Increased frequency and vasculogenic potential of endothelial colony-forming cells in patients with Kaposi's sarcoma

Francesca Calcaterra,[1,2] Lucia Brambilla,[3] Elena Colombo,[1,2] Athanasia Tourlaki,[3] Stefano Veraldi,[3,4] Claudia Carenza,[1,2] Domenico Mavilio,[1,2,*] Silvia Della Bella[1,2,*]

[1]Department of Medical Biotechnologies and Translational Medicine, University of Milan, Italy. [2]Unit of Clinical and Experimental Immunology, Humanitas Clinical and Research Center, Rozzano, Milan, Italy. [3]Unit of Dermatology, Fondazione IRCCS Ca' Granda - Ospedale Maggiore Policlinico, Milan, Italy. [4]Department of Pathophysiology and Transplantation, University of Milan, Italy.

* D. Mavilio and S. Della Bella are equally contributing senior authors.

ORCID numbers: Francesca Calcaterra: 0000-0002-7625-1979**;** Domenico Mavilio: 0000-0001-6147-0952**;** Silvia Della Bella: 0000-0003-0818-5049.

Corresponding author: Silvia Della Bella, MD, PhD; Department of Medical Biotechnologies and Translational Medicine, University of Milan, and Lab of Clinical and Experimental Immunology, Humanitas Clinical and Research Center, via Manzoni 113, 20089 Rozzano (MI), Italy; e-mail: silvia.dellabella@unimi.it; phone: +39-2-82245144; fax: +39-2-82245101

Short title: ECFCs as precursors of KS spindle cells

Abbreviations: cKS, classic Kaposi's sarcoma; ECFC, endothelial colony-forming cell; HHV8, human herpesvirus-8; LANA-1, latency-associated nuclear antigen-1; PBMC, peripheral blood mononuclear cell.

ABSTRACT

Kaposi's sarcoma (KS) is characterized by hyperproliferation of spindle cells that have an endothelial origin and assume their characteristic features upon infection with human herpesvirus-8 (HHV8), the causative agent for KS. The multifocal nature of KS suggests that spindle cells derive from circulating HHV8-infected precursors that yet lack identification. We investigated whether ECFCs obtained from KS patients may be putative precursors of spindle cells, by assessing whether their in vitro behavior may evoke the in vivo behavior of KS spindle cells. We isolated and cultured ECFCs from the blood of 83 patients with classic KS (cKS) and compared them with ECFCs obtained from 86 healthy donors. ECFCs were highly increased in the blood of cKS patients; they showed higher proliferative and vasculogenic potential and higher production of interleukin-6 than control ECFCs. Similarly to spindle cells in KS lesions, a variable proportion of cells within each ECFC colony expressed the HHV8 latency-associated nuclear antigen. All together, ECFCs obtained from cKS patients evoked KS spindle cell behavior, thus supporting the hypothesis that ECFCs may be putative precursors of spindle cells. ECFCs can therefore represent a non-invasive tool for studying KS and screening drug activity, thus possibly guiding personalized care for KS patients.

INTRODUCTION

Kaposi's sarcoma (KS) is an angioproliferative disease of the skin and mucosa characterized by hyperproliferation of spindle-shaped cells, angiogenesis and inflammation. Spindle cells, which represent the typical and predominant cells of KS lesions, have an endothelial origin and undergo typical changes in their morphology, growth rate, life span, and gene expression upon infection with human herpesvirus-8 (HHV8), the etiological agent of KS (Moses et al., 1999; Ciufo et al., 2001; Gramolelli et al., 2015). Notably, HHV8 infection is necessary but not sufficient for the development of KS. Indeed, genetic, immunological and environmental factors are needed and differentially contribute to the development of the four different clinical and epidemiological forms of the disease consisting of classic, epidemic (AIDS-associated), iatrogenic and African endemic KS. Cofactors can contribute to KS development by many different mechanisms. They can act as HHV8 activators by promoting viral lytic replication, as is the case of HIV coinfection in epidemic KS or by hypoxia in classic KS (cKS) (Harrington et al., 1997; Davis et al., 2001). They can act as vasoactive agents by promoting angiogenesis, as suggested for ACE inhibitors in cKS and iatrogenic KS (Bilen et al., 2002). Finally, they can act by affecting the immune system, either by causing immunosuppression or immunodeficiency, as it occurs in epidemic and iatrogenic KS, or by promoting inflammation that is a key feature of KS lesions (Ganem, 2010).

The pathogenetic relevance of cofactors acting in the local microenvironment is strongly supported by the observation that KS lesions preferentially develop at sites of local hypoxia, as occurring in the presence of venous insufficiency of the lower extremities in cKS, or at sites of previous trauma or inflammation, as occurring in Koebner phenomenon (Ruocco et al., 2013). Therefore, although it is possible that KS spindle cells originate from mature endothelial cells that acquire HHV8 infection at the site of the lesion, it seems more likely that KS lesions may derive from the seeding of previously infected circulating endothelial progenitor cells that, upon localization in peripheral tissues, may differentiate into the typical

spindle cells and undergo intense proliferation and angiogenesis under the effects of the above described cofactors. The multiclonal origin of KS lesions, which has been demonstrated not only in multifocal disseminated tumors in the same patient, but even within each single advanced KS lesion (Duprez et al.,2007), seems to strongly support this latter hypothesis. Other lines of evidence supporting the involvement of HHV8-infected endothelial progenitors in the pathogenesis of KS include the evidence of uninfected endothelial progenitor cells in KS biopsies (Pyakurel et al., 2006), clearly indicating that these cells can be recruited in KS lesions. Because HHV8 infection can be detected in the bone marrow of patients with KS (Corbellino et al., 1996), the possibility that bone marrow-derived endothelial progenitors acquire HHV8 infection in the bone marrow before seeding in peripheral tissues seems quite conceivable. Accordingly, in a previous study we demonstrated that endothelial colony-forming cells (ECFCs) obtained from the blood of cKS patients are HHV8-infected as they harbor HHV8-genomes and can support viral lytic replication (Della Bella et al., 2008).

