Characterization and clinical relevance of circulating and biopsy-derived endothelial progenitor cells in lymphoma patients

Cátia Igreja, Margarida Courinha, Ana Sofia Cachaço, Teresa Pereira, José Cabeçadas, Maria Gomes da Silva, Sérgio Dias

From the Angiogenesis Laboratory, Centro Investigação em Patobiologia Molecular (CIPM), Instituto Português de Oncologia Francisco Gentil, Centro de Lisboa (IPOFG), Lisboa, Portugal (CI, MC, ASC, SD); Serviço de Anatomia Patológica, IPOFG (TP, JC); Departamento de Hemato-Oncologia, IPOFG (MGDS); Instituto Gulbenkian de Ciência, Oeiras, Portugal (SD); Instituto de Medicina Molecular, Faculdade de Medicina de Lisboa, Portugal (SD).

Correspondence:
Sérgio Dias, Angiogenesis Laboratory, CIPM, Instituto Português de Oncologia Francisco Gentil, Centro Regional de Oncologia de Lisboa, Rua Professor Lima Basto, 1099-023 Lisboa, Portugal.
E-mail: sergidias@ipolisboa.min-saude.pt

ABSTRACT

Background and Objectives

Endothelial progenitor cells (EPC) have been proven to be essential for tumor angiogenesis and growth in animal tumor models. However, the involvement and relevance of EPC in human cancers remain poorly studied. We, therefore, investigated the presence, differentiation potential and molecular characteristics of EPC in lymphoma patients.

Design and Methods

EPC ($CD133^+CD34^+KDR^+$ cells) were detected in peripheral blood (PB) and lymph node (LN) biopsy samples of 70 lymphoma patients by reverse transcription-polymerase chain reaction (RT-PCR) and flow cytometry. Magnetically isolated EPC (PB and LN-derived) were tested, *in vitro*, for endothelial differentiation potential and RNA was collected to study their gene expression profiles by Affymetrix oligonucleotide arrays. Lymphoma patients were classified according to disease aggressiveness (indolent vs aggressive lymphoma) and their data (tumor angiogenesis, tumor stage and clinical treatment) were related to the presence or absence of EPC in the circulation or in tumor samples.

Results

Circulating EPC (CEPC) were more frequent in patients than in healthy controls and more frequent in younger patients than in older patients and in those with aggressive lymphomas. The levels of CEPC decreased in patients with complete response to treatment, but were sustained or increased in the non- or partial- responders to lymphoma therapy. Notably, EPC in the LN (LN-EPC) were more frequently detected than CEPC; LN-EPC were detected in vascular structures and also in the stroma, and after isolation differentiated into endothelial cells *in vitro*. The presence of LN-EPC correlated with lesion size and with increased angiogenesis in indolent lymphomas. CEPC and LN-EPC share endothelial markers but can be identified and quantified separately, since they express different CD133 isoforms. Gene expression profiling of isolated LN-EPC revealed the expression of pro-angiogenic and tumor growth factors that may influence lymphoma growth.

Interpretation and Conclusions

EPC are present in the circulation and in tumor samples from patients with non-Hodgkin's lymphoma. Since there are relationships between EPC and various characteristics of lymphoma, our research has demonstrated the clinical and biological relevance of studying CEPC and LN-EPC in lymphoma patients.

Key words: endothelial progenitors, non-Hodgkin's lymphoma, molecular characterization.

Neoplastic growth is angiogenesis-dependent.[1,2] The increase in tumor mass during the initial stages of tumor growth results in the creation of a hypoxic environment, which leads to the production of pro-angiogenic growth factors, and the onset of the angiogenesis switch.[1-3] Recently, there has been great interest in determining the origin of the endothelial cells that compose the newly formed vessels of growing tumors. This has resulted in the discovery of endothelial precursor/progenitor cells (EPC), a bone marrow (BM)-derived population that is recruited to sites of neo-angiogenesis and contributes to the formation of the neo-vasculature.[4-6] Murine tumor models have demonstrated that recruitment and incorporation of EPC is essential for tumor angiogenesis and growth.[7-9] Moreover, the contribution of EPC towards formation of neo-vessels was shown to be particularly relevant in models of lymphoma[7,10,11,] while the presence of circulating EPC correlated with angiogenic activity during tumor growth[12,13,] and anti-angiogenic therapies were shown to reduce the number of circulating EPC in patients with renal cancer.[14] Nevertheless, the molecular mechanisms involved in the recruitment, incorporation and differentiation of EPC into functional mature endothelial cells are still poorly understood.[5, 15] Similarly, the precise entity (molecular markers/signature) of EPC has not been fully determined, since numerous studies have suggested different surface markers may be used to isolate and characterize such cells. There are, however, some *consensual* markers for defining EPC, such as CD133,[16] vascular endothelial growth factor receptor-2 (VEGFR3, KDR/Flk-1[16]), and CD34,[17] but several others have been under intense scrutiny.[5,6] With regard to studies on the importance of EPC for tumor growth in humans, information is clearly lacking, although one study confirmed that EPC are incorporated in the neo-vessels of human tumors,[18, 19] and others suggested that the presence of circulating EPC in patients with multiple myeloma or non-small cell lung cancer correlates with clinical outcome.[20, 21] Based on these facts, we sought to define the presence, the molecular features and the clinical relevance of EPC in lymphoma patients, given the possibility of obtaining parallel BM, peripheral blood (PB) and tumor biopsy samples. We also characterized the molecular differences between EPC found in the circulation (CEPC) and present in tumor lymph node samples (LN-EPC), by oligonucleotide microarray technology (Affymetrix). Our study demonstrates the feasibility and significance of studying circulating and tissue-bound EPC populations in lymphoma patients, as surrogate markers of tumor angiogenesis and growth.

