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Advances in Brief

Kinetics and Viability of Circulating Endothelial Cells As Surrogate Angiogenesis Marker in an Animal Model of Human Lymphoma[1]

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Abstract

Circulating endothelial cells (CECs) were evaluated by flow cytometry in immunodeficient mice bearing human lymphoma. A trend toward higher CEC values was observed on days 7 and 14 after transplant, and differences *versus* controls were highly significant on day 21 ($P = 0.0061$). A strong correlation was found between CEC and tumor volume (r, 0.942; $P = 0.004$) and between CEC and tumor-generated VEGF (r, 0.669; $P = 0.02$). In mice given cyclophosphamide, most of the circulating apoptotic cells were hematopoietic and not endothelial. Conversely, in mice given endostatin, all of the increase in apoptotic cells was in the endothelial cell compartment. CEC evaluation is promising as a noninvasive, surrogate angiogenesis marker.

Introduction

The generation of new blood vessels (angiogenesis) is considered an essential step in tumor growth and metastasis (1), and a new class of drugs with targeted activity on angiogenic vessels has been developed to control cancer progression (2). At the present time, measurement of tumor angiogenesis to predict and/or assess the efficacy of antiangiogenic therapies is mainly based on the evaluation of MVD.[3] In this procedure, blood vessels of tumor samples are stained with antibodies and counted by light microscopy. This approach is invasive, MVD of tumor biopsy might not correlate with MVD of the whole tumor specimen, and the correlation with the clinical outcome is still uncertain in most tumor types (3). We have recently found that CECs, measured by FC, were significantly increased in the PB of untreated lymphoma and breast cancer patients. In lymphoma patients achieving complete remission after chemotherapy, CECs were reduced to the values observed in healthy controls, and activated CECs were found to decrease in breast cancer patients evaluated before and after quadrantectomy (4). To better understand CEC relevance as a noninvasive, surrogate angiogenesis marker, we evaluated CEC kinetic in an animal model of human lymphoma recently developed in our laboratory (5, 6). In parallel, we evaluated CEC viability to ascertain whether this measurement may reflect the antiangiogenic properties of a given drug.

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[3] The abbreviations used are: MVD, microvessel density; CEC, circulating endothelial cell; VEGF, vascular endothelial growth factor; FC, flow cytometry; PB, peripheral blood; NOD/SCID, nonobese diabetic/severe combined immunodeficiency; CTX, cyclophosphamide; MTD, maximum tolerable dose; IHC, immunohistochemistry; FLK, VEGF receptor 2 fetal liver kinase 1; 7AAD, 7-aminoactinomycin D.

Materials and Methods

Animal Model. As described previously (5, 6), 6–8-week-old NOD/SCID mice were given i.p. injections of 10×10^6 Namalwa cells (phenotype CD3−, CD10+, CD13−, CD19+, CD20+, GlyA−). Namalwa cells were derived from an EBV+ Burkitt's lymphoma, obtained from American Type Culture Collection (Manassas, VA) and cultured in RPMI-8% fetal bovine serum (HyClone, Logan, UT). Tumor growth was evaluated every other day, tumors were measured by calipers, and the formula ($\text{width}^2 \times \text{length} \times 0.52$) was applied to approximate the volume of a spheroid (6, 7). To evaluate CECs and circulating human VEGF, Namalwa tumor-bearing NOD/SCID mice and untransplanted controls ($n = 6$ per study group) were bled from the lateral tail vein before, and on days 7, 14, and 21 after, transplant. All of the procedures involving animals were done in accordance with national and international laws and policies.

CTX and Endostatin Treatment. In drug treatment studies, on day 21 after tumor injection, drugs were supplied at a site remote from the inoculated tumor. According to previous studies (6), the cytotoxic drug CTX (Sigma Chemical Co., St. Louis, MO) was given i.p. at the MTD of 150 mg/kg as a single administration ($n = 6$), the antiangiogenic drug endostatin (Calbiochem, San Diego, CA) was given s.c. as a single dose of 150 μg/mouse ($n = 6$). As a control, tumor-bearing mice received i.p. or s.c. PBS ($n = 6$ per study group). Before treatment, and 24 h after, mice were bled from the lateral tail vein for CEC evaluation.