All together, these observations strongly support the hypothesis that ECFCs may represent potential virus reservoirs and putative precursors of KS spindle cells. In this study we add a further piece of evidence in favor of this hypothesis. In fact, we isolated and characterized ECFCs from cKS patients and demonstrated that their frequency in the peripheral blood was considerably higher compared with healthy donors and that they showed an in vitro behavior, in terms of proliferative and vasculogenic potential, cytokine production and HHV8 infection, that may evoke the *in vivo* behavior of KS spindle cells.

RESULTS

The frequency of circulating ECFCs is highly increased in patients with cKS

To assess whether the number of ECFCs in the peripheral blood may be altered in patients with cKS, we compared the appearance of ECFC colonies obtained after seeding peripheral blood mononuclear cells (PBMCs) isolated from 83 cKS patients and 86 healthy donors on fibronectin-coated plates, by applying a method recently optimized in our lab (Colombo et al., 2013). The proportion of donors giving rise to at least one ECFC colony was similar in cKS patients and controls (81 and 73%, respectively). However, as shown in Figure 1a, the frequency of ECFCs in PBMCs was strikingly higher in cKS patients than controls. Moreover, ECFC colonies isolated from cKS patients appeared some days earlier than those from healthy donors (Figure 1b).

Within the group of cKS patients, the increased frequency of ECFC colonies was more pronounced in patients with rapidly evolving (all stages B, n=52) than slowly evolving (all stages A, n=15) disease (Figure 1c). No correlation between the frequency of ECFC colonies and KS clinical stage (I, II, III, IV) was observed.

In order to assess whether the use of collagen rather than fibronectin used for coating plastic plates may differently affect the efficiency of ECFC colony isolation from cKS patients, in few experiments we compared the appearance of ECFC colonies obtained after seeding PBMCs from cKS on either substrate. To this aim, the PBMCs obtained from 3 cKS patients were divided into portions, with half of them seeded on fibronectin-coated plates and the rest seeded on collagen-coated plates. Similarly to our previous observation in healthy donors (Colombo et al., 2013), also ECFCs from cKS patients were isolated more efficiently when fibronectin was used, as ECFC colonies appeared some days later when PBMCs were seeded on collagen instead of fibronectin (mean±SEM, collagen vs fibronectin: 19.1±2.7 vs 15.1±2.0 days, $P<0.05$).

ECFCs isolated from cKS patients are endowed with higher proliferative and vasculogenic potential

In order to assess whether ECFCs obtained from cKS patients may have an *in vitro* behavior that may evoke the *in vivo* behavior of KS spindle cells, we first evaluated the proliferative potential of ECFC colonies obtained from cKS and healthy donors. To this aim, we analyzed and compared 45 ECFC colonies obtained from each group. As shown in Figure 2a, ECFCs from cKS patients displayed at the end of the culture more than tenfold higher cell yield than ECFCs obtained from healthy donors, indicating that ECFCs from cKS patients were endowed with higher proliferative activity. As shown in Figure 2b, the higher proliferative activity of ECFCs from cKS patients was evident from the initial phases of cell culture, as ECFCs obtained from cKS patients gave rise to a significantly higher number of cells than control ECFCs starting from the first week of culture. Within the group of cKS patients, the proliferative activity of ECFCs did not differ significantly according to clinical features, although it tended to be higher in patients with rapidly evolving (n=24) than slowly evolving (n=21) disease (Figure 2c).

Notably, similar to ECFCs obtained from healthy donors, those obtained from cKS patients maintained stable endothelial morphology and exhibited features of primary cells by ultimately becoming senescent after long-term culture. ECFCs from cKS patients and healthy donors could be passaged in some cases up to passage 13 and 11, respectively, with ECFCs from cKS patients achieving an overall slightly higher number of passages compared with control ECFCs (mean±SEM, cKS vs controls: 8.0±0.4 vs 6.8±0.4, $P<0.05$).

Next, we evaluated the vasculogenic activity of ECFCs by assessing the ability of ECFCs to form tubules when seeded in Matrigel matrix, the most standard in vitro angiogenesis assay (Moore et al., 2015). As shown in Figure 2d, ECFCs from both cKS patients and healthy donors gave rise to tubular networks when assayed in Matrigel. However, ECFCs isolated

from cKS patients (n=32) showed a higher vasculogenic activity than ECFCs isolated from healthy donors (n=27), as they formed a significantly higher number of tubules (Figure 2e), thus confirming their higher vasculogenic activity compared with ECFCs obtained from healthy donors.

ECFCs isolated from cKS patients produce higher levels of IL-6

We next investigated whether ECFCs obtained from cKS patients were endowed with increased production of angiogenic cyokines that have been suggested to play a role in the development of KS lesions (reviewed in Mesri et al., 2010). In particular, because in a previous study we demonstrated that IL-6 and IL-8 are produced by ECFCs and sustain the proliferation of ECFCs (Colombo et al., 2013), and both cytokines are produced by endothelial cells upon HHV8 infection and sustain KS pathogenesis by supporting spindle cell growth (An et al., 2002; Masood et al., 2001), we investigated whether ECFCs obtained from cKS patients may produce higher levels of these proangiogenic cytokines than ECFCs obtained from healthy donors. To this aim, we measured the levels of IL-6 and IL-8 in the supernatants of ECFC colonies obtained from both groups and recovered from each ECFC culture just before cell passaging. As shown in Figure 3a, the concentration of IL-6 increased with culture progression in both patients and controls and was higher in cultures obtained from cKS patients from the initial phases of cell culture. The comparison of the maximum levels of IL-6 reached in the supernatants during ECFC culture further indicated that the levels of IL-6 were higher in ECFC cultures obtained from cKS patients (n=34) than healthy donors (n=34) (Figure 3b). Within the group of cKS patients, the levels of IL-6 did not differ significantly according to clinical features, although they tended to be higher in patients with rapidly evolving (n=17) than slowly evolving (n=17) disease (Figure 3c). The levels of IL-8 were similar in ECFCs obtained from cKS patients and healthy donors (mean±SEM, cKS vs

controls: 14924±2054 vs 10707±1158, *P*=n.s.). Other proangiogenic cytokines that may play a role in KS lesions, namely CXCL-1, angiopoietin-2 (ANGPT2) and platelet-derived growth factor (PDGF), did not differ significantly between ECFCs from cKS patients and controls (CXCL-1: 16184±2734 vs 8924±1151, *P*=n.s.; ANGPT2: 22207±2555 vs 28534±4292, *P*=n.s.; PDGF: 330±93 vs 269±76, *P*=n.s.). Vascular-endothelial growth factor (VEGF) was undetectable in most ECFC cultures from both patients and controls, according to previous reports by us and others in healthy donors (Colombo et al., 2013; Hur et al., 2004).