Design and Methods

Samples

All samples from lymphoma patient (n=70) were collected after informed consent, according to IPOFG-EPE guidelines (approval from the Institutional Review Board and Ethics Committee). Of the 70 patients studied, 10 bone marrow (BM), 41 peripheral blood (PB) and 39 lymph node (LN) biopsies were collected and analyzed. In parallel, PB samples from 11 healthy individuals were also studied. Mononuclear cells (MNC) were separated by density gradient centrifugation. In the case of LN biopsies, mechanical dissociation was also used, to obtain a single cell suspension. See supplementary information for a detailed description of the lymphoma patient population (*supplementary Table 2*). Except for the studies of EPC as indicators of tumor response to treatment, all samples were collected prior to any treatment and none of the patients received any mobilization treatment at any stage that may have influenced EPC levels.

Flow cytometry analysis and EPC isolation

For fluorescence-activated cell-sorting (FACS) analysis we used 100 µL of PB sample and tumor-derived cells resuspended in 100 µL of FACS buffer (PBS 1x, 2mM EDTA, 0.5% BSA). For the identification of EPC, immunofluorescent staining was performed, according to the manufacturer instructions, with the use of the following fluorescent conjugated antibodies: CD34-R-PE, CD133-PE and KDR-APC (BD Biosciences), CD19-FITC (Immunotech, Emeryville, CA, USA). CD133+ cells were isolated from LN biopsies using the mini-MACS immunomagnetic separation system (Miltenyi Biotec), according to the manufacturer instructions. Isolated EPC purity was determined by FACS (CD34-PERCP and CD133-PE antibodies) or reverse-transcriptase polymerase chain reaction (RT-PCR) (CD133, KDR, c-Kit, CXCR-4).

RNA extraction, cDNA synthesis RT-PCR and real time PCR (RQ-PCR)

The expression of EPC markers was assessed by RT-PCR in PB and LN biopsies from lymphoma patients and healthy controls. RNA extraction, cDNA synthesis and RT-PCR were performed following conventional protocols.[19] VEGF mRNA quantification was performed using an ABI Prism 7700 Sequence Detection System and TaqMan Universal PCR Master Mix (Applied Biosystems, Foster City, CA, USA). 18S rRNA (Human 18 S rRNA – 20x, Applied Biosystems) was used as standard reference. The relative expression of VEGF was calculated by using the comparative threshold cycle method. Primers and probe sequences for RT and RQ-PCR are described in *Supplementary Table 1*.

Rapid amplification of cDNA end (5' RACE) RT-PCR and DNA sequencing

5' RACE of total RNA from PB, BM and LN samples was performed using the SMART™ RACE cDNA amplification and the BD Advantage™ 2 PCR kits (BD Biosciences Clontech, Palo Alto, CA, USA), according to

Table 1. Frequency of CEPC and LN-EPC in lymphoma patients.

Patients' characteristics	EPC*	
	PB	LN
Median age, year (range)	53 (29-76)	60 (28-82)
Male	33% (7/21)	66% (10/15)
Female	45% (9/20)	58% (14/24)
Indolent lymphoma	38% (5/15)	71%* (10-14)
Aggressive lymphoma	42% (11/26)	64% (16/25)
BM infiltration	23%	64%
Total	39% (16/41)	77% (30/39)

*RT-PCR and flow cytometry screening of EPC markers (CD133, CD34, KDR) in PB samples from 41 lymphoma patients (20 females, 21 males), 11 healthy individuals (data not shown) and LN biopsies from 39 lymphoma patients (24 females, 15 males). The CEPC occured in a greater proportion of lymphoma patients than in healthy controls (39% vs. 18%). LN-EPC were detected more frequently than CEPC (77% vs. 39% of the samples) particularly in patients with indolent lymphoma (38% vs. 75%). There were no significant differences between the presence of EPC in PB or in LN and patients sex. * p=0.02; χ^2 test.*
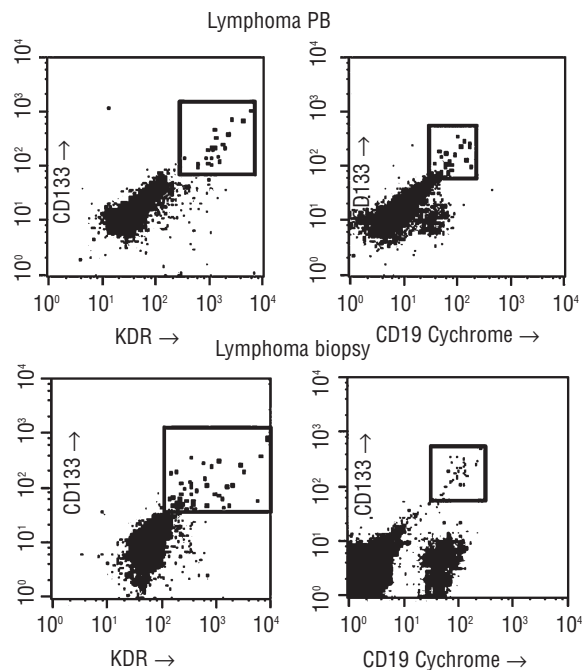

