Strategies to Investigate Circulating Endothelial Cells in Cancer

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Key Words

Circulating endothelial cells · Cancer

Abstract

There is an increasing evidence of the crucial role of angiogenesis in cancer, and randomized studies have indicated that a "pure" anti-angiogenic drug (the anti-VEGF antibody Avastin) is very effective in colorectal cancer. In addition, this and other anti-angiogenic drugs have demonstrated activity and are currently under clinical investigation in a variety of other cancer types. At the present time, however, there is a scarcity of useful endpoints for treatment outcome beside survival. Using flow cytometry, quantitative PCR and cell culture we have found that circulating endothelial cells and progenitors are increased in cancer patients, and that measuring their viability and kinetics may offer significant clinical insight in the management of cancer patients.

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Introduction

The generation of new blood vessels is a crucial 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 anti-angiogenic therapies is mainly based on the evaluation of microvessel density (MVD). 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, MVD might not be useful to predict the efficacy of an anti-angiogenic drugs [3], and the correlation between MVD and the clinical outcome is still uncertain in most tumor types [4].

It has been reported in the past that circulating endothelial cells (CECs) are increased in the peripheral blood (PB) of patients affected by sickle cell anemia [5], cytomegalovirus [6] or rickettsial [7] infection, myocardial infarction and endotoxemia [8,9]. Moreover, increased CECs have been reported in patients bearing intravascular instrumentation [10]. We have developed novel flow cytometry and quantitative PCR procedure to measure CECs and circulating endothelial progenitors (CEPs) in cancer pre-clinical models and in cancer patients.

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CECs as Marker of Tumor Growth in Preclinical Studies

In mice bearing human lymphoma, murine CECs were enumerated by four-color flow-cytometry using a panel of monoclonal antibodies reacting with murine CD45 (to exclude hematopoietic cells) and endothelial murine markers VEGF receptor 2 fetal liver kinase 1 (FLK), CD105, VE cadherin, MECA-32, CD13, CD31 and CD34 (PharMingen BD, San Diego, CA) [14-16]. 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. After acquisition of at least 100,000 cells per sample, analyses were considered as informative when adequate numbers of events (ie >50, typically 100-200) were collected in the CEC enumeration gates. The percent of stained cells was determined as compared with appropriate negative controls. Positivity was defined as being greater than non-specific background staining. According to the method of Philpott et al [13], annexin V and 7AAD were used to depict apoptotic and dead cells. A trend toward higher CECs values was observed in mice xenografted with human lymphoma on day 7 and 14, and differences were highly significant on day 21, when in xenografted mice mean CECs/ μ L were 10.2 (95% confidence limit 1.2-19.3, $p=0.0061$ vs controls). A strong correlation was found between CECs and tumor volume ($r=0.942$, $p=0.004$ by Spearman Rank test) and between CEC and tumor weight on day 21 ($r=0.885$, $p=0.01$). 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 ($r=0.948$, $p=0.05$).

CECs as Marker of Antiangiogenic Activity in Preclinical Studies

Opposite Effects of High- vs Low-dose Chemotherapy on CEPs

We showed that the administration of cyclophosphamide (CTX) at the maximum tolerable dose with 21-day breaks or at more frequent low-dose (metronomic) schedules have opposite effects on the mobilization and viability of CEPs in immunodeficient mice bearing human lymphoma cells [16]. Animals treated with the maximum tolerable dose CTX experienced a robust CEPs mobilization a few days after the end of a cycle of drug administration, and tumors rapidly became drug resistant. Conversely, the administration of metronomic CTX was associated with a consistent decrease in CEPs numbers and viability and with more durable inhibition of tumor growth. Our findings suggest that metronomic low-dose chemotherapy regimens are particularly

promising for avoiding CEP mobilization and, hence, to potentially reduce vasculogenesis-dependent mechanisms of tumor growth.

Effect of Endostatin on CECs and CEPs

We investigated the effect of endostatin on differentiation, mobilization and clonogenic potential of CEPs and whether the effect of endostatin was improved by continuous infusion (CI) versus bolus administration [15]. CEPs were studied in tumor free mice, tumor bearing immunodeficient mice and in mice xenotransplanted with human bone marrow cells by flow-cytometry and endothelial cell cultures. We observed that endostatin significantly reduced the number of circulating EC progenitors in tumor-free BALB/c mice. The effect of endostatin on EC progenitors was enhanced significantly in mice treated with CI drug treatment. When immunodeficient mice xenotransplanted with human BM cells were treated with CI of endostatin we observed a significant decrease in the engraftment and differentiation of human BM-derived EC progenitors. Numbers of circulating EC progenitors increased 7-fold in immunodeficient mice bearing human lymphoma. In this preclinical model, treatment with CI of endostatin inhibited host murine EC progenitor mobilization and human tumor growth. Furthermore, the clonogenic potential of EC progenitors was impaired severely.