ECFCs isolated from cKS patients are latently infected by HHV8

In a previous study we demonstrated that ECFC colonies obtained from cKS patients are HHV8-infected, as they harbor viral DNA (Della Bella et al., 2008). However, because we evaluated HHV8-infection by performing PCR on bulk cultures, in that study we could not assess the proportion of infected cells within each ECFC colony. In the present study we assessed HHV8 infection of ECFCs as expression of latency-associated nuclear antigen-1 (LANA-1), a nuclear antigen typically expressed during the latent phase of HHV8 infection. As shown in Figure 4a, LANA-1 immunofluorescence assay performed on body-cavity-based lymphoma cell line-1 (BCBL-1) cells, used as a positive control, showed a speckled pattern of nuclear reactivity on the majority of the cells, as expected (Dupin et al., 1999). The same immunofluorescence assay applied to our ECFC cultures showed the expression of LANA-1 in all the analyzed ECFC colonies obtained from cKS patients (n=6). Notably, within each ECFC colony the expression of LANA-1 was limited to a small proportion of cells, ranging from 5% to 15% ECFCs (mean±SEM, 8.7±1.5). As expected, all the screened ECFC colonies isolated from HHV-8 seronegative healthy donors (n=6) were negative for LANA-1 expression. Representative images of LANA-1 immunofluorescence assay applied to ECFCs from healthy and cKS donors are shown in Figures 4b and 4c, respectively.

DISCUSSION

The term endothelial progenitor cells is a generic term that is largely used to describe many different cell types endowed with proangiogenic activity. Indeed, there is by now general consensus that only ECFCs, a unique endothelial stem cell population that can be isolated and cultured from the blood, are true endothelial progenitor cells. In fact, they contain a complete hierarchy endowed with typical endothelial morphology, expression of endothelial but not hematopoietic markers, clonal proliferative potential, ability to migrate to vascular chemokines and ability to perform angiogenesis and vasculogenesis in vivo (Mund and Case, 2011; Moschetta et al., 2014). For this reason, the demonstration in the present study that patients with cKS have a markedly higher frequency of ECFCs compared with healthy donors, and that patients with rapidly evolving disease have higher levels of circulating ECFCs than patients with slower evolving disease, is particularly relevant for exploiting the potential of ECFCs as putative precursors of KS spindle cells. By contrast, other cell populations that have been labeled as endothelial progenitor cells but are not functionally integrated into the blood vessels may contribute only with an ancillary role to the development of KS lesions, just by providing proangiogenic factors. This may be the case of $CD45^{dim}/CD34^{+}/KDR^{+}$ cells, that we previously reported to be increased in cKS patients (Taddeo et al., 2008), and are by now considered a main population of hematopoietic precursors that contribute to angiogenesis by secreting proangiogenic paracrine factors (Moschetta et al., 2014).

In this study, we further demonstrated that ECFCs obtained from cKS patients are endowed with a higher proliferative and vasculogenic potential than healthy donors. This finding is highly relevant to the possible role of ECFCs in KS pathogenesis. In fact, while in other types of cancer ECFCs are thought to support the tumor growth just by contributing to tumor

vascularization (Moccia et al., 2015), in KS - because of the endothelial origin of KS - ECFCs may participate to the tumor growth likely by acting as the precursors of the tumor cells themselves. Notably, the higher proliferative activity of ECFCs from cKS patients was likely independent from the substrate used for cell culture, as in a previous study aimed at validating the use of synchrotron microtomography for the analysis of cell proliferation on bioscaffolds, an ECFC colony obtained from a cKS patient similarly showed a higher proliferation rate than a control ECFC (Giuliani et al., 2014). Our observation that ECFCs isolated and cultured from KS patients underwent cell senescence, albeit slightly delayed compared with control ECFCs, is in accordance with the notion that the majority of spindle cells isolated from primary KS lesions are not immortalized (Ganem, 2010). Accordingly, KS spindle cells injected alone in nude mice are unable to produce persistent tumors (Salahuddin et al., 1988).

The increased proliferative activity of ECFCs observed in cKS patients, likely reflected in the increased frequency of ECFCs isolated from the peripheral blood, may be sustained by HHV8 infection. In fact, a number of HHV8 gene products expressed during latent viral infection, which predominates in KS lesions, can promote cell proliferation (Klass et al., 2005). They include LANA-1, kaposin A and v-cyclin (Klass et al., 2005). Indeed, we demonstrated in a previous study that ECFC colonies obtained from cKS patients are consistently infected by HHV8, as they contain viral DNA (Della Bella et al., 2008). By performing an immunofluorescence assay of LANA-1 expression, in this study we could further assess the proportion of latently infected cells within each ECFC colony, and observed that HHV8 infection was confined to a small proportion of cells, ranging between 5 and 15% of ECFCs. This finding is in accordance with a previous study on AIDS-related and African KS patients reporting that LANA-1, considered a pathognomonic marker for KS, was expressed in all KS lesions though on variable percentages of spindle cells, ranging from 18 to 56% in cutaneous lesions (Pak et al., 2007). This observation suggested that non-infected cells may be recruited

during KS development and that HHV8-infection may affect cell behavior with paracrine mechanisms, as well, in a model that has been coined paracrine neoplasia (Cesarman et al., 2000). It is likely that the low proportion of HHV8-infected cells observed in our ECFC cultures may be related to the type of KS patients selected for this study. In fact, we analyzed ECFCs in patients with the classic variant of KS, in order to avoid the confounding effects of HIV co-infection or immunosuppressive therapy that are present in AIDS-associated or iatrogenic KS. Compared with those clinical variants, cKS is typically an indolent disorder characterized by a poorly aggressive course and a relatively low viral load (Antman and Chang, 2000; Pellet et al., 2006). Notably, the low percentage of LANA-1 positive ECFCs in our patients was not unexpected for us, as it could be predicted by the detection of moderate amounts of viral genomes in ECFCs from cKS patients that we reported in our previous study (Della Bella et al., 2008).