Figure 1. Detection of circulating and LN-CD133+ cells in lymphoma patients. To identify the cells expressing CD133, blood and biopsy cells were analyzed for the presence of lineage (CD133, CD34, KDR) antigens by flow cytometry in paired samples from four lymphoma patients. Here we show representative matched data from one of the analyzed patients. Circulating and LN-CD133+ cells express stem cell (CD34) and endothelial (KDR) markers.

the manufacturer's instructions. See *Supplementary Table 1* for a description of the primers: CD133-5' F, CD133 - exon 4 and CD133-3' R. PCR products were sequenced on an ABI Prism 377 DNA Sequencer with DNA Sequencing kit (Applied Biosystem, UK) according to the manufacturer instructions.

Endothelial differentiation culture conditions and identification of mature endothelial cells

Isolated EPC were transferred onto 1% gelatine coated 24-well plates and incubated in complete endothelial cells medium (EGM-2) supplemented with bovine brain extract (BBE), Bullet kit growth factors (all from Clonetics, Cambrex, USA) and 5% FBS (Sigma Aldrich, Madrid, Spain). Every other day the medium was supplemented with 10 ng/mL VEGF, 10 ng/mL bFGF, 5 U/mL heparin and 1 ng/mL Kit-L (all products from Sigma Aldrich). After differentiation, cells were stained with mouse anti-human vascular endothelial (VE)-cadherin (1:500) or rabbit anti-human KDR (1 µg/ mL) (Santa Cruz Biotechnology, Santa Cruz, USA), followed by goat anti-mouse Alexa Fluor 488 or goat anti-rabbit Alexa Fluor 594 (1:100, Molecular Probes, Eugene, OR, USA). Cells were examined using a fluorescence microscope (Axioplan Microscope, Zeiss, Germany).

Immunohistochemical staining of LN samples

LN-EPC localization was assessed in frozen 5 µm sections (acetone fixed) of LN biopsies incubated with anti-human CD133 (Miltenyi Biotec, 1:5). Vessel density was determined in paraffin-embedded tumor sections stained for Factor VIII (Dako Cytomation M0616, clone F8/86; 1:30). Angiogenic index was determined as the number of vessels per 5 high power fields (hpf) (amplification: 200×) in each section. Lesion size was determined by measuring the longest axis of the LN biopsy, in the cases of total LN excision; the predicted lesion size should correspond to the total affected LN.

Microarray analysis – Affymetrix

RNA was extracted from isolated parallel LN-EPC and CEPC from two different lymphoma patients. Each experiment (hybridization) was done using 100ng of total RNA. Amplified probes were prepared by *in vitro* transcription and hybridized to Affymetrix oligonucleotide microarrays (HG-U133A) containing 14500 genes. dChip software, a statistical program for model-based expression analysis, was used to obtain expression indices that identify transcripts enriched in the different cell populations.

Assessment of VEGF and SDF-1α levels

Plasma VEGF and SDF-1α levels were measured in plasma samples from lymphoma patients and healthy controls, by ELISA (Calbiochem, Dalmstadt, Germany and R&D Systems, USA respectively), following the manufacturer protocols.

Statistical analysis

Results are expressed as mean ± standard deviation (SD). Data were analyzed using the unpaired two-tailed Student's *t* test or χ^2 analysis. *p* values of less than 0.05 were considered statistically significant.

Results

As shown in murine models, lymphomas are one of the types of tumors in which the contribution of BM-derived cells to tumor angiogenesis is greatest.[5,7] Thus, we investigated the *systemic* (circulating) and the *local* (tumor-incorporated) presence of EPC (CD133+KDR+ cells), in lymphoma patients, based also on the accessibility of BM, PB samples and LN biopsies from the same patient, and on the possibility of studying sequential samples before and after treatment.

