Circulating Endothelial Cells in Patients With Chronic Lymphocytic Leukemia

Clinical-Prognostic and Biologic Significance

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BACKGROUND: In patients with cancer, circulating endothelial cells (CECs) are increased and are correlated with an aggressive disease course. However, the clinical and biologic significance of CECs in chronic lymphocytic leukemia (CLL) remains uncertain.

METHODS: In 170 patients with CLL, CEC levels were quantified by flow cytometry and were correlated with clinical and biologic data. In addition, CECs were characterized by immunophenotypic, fluorescence in situ hybridization (FISH), and gene expression profile analyses.

RESULTS: In patients with CLL, CECs were increased compared with controls. A higher level of CECs (>20/μL) identified a subset of patients with a more aggressive disease course characterized by a shorter time to first treatment both in univariate and multivariate analyses. In FISH analysis, 7 patients had a significant proportion of CECs and presented with the same cytogenetic lesion of neoplastic lymphocytes and immunophenotypic features of endothelial progenitor cells. The gene expression profile of sorted CECs revealed a molecular pattern, suggesting a derivation from CLL leukemic cells with increased cell survival and proliferation, diminished cell adhesion to extracellular matrix, and enhanced proangiogenic function compared with their normal counterparts.

CONCLUSIONS: The current data suggest that, in CLL, CECs may represent a biologic marker of aggressiveness and disease progression to be considered for new, targeted antiangiogenic treatments.

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An increased number of circulating endothelial cells (CECs) has been reported in many pathologic conditions, including hematopoietic neoplasms.1-5 However, the role of such endothelial cells (ECs) in tumor angiogenesis remains controversial.6,7 Several reports have suggested that, in cancers, ECs may be genetically altered.5,8-12

The origin of tumor-related ECs, the definition of CECs, and the methodology of their enumeration are a matter of debate, and research is aimed at clarifying whether CECs could be used as a marker of angiogenesis for clinical purposes and for monitoring antiangiogenic treatments.13,14 In chronic lymphocytic leukemia (CLL), experimental and clinical data suggest that angiogenesis may have a role in the pathogenesis and progression of the disease.15 In addition, it has been demonstrated that several biologic markers of angiogenesis may have prognostic relevance.16-21 To better understand the role of cells with the EC phenotype, in 170 consecutive patients with CLL, CEC levels were correlated with clinical-biologic features with particular reference to their cytogenetic and gene expression profiles.

MATERIALS AND METHODS

Patients

One hundred seventy consecutive patients with a diagnosis of CLL according to National Cancer Institute Working Group (NCI-WG) guidelines22 were treated between 2003 and 2008 and were enrolled prospectively in the current study. All patients provided written informed consent. The study was approved by the local ethics committee.

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Original Article

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Peripheral blood (PB) samples were obtained at the time of presentation for the purpose of quantifying CECs. Indications for treatment included an increased white blood cell (WBC) count with a <6-month lymphocyte doubling time; the development of anemia, neutropenia, or thrombocytopenia because of bone marrow infiltration; or autoimmune phenomena that did not respond to steroid drugs (disease progression according to the Rai staging system). Patients were treated according to the guidelines that were in use at our institutions. Fludarabine-containing regimens were used as front-line treatment, and intermittent chlorambucil was used as first-line therapy in some elderly patients (aged >70 years). Response to treatment was evaluated according to NCI-WG guidelines.

**Flow Cytometry and Circulating Endothelial Cell Quantification**

The following markers were tested in all patients by multicolor flow cytometry analysis, as described previously, using a 30% cutoff level for positivity in the lymphocyte gate: cluster of differentiation 5 (CD5)-phycoerythrin (PE), CD5 allophycocyanin (APC), CD19-fluorescein isothiocyanate (FITC), CD19-peridinin chlorophyll protein (PerCP)-cyanine 5.5 (Cy5.5), CD19-PE-Cy7, CD22-PE, CD23-PE, CD38-PE, anti-κ-FITC, anti-λ-PE (Becton Dickinson, Milan, Italy), CD10-FITC, FMC7-FITC, immunoglobulin G (IgG)-FITC, IgM-FITC, IgA-FITC, IgD-FITC (Dako, Milan, Italy), and ζ-chain–associated protein kinase 70 (ZAP70)-AlexaFluor-488 (Valter Occhiena, Torino, Italy).

CECs were quantified by using 4-color flow cytometry as described previously. Whole PB samples were analyzed within 3 hours of collection using a “lyse-no-wash” procedure. Acquisitions were performed with a FACSCalibur flow cytometer running Cellquest software (Becton Dickinson). At least 250,000 events were acquired for each sample. For CEC enumeration, the following antibodies were used according to the manufacturers’ instructions: CD34-PerCP, CD45-APC (Becton Dickinson), CD133-PE (Miltenyi Biotec, Bergisch Gladbach, Germany), and rabbit antivascular endothelial growth factor receptor 2 (anti-VEGFR-2) (Santa Cruz Biotechnology, Santa Cruz, Calif) followed by swine antirabbit IgG-FITC (Dako) as secondary reagent. Isotype-matched negative controls were used in all assays. The analysis was performed excluding cellular debris in a side scatter/forward scatter dot plot. CECs were defined as cells with negative expression for CD45 and positive expression for CD34 and VEGFR-2. Endothelial progenitor cells (EPCs) were defined as CD45-negative, CD34-positive, VEGFR-2-positive, and CD133-positive cells (Fig. 1A,B). The percentage of positive cells was calculated by subtracting the value of the appropriate isotype controls. The percentage of positive cells was converted into the absolute number of positive cells per μL using the following formula: percentage of positive cells × WBC count × 1000. Twenty age-matched and sex-matched controls were evaluated for comparison.