HHV8 may also affect ECFC behavior through the expression of other viral gene products that can subvert host signaling pathways leading to the expression and secretion of angiogenic, inflammatory and proliferative factors (Mesri et al., 2010).

Our observation that ECFCs obtained from KS patients are endowed with higher IL-6 production than ECFCs obtained from healthy donors may suggest that the increased proliferative activity showed by ECFCs of KS patients may be supported at least in part by HHV8-induced production of IL-6. In fact, in a previous study we demonstrated that IL-6 autocrinally promote ECFC proliferation (Colombo et al., 2013). Notably, the increased production of IL-6 observed in ECFCs of cKS patients may be particularly relevant to our comprehension of KS pathogenesis. Beyond being a prototypical inflammatory cytokine, IL-6 is a major tumor-promoting cytokine endowed with potent pro-angiogenic activity (Gopinathan et al., 2015). In particular in KS, IL-6 has a prominent pathogenic role. Increased IL-6 expression is a hallmark indication of HHV8 infection and used for clinical diagnosis

and therapeutic targeting (Polizzotto et al., 2013; Robey and Bower, 2015). Increased IL-6 levels contribute to the inflammatory and pro-angiogenic environment that characterizes KS lesions (Ganem, 2010). More importantly, IL-6 is highly expressed by KS spindle cells and, by acting as an autocrine growth factor on these cells, it fosters spindle cell proliferation (Miles et al., 1990). The observation that ECFCs from cKS patients share this same property with KS spindle cells may support our hypothesis that ECFCs may be precursors of spindle cells. Further corroborating this hypothesis, we observed that ECFCs obtained from cKS patients, similarly to control cells, express CXCR4 (data not shown). Because the CXCR4/SDF-1 axis has been demonstrated to be crucial in recruiting HHV8-infected cells into the extravascular cutaneous space and in dictating the preferential localization of KS lesions in the skin (Yao et al., 2003; Desnoyer et al., 2016), CXCR4 expression may render ECFCs favourite candidates as putative precursors of spindle cells.

In conclusion, in this study we demonstrated that patients with cKS have increased frequency of circulating ECFCs that are endowed with latent HHV8-infection, increased proliferative and vasculogenic potential, and increased production of IL-6. All these features are shared with KS spindle cells and are crucial to the ability of the spindle cells to sustain KS development. Therefore, the results of this study provide important insights into the comprehension of the possible contribution of endothelial progenitor cells to KS development, and further support the hypothesis that ECFCs are HHV8-infected circulating precursors of KS spindle cells. As important translational implication of this study, ECFCs can represent a non-invasive tool for studying KS. Used for screening drug activity and optimizing drug combinations, ECFCs may predict cellular responses and therefore be used for guiding personalized treatment plans for KS patients.

MATERIALS AND METHODS

Study population

Eighty-three cKS patients were included in the study. All patients had histologically confirmed diagnosis of KS, were positive for anti- HHV-8 antibody and negative for HIV. Staging was performed in accordance with our classification that takes into account the prevalent type of lesions, localization, clinical behavior, evolutive pattern (A and B, indicating slowly and rapidly evolving disease, respectively) and presence of complications (Brambilla et al., 2003; Della Bella et al., 2006; Cappelletti et al., 2012). Patients in systemic chemotherapy were excluded. Isolation and culture of ECFCs was performed at a single time point on fresh peripheral blood samples; staging at this time is reported in Table 1. Eighty-six healthy HHV-8-seronegative donors were included as controls. None of the subjects had suffered from chronic inflammatory, autoimmune and cancer disease other than KS, nor they had clinically evident infections. None of the subjects were under treatment with drugs that have been reported to affect the number and function of endothelial progenitor cells. Ethics approval was obtained from the Ethics Committee of Fondazione IRCCS Ca' Granda - Ospedale Maggiore Policlinico, and written informed consent was provided by all participants prior to inclusion in the study. Peripheral blood samples were obtained by venipuncture and collected into heparin Vacutainer tubes (BD Biosciences, San Jose, CA).

Isolation and culture of ECFCs

ECFCs were isolated and cultured from PBMCs obtained from cKS and healthy donors according to methods described in Supplementary Materials and Methods.

In vitro tube formation assay

The ability of ECFCs to form tubes in vitro was assessed using Matrigel, using protocols described in Supplementary Materials and Methods.

Measurement of angiogenic cytokines in ECFC supernatants

The amount of IL-6, IL-8, CXCL-1, ANGPT2, PDGF and VEGF in culture supernatants were determined by DuoSet ELISA kits (R&D Systems). All individual steps were performed according to the manufacturer's instructions.

Assessment of HHV8 latent infection of ECFCs

HHV8 latent infection of ECFC colonies was assessed as expression of LANA-1 by using an immunofluorescence assay described in Supplementary Materials and Methods.

Statistical analysis

Data were shown as mean ± SEM. The Mann-Whitney U-test was used for comparisons between samples. All statistical analyses assumed a 2-sided significance level of 0.05. Data analyses were performed with Openstat software.

CONFLICT OF INTEREST

The authors state no conflict of interest.

ACKNOWLEDGMENTS

We thank Marco Borsani and Elisa Zaghi for expert technical assistance. This work was supported by grants from Italian Ministry of Education, Universities and Research (2008A3E9WM-003 to S. Della Bella); University of Milan (2009ATE0573 to S. Della Bella); Italian Association for Cancer Research (AIRC IG 14687 to D. Mavilio); Italian Ministry of Health (GR-2013-02356522); the intramural research program of Humanitas Clinical and Research Center (to D. Mavilio). F. Calcaterra was recipient of a fellowship awarded by the Italian Foundation for Cancer Research.

REFERENCES

An J, Lichtenstein AK, Brent G, Rettig MB. The Kaposi sarcoma-associated herpesvirus (KSHV) induces cellular interleukin 6 expression: role of the KSHV latency-associated nuclear antigen and the AP1 response element. Blood 2002;99:649-54.

Antman K, Chang Y. Kaposi's sarcoma. N Engl J Med 2000;342:1027-38.

Bilen N, Bayramgurler D, Aydeniz B, Apaydin R, Ozkara SK. Possible causal role of lisinopril in a case of Kaposi's sarcoma. Br J Dermatol 2002;147:1042-4.