Detection of EPC in PB and LN samples from lymphoma patients

To assess the presence of EPC in PB and tumor samples from lymphoma patients we used RT-PCR and flow cytometry analysis (FACS) to detect CD133+CD34+KDR+ cells, due to the rarity of this cell population. A patient's sample was only considered CEPC or LN-EPC positive if all EPC markers were detected by both methods, in all the analyses performed. As determined by RT-PCR and FACS analysis, 16/41 (39%) of the lymphoma patients studied had CEPC (CD133+KDR+CD34+ cells) (Table 1 and Figure 1). This frequency was twice that in normal controls; similarly, levels of circulating VEGF were significantly higher in lymphoma patients than in normal controls (522.5±459.3 pg/mL (n=31) vs. 59.7±20.7 pg/mL (n=5), respectively). Regarding disease subtype, the frequency of CEPC-positive samples in lymphoma patients with aggressive or indolent lymphomas was similar (42% vs. 38%, respectively), that is, the presence of CEPC was independent of lymphoma type (the study population from which PB samples were taken comprised 67% of patients with aggressive lymphoma and 33% of patients with indolent lymphoma subtypes (*Supplementary Table 1*). Interestingly although not statistically significant (*p*=0.17), when the lymphoma patient population was divided (into quartiles) by age we observed that the younger the patients (11 patients were between 20 and 50 years old, while a second group of 28 patients were 50 to 80 years old) the higher the frequency of CEPC (55% positive samples in the youngest group versus 32% in the older patients). In contrast, EPC detection was not influenced by sex, BM infiltration or stage of the disease. As for the CEPC, we also investigated the presence of EPC in lymphoma (LN) biopsies, which were available from 39 patients. As summarized in Table 1, LN-EPC were more frequent than CEPC (77% vs. 39%) and were also more frequently detected in indolent lymphoma samples (71% of indolent lymphomas had EPC; *p*=0.02 compared to the prevalence in the remaining lymphomas). Importantly, the percentage of CD133+CD34+KDR+ cells, as determined by FACS analysis, was small in PB and LN samples (ranging from median values of 0.09 to 1.4%) (Table 2).

EPC present in biopsies differentiate into functional endothelial cells

After detecting CEPC and LN-EPC in lymphoma patients, we studied some of the biological properties of EPC after their incorporation into the affected LN. First, we verified the capacity of these cells to differentiate into endothelial cells *in vitro*; as shown in Figure 2 A-B and Table 3, isolated LN-EPC differentiate into KDR and VE-cadherin positive endothelial cells, concomitant with the loss of CD133 expression after differentiation. Morphologically, the differentiated cells show endothelial morphology, and express endothelial-specific markers (VE-cadherin, KDR). As shown by immunohistochemical staining against CD133, LN-EPC were detected in different areas of the affected LN, both incorporated into vascular structures and scattered throughout the tissue (Figure 2, C-D). The frequency of CD133+ cells localized to vascular structures and scattered throughout the tissue was identical (*data not shown*) in the biopsies studied. As shown in Supplementary Figure 2, immunofluorescent staining of CD133 and KDR in sequential tumor cryosections indicates that CD133+ cells are present singly or in clusters throughout the section, and that some of the KDR+ cells also have the same pattern of staining and distribution.

CEPC levels correlate with response to treatment

Following the detection of CEPC in lymphoma patients, we next sought to define a relationship between clinical behavior (response to treatment) and CEPC detection. As shown in *Supplementary Table 2*, among the 11 patients analyzed for the number of CEPC before and after treatment, 54.5% had aggressive forms of the disease whereas the remaining had indolent lymphomas. After treatment, 54.5% (6/11) of the patients achieved complete remission, whereas 36% (4/11) and 9% (1/11) had partial remission or active disease, respectively. Interestingly, lymphoma patients who achieved complete responses had reduced or sustained (compared to the first samples) CEPC levels (this was observed in six out of six patients) (Figure 3 and *Supplementary Table 2*). In contrast, patients who did not respond or responded only partially to induction therapy had higher numbers (four out of five patients) of CEPC in the later PB analysis (Figure 3 and *Supplementary Table 2*). The quan-

Table 2. Percentage of EPC in PB and biopsy samples from lymphoma patients.

Lymphoma type	Aggressive	Indolent	p
x LN-EPC (%)	0.23 (n=6)	0.18 (n=6)	0.55
x CEPC (%)	1.4 (n=6)	0.09 (n=3)	0.46
p	0.34	0.11	