Tumor Evaluation. On day 22, mice were killed by CO_2 inhalation. Tumors were collected from all of the mice and evaluated by histology, IHC, and FC as described previously (5, 6). For histology and IHC evaluation, Namalwa tumor samples were fixed in 10% buffered formalin and embedded in paraffin. Tumor sections (4 μm thick) were stained with H&E and Giemsa for conventional histology. For IHC, sections were immunostained with anti-CD10 and -CD20 monoclonal antibodies by DAKO (Glostrup, Denmark). Tumor expression of human CD19 and CD20 antigens was also evaluated by FC using BD (Mountain View, CA) monoclonal antibodies. MVD was evaluated as described previously (5). To detect the area with the highest MVD (hot spot), H&E-stained slides were evaluated at ×40 and ×100. Three microscopic fields were then examined in this area at ×250 (each field representing an area of 0.72 mm^2), and the mean MVD value was recorded. Any endothelial cell or endothelial cell cluster that was clearly separated from adjacent microvessels was considered a single, countable microvessel.

Measurement of CEC Number and Viability by FC. CECs in the PB were enumerated by three-color FC using a panel of monoclonal antibodies reacting with murine CD45 (to exclude hematopoietic cells; Ref. 4) and endothelial murine markers FLK, CD105, VE cadherin, MECA-32, CD31, and CD34 (PharMingen BD, San Diego, CA). After red cell lysis, cell suspensions were evaluated by a FACSCalibur (BD, San Jose, CA) using analysis gates designed to remove dead cells, platelets, and debris (Fig. 1). After acquisition of at least 100,000 cells per sample, analyses were considered as informative when adequate numbers of events (*i.e.,* >50, typically 100–200) were collected in the CEC enumeration gates. The percentage of stained cells was determined as compared with appropriate negative controls. Positivity was defined as being greater than nonspecific background staining. According to the method of Philpott *et al.* (8), annexin V and 7AAD were used to depict apoptotic and dead cells (5, 6, 8).

Evaluation of Human VEGF in Mouse PB. Human VEGF (known to be produced by Namalwa cells; Ref. 5) was measured in the PB of tumor-bearing

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mice and controls by commercial ELISA kits (R&D, Minneapolis, MN) as described previously (5).

Statistical Analysis. CEC kinetics were compared in tumor-bearing mice and untransplanted controls over the entire period of observation. Statistical comparisons were performed using the *t* test, ANOVA, and linear regression when data were normally distributed and the nonparametric analyses of Spearman and Mann-Whitney when data were not normally distributed. Values of *P* lower than 0.05 were considered as statistically significant.

Results

CEC Kinetics in Controls and Xenografted Mice. Fig. 1 shows the FC evaluation of murine CD45−, FLK+, CD31+, CD34+, VE-cadherin+, MECA-32+ CECs and of the endothelial cell activation marker CD105 (4, 9). In the CD45 negative fraction of nonhematopoietic cells, expression of CD31, CD34, VE-cadherin, and MECA-32 was similar to FLK expression. Thus, throughout the study, CECs are reported as CD45− FLK+ cells. Before transplant, mean CECs/μl were 0.9 (95% confidence interval, 0.6–1.1). As indicated in Fig. 2, a trend toward higher CEC values was observed in xenografted mice on days 7 and 14, and differences were highly significant on day 21, when in xenografted mice, mean CECs/μl were 10.2 (95% confidence interval, 1.2–19.3; $P = 0.0061$ *versus* controls). A strong correlation was found between CEC and tumor volume (*r*, 0.942; $P = 0.004$, by Spearman Rank test) and between CECs and tumor weight on day 21 (*r*, 0.885; $P = 0.01$). In control mice, circulating human VEGF was undetectable, in tumor-bearing mice mean human VEGF was 14 pg/ml (95% confidence interval, 6–22). A positive correlation was found in tumor-bearing mice between CECs and human VEGF (*r*, 0.669; $P = 0.02$) and between MVD and tumor volume (*r*, 0.948; $P = 0.05$). The correlation between CECs and MVD was slightly weaker (*r*, 0.737; $P = 0.26$). As indicated in Fig. 1, 30–60% of CECs expressed the activation marker CD105, and differences between tumor-bearing mice and controls were not significant.