Quantitative 4-color flow cytometry experiments were performed in 29 CLL patients by comparing CD61 expression (CD61-FITC; Becton Dickinson) on CECs (positive for CD45-APC, CD19-PerCP, CD5-PE, and CD61-FITC) and CLL-CECs (negative for CD45-APC and positive for CD34-PerCP, CD146-PE, and CD61-FITC) and FMS-like tyrosine kinase (flt1) expression (RR9S; Santa Cruz Biotechnology; followed by goat antimouse IgG-FITC) on CECs (negative for CD45-APC and positive for CD34-PerCP, CD146-PE, and flt1) from patients with CLL and from normal individuals (n = 8 and n = 4, respectively). FITC-labeled fluorescent beads (Fluorospheres; Dako) were used to quantify flow cytometry determinations by calculating the molecular equivalent of soluble fluorescence (MESF), as described previously.

**Cell Isolation and Circulating Endothelial Cell Characterization**

Peripheral blood mononuclear cells were isolated by density gradient separation (Lympholyte-H Cedarlane; Cellbio S.p.a., Milan, Italy). Cells subsequently were enriched by immunomagnetic sorting with Dynabeads Pan Mouse IgG (Dynal A.S., Oslo, Norway). In patients with CLL, mouse anti-CD19 (clone HD37; Dako) and anti-CD14 (clone TUK4; Dako) monoclonal antibodies were used for the selection of CD19-positive cells and CD14-positive monocytes, respectively. The purity of sorted cells was >95% as determined by flow cytometry.

CECs were isolated as described previously. To eliminate hematopoietic cells that were CD45-positive, first, we performed a negative selection using Dynabeads that were coated with anti-CD45 (clone 2D1; Becton Dickinson). CD45-negative cells subsequently were subjected to a positive selection using Dynabeads that were coated with anti-CD146 antibody (clone P1H12; Becton Dickinson). Finally CECs were characterized immunologically by double-staining experiments, as described previously, with CD45-FITC and CD14-PE (Becton
Dickinson) and with EC markers that included Ulex Europaeus agglutinin (UEA-1), von Willebrand factor (Dako), VEGFR-2 and with the CLL markers CD19-PE (Beckman Coulter Milan, Italy) and CD5-PE. Evaluation of immunophenotypic results was performed using a Nikon fluorescence-equipped microscope with a charge-coupled black-and-white camera device (Cytovision System; Applied Imaging; Nikon, Florence, Italy).

**Molecular-Cytogenetic Studies**

Interphase FISH (I-FISH) was performed on PB samples that were obtained at diagnosis or before therapy in 154 patients and, in 7 patients, on sorted CECs using probes for the following regions: 13q14, 12q13; 6q21; 11q22/ataxia telangiectasia (ATM); and 17p13/tumor protein 53 (TP53) (Vysis/Abbott, Downers Grove, Ill), as described elsewhere. Before hybridization, CECs were stained with CD133-PE (I-FISH), as described previously. Dual-color FISH using control and test probes was performed on PB cells, and signal screening was carried out on at least 200 cells. Evaluation of FISH results was performed using a Nikon fluorescence-equipped microscope with a charge-coupled black-and-white camera device. To prevent data misinterpretation, only those areas in which >80% of cells had 2 control signals were analyzed. Cutoff

Figure 1. Circulating endothelial cells (CECs) are illustrated in patients with chronic lymphocytic leukemia (CLL). Flow cytometric evaluations of CECs in (A) a normal individual and (B) a patient with chronic lymphocytic leukemia are shown. After gating on (i) forward scatter (FCS) and side scatter (SSC), (ii) CECs are identified as CD45-negative and CD34-positive cells that (iii) coexpress vascular endothelial growth factor receptor-2 (VEGFR-2). Endothelial progenitor cells (EPCs) were identified as CECs that expressed cluster of differentiation 133 (CD133) (glycoprotein Prominin 1). (C) CEC levels were higher in patients with CLL compared with age-related and sex-related controls. (D) The time to first treatment is shown by CEC level. FSC-H indicates forward side-scattered light height; SSC-H, side-scattered light height; CD34, human gene that encodes the CD34 protein; PerPC, peridinin chlorophyll protein complex; CD45, protein tyrosine phosphatase, receptor type C; APC, allophycocyanin; PE, phycoerythrin; FITC, fluorescein isothiocyanate.
points for positivity were previously reported. The sensitivity limits on I-FISH preparations for the detection of trisomy 12, deletion (del)17p, and del11q/del13q were 11%, 16%, and 13% of cells, respectively.