Numbers of EPC were determined, by flow cytometry analysis, in PB (CEPC) and tumor samples (LN-EPC) from patients with aggressive and indolent types of lymphoma. x: median. The percentages of EPC in those patients (positive patients) with EPC marker expression detected by RT-PCR and flow cytometry are shown. Associations between variables were evaluated by Student's t test.
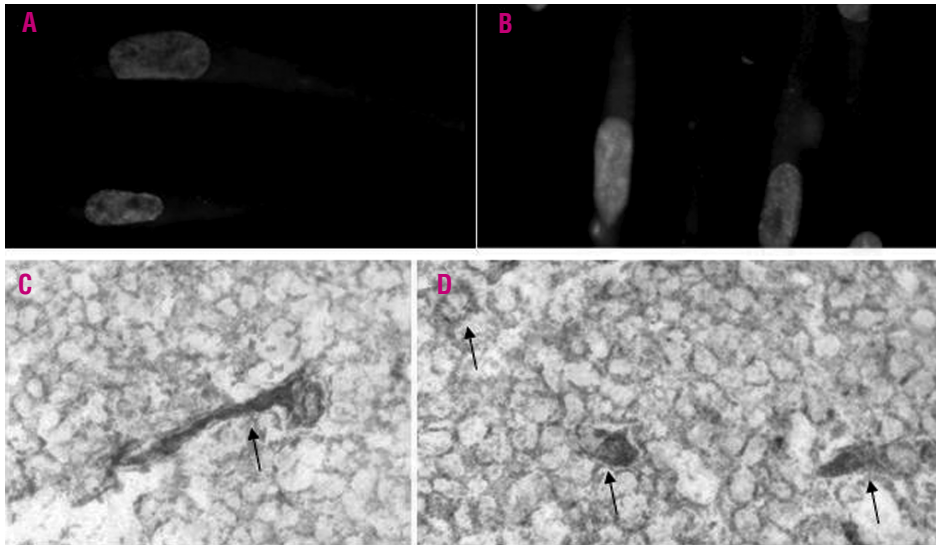
Figure 2. EPC present in LN differentiate into mature endothelial cells and co-localize with vascular structures or can be found dispersed throughout the tumor. **A-B.** Immunofluorescence images of LN-EPC collected after differentiation cultures and stained for endothelial markers: KDR (A) and VE-cadherin (B). DNA (blue-DAPI). Magnification: 630×. **C-D.** Immunohistochemistry images of LN-CD133+ cells stained with FastRed and counterstained with hematoxylin. CD133+ cells, identified by arrows, are present in tumor vessels (C) or dispersed in the tumor bed (D). Magnification: 630×.

Table 3. RT-PCR screening of EPC and endothelial cells markers in LN-CD133+ cells before (D0) and after EPC differentiation culture (D15).

EPC	KDR	VE-cadherin	c-Kit	FLT-1	CD133
D0	+	–	+	+	+
D15	+	+	+	–	–

Figure 3. CEPC levels correlate with response to treatment. PB samples from 11 lymphoma patients were analyzed, before and after treatment, for CEPC levels, by flow cytometry analysis and RT-PCR. This graph represents the observed variation in CEPC levels after therapy and its relation with clinical outcome (CR: complete remission; PR: partial remission; disease). According to the Kruskal-Wallis one way analysis of variance on ranks, the differences in the median values among the treatment groups are greater than would be expected by chance so there is a statistically significant difference (p=0.047).

titative increase in circulating CD133+ transcripts, as determined by RQ-PCR, in a patient who responded partially to treatment is shown in *Supplementary Figure 1*. Notably, systemic levels of VEGF or SDF-1 were not related to the response to treatment or with the presence of CEPC. As summarized in *Supplementary Table 2*, in the group of patients who had a complete response to treatment (n=6), VEGF levels increased in three of five patients (one of the other two patients had sustained VEGF levels and the other had decreased VEGF levels). SDF-1 levels increased in only one of four patients, decreased in one and remained almost constant in the other two. In the group of patients with partial responses to therapy (n=4), levels of circulating VEGF and SDF-1 also varied after therapy (increased in one patient, decreased in two and remained substantially stable in the other). The only patient who did not respond to therapy had decreased levels of VEGF and SDF-1 after therapy. These data suggest that in the lymphoma patients studied, VEGF and/or SDF-1 levels are not related to the type of disease or to the response to therapy.

LN-EPC correlate with lesion size, angiogenic index, VEGF levels, response to treatment and vessel sprouting

Having studied the clinical relevance of CEPC, we next determined the biological importance of detecting EPC in LN biopsies. The presence of EPC in the LN correlated with a higher angiogenic index in indolent lymphomas (Figure 4A, median vessel score), and significantly correlated with increased tumor size (Figure 4B,
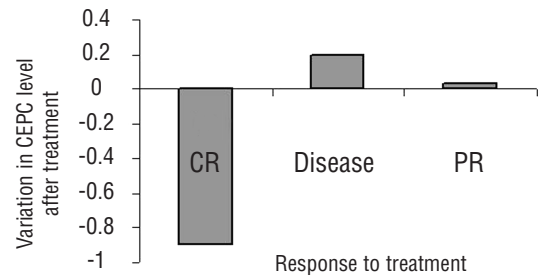
axis length; p=0.017, Student's t test) and VEGF mRNA levels (Figure 4C) in both aggressive and indolent lymphomas. Taken together, these data suggest that EPC in lymphoma biopsies may be quantified, and correlate with lesion size and angiogenesis in the affected LN. As for CEPC, we attempted to define the relationship between LN-EPC and clinical response in the different patients. LN-EPC were detected in all patients in clinical relapse (in contrast to patients at diagnosis or with disease progression - Figure 4D). We did not observe a correlation between lymphoma stage and LN-EPC frequency (see Supplementary Figure 3 for a description of lymphoma stage (I-IV) according to lymphoma type (indolent vs. aggressive) and the presence of LN-EPC). On the other hand, as shown in Figure 4E, LN-EPC evidenced a significantly higher potential to induce the formation of capillary-like tubes on HMVEC-LBI, compared to CEPC or HMVEC alone.