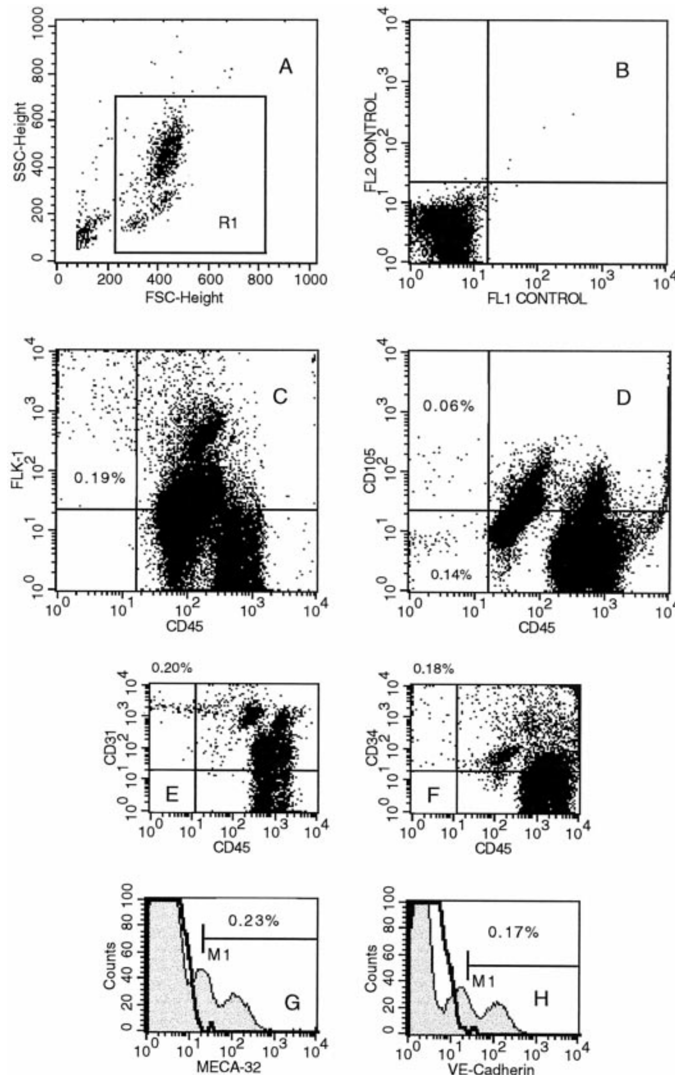
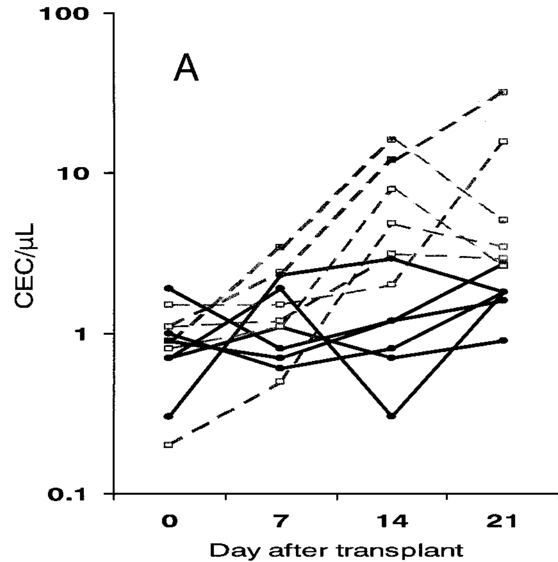


Fig. 1. Representative CEC evaluation by FC. *A,* the analysis gate used to exclude platelets, dead cells, and debris. *B,* negative controls. *C,* the frequency of CD45− and FLK+ cells. *D,* the distribution of the murine endothelial cell activation marker CD105 among nonhematopoietic (CD45−) cells. *E,* the frequency of CD45− and CD31+ cells. *F,* the frequency of CD45− and CD34+ cells. *G* and *H,* the expression of murine endothelial markers MECA-32 and VE-cadherin, respectively.



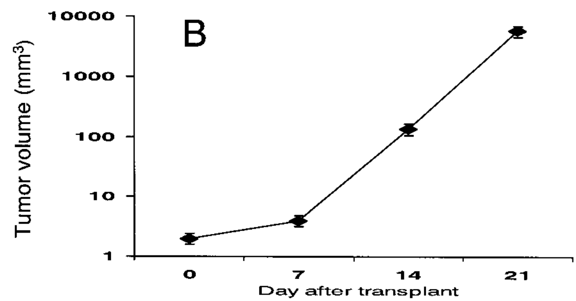


Fig. 2, *A* and *B*. *A,* CEC kinetics in NOD/SCID mice that were given injections of Namalwa lymphoma cells ($n = 6$) and in controls ($n = 6$). Despite repeated bleeding, CECs were stable in untransplanted control mice (———). In tumor-bearing animals (····), a trend toward increased CEC numbers was already observed on days 7 and 14 after transplant; and on day 21, differences were highly significant ($P = 0.0061$ *versus* control). *B,* Namalwa tumor growth curve. Values, mean ± 1 SD.

