the most appropriate phenotype to identify EPCs. This conclusion is in accordance with the recent demonstration that endothelial outgrowth cells do not derive from $CD133^+$ cells (Timmermans *et al.*, 2007).

Given the angioproliferative nature of the disease, a number of soluble factors may be involved to sustain the proliferation and mobilization of EPCs in subjects with KS (Ensoli *et al.*, 2001). To investigate their possible role in our patients, we measured the plasmatic levels of vascular endothelial growth factor, tumor necrosis factor-α, and GM-CSF by using specific commercial enzyme-linked immunosorbent assays (all from R&D Systems, Minneapolis MN). In part, confirming our previous results (Della Bella *et al.*, 2006), we found that vascular endothelial growth factor levels did not differ between patients and controls, and tumor necrosis factor-α levels were below or close to the sensitivity limits of the assays in both groups, possibly related to the low aggressiveness of cKS compared with the other clinical variants of the disease. GM-CSF was detectable in 63.2% of the patients and 38.5% of the controls, with plasmatic levels significantly higher in cKS patients than in healthy individuals (*t*-test $P=0.009$). However, the levels of GM-CSF did not correlate with the frequency of circulating EPCs (data not shown). It is possible that other soluble factors may be involved in the increase of EPCs observed in our patients. Another possibility is that the increased frequency of EPCs in cKS patients may be related to direct or indirect effects of viral environment. In facts, KS is strictly associated with infection by HHV-8, the causative agent for KS (Dupin and Grange, 2006). Although in this study we did not succeed in visualizing HHV-8 infection of EPCs by flow cytometry, according to Pellet *et al.* (2006), the demonstration that circulating $CD34^+$ cells as well as $CD146^+$ cells from KS patients harbor the virus (Henry *et al.*, 1999; Pellet *et al.*, 2006), together with our observation that late-EPCs cultured from the peripheral blood of cKS patient are HHV-8-infected (Della Bella *et al.*, 2008), may support the involvement of HHV-8 in the biology of EPCs. A probable scenario may be that EPCs may act as preferential

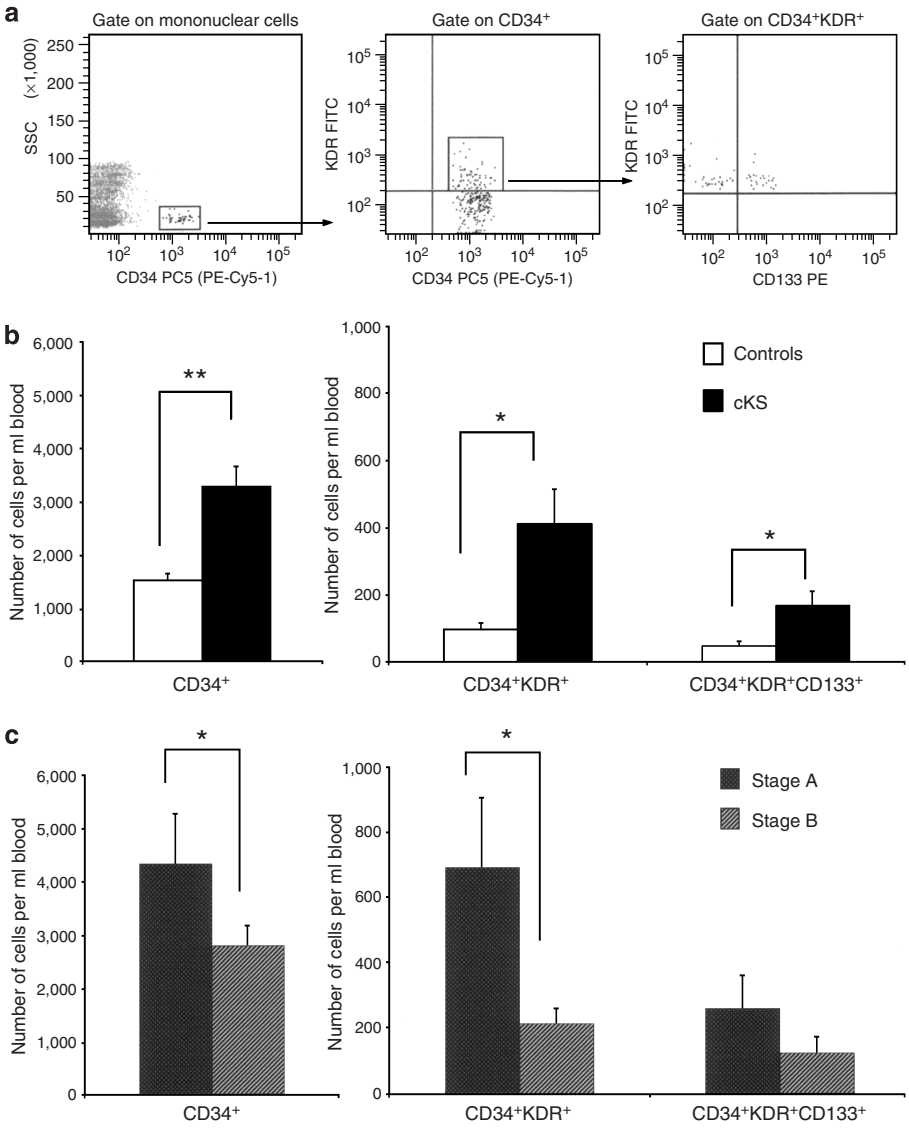

Figure 1. Analysis of CD34$^+$ progenitors and EPCs in the peripheral blood of cKS patients and healthy controls. (**a**) Representative flow-cytometry gating strategy for identification of CD34$^+$, CD34$^+$KDR$^+$, and CD34$^+$KDR$^+$CD133$^+$ cells. (**b**) The number of CD34$^+$ progenitors and EPCs per milliliter of whole blood in cKS patients compared with matched healthy individuals, and (**c**) in slowly evolving cKS patients (all stages A) compared with rapidly evolving patients (all stages B). Data presented as mean ± SE. Statistical significance was determined by two-tailed Student's *t*-test. *$P<0.05$ and **$P<0.001$ between patients with cKS ($n=29$) and controls ($n=27$), or patients with disease in stage A ($n=8$) and stage B ($n=21$).

HHV-8 reservoirs and, whether infected, may home to permissive sites and propagate to produce KS lesions (Gill, 2007).

In conclusion, we provide evidence that EPCs are increased in the peripheral blood of patients with KS, and to our knowledge this is previously unreported. The results of this study may also suggest that circulating EPCs, identified as CD34$^+$KDR$^+$ cells, may represent a sensitive tool for monitoring the evolutive trend of the disease. Future studies involving a larger cohort of prospectively observed patients will be needed to confirm the findings reported in this study and to investigate whether changes in the frequency of EPCs may predict disease progression and may therefore be proposed as a biomarker in the follow-up of KS patients.

CONFLICT OF INTEREST

The authors state no conflict of interest.

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Adriano Taddeo[1,3], Pietro Presicce[1,3], Lucia Brambilla[2], Monica Bellinvia[2], Maria L. Villa[1] and Silvia Della Bella[1]

[1]Laboratory of Immunology, Dipartimento di Scienze e Tecnologie Biomediche, Università degli Studi di Milano, Milan, Italy and [2]Department of Dermatology, IRCCS Ospedale Maggiore, Milan, Italy
E-mail: adriano.taddeo@unimi.it
[3]Supported by a fellowship of the Doctorate School of Molecular Medicine, University of Milan, Milan, Italy.

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