CEPC and LN-EPC cells co-exist in patients with lymphoma and can be distinguished based on the expression of CD133 mRNA isoforms

The results obtained from 41 PB samples and 39 LN biopsies suggest that circulating and LN-EPC may coexist in the same patient during tumor growth. To test this, we studied the presence of EPC in PB and LN samples obtained from the same patient at the same time. We obtained paired samples (PB and LN biopsy) from ten patients. As presented in Supplementary Table 4, CEPC and LN-EPC were simultaneously detected (samples collected at the same time) in 40% of these patients. Notably, the two cell populations (CEPC and LN-EPC) express distinct CD133 isoforms. As previously shown, the CD133 gene generates several isoforms, by alternative splicing, which differ for the presence of 5´ or 3´exons.[23,24] As illustrated in Supplementary Figure 4 and shown in Table 4, CEPC from patients' PB and BM samples express two CD133-5' and two CD133-3' mRNA isoforms. The main isoforms expressed by BM samples are A and C, while EPC isolated from LN biopsies express only the A isoform. CEPC and LN-EPC share several lineage markers, but upon incorporation into the affected LN, the pattern of alternative splicing may change, giving raise to a distinct CD133 mRNA isoform expression profile. In addition, these data also demonstrate the possibility of identifying and distinguishing CEPC and LN-EPC.

Gene expression profile of LN-EPC suggests different roles during lymphoma growth

In order to understand a putative role of LN-EPC during lymphoma growth, we characterized, by Affymetrix microarray analysis, the gene expression profile of isolated LN-EPC and compared it with that of CEPC. Globally, LN-EPC expressed higher levels of genes belonging to different categories, such as cell adhesion, signaling, angiogenesis and chemokines and receptors (Table 5, and *Supplementary Table 5*). The function of some of the proteins encoded by these genes has been well demonstrated in the context of tumor growth in other tumor models.[1,2] For instance, the up-regulation of *VCAM-1, HGF receptor* (*MET*), *IL-8* and *CXCR-4* has been implicated in processes such as inflammation, angiogenesis, and also recruitment of EPC.[25-29] To validate some of the results obtained with Affymetrix technology we performed RQ-PCR for two genes reported as being upregulated in LN-EPC. As depicted in Figure 5, the relative mRNA expression of CXCR4 (Figure 5A) and VCAM-1 (Figure 5B) was 1.5x and 2x, respectively, higher in LN-isolated EPC than in isolated CEPC. Little is known regarding a putative function for genes such as
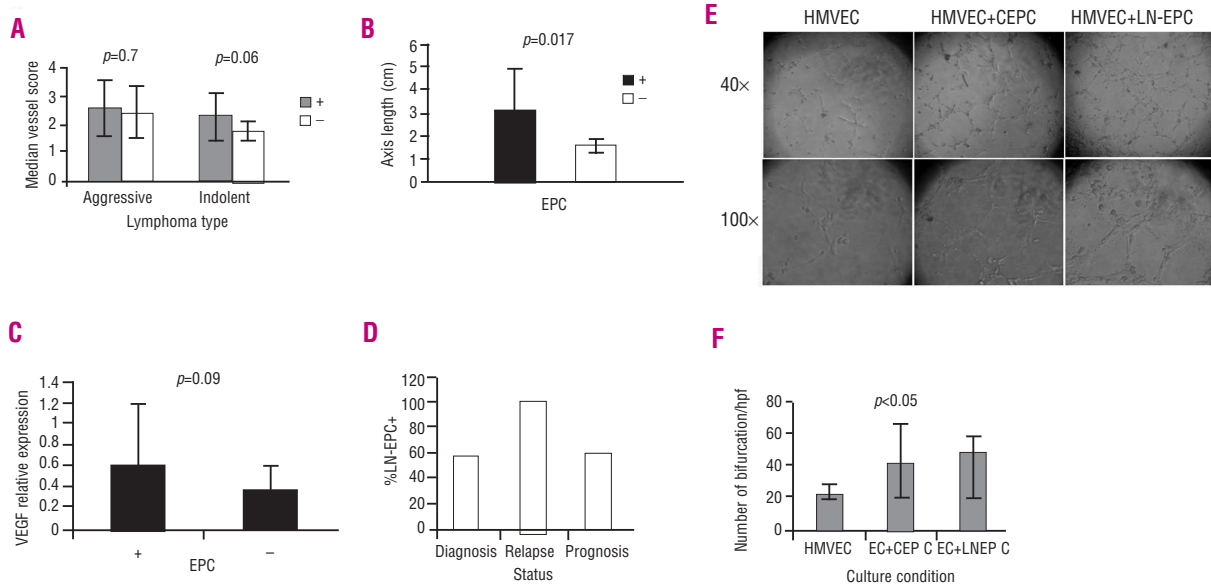