Brambilla L, Boneschi V, Taglioni M, Ferrucci S. Staging of classic Kaposi's sarcoma: a useful tool for therapeutic choices. Eur J Dermatol 2003;13:83-6.

Cappelletti M, Taddeo A, Colombo E, Brambilla L, Bellinvia M, Pregliasco F, et al. Immunogenicity and safety of seasonal influenza vaccination in patients with classic Kaposi's sarcoma. J Invest Dermatol 2012;132:2414-21.

Cesarman E, Mesri EA, Gershengorn MC. Viral G protein-coupled receptor and Kaposi's sarcoma: a model of paracrine neoplasia? J Exp Med 2000;191:417-22.

Ciufo DM, Cannon JS, Poole LJ, Wu FY, Murray P, Ambinder RF, et al. Spindle cell conversion by Kaposi's sarcoma-associated herpesvirus: formation of colonies and plaques with mixed lytic and latent gene expression in infected primary dermal microvascular endothelial cell cultures. J Virol 2001;75:5614-26.

Colombo E, Calcaterra F, Cappelletti M, Mavilio D, Della Bella S. Comparison of fibronectin and collagen in supporting the isolation and expansion of endothelial progenitor cells from human adult peripheral blood. PLoS One 2013;8:e66734.

Corbellino M, Poirel L, Bestetti G, Pizzuto M, Aubin JT, Capra M, et al. Restricted tissue distribution of extralesional Kaposi's sarcoma-associated herpesvirus-like DNA sequences in AIDS patients with Kaposi's sarcoma. AIDS Res Hum Retroviruses 1996;12:651-7.

Davis DA, Rinderknecht AS, Zoeteweij JP, Aoki Y, Read-Connole EL, Tosato G, et al. Hypoxia induces lytic replication of Kaposi sarcoma-associated herpesvirus. Blood 2001;97:3244-50.

Della Bella S, Nicola S, Brambilla L, Riva A, Ferrucci S, Presicce P, et al. Quantitative and functional defects of dendritic cells in classic Kaposi's sarcoma. Clin Immunol 2006;119:317-29.

Della Bella S, Taddeo A, Calabro ML, Brambilla L, Bellinvia M, Bergamo E, et al. Peripheral blood endothelial progenitors as potential reservoirs of Kaposi's sarcoma-associated herpesvirus. PLoS One 2008;3:e1520.

Desnoyer A, Dupin N, Assoumou L, Carlotti A, Gaudin F, Deback C, et al. Expression pattern of the CXCL12/CXCR4-CXCR7 trio in Kaposi's sarcoma skin lesions. Br J Dermatol 2016; early view.

Douglas JL, Gustin JK, Moses AV, Dezube BJ, Pantanowitz L. Kaposi Sarcoma pathogenesis: a triad of viral infection, oncogenesis and chronic inflammation. Transl Biomed 2010;1.pii:172.

Dupin N, Fisher C, Kellam P, Ariad S, Tulliez M, Franck N, et al. Distribution of human herpesvirus-8 latently infected cells in Kaposi's sarcoma, multicentric Castleman's disease, and primary effusion lymphoma. Proc Natl Acad Sci U S A 1999;96:4546-51.

Duprez R, Lacoste V, Briere J, Couppie P, Frances C, Sainte-Marie D, et al. Evidence for a multiclonal origin of multicentric advanced lesions of Kaposi sarcoma. J Natl Cancer Inst 2007;99:1086-94.

Ganem D. KSHV and the pathogenesis of Kaposi sarcoma: listening to human biology and medicine. J Clin Invest 2010;120:939-49.

Giuliani A, Moroncini F, Mazzoni S, Belicchi MLC, Villa C, Erratico S, et al. Polyglycolic acid-polylactic acid scaffold response to different progenitor cell in vitro cultures: a demonstrative and comparative x-ray synchrotron radiation phase-contrast microtomography study. Tissue Eng Part C Methods 2014;20:308-16.

Gopinathan G, Milagre C, Pearce OM, Reynolds LE, Hodivala-Dilke K, Leinster DA, et al. Interleukin-6 stimulates defective angiogenesis. Cancer Res 2015;75:3098-107.

Gramolelli S, Schulz TF. The role of Kaposi sarcoma-associated herpesvirus in the pathogenesis of Kaposi sarcoma. J Pathol 2015;235:368-80.

Harrington WJr, Sieczkowski L, Sosa C, Chan-a-Sue S, Cai JP, Cabral L, et al. Activation of HHV-8 by HIV-1 tat. Lancet 1997;349:774-5.

Hur J, Yoon CH, Kim HS, Choi JH, Kang HJ, et al. Characterization of two types of endothelial progenitor cells and their different contributions to neovasculogenesis. Arterioscler Thromb Vasc Biol 2004;24:288-93.

Klass CM, Offermann MK. Targeting human herpesvirus-8 for treatment of Kaposi's sarcoma and primary effusion lymphoma. Curr Opin Oncol 2005:17:447-55.

Masood R, Cai J, Tulpule A, Zheng T, Hamilton A, Sharma S, et al. Interleukin 8 is an autocrine growth factor and a surrogate marker for Kaposi's sarcoma. Clin Cancer Res 2001;7:2693-702.

Mesri EA, Cesarman E, Boshoff C. Kaposi's sarcoma and its associated herpesvirus. Nat Rev Cancer 2010;10:707-19.

Miles SA, Rezai AR, Salazar-Gonzalez JF, Vander Meyden M, Stevens RH, Logan DM, et al. AIDS Kaposi sarcoma-derived cells produce and respond to interleukin 6. Proc Natl Acad Sci U S A 1990;87:4068-72.

Moccia F, Zuccolo E, Poletto V, Cinelli M, Bonetti E, Guerra G, et al. Endothelial progenitor cells support tumour growth and metastatisation: implications for the resistance to anti-angiogenic therapy. Tumour Biol 2015;36:6603-14.

Moore MC, Pandolfi V, McFetridge PS. Novel human-derived extracellular matrix induces in vitro and in vivo vascularization and inhibits fibrosis. Biomaterials 2015;49:37-46.

Moschetta M, Mishima Y, Sahin I, Manier S, Glavey S, Vacca A, et al. Role of endothelial progenitor cells in cancer progression. Biochim Biophys Acta 2014;1846:26-39.