CECs and CEPs in Cancer Patients

Flow cytometry and antibodies (including anti-CD45 to exclude hematopoietic cells, anti-CD31, -CD133, -P1H12, and the apoptosis marker 7AAD) have been used to enumerate viable and apoptotic CECs and CEPs in cancer patients. Cell suspensions were evaluated after red cell lysis by a FACSCalibur equipped with a second red-diode laser (BD, San Jose, CA). After acquisition of at least 100,000 cells per blood sample, analyses were considered as informative when adequate numbers of events (ie >100, typically 3-400) were collected in the CECs enumeration gates. CECs were defined as negative for hematopoietic marker CD45, positive for endothelial markers P1H12 and CD31 and negative for the progenitor marker CD133. CEPs were depicted by expression of CD133.

In breast cancer (BC) and lymphoma patients, CECs were increased by 5-fold ($P < .0008$ vs control). CECs significantly correlated with plasma levels of VCAM-1 and VEGF. CECs were similar to healthy controls in 7 lymphoma patients achieving complete remission after chemotherapy. Repeated CECs measurements in patients and controls indicated a low longitudinal CEC variation,

and the count of resting and activated CECs did not correlate with the count of white cells, red cells or platelets.

We are currently evaluating CECs and CEPs in different clinical trials involving the administration of different anti-angiogenic drugs. Preliminary results indicate that CECs and CEPs are reduced to normal values in most patients achieving a clinical response or a stable disease, and that a rise in CECs numbers may predict disease recurrence.

Quantitative RT-PCR of the VE-Cadherin Gene, a Novel Surrogate Angiogenesis Marker

Using a real-time PCR approach, we found that circulating RNA levels of CEP- or endothelial cell-specific CD133, Tie-2 and VEGFR2 genes were not significantly increased in pregnant women or cancer patients, whereas circulating VE-Cadherin (VE-C) RNA was significantly increased in pregnant women and cancer patients before therapy ($p<0.001$).

The mean percentage (%) VE-C/beta actin ratio was 0.2 ± 0.1 in controls and 10-fold increased (ie 2.1 ± 1.3) in cancer-bearing patients evaluated before therapy ($p=0.0003$). In the subgroup of patients who achieved a partial remission the mean ratio was 0.9 ± 0.3 ($p=0.01$ vs healthy controls). In the subgroup of 14 patients who achieved a complete remission the mean ratio was 0.5 ± 0.3 , ie similar to healthy controls ($p=0.14$) and significantly lower than cancer-bearing patients ($p=0.0004$). When evaluated before therapy, patients affected by lymphoproliferative malignancies or acute leukemia had mean ratios of 2.0 ± 1.1 and 3.1 ± 1.7 , respectively ($p<0.001$ vs healthy controls). The mean ratio of solid cancer patients evaluated before therapy was 0.7 ± 0.2 , ie lower than that of patients affected by hematological diseases, albeit still significantly higher than healthy controls ($p=0.03$).

A switch to increased CEC, VEGF levels and VE-C ratio was observed in patients affected by lymphoproliferative malignant diseases. The flow cytometric evaluation of the apoptosis marker 7AAD indicated that CEC viability was significantly increased in cancer patients compared to

healthy controls ($p<0.01$). When culture assays were performed in 12 healthy controls and 28 cancer patients, a significant increase in EC colonies (and in EC colonies including CD133+ CEPs) was found in cancer patients compared to healthy controls and patients in complete remission. A significant correlation was found between endothelial colonies and the number of viable (7AAD-negative) CEC evaluated by flow cytometry ($r=0.43$, $p=0.04$).

In apoptotic (starved) HUVEC, we observed a 2-300 fold reduction of VE-C RNA compared to viable cells. Thus, we correlated VE-C RNA and the number of viable CEC (enumerated by flow cytometry) in patients affected by hematological malignancies. This correlation was highly significant ($r=0.86$, $p=0.008$). Along this line, in this subgroup of patients evaluated before therapy a three-dimensional plot indicated a switch toward increased CEC numbers, increased CEC viability and increased circulating VE-C RNA.

Quantitative VE-C RNA evaluation offers some distinct advantages over flow cytometric CEC enumeration and culture assays. VE-C RNA enumeration can be performed in large series of frozen samples. Inter-laboratories standardization seems to be more easily achievable, and our data indicate an interesting correlation between VE-C RNA and CEC viability. On the other hand, flow cytometric CEC enumeration and culture assays can probably discriminate more accurately than VE-C RNA evaluation between cancer patients with a prevalent angiogenic (ie mature endothelial cell-driven) phenotype and patients with a prevalent vasculogenic (ie endothelial progenitor cell-driven) phenotype.

Conclusion

Taken together, our data indicate that VE-C RNA, flow cytometric CEC and CEP enumeration and culture assays will offer some non-overlapping series of clinical information, and a multi-faceted evaluation of these surrogate markers will be of help to design patient-tailored therapies for neoplastic and non-neoplastic patients.