CEC Viability. As shown in Fig. 3, in control mice most CECs were annexin V+, and 30–50% of them also expressed intermediate 7AAD staining. These findings suggest that in healthy mice most CEC may have initiated an apoptotic process. In tumor-bearing mice, the mean frequency of annexin V+ or 7AAD+ CEC was 29% (95% confidence interval, 23–35%; $P < 0.01$ *versus* control). This finding indicates improved CEC viability in tumor-bearing mice, possibly because of high VEGF levels generated by tumor cells.

As indicated in Fig. 4, the administration of the cytotoxic drug CTX at MTD ($n = 6$ CTX-treated mice and 6 tumor-bearing, untreated mice evaluated as control) was associated 24 h after treatment with a significant increase of circulating cells showing intermediate and high

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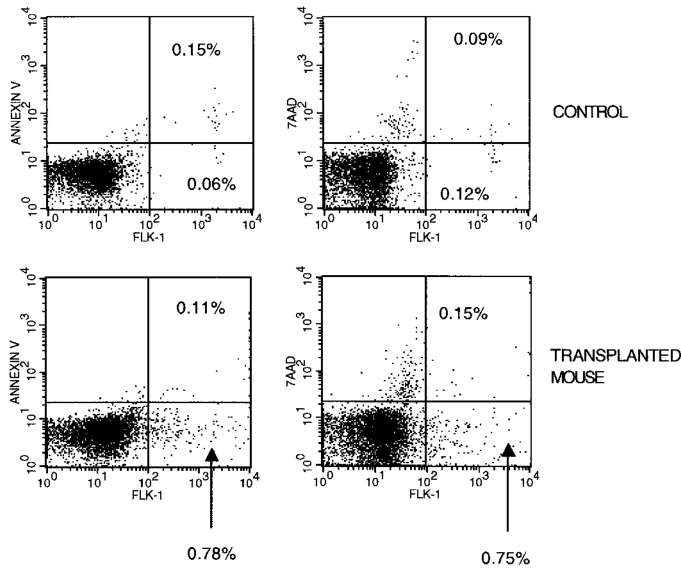

Fig. 3. Representative evaluation of CEC viability in control NOD/SCID mice (*top panels*) and in mice bearing Namalwa lymphoma (*bottom panels*). In control mice, the majority of CECs were annexin-V positive, and 30–50% of them also expressed intermediate 7AAD staining. Accordingly, in control mice, most CECs may have initiated an apoptotic process. In tumor-bearing mice, the frequency of annexin V and 7AAD indicated that most CECs were viable.

levels of 7AAD staining. After CTX administration at MTD, ~85% of the 7AAD+ apoptotic cells were hematopoietic and not endothelial (CD45+ FLK−), and ~40% of FLK+ CECs were still viable (7AAD−). Conversely, 24 h after the administration of the antiangiogenic drug endostatin ($n = 6$ endostatin-treated mice and 6 tumor-bearing, untreated mice evaluated as control), all of the increase in 7AAD+ circulating cells was in the endothelial (CD45− FLK+) cell compartment, and most of the CECs were apoptotic or dead, *i.e.,* expressing high 7AAD staining ($P < 0.001$ *versus* controls).

Discussion

In a previous clinical study, we found that CECs were increased in untreated breast cancer and lymphoma patients and reduced to normal values in patients achieving complete remission (4). We took advantage of an animal model of human lymphoma (5, 6) to evaluate CEC kinetics and viability before and after drug therapy. Present data indicate a stable CEC increase after lymphoma transplant, and the strong correlation observed between CEC and tumor volume indicates that CEC increase may parallel tumor progression. As shown in Fig. 2, some variability in CEC numbers was found among different tumor-bearing mice. The correlation observed between CEC and circulating levels of human VEGF (produced by transplanted Namalwa cells; Ref. 5), suggests that this variable may also play a role in determining CEC frequency in tumor-bearing mice. The role of VEGF is particularly interesting, because we have found that in control animals, most CECs seem to have initiated an apoptotic program, whereas CEC viability is markedly improved in tumor-bearing mice. Considering the antiapoptotic properties of VEGF (10), this growth factor may play a relevant role in preserving CEC from apoptosis.