Conventional chromosome analysis was performed as described elsewhere. A “mixed” cytogenetic/FISH classification was adopted, as reported previously, and each patient was categorized into a cytogenetic risk group according to the following hierarchical classification: unfavorable group (11q−, 17p−, or complex karyotype, ie, at least 3 chromosome aberrations in the karyotype); intermediate-favorable group (+12, 6q−, or 1-2 chromosome aberrations in the karyotype; 13q− as a single abnormality, absence of detectable abnormalities by FISH, and absence of karyotypic aberrations).

We performed a set of 8 family-specific polymerase chain reactions to isolate the clonally expressed VHDHJH patterns, as described previously. Sequence alignment was determined according to the Entrez database (National Center for Biotechnology Information [NCBI], Bethesda, Md) and V Base (Medical Research Council Center for Protein Engineering, Cambridge, United Kingdom). The cutoff of 98% homology to the germline sequence was chosen to discriminate between mutated (<98% homology) and unmutated (>98% homology) samples, as reported previously.

**Genome-Wide Expression Profiling**

Large-scale, genome-wide expression profiling (GEP) was performed on 12 CEC samples, 12 CD19-positive samples, and 10 CD14-positive samples that were purified from patients with CLL (CLL-CECs) and on 2 CEC samples from normal controls (nCECs). Total RNA was isolated using the RNeasy Kit (QIAGEN, Valencia, Calif). High-quality RNA was amplified and labeled with Cy5 using the Low RNA Input Fluorescent Linear Amplification Kit (Agilent Technologies, Palo Alto, Calif). Universal Human Reference RNA (Stratagene, Cedar Creek, Tex), consisting of equal amounts of total RNA from 10 human cancer cell lines, was used as a reference control in GEP experiments. Amplified reference complementary RNA (cRNA) was labeled with Cy3-camptothecin (CTP) (Agilent Technologies). Two-color Spike-In was added to provide positive controls for monitoring the microarray workflow from sample amplification and labeling to microarray processing. It contains 10 in vitro synthesized, polyadenylated transcripts derived from the adenovirus E1A transcriptome that are premixed at various ratios.

cRNA products were purified using RNeasy columns (QIAGEN). Samples had to contain at least 10 to 15 pmol of cyanine dye per μg of cRNA to be considered suitable for hybridization. Eight hundred twenty-five nanograms of Cy5-labeled cRNA were mixed with the same amount of Cy3-labeled reference cRNA, and the cRNA mixtures were fragmented to an average size of 50 nanonesia (nt) to 100 nt by incubation at 60°C for 30 minutes using an In Situ Hybridization Kit+ (Agilent Technologies). Samples were hybridized for 17 hours at 65°C on a 4 × 44 K Whole Human Genome Microarray (Agilent Technologies), which comprises >33,000 (60 mer) experimentally validated oligonucleotide probes. Then, the samples were scanned using a confocal laser scanner (Agilent Technologies).

**Genome-Wide Expression Profiling Analysis**

Fluorescence data were analyzed with Feature Extraction Software version 9.1 (Agilent Technologies). Data from each scan (Log_{10} Cy5/Cy3) were imported into the GEP analysis software Luminator (Rosetta Bio Software, Seattle, Wash). The data discussed in this publication have been deposited in the NCBI Gene Expression Omnibus (GEO) (available at: http://www.ncbi.nlm.nih.gov/geo/ accessed on August 15, 2009) and are accessible through GEO Series accession number GSE14853. Two-dimensional clustering analysis was performed using an agglomerative algorithm with an average link heuristics and a correlation with mean subtraction. The identification of genes that were expressed differentially between subgroups was performed using both a significant analysis of microarray (SAM) algorithm with a false-discovery rate of <5% and an enhanced analysis of variance (ANOVA) with P values <.001. To increase the statistical power, as input data, the enhanced ANOVA uses both the expression level and the estimated technology error associated with the expression level. Consequently, the false-positive rate is reduced when the number of replicates is small and, then, sensitivity detection is increased. A complete description of the statistical methods that we used is available in the technology section of the Rosetta Bio Software website (available at: http://www.rosettabio.com/tech/ default.htm accessed on August 15, 2009). The PANTHER (Protein A Nalysis T Hrough Evolutionary R elationships) classification system (Applied Biosystems, Foster City, Calif; available at: http://www.pantherdb.org/ accessed on August 15, 2009) was used to determine the cellular pathways of genes identified.
**Statistical Analysis**
Quantitative variables were reported as mean values with standard deviations (SDs) and were compared using the Mann-Whitney test. The Fisher exact test was used for categorical variables. All tests were 2-sided. The time to first treatment (TFT) was calculated as the interval between diagnosis and the start of first-line treatment. Survival curves were compared by using the log-rank test. A *P* value < .05 was used as a criterion for statistical significance. Proportional hazards regression analysis was used to identify the most significant independent prognostic variables on TFT. Statistical analyses were performed using Stata software release 8.0 (Stata Corporation, College Station, Tex).

**RESULTS**

**Hematologic and Clinical Findings**
Patients’ hematologic data are provided in Table 1. Molecular cytogenetic data were available on 154 patients. Thirty-two patients had either a complex karyotype and/or 11q− or 17p− deletions by FISH and were allocated to the unfavorable risk group, whereas all other patients were classified as favorable-intermediate risk by using molecular cytogenetics.