References

- 1 Folkman J. Angiogenesis in cancer, vascular, rheumatoid and other diseases. *Nat Med* 1995;1:27-31.
- 2 Folkman J, Hahnfeltdt P, Hlatky J. Cancer: looking outside the genome. *Nat Rev Mol Cell Biol* 2000;1:76-79.
- 3 Hlatky L, Hahnfeltdt P, Folkman J. Clinical Application of Antiangiogenic Therapy: Microvessel Density, What it does and doesn't tell us. *J Natl Cancer Inst* 2002;94:883-893.
- 4 Weidner N. Angiogenesis as a predictor of clinical outcome in cancer patients. *Hum. Pathol* 2000;31:403-405.
- 5 Solovey A, Lin Y, Browne P, Choong S, Wayner E, Heibel RP. Circulating endothelial cells in sickle cell anemia. *New Engl J Med* 1997;337:1584-1590.
- 6 Grefte A, Van der Giessen M, van Son W, The TH. Circulating cytomegalovirus-infected endothelial cells in patients with an active CMV infection. *J Infect Dis* 1993;167:270-277.
- 7 Drancourt M, George F, Brouqui P, Sampil J, Raoult D. Diagnosis of Mediterranean spotted fever by indirect immunofluorescence of *Rickettsia conorii* in circulating endothelial cells isolated with monoclonal antibody-coated immunomagnetic beads. *J Infect Dis* 1992;166:660-663.

- 8 Mutin M, Canavy I, Blann A, Bory M, Sampol J, Dignat-George F. Direct evidence of endothelial injury in acute myocardial infarction and unstable angina by demonstration of circulating endothelial cells. *Blood* 1999;93:2951-2958.
- 9 Lin Y, Weisdorf D.J, Solovey A, Hebbel RP. Origins of circulating endothelial cells and endothelial outgrowth from blood. *J Clin Invest* 2000;105:71-77.
- 10 George F, Poncelet P, Laurent J.C, Massot O, Arnoux D, Lequeux N, Ambrosi P, Chicheportiche C, Sampol J. Cytofluorometric detection of human endothelial cells in whole blood using S-Endo 1 monoclonal antibody. *J Immunol Methods* 1991;139:65-75.
- 11 Fusetti L, Gobbi A, Rabascio C, Pruneri G, Carboni N, Peccatori F, Martinelli G, Bertolini F. Human Myeloid and Lymphoid Malignancies in the Non-Obese Diabetic/Severe Combined Immunodeficiency Mouse Model: Frequency of Apoptotic Cells in Solid Tumors and Efficiency and Speed of Engraftment Correlate with Vascular Endothelial Growth Factor Production. *Cancer Res* 2000;60:2527-2534.
- 12 Bertolini F, Fusetti L, Mancuso P, Gobbi A, Corsini C, Ferrucci P.F, Martinelli G, Pruneri G. Endostatin, an antiangiogenic drug, induces tumor stabilization after chemotherapy or anti-CD20 therapy in a NOD/SCID mouse model of human high-grade non-Hodgkin's lymphoma. *Blood* 2000;96:282-287.
- 13 Philpott NJ, Turner AJC, Scopes J, Westby M, Marsch JCW, Gordon-Smith EC, Dalglish AG, Gibson FM. The use of 7-amino actinomycin D in identifying apoptosis: Simplicity of use and broad spectrum of application compared with other techniques. *Blood* 1996;87:2244-2251.
- 14 Monestiroli S, Mancuso P, Burlini A, Pruneri G, Dell'Agnola C, Gobbi A, Martinelli G, Bertolini F. Kinetics and viability of circulating endothelial cells as surrogate angiogenesis marker in an animal model of human lymphoma. *Cancer Res* 2001;61:4341-4344.
- 15 Capillo M, Mancuso P, Gobbi A, Monestiroli S, Pruneri G, Dell'Agnola C, Martinelli G, Shultz L, Bertolini F. Continuous infusion of endostatin inhibits differentiation, mobilization and clonogenic potential of endothelial cell progenitors. *Clin Cancer Res* 2003;9:377-382.
- 16 Bertolini F, Paul S, Mancuso P, Monestiroli S, Gobbi A, Shaked Y, Kerbel RS. Maximum tolerable dose and low-dose metronomic chemotherapy have opposite effects on the mobilization and viability of circulating endothelial progenitor cells. *Cancer Res* 2003;63:4342-4346.
- 17 Mancuso P, Burlini A, Pruneri G, Goldhirsch A, Martinelli G, Bertolini F. Resting and activated endothelial cells are increased in the peripheral blood of cancer patients. *Blood* 2001;97:3658-3661.
- 18 Rafii S. Circulating endothelial precursors: mystery, reality, and promise. *J Clin Invest* 2000;105:17-19.
- 19 Rabascio C, Muratori E, Mancuso P, Calleri A, Raia V, Fouts T, Cinieri S, Veronesi G, Pruneri G, Lampertico P, Iavarone M, Martinelli G, Goldhirsch A, Bertolini F. Assessing tumor angiogenesis: Increased circulating VE-cadherin RNA in patients with cancer indicates viability of circulating endothelial cells. *Cancer Res* 2004; in press.