Figure 4. EPC in biopsies correlate with lymphoma size and angiogenesis. Tumor size and angiogenesis were determined in LN biopsies to evaluate whether the presence of EPC was associated with increased tumor growth. (A) Vessel density was determined in lymphoma biopsies (indolent and aggressive types; n=23) after immunohistochemical staining for blood vessels (factor VIII). Values represent the median vessel score determined as the average number of blood vessels detected in five high power fields (hpf=200x magnification) per tumor sample. (B) Tumor size was evaluated in EPC-positive and EPC-negative LN biopsies by measuring the length (cm) of the longest axis of the affected LN (n=20). (C) VEGF mRNA levels were quantified by RQ-PCR in LN biopsies (EPC⁺ vs. EPC⁻) from 22 lymphoma patients. The histograms represent the VEGF/ 18S rRNA ratio (VEGF relative expression) in lymphoma biopsies with and without detectable EPC. An association between the variables was evaluated by Student's t test. (D) LN-EPC were detected in all patients in clinical relapse. LN-EPC markers were analyzed by RT-PCR and flow cytometry in lymphoma biopsies (n=28) and the presence of these cells was correlated with the clinical status of the patient at the time of the analysis. (E) Phase contrast micrograph (magnification: 40× and 100×) comparison of HMVEC-LBI morphological capillary differentiation on matrigel in the presence of CEPC (HMVEC+CEPC) or LN-EPC (HMVEC+LN-EPC) (1×10⁵ cells/well) for 4 hours. (F) After 18 hours, tube formation was scored in 4× microscopic field as tree branch point events. Data are shown as the mean ± SD of one experiment with all the samples in triplicate. Associations between variables were evaluated by one-way ANOVA. Error bars represent standard deviation (SD).

SYNDECAN 2, BMP receptor, CXCL-2 or *CCL4,* in either EPC recruitment or lymphoma growth. Taken together, the data from gene expression profiling of LN-EPC suggest that these cells have molecular characteristics that differ from their circulating counterparts (CEPC). In addition, gene expression profiling identified several genes whose resulting functions may contribute towards lymphoma growth and angiogenesis.

Discussion

The importance of angiogenesis for the growth and expansion of solid and hematologic neoplasias has been amply demonstrated. Moreover, evidence from clinical studies and animal models suggests that targeting angiogenesis pathways may be of therapeutic benefit. Recently, the discovery of circulating, BM-derived EPC, and the demonstration of their importance for tumor angiogenesis,[5-9] revealed a novel therapeutic target, while providing an attractive surrogate marker to monitor tumor responses to conventional or experimental therapies. Several pre-clinical tumor models demonstrated the importance of monitoring the levels of CEPC during disease progression or after therapy.[11,12,14,18,20,30-33] With the present research we addressed the putative importance of measuring EPC levels in a clinical setting, and studied, in detail, patients with different lymphomas. This is a very important field of research since existing anti-lymphoma therapies still do not cure a large number of patients with lymphoma. Moreover, besides studying CEPC, we also attempted to study those cells already incorporated into the affected LN. For this purpose, we obtained samples from PB and LN biopsies. Whenever possible, we studied all such samples from the same patient, taken at the same time; in this way, we sought to define the relative importance of CEPC versus EPC located in affected tissues. We demonstrate that CEPC are increased in lymphoma patients, and correlate with the levels of circulating VEGF, as observed in other studies,[12,34] but not the plasma levels of SDF-1. It remains to be determined whether the circulating VEGF is produced by the CEPC themselves, or whether it is a consequence of disease progression or dissemination. While studying the clinical relevance of CEPC, we observed decreased or stable levels of CEPC in patients who achieved complete remission after a first line of chemotherapy. In contrast, patients who did not respond or only partially responded to first-line therapy tended to have increased CEPC levels: indeed, CEPC were detected for the first time in one patient after therapy had failed and concomitant with disease progression. These data are in agreement with several pre-clinical studies demonstrating the relevance of measuring CEPC as a surrogate marker of sensitivity to chemotherapy.[11-13,30-32] Our study provides the first demonstration of such principle in lymphoma patients. After determining, in detail, the lev-
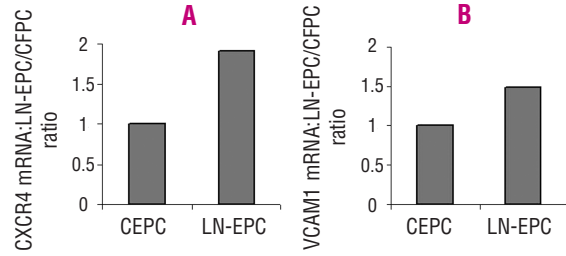
Figure 5. Validation of gene expression profile results by RQ-PCR: upregulation of CXCR-4 mRNA and VCAM-1 mRNA in LN-EPC compared to in CEPC. Relative mRNA expression was evaluated in magnetically isolated CD133⁺ LN- and PB-derived cells from two lymphoma patients, after RNA extraction. The histograms represent the ratio between relative mRNA levels of LN-EPC and CEPC.