**Circulating Endothelial Cell Quantification**
Patients with CLL had significantly elevated levels of CECs (mean ± SD, 18.4 ± 28.4 CECs per μL) and EPCs (mean ± SD, 17.6 ± 27.4 EPCs per μL) compared with normal age-related and sex-related controls (mean ± SD CEC value, 3.3 ± 1.1 CECs per μL; *P* < .0001 [Fig. 1C]; mean ± SD EPC value, 2.8 ± 0.9 EPCs per μL; *P* < .0001).

Next, patients were subdivided into 2 groups according to the upper quartile CEC level (cutoff value, 20 CECs per μL). Patients with high CEC levels were more likely to be men, to have higher WBC counts, to require treatment, and to have a worse response to treatment. No correlation was observed between CEC levels and disease stage, CD38 or ZAP70 positivity, FISH findings, and Ig variable heavy chain (IgVH) mutation status (Table 2). Similar results were observed concerning EPCs and clinicobiologic findings (data not shown).

**Time to First Treatment**
The median follow-up for all 170 patients was 27.5 months. On univariate analysis, significantly shorter TFT was observed in patients with Rai stage 2 through 4 disease, CD38 and ZAP70 positivity, unfavorable cytogenetics, and higher CEC levels (Table 3, Fig. 1D). On multivariate analysis, the following factors were independently predictive of shorter TFT: advanced disease stage, CD38 positivity, high CEC level, and unfavorable cytogenetics (Table 4). IgVH mutations were not included in the survival analysis, because the data were not available for all patients.

**Immunophenotypic and Fluorescence In Situ Hybridization Characterization of Circulating Endothelial Cells**
In all experiments, >95% of sorted cells had the features of ECs, as demonstrated by phenotypic analyses (Table 5). In 7 patients who had CLL with known cytogenetic aberrations, FISH analysis revealed that a significant proportion of sorted CECs (40.7%; range, 20%-78%; 200 cells observed in each patient) harbored the same genetic lesion that was observed in neoplastic CLL cells (Table 5). Overall, 98.1% of CLL-CECs with genetic lesions were positive for CD133 (Fig. 2A).

**Genome-Wide Expression Profiling Analyses**
Unsupervised hierarchical clustering
Unsupervised clustering analysis was performed by using 2 filtered subsets of genes. First, a Probe Set 1 was obtained by selecting genes based on a *P* value ≤ .001 and a fold change ≥ 2 in at least 10 cases (n = 15,281).
basis of this cluster analysis, 3 main clusters were identified: unsupervised cluster 1 (UnC1), UnC2, and UnC3 (Fig. 2B). CEC samples obtained from healthy controls (nCECs) clustered in cluster 1 (UnC1) together with CLL-CECs obtained from Patient 5. Almost all CD14-positive samples clustered in cluster 2 (UnC2), whereas cluster 3 (UnC3) included CD19-positive CLL cells and CLL-CECs. CLL-CECs did not generate a separate cluster but were intermingled with the CD19-positive CLL cells. Second, to define the reciprocal relation between CECs, CD19-positive groups, and CD14-positive groups, we generated a list of genes (Probe Set 2) with expression that varied significantly across samples by using a 1-way ANOVA. The, an agglomerative hierarchical algorithm was run using the selected list of genes (n = 9400; P < .01). The overall organization of UnC1 and UnC2 was maintained (Fig. 2C,D). A 2-dimensional matrix provided an overview of GEP among clusters. It suggested a relatively homogeneous GEP among CD19-positive samples and CLL-CECs. Conversely, distinct GEP characterized CD14-positive samples from CLL patients and nCECs.

Table 2. Hematologic and Clinical Data by Circulating Endothelial Cell Level

| Variable                          | Low CECs (n=125) | High CECs (n=45) | P   |
|-----------------------------------|------------------|------------------|-----|
| Age, y                            | 64.8±10.9        | 63.6±11.17       | NS  |
| No. of men/women                  | 63/62            | 31/14            | .032|
| WBC, ×10^9/L                      | 20.75±17.0       | 32.2±25.1        | .0009|
| Hemoglobin, g/dL                  | 13.5±2.0         | 13.3±2.3         | NS  |
| Platelets, ×10^5/L                | 219±84           | 203±62           | NS  |
| No. with Rai stage 0-1/2-4        | 89/36            | 32/13            | NS  |
| No with <30%/≥30% CD38 (n=168)    | 89/34            | 36/9             | NS  |
| No. with <30%/≥30% Zap70 (n=151)  | 71/36            | 24/20            | NS  |
| No. with favorable-intermediate/  | 92/21            | 30/11            | NS  |
| unfavorable FISH results (n=154)   | 36/42            | 14/16            | NS  |
| No. with mutated/unmutated IgVH   | 53/72            | 28/17            | .022|
| No. who received/did not receive therapy | 50/8             | 18/9             | .036|
| Response to treatment: CR and PR/SD and PD (n=85) |                         |                   |     |

SD indicates standard deviation; CECs, circulating endothelial cells; NS, nonsignificant; WBC, white blood cells; CD38, cluster of differentiation 38 (cyclic ADP-ribose hydrolase); Zap70, ζ-chain-associated protein kinase 70; FISH, fluorescence in situ hybridization; IgVH, immunoglobulin G variable heavy chain; CR complete response; PR partial response; SD stable disease; PD progressive disease.