Moses AV, Fish KN, Ruhl R, Smith PP, Strussenberg JG, Zhu L, et al. Long-term infection and transformation of dermal microvascular endothelial cells by human herpesvirus 8. J Virol 1999;73:6892-902.

Mund JA, Case J. The ontogeny of endothelial progenitor cells through flow cytometry. Curr Opin Hematol 2011;18:166-170.

Pak F, Mwakigonja AR, Kokhaei P, Hosseinzadeh N, Pyakurel P, Kaaya E, et al. Kaposi's sarcoma herpesvirus load in biopsies of cutaneous and oral Kaposi's sarcoma lesions. Eur J Cancer 2007;43:1877-82.

Pellet C, Kerob D, Dupuy A, Carmagnat MV, Mourah S, Podgorniak MP, et al. Kaposi's sarcoma-associated herpesvirus viremia is associated with the progression of classic and endemic Kaposi's sarcoma. J Invest Dermatol 2006;126:621-7.

Polizzotto MN, Uldrick TS, Wang V, Aleman K, Wyvill KM, Marshall V, et al. Human and viral interleukin-6 and other cytokines in Kaposi sarcoma herpesvirus-associated multicentric Castleman disease. Blood 2013;122:4189-98.

Pyakurel P, Pak F, Mwakigonja AR, Kaaya E, Heiden T, Biberfeld P. Lymphatic and vascular origin of Kaposi's sarcoma spindle cells during tumor development. Int J Cancer 2006;119:1262-7.

Robey RC, Bower M. Facing up to the ongoing challenge of Kaposi's sarcoma. Curr Opin Infect Dis 2015;28:31-40.

Ruocco E, Ruocco V, Tornesello ML, Gambardella A, Wolf R, Buonaguro FM. Kaposi's sarcoma: etiology and pathogenesis, inducing factors, causal associations, and treatments: facts and controversies. Clin Dermatol 2013;31:413-22.

Salahuddin SZ, Nakamura S, Biberfeld P, Kaplan MH, Markham PD, Larsson L, et al. Angiogenic properties of Kaposi's sarcoma-derived cells after long-term culture in vitro. Science 1988;242:430-3.

Taddeo A, Presicce P, Brambilla L, Bellinvia M, Villa ML, Della Bella S. Circulating endothelial progenitor cells are increased in patients with classic Kaposi's sarcoma. J Invest Dermatol 2008;128:2125-8.

Yao L, Salvucci O, Cardones AR, Hwang ST, Aoki Y, De La Luz Sierra M, et al. Selective expression of stromal-derived factor-1 in the capillary vascular endothelium plays a role in Kaposi sarcoma pathogenesis. Blood 2003;102:3900-5.

Table 1. Clinical characteristics of controls and cKS patients

Characteristics	Controls	Overall patients	Evolutive pattern	
No. of patients	86	83		
Age, yr[A]	23-80	49-93		
Sex, no.				
Male	55	68		
Female	31	15		
KS stage[B], no.				
			A (slow)	B (rapid)
I: maculo-nodular			12	19
II: infiltrative			3	21
III: florid			2	16
IV: disseminated			1	9
Total			18	65

[A] Range

[B]cKS patients were classified according to our classification that takes into account the prevalent type of lesions, localization, clinical behaviour, evolutive pattern and presence of complications.

A indicates 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 three months following the last examination

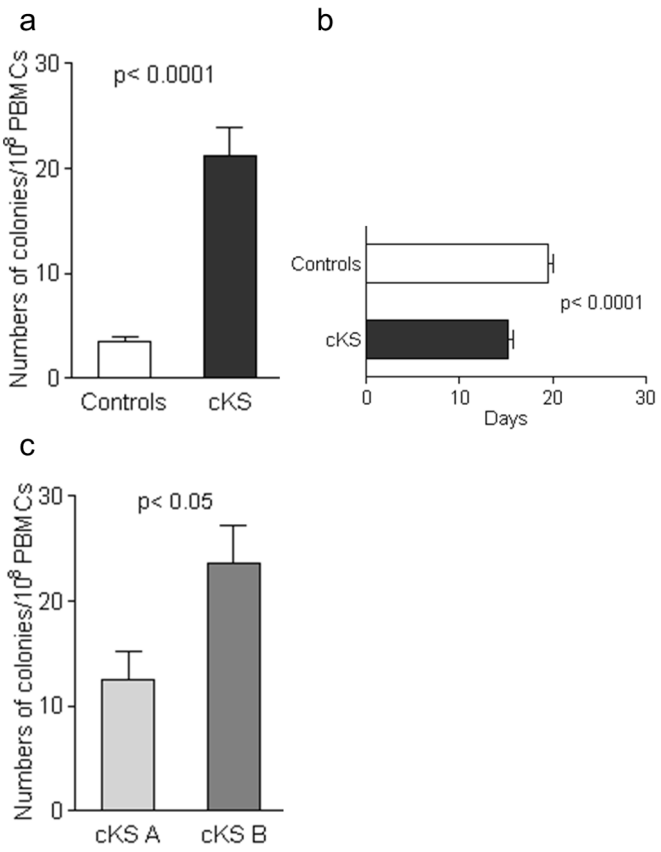
FIGURE LEGENDS

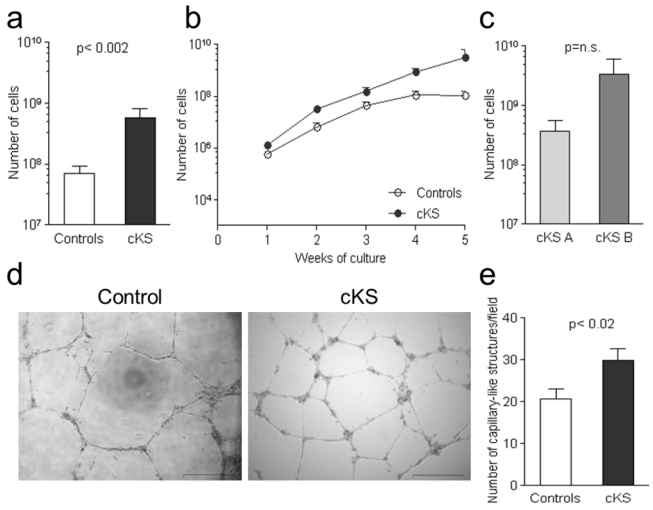
Figure 1. The frequency of circulating ECFCs is highly increased in patients with cKS. (**a**) The number of ECFC colonies isolated from the peripheral blood was much higher in cKS patients (n=86) than healthy controls (n=83). (**b**) ECFC colonies isolated from cKS patients appeared some days earlier than those from healthy donors. (**c**) Within the group of cKS patients, the increased frequency of ECFC colonies was more pronounced in patients with rapidly evolving (cKS B, n=52) than slowly evolving (cKS A, n=15) disease. Data presented as mean ± SEM. *P* values calculated by the Mann-Whitney U-test.