In a previous work, the frequency of endothelial apoptotic cells (measured by anti-FLK monoclonal antibodies, 7AAD staining, and FC) was found to be significantly increased in Namalwa tumors removed from NOD/SCID mice treated with the antiangiogenic drug endostatin (6). In the present work, we evaluated CEC viability before and after drug therapy and found that the cytotoxic drug CTX at MTD induces apoptosis of circulating hematopoietic and (to a lesser extent) endothelial cells. Conversely, the antiangiogenic drug endostatin specifically targets endothelial and not hematopoietic cells. Our finding confirms recent data about the antiangiogenic activity of some cytotoxic drugs including CTX (11, 12), and antiangiogenic drugs such as endostatin and angiostatin are currently in Phase I-II clinical trials. Thus, the measurement of CEC viability during clinical studies may be of relevant help to define the balance between cytotoxic and antiangiogenic activity of different drug schedules.

CEC increase in cancer patients may be attributable to at least three different causes: CECs may derive from the lining of angiogenic tumor vessels, represent ingress of proliferating endothelial cells from neighboring normal tissue, or derive from distant uninvolved vessels activated by the derived cytokines of the tumor. Chang *et al.* (13) have recently provided evidence indicating that tumor blood vessels may be mosaics in which both endothelial and tumor cells form the luminal surface. Although controversies still exist about the frequency of tumor cells contributing to this phenomenon (14), data by Chang *et al.* (13) provide a possible explanation for the finding of high CEC numbers in cancer patients. In their colon tumor xenograft model, in fact, they collected morphological evidence of endothelial cells shedding from the lining of tumor vessels. Commenting on data on mosaic tumor vessels, Folkman (15) has indicated that in tumor angiogenic vessels the endothelial cell lining is continuously migrating, whereas in mature, quiescent vessels there is little or no endothelial cell turnover. Again, this picture fits well with our present data on CEC kinetics.

Taken together, our findings support CEC evaluation as a surrogate, noninvasive angiogenesis marker that may contribute to the existing panel of angiogenesis assays (16). The measurement of CEC viability by FC seems a useful, noninvasive tool to evaluate the efficacy of targeted antiangiogenic drugs in preclinical models of human disease as well as in clinical trials. Considering that CECs correlate well with tumor volume and circulating VEGF, known to be a relevant prognostic factor in human lymphoma (17, 18), we are evaluating CECs longitudinally in patients enrolled in different clinical trials.

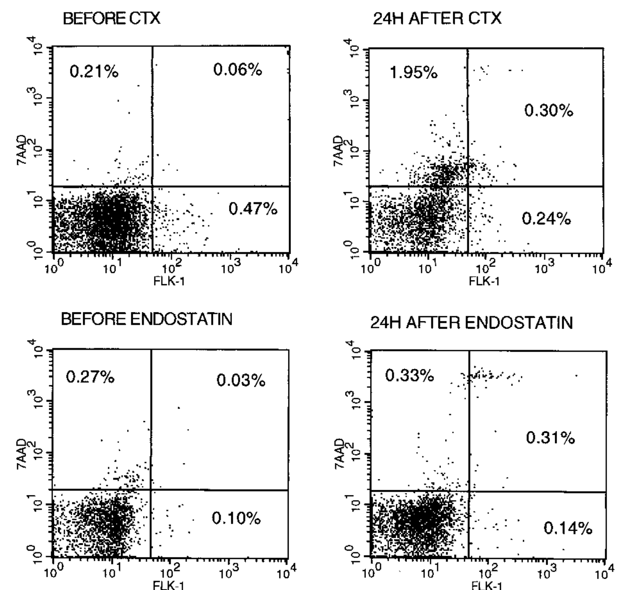

Fig. 4. Representative evaluation of CEC viability before (*left*) and after (*right*) administration of the cytotoxic drug CTX (*top*) and of the antiangiogenic drug endostatin (*bottom*). In mice given CTX at MTD, most of the circulating apoptotic (7AAD+) cells were hematopoietic (FLK−) and not endothelial (FLK+), and a relevant frequency of CECs were still viable (FLK+ 7AAD−). In mice given endostatin, all of the increase in circulating apoptotic cells was in the endothelial (FLK+) cell compartment, and most FLK+ CECs were apoptotic (intermediate 7AAD staining) or dead (high 7AAD staining).

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Acknowledgments

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