Table 3. Factors That Affected the Time to First Treatment in Univariate Analysis

| Univariate Analysis Variable     | No. of Patients | Median±SE Time to First Treatment, mo | P   |
|----------------------------------|-----------------|---------------------------------------|-----|
| Rai stage 0-1/2-4 (n=170)        | 121/49          | 54±3.8/12±2.8                          | <.0001|
| CD38 <30%/≥30% (n=168)           | 125/43          | 58±4.4/15±3.3                          | <.0001|
| Zap70 <30%/≥30% (n=151)          | 95/56           | 53±4.1/22±2.7                          | .0006|
| Favorable-intermediate/unfavorable FISH results (n=154) | 122/32          | 53±4.0/12±1.0                          | <.0001|
| Low/high CECs (n=170)            | 125/45          | 53±4.3/35±3.7                          | .0027|

SE indicates standard error; CD38, cluster of differentiation 38 (cyclic ADP-ribose hydrolase); Zap70, ζ-chain-associated protein kinase 70; FISH, fluorescence in situ hybridization; CECs, circulating endothelial cells.

Table 4. Factors That Affected the Time to First Treatment in Multivariate Analysis

| Multivariate Analysis Variable   | HR (SE)         | P   |
|----------------------------------|-----------------|-----|
| Rai stage 0-1/2-4                | 2.38 (0.61)     | .001|
| CD38 <30%/≥30%                   | 2.38 (0.62)     | .001|
| Favorable-intermediate/unfavorable FISH results | 1.76 (0.47)   | .033|
| Low/high CECs                    | 1.97 (0.56)     | .007|

HR indicates hazard ratio; SE, standard error; CD38, cluster of differentiation 38 (cyclic ADP-ribose hydrolase); FISH, fluorescence in situ hybridization; CECs, circulating endothelial cells.
Altered expression of 4419 genes differentiates CLL-CECs from nCECs

On unsupervised analysis, all but 1 CLL-CEC clustered together, whereas nCECs displayed a highly similar GEP and clustered together with CLL-CECs from Patient 5. Inspection of the GEP matrix revealed both a homogeneous GEP among CLL-CECs and a deep difference between CLL-CECs and nCECs. Supervised analyses identified 4419 genes that were expressed differentially in CLL-CECs (a sample of CLL-CECs from Patient 5 was excluded from analysis) compared with nCECs. Differentially expressed genes comprised 2285 up-regulated genes and 2134 down-regulated genes in CLL-CECs compared with nCECs.

The GEP of CLL-CECs and nCECs revealed differences in several genes that are involved in angiogenesis, in blood vessel regulation and maturation, and in the signaling pathways of the wingless-type mouse mammary tumor virus integration site family (Wnt)/β-catenin, notch, apoptosis, integrins, cytokines, and chemokines (Table 6).

By contrast, CLL-CECs and nCECs had similar GEPs for several genes that characterized endothelial function, including CD144, CD34, CD133, CD31, VEGFR2, VEGFR3, von Willebrand factor (VWF), and endothelium-specific receptor tyrosine kinase 2 (TIE2).

Altered expression of 1029 genes differentiates CD19-positive chronic lymphocytic leukemia samples from chronic lymphocytic leukemia circulating endothelial cell samples

A comparison between CLL-CECs and CD19-positive cells derived from the same patients with CLL identified 1029 differentially expressed genes, comprising 183 up-regulated genes and 846 down-regulated genes in CLL-CECs compared with CD19-positive CLL cells.

When these genes were analyzed for relevant processes and pathways, we identified several genes involved in the Wnt, transforming growth factor β (TGF-β), and integrin signaling pathways. Moreover, genes involved in the cell cycle, such as cyclin G1, C, T2, and G2 as well as the VAV2 gene, were down-regulated; whereas VWF was up-regulated in CLL-CECs compared with CD19-positive CLL cells (Table 7).

Quantitative Flow Cytometry

Quantitative 4-color flow cytometry demonstrated that CD61 was expressed significantly more in CLL-CECs than in CLL leukemic cells (n = 29; mean ± SD, 14,140
7828 MESF vs 5920 ± 456 MESF; *P* < .001) and that flt1 was over-expressed on CLL-CECs (n = 8; mean ± SD, 8942 ± 227 MESF) compared with nCECs (n = 4; mean ± SD, 5369 ± 318 MESF; *P* = .01). These results are in keeping with GEP data.

**DISCUSSION**

In patients with CLL, CECs are increased compared with normal individuals* and may correlate with advanced-stage disease. However, the clinical and biologic significance of CECs in CLL remains uncertain.