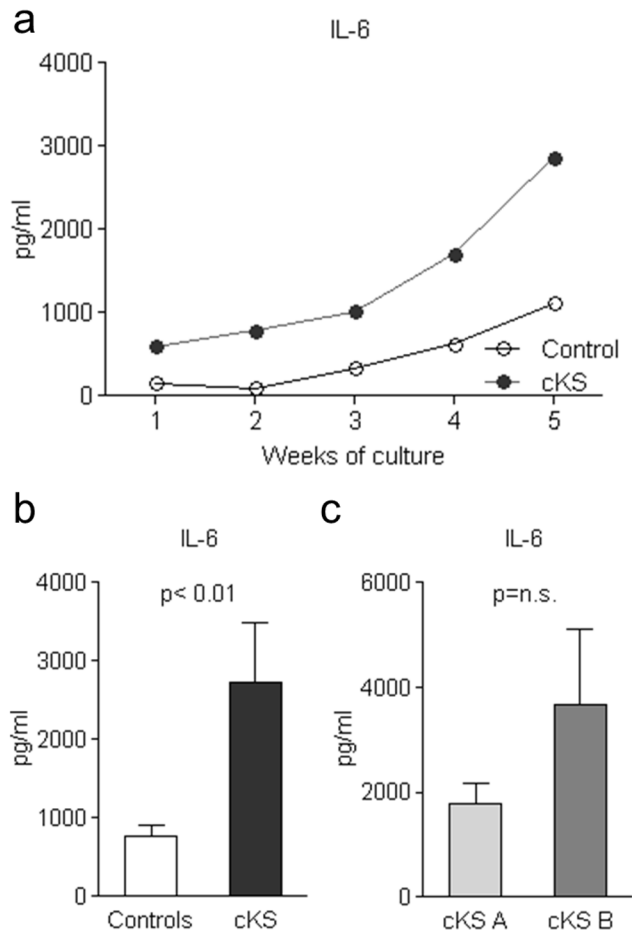
Figure 2. ECFCs isolated from cKS patients are endowed with higher proliferative and vasculogenic potential. (**a**) ECFCs from cKS patients (n=45) displayed at the end of the culture more than tenfold higher cell yield than ECFCs obtained from healthy donors (n=45). (**b**) The higher cell yield of ECFCs from cKS patients was evident from the initial phases of cell culture, as ECFCs obtained from cKS patients gave rise to a higher number of cells than control ECFCs starting from the first week of culture. (**c**) Within the group of cKS patients, the cell yield of ECFCs tended to be higher in patients with rapidly evolving (cKS B, n=24) than slowly evolving (cKS A, n=21) disease. (**d**) Representative photographs of tubules formed in Matrigel matrix by ECFCs obtained from one healthy donor and one cKS patient (original magnification x4, scale bar = 500 μm). (**e**) ECFCs isolated from cKS patients (n=32) formed a significantly higher number of tubules than healthy donors (n=27). Data presented as mean ± SEM. *P* values calculated by the Mann-Whitney U-test.

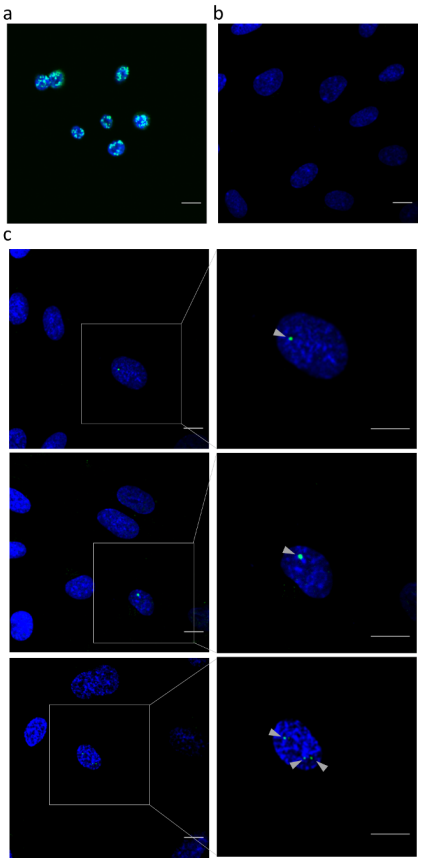
Figure 3. ECFCs isolated from cKS patients produce higher levels of IL-6. (**a**) Representative curves from one healthy donor and one cKS patient are presented, showing that the levels of IL-6 in ECFC supernatants increase progressively along with culture progression. (**b**) ECFC cultures obtained from cKS patients (n=34) reached higher levels of IL-6 than ECFCs obtained from healthy donors (n=34). (**c**) Within the group of cKS patients, the levels of IL-6 tended to be higher in patients with rapidly evolving (cKS B, n=17) than slowly evolving (cKS A, n=17) disease (*P*=0.24). Data presented as mean ± SEM. *P* values calculated by the Mann-Whitney U-test.