Table 4. CD133 mRNA expression determined by RT-PCR and DNA sequencing.

Samples	RT-PCR products	Band size (BP)	Sequence	CD133-3' isoform	CD133-5' isoform	CD133 isoforms
PB	2	331	exons 32-37	A	1	s1, s2, s5, s7
		262	without exon 35	C	2	
BM	2	331	exons 32-37	A	1	s1, s2, s5, s7
		262	without exon 35	C	2	
LN	2	331	exons 32-37	A	1	s2
K231	3	331	exons 32-37	A	ND	ND
		262	without exon 35	C		
		238	without exons 34 and 35	B		

CD133 mRNA isoform expression determined by RT-PCR was confirmed with RACE (combination of 5' and 3' alternative splicing) and DNA sequencing in PB (n=41), BM (n=10) and LN (n=39) samples. ND: not determined. Specific CD133 mRNA isoforms (s5 and s7) could only be detected in circulating and BM-derived CD133⁺ cells.

Table 5. Gene expression profile of biopsy-EPC suggests different roles during lymphoma growth.

Category	Genes
Cell adhesion	VCAM-1, Protocadherin β11, Syndecan 2, secreted Phosphoprotein 1
Signaling	v-ERB-B2 (homolog 2), PHOSPHOLIPASE C γ2, MET, BMP receptor (type IA), IGF-binding protein 4
Angiogenensis	IL-8, Cathepsin L, THBS 1, IL-1β/4, CXCL-2, CCL-2
Chemokines + receptors	CCL4, CXCL3, CXCL9, CXCL10, IL2/13/21 receptors, IL-10, CCR5, CXCR-4

The gene expression profile of lymphoma-incorporated CD133⁺ cells was compared, by Affymetrix oligonucleotide microarray technology, with that of CEPC. The table shows those genes, classified by gene category according to attributed function, which were upregulated in tumor-incorporated CD133⁺ cells (LCB>2).

els and relevance of CEPC in the different patients, we proceeded with the detection and quantification of EPC incorporated into affected LN (LN-EPC), in parallel with CEPC. Surprisingly, the presence and the levels of LN-EPC did not correlate significantly with the type of lymphoma (although they were more frequently detected in indolent lymphomas), but did correlate significantly with greater LN angiogenesis, tumor (lesion) size and the patients' clinical status. Taken together, these data indi-

cate that, in the same lymphoma patient, quantification of CEPC and LN-EPC may provide complementary and clinically relevant information. Interestingly, LN-EPC were not located solely around vessels, but could also be found scattered throughout the tissue. Whether this observation means that we detected EPC *in transit* as well as those incorporated into the growing neo-vessels, or whether the two localizations reflect the presence of at least two separate cell populations, is not known. Since we detected CEPC and those incorporated into affected LN simultaneously, we reasoned that the two cell populations should share endothelial-specific lineage markers, but might also have distinct molecular features and phenotypic properties. In agreement with this hypothesis, CEPC and LN-EPC both express CD133, KDR, CD34, and CXCR4 (EPC markers). Surprisingly, however, we observed that CEPC and LN-EPC differ in their expression of CD133 mRNA isoforms. As previously shown, the CD133 gene is complex, and is regulated at the promoter level (tissue specific promoters have been identified), but may also be regulated at the post-transcriptional level by alternative splicing.[23, 24] We clearly demonstrate that PB and BM-derived EPC express specific CD133 mRNA isoforms, in contrast to LN-EPC which lose the expression of at least one of the isoforms. The mechanism whereby the CD133 gene undergoes a process of alternative splicing when CEPC become incorporated into an affected LN, and the relevance of such a change in CD133 isoform expression, remain unknown. Nevertheless, the possibility of distinguishing and quantifying circulating versus LN-EPC may be of clinical relevance. To characterize the putative involvement of LN-EPC during lymphoma growth we defined the gene expression profile of EPC isolated from differ-ent biopsy samples, and compared it to the corresponding CEPC profile. We found that LN-EPC express a number of genes whose protein products may in fact contribute to the expansion of the lymphoma mass, including several proteins involved in angiogenesis, activation of specific signaling pathways and production of chemoattractant cytokines. We are currently exploring the importance of each gene/gene family in the process of lymphoma growth and exit of lymphoma cells into the PB.

Taken as a whole, our study provides the first demonstration of the feasibility of determining the levels of CEPC and the levels of EPC already incorporated into affected lymph nodes, in lymphoma patients. The simultaneous detection of CEPC and LN-EPC, and the correlation between these two cell populations and clinical parameters, reveals novel surrogate markers that may be used to monitor disease progression, aggressiveness or response to treatments. Moreover, in tumors in which tumor biopsies are easily obtainable, we suggest that quantification of CEPC and LN-EPC may provide clinically relevant and complementary information.

Authors' Contributions

Conflict of Interest

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