In the current study, we demonstrated that, in patients with CLL, CECs and EPCs are increased compared with healthy controls and that higher levels of CECs define a subset of patients that have a more aggressive disease course as indicated by the shorter TFT, a parameter related strictly to prognosis. No correlation was observed between CECs and other predictors of a worse outcome, including markers of tumor mass or biologic aggressiveness, such as stage, positive CD38 and ZAP70 status, cytogenetics, and Ig mutation status. It is noteworthy that several reports have suggested that CECs and EPCs are increased in patients with cancer and are correlated with a more aggressive disease course.*2-4

Overall, these findings suggest that CECs may be related to the so-called angiogenic switch, a phenomenon that characterizes the shift to a more aggressive course of

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*Figure 2. Fluorescent in situ hybridization (FISH) and gene expression profile (GEP) analyses are illustrated in patients with chronic lymphocytic leukemia (CLL). (A) FISH analysis of sorted circulating endothelial cells (CECs) demonstrated 1 normal CEC (nCEC) (left) with a diploid copy number of chromosome 12 (2 red signals) and 1 cytogenetically abnormal CEC (right) with trisomy of chromosome 12 (3 red signals). In patients with CLL, the majority of CECs are endothelial progenitor cells (EPCs), because they express cluster of differentiation 133 (CD133/glycoprotein Prominin 1) (positive surface red staining for CD133-phycocerythrin). Note that several Dynabeads are attached to the cells. (B) An unsupervised cluster analysis (Probe Set 1) identified 3 main clusters (UnC1, UnC2, and UnC3). (C) The overall organization of UnC1 and UnC2 is maintained when using a second list of genes (Probe Set 2) in which expression varied significantly across samples in a 1-way analysis of variance using variance that was computed by applying a technology-specific, error-weighting model. (D) This is a thumbnail overview of the 2-way (genes vs samples) hierarchical clustering of samples. The 2-dimensional matrix shows relatively homogeneous GEPs among samples that were positive for the human CD19 protein (CD19-positive [+]) and CECs from patients with CLL. Distinct GEPs characterize CD14+ samples from patients with CLL and normal CECs.*

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Table 6. List of Some Genes That Are Expressed Differentially Between Circulating Endothelial Cells From Patients With Chronic Lymphocytic Leukemia and From Normal Controls

| Signaling Pathway                  | Genes                          | Expression |
|-----------------------------------|--------------------------------|------------|
| Angiogenesis                      | FLT1, TGFβ11, FG2, VEGFB       | Up         |
|                                   | FGF4, EPHRA4, EPHRB1, EPHRB2, ANGPT1 | Down       |
| Wnt                               | WNT3, WNT10A, WNT16, FRZB, DACT1, PPARD, HELLS, ARID1B, NFATC1, NFATC2, SMARCB1, AES, TLE1, HOXB7, LRPSL | Up         |
|                                   | WNT1, SMARCD2, SMARCD3, GSK3β, CTBP2, SMAD1, SMAD3, FZD2, CSNK1A1, TLE2 | Down       |
| Apoptosis                         | RELB, ATF1, BCL2               | Up         |
|                                   | TNFRSF1A, TNFRSF10, BIRC4, CASP10, APAF1, MCL1, FAS | Down       |
| Notch                             | NOTCH2, NOTCH4, PSEN1, JAG1, ADAM10, NUMB | Up         |
| Integrin                          | LAMC3, LAMAS, COL9A3, COL9A4, COL19A1, TGFβ1 | Up         |
| Cytokine and chemokine            | PXN, VCL, ACTN1, ACTN2, ITG4, ITGAV, ITGAX | Up         |
| Blood vessel regulation           | IL7, IL12A, CCR1, CCL27        | Down       |
|                                   | IL13RA1, IL6R, IL4, IL15, CCR2, CCR5, CCR1, CX3CR1, CCL2 | Up         |
|                                   | EDN1, EPAS1                    | Up         |
|                                   | ECGF1, EDG2                    | Down       |