Figure 4. ECFCs isolated from cKS patients are latently infected by HHV8. Photographs of LANA-1 staining observed in: (**a**) the body-cavity-based lymphoma cell line BCBL-1 latently infected by HHV8, used as positive control (magnification, x120); (**b**) ECFCs obtained from one representative of six healthy HHV8-seronegative donor (magnification x120); (**c**) ECFCs obtained from three representative of six different cKS patients, showing positive LANA-1 expression in a small proportion of cells in each colony (magnification x120, scale bar = 10 μm). Insets for cKS patients shown at higher magnification (magnification, x240). Arrows indicate the localization of LANA-1 staining in ECFCs.


a
b
c
p< 0.0001
Numbers of colonies/10^8 PBMCs
30
20
10
0
Controls
cKS
Days
p< 0.0001
p< 0.05
cKS A
cKS B


a
p< 0.002
Number of cells
Controls
cKS
b
Number of cells
Controls
cKS
Weeks of culture
c
p=n.s.
Number of cells
cKS A
cKS B
d
Control
cKS
e
p< 0.02
Number of capillary-like structures/field
Controls
cKS


a
IL-6
4000
3000
2000
1000
0
pg/ml
Control
cKS
1
2
3
4
5
Weeks of culture
b
IL-6
p< 0.01
4000
3000
2000
1000
0
pg/ml
Controls
cKS
c
IL-6
p=n.s.
6000
4000
2000
0
pg/ml
cKS A
cKS B


a
b
c

SUPPLEMENTARY INFORMATION

Increased frequency and vasculogenic potential of endothelial colony-forming cells in patients with Kaposi's sarcoma

Francesca Calcaterra, Lucia Brambilla, Elena Colombo, Athanasia Tourlaki, Stefano Veraldi, Domenico Mavilio, Silvia Della Bella

Content:

MATERIALS AND METHODS

Isolation and culture of ECFCs

ECFCs were isolated and cultured from PBMCs obtained from cKS and healthy donors according to methods previously described (Colombo et al., 2013). Briefly, PBMCs obtained by Ficoll density gradient centrifugation (Cedarlane, Hornby, Canada) were resuspended in EGM-2 medium (Lonza Inc, Allendale, NJ) and seeded onto 24-well tissue culture plates previously coated with human fibronectin (Sigma-Aldrich, St. Louis, MO). Nonadherent cells and debris were aspirated after one day of culture; adherent cells were washed and cultured in EGM-2 medium that was changed every 2 days until the first passage. Culture plates underwent daily inspection using an inverted microscope. The appearance of visible colonies was identified as clearly circumscribed monolayers of cobblestone-like cells; the first day of ECFC colony appearance was recorded. The frequency of ECFC colonies was determined by measuring the number of colonies in primary cultures within 30 days after PBMC seeding. ECFC colonies were released from the original tissue culture plates by trypsinization (Euroclone, Wetherby, UK). Cells were resuspended in EGM-2

medium and plated onto 6-well tissue culture plates previously coated with type-I collagen (Corning Inc., Corning, NY). Subconfluent cells were further subpassaged in flasks and expanded until cell senescence. Cell senescence was assessed by morphology changes, decrease in proliferation, and positive staining for senescenceassociated ß-galactosidase (Millipore, Billerica, MA) (Colombo et al., 2013; Thill et al., 2008). Cell count and viability was assessed at each passage. Cell-free culture supernatants were filtered and stored at –20°C until use.

3

In vitro tube formation assay

The ability of ECFCs to form tubes in vitro was assessed using Matrigel, as previously described (Della Bella et al., 2008; Colombo et al., 2013). Briefly, multiwell dishes were coated with 70 mL of Matrigel (BD Biosciences) for 45 minutes at 37°C. ECFCs were seeded in triplicate at a density of $15x10_3$ per well in EGM-2 medium. Cells were cultured for 24 hours, and inverted phase-contrast microscopy was used to assess the number of endothelial tube-like structures (Arnaoutova and Kleinman, 2010). Five photographs from 5 different microscopic fields were taken for each well. Images were analyzed with FIJI ImageJ (NIH) software (Alonso et al., 2016).

Assessment of HHV8 latent infection of ECFCs

In order to determine the proportion of HHV8 latently-infected cells in ECFC colonies, the expression of latency-associated nuclear antigen-1 (LANA-1) was analyzed by LANA immunofluorescence assay (Paudel et al., 2012). Briefly, cells cultured on collagen-coated coverslips were fixed with 4% formaldehyde and permeabilized with 0,3% Triton X-100 for 20 minutes. The cells were blocked with 2% bovine serum albumin and 5% normal goat serum in PBS for 20 minutes followed

by incubation with a rat anti-LANA-1 primary antibody (Advanced Biotechnologies Inc, Eldersburg, MD) at 4°C overnight. Specific signal was detected by incubating the cells with a secondary AlexaFluor488-conjugated goat-anti-rat antibody (Invitrogen, Carlsbad, CA) for 1 hour at RT. Coverslips were mounted in anti-fading mounting media containing DAPI and analysed on the confocal microscope. Fifteen casual microscopic fields (60x magnification) were acquired and analyzed for each sample. Body-cavity-based lymphoma cell line-1 (BCBL-1) was used as positive control;

4

BCBL-1 were kindly provided by Dr. Roberta Mancuso (Don C. Gnocchi Foundation, Milan, Italy) and used from up to passage 6.

SUPPLEMENTARY REFERENCES

Alonso F, Domingos-Pereira S, Le Gal L, Derré L, Meda P, Jichlinski P, et al. Targeting endothelial connexin40 inhibits tumor growth by reducing angiogenesis and improving vessel perfusion. Oncotarget 2016;7:14015-28.

Arnaoutova I, Kleinman HK. In vitro angiogenesis: endothelial cell tube formation on gelled basement membrane extract. Nat Protoc 2010;5: 628-35.

Colombo E, Calcaterra F, Cappelletti M, Mavilio D, Della Bella S. Comparison of fibronectin and collagen in supporting the isolation and expansion of endothelial progenitor cells from human adult peripheral blood. PLoS One 2013;8:e66734.

Della Bella S, Taddeo A, Calabro ML, Brambilla L, Bellinvia M, Bergamo E, et al. Peripheral blood endothelial progenitors as potential reservoirs of Kaposi's sarcoma-associated herpesvirus. PLoS One 2008;3:e1520.

Paudel N, Sadagopan S, Balasubramanian S, Chandran B. Kaposi's sarcomaassociated herpesvirus latency-associated nuclear antigen and angiogenin

interact with common host proteins, including annexin A2, which is essential for survival of latently infected cells. J Virol 2012;86:1589-607.

Thill M, Strunnikova NV, Berna MJ, Gordiyenko N, Schmid K, Cousins SW, et al. Late outgrowth endothelial progenitor cells in patients with age-related macular degeneration. Invest Ophtalmol Vis Sci 2008;49: 2696–708