FLT1 indicates fms-related tyrosine kinase 1 (vascular endothelial growth factor/vascular permeability factor receptor); TGFβ11, transforming growth factor β1-induced transcript 1; FG2, fibroblast growth factor 2; VEGFB, vascular endothelial growth factor B; FGF4, fibroblast growth factor receptor 4; EPHRA4, ephrin receptor type A, receptor 4; EPHRB1, ephrin receptor type B, receptor 1; EPHRB2, ephrin receptor type B, receptor 2; ANGPT1, angiopoeitin-1; WNT3, wingless type mouse mammary tumor virus integration site family (WNT), member 3; WNT10A, WNT member 10A; WNT16, WNT member 16; FRZB, frizzled-related protein; DACT1, dapper, antagonist of β-catenin, homolog 1; PPARD, peroxisome proliferator-activated receptor β; HELLS, helicase, lymphoid-specific; ARID1B, AT-rich interactive domain 1B (switch 1-like); NFATC1, nuclear factor of activated T-cells; NFATC2, nuclear factor of activated T-cells 2; SMARCB1, switch/sucrose nonfermentable (SWI/SNF)-related, matrix-associated, actin-dependent regulator of chromatin, subfamily b, member 1; AES, amino-terminal enhancer of split; TLE1, transducin-like enhancer of split 1; HOXB7, homeobox B7; LRPSL, low-density lipoprotein receptor-related protein 5-like; WNT1, wingless type mouse mammary tumor virus integration site family, member 1; SMARCD2, SWI/SNF-related, matrix-associated, actin-dependent regulator of chromatin, subfamily d, member 3; GSK3β, glycogen synthase kinase β; CTBP2, C-terminal binding protein 2; SMAD1, Smo/mothers against decapentaplegic 1; SMAD3, Smo/mothers against decapentaplegic 3; FZD2, frizzled homolog 2; CSNK1A1, casein kinase 1, x1; TLE2, transducin-like enhancer of split 2; RELB, v-rel reticuloendotheliosis viral oncogene homolog B; ATF1, activating transcription factor 1; BCL2, B-cell chronic lymphocytic leukemia/lymphoma 2; TNFRSF1A, tumor necrosis factor receptor superfamily, member 1A; TNFβ10, tumor necrosis factor (ligand) superfamily, member 10; BIRC4, baculoviral IAP repeat-containing protein 4; CASP10, caspase 10, apoptosis-related cysteine peptidase; APAF1, apoptotic peptidase activating factor 1; MCL1, myeloid cell leukemia sequence 1 (BCL2-related); FAS, fatty acid synthase; NOTCH2, Notch homolog 2; NOTCH4, Notch homolog 4; PSEN1, presenilin 1; JAG1, jagged 1; ADAM10, a disintegrin-like and metalloprotease (ADAM) metalloprotease domain 10; NUMB, numb homolog; LAMC3, laminin γ3; LAMAS, laminin γ5; COL9A3, collagen type IX, x3; COL9A2, collagen type IX, x2; COL9A4, collagen type IX, x4; COL19A1, collagen type XIX, x1; TGFβ1, transforming growth factor β1; PXN, paxillin; VCL, vinculin; ACTN1, actinin α1; ACTN2, actinin α2; ITGAV, integrin αv; ITG4, integrin δ4; ITGA6, integrin α6; ITGAX, integrin α5; IL7, interleukin 7; IL12A, interleukin 12A; CCR1, chemokine (C-C motif) receptor-like 1; CCL27, chemokine (C-C motif) ligand 27; IL13RA1, interleukin 13 receptor, α1; IL6R, interleukin 6 receptor; IL4, interleukin 4; IL15, interleukin 15; CCR2, chemokine (C-C motif) receptor 2; CCR5, chemokine (C-C motif) receptor 5; CR1, chemokine (C-C motif) receptor 1; CX3CR1, chemokine (C-X3-C motif) receptor 1; CCL2, chemokine (C-C motif) ligand 2; EDN1, endothelin 1; EPAS1, endothelial PAS domain protein 1 (where PAS indicates period circadian protein [Per]), Ah receptor nuclear translocator protein [Arnt], single-minded protein [Sim]); ECGF1, endothelial differentiation gene 2; EDG2, endothelial differentiation gene 1.

the disease, and support the idea that, in CLL, CECs may represent a new and easy-to-use prognostic marker to be tested in large multicenter series of patients for correlation with prognosis. It remains to be determined whether, in patients with CLL, CECs can be used as a surrogate marker to monitor the efficacy of therapy and whether patients with high CEC levels may represent a subset of patients who are eligible for antiangiogenic treatments, including new drugs like lenalidomide.31

Some limitations should be considered in the evaluation of these results: 1) The definitions of CECs and EPCs remain matters of debate,32 because some of the markers that are used to identify CECs are not specific to CECs and are expressed by subsets of hematopoietic cells. 2) Functional EC assays were not evaluated in this analysis, because standardized functional assays that correlate with immunophenotypic data still are lacking.33 3) Finally, it was demonstrated previously that cells similar to ECs deriving from sites outside the bone marrow may contribute to postnatal neovascularization and possibly to tumor angiogenesis.34

Angiogenesis and genetic aberrations reflect different biologic events that occur in tumor development and in the definition of prognosis and treatment response. In the current analysis, we demonstrated that, in patients with CLL, a significant proportion of cells with phenotypic features of CECs harbors the same cytogenetic aberration as the neoplastic cells. These data are in line with previous studies, which reported a similar phenomenon in other hematologic neoplasms.10-12

Because different populations of cells with the EC phenotype and angiogenic properties have been described in the PB,5,32,34 to better clarify the nature and the origin of these “neoplastic CECs,” we evaluated the GEP of
CLL-CECs and nCECs as well as the GEP of CD19-positive and CD14-positive cells from the same patients with CLL. Unsupervised cluster analyses revealed that CLL-CECs did not generate a separate cluster but were intermingled with CD19-positive CLL samples, whereas separated clusters were generated for nCECs and for CD14-positive cells, excluding the possibility that CLL-CECs may represent monocytic cells.\textsuperscript{35} A comparison between CLL-CECs and nCECs highlighted strong differences in the overall GEP, indicating increased cell survival and proliferation, diminished cell adhesion to extracellular matrix, and enhanced proangiogenic function in CLL-CECs compared with nCECs. No differences were observed concerning several angiogenic markers that were used for immunomagnetic sorting and immunophenotypic studies, confirming the validity of our selection method. GEP data also were correlated with immunophenotypic studies.

Among the genes that were expressed differentially in CLL-CECs compared with nCECs, we identified genes that were involved in the Wnt and Notch signaling pathways. Altered Wnt/β-catenin signaling and Notch signaling have been described in cancer\textsuperscript{36,37} and in patients with CLL\textsuperscript{38} and have been proposed as innate resistance mechanisms against radiation-induced and chemotherapy-induced cancer cell death.\textsuperscript{39} Moreover, functional studies in tumor models have indicated that Notch signaling is involved in EC proliferation and in the control of blood vessel sprouting and branching during tumor angiogenesis, although, paradoxically, the Notch pathway may be involved in both induction and inhibition of angiogenesis.\textsuperscript{38,40} The results of our GEP analyses demonstrate that, in CLL-CECs, the Wnt signaling pathway is active, whereas several genes of the Notch pathway are down-regulated, suggesting a significant activation of the angiogenic process. Finally, we observed that the integrin pathway was heavily dysregulated in CLL-CECs, which was in keeping with recent data indicating that angiogenesis is regulated by integrins.\textsuperscript{41} Taken together, our FISH and GEP data suggest that, in patients with CLL, a significant proportion of CECs may be derived from neoplastic CLL cells, as demonstrated not only by the presence of the same genetic lesion of the CLL clone but also by the results from a GEP analysis comparing CLL-CECs with both nCECs and CD19-positive CLL cells. Therefore, it is possible to speculate that these disguised CECs may mimic the phenotypic profile of CECs/EPCs and possibly, through the activation of genetic angiogenic programs, may act as a sort of bridgehead that, through the recruitment of nonclonal, bone marrow-derived ECs, favors tumor neovascularization and disease progression.\textsuperscript{10} Mathematical models support this hypothesis by demonstrating that CECs with an EPC phenotype have a significant effect on tumor growth and angiogenesis primarily by their localization in the tumor as opposed to their proliferation.\textsuperscript{42}

### Table 7. List of Some Genes That Are Expressed Differentially Between Circulating Endothelial Cells and CD19-Positive Cells From Patients With Chronic Lymphocytic Leukemia

| Signaling Pathway | Genes | Expression |
|-------------------|-------|------------|
| Wnt               | BTA1F, SMARCA5, SIAH1, CSNK1A, CSNK1G3, CSNK1A1L | Down |
|                   | ACVR1B, CTBP2 | Up |
| Ubiquitin-proteasome | EDD1, UBE2E1, UBE3A, UBE2NL, UBE1C | Down |
| TGF-beta          | SMURF2, GDF9, RAB28, RRA52 | Down |
| Integrin          | ACTN1, ITGB3, COL10A1 | Up |
| Blood coagulation | VWF, HOXB7, EVX1 | Up |
| Transcription factors | CREB1, MLLT10, TGF, HESX1, KRAB box transcription factors | Down |
| Others            | EPAS1, CCNG1, TNFRSF10A, CCNG2, PRKAB2, SUMO1 | Down |

BTA1F indicates BTA1 RNA polymerase II, B-transcription factor II D-associated (molybdate transporter 1 homolog, S. cerevisiae); SMARCA5, switch/sucrose nonfermentable (SWI/SNF)-related, matrix-associated, actin-dependent regulator of chromatin, subfamily a, member 5; SIAH1, seven in absentia homolog 1; CSNK1A, casein kinase 1, α; CSNK1G3, casein kinase 1, γ; CSNK1A1L, casein kinase 1, γ-1-like; ACVR1B, activin A receptor, type IB; CTBP2, C-terminal binding protein 2; EDD1, ubiquitin protein ligase E3 component n-recognin 5; UBE2E1, ubiquitin-conjugating enzyme E2E 1; UBE3A, ubiquitin protein ligase E3A; UBE2NL, ubiquitin-conjugating enzyme, E2N-like; UBE1C, ubiquitin-like modifier activating enzyme 3; TGF, transforming growth factor; SMURF2, Sma/mothers against decapentaplegic (SMAD)-specific E3 ubiquitin protein ligase 2; GDF9, growth differentiation factor 9; RAB28, member, RAS oncogene family; RRA52, related RAS viral (r-ras) oncogene homolog 2; ACTN1, actinin, α 1; ITGB3, integrin β 3; COL10A1, collagen, type X, α 1; VWF, von Willebrand factor; HOXB7, homeobox B7; EVX1, even-skipped homolog 1; CREB1, cyclic adenosine monophosphate responsive element-binding protein 1; MLLT10, myeloid/lymphoid or mixed-lineage leukemia translocated to 10; TGIF, transforming growth factor B-induced factor; HESX1, homeobox ES cell expressed 1; KRAB, Kruppel-associated box; EPAS1, endothelial PAS domain protein 1 (where PAS indicates period circadian protein [Per], Ah receptor nuclear translocator protein [Arnt], single-minded protein [Sim]); CCNG1, cyclin G2; TNFRSF10A, tumor necrosis factor receptor superfamily, member 10A; CCNG2, cyclin G2; PRKAB2, protein kinase, adenosine monophosphate-activated, β γ noncatalytic subunit; SUMO1, small ubiquitin-related modifier 1.
In conclusion, we demonstrated that the flow cytometric evaluation of cells with the CEC phenotype may identify a subset of patients with a more aggressive disease course and that, in FISH and GEP analyses, CECs have a molecular pattern that suggests a possible derivation from CLL leukemic cells. These observations may represent a rationale for the development of new treatment strategies targeting the angiogenic process.

CONFLICT OF INTEREST DISCLOSURES

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