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Differential CD146 Expression on Circulating Versus Tissue Endothelial Cells in Rectal Cancer Patients: Implications for Circulating Endothelial and Progenitor Cells As Biomarkers for Antiangiogenic Therapy

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Abstract

Purpose—Circulating endothelial cells (CECs) and progenitor cells are currently evaluated as potential biomarkers of antiangiogenic therapy. CD146 is considered a panendothelial-specific marker, but its utility as a CEC marker in cancer patients remains unclear.

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Authors' Disclosures of Potential Conflicts of Interest

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Patients and Methods—We analyzed the expression of CD146 on mononuclear blood cells, primary tissue endothelial cells, and malignant and normal tissues by flow cytometric and immunohistochemical analyses. Furthermore, we measured the circulation kinetics of CD146⁺ cells before, and then 3 and 12 days after vascular endothelial growth factor (VEGF) antibody blockade by bevacizumab infusion in rectal cancer patients enrolled in a phase I trial.

Results—In the peripheral blood of these cancer patients, over 90% of the CD146⁺ cells were CD45⁺ hematopoietic cells. CD146 expression was primarily detected on a subset of CD3⁺CD4⁺ lymphocytes, and was undetectable on CD34⁺CD133⁺CD45^{dim} progenitor cells or CD31^{bright}CD45[−] viable CECs. In contradistinction, CD146 was detectable in tissues on both cellular components of tumor vessel wall: CD31^{bright}CD45[−] endothelial cells and α -SMA⁺ pericytes. Unlike viable CECs and progenitor cells, CD146⁺ cell concentration in the peripheral blood of cancer patients did not decrease during VEGF blockade.

Conclusion—CD146 is fairly homogeneously expressed on vascular endothelium but not on viable CECs or progenitor cells. The vast majority of CD146⁺ blood cells are lymphocytes, and VEGF blockade by bevacizumab did not significantly change their number in rectal cancer patients. These results underscore the need for further characterization and identification of new markers for CEC subpopulations for their development as biomarkers of antiangiogenic therapy.

INTRODUCTION

The clinical utility of antiangiogenic therapy has recently been established. The addition of vascular endothelial growth factor (VEGF) -specific antibody (bevacizumab; Avastin, Genentech, South San Francisco, CA) to contemporary therapy for colon and rectal, lung, and breast cancer patients increased survival rates in phase III clinical trials.^{1,2} Positive data have also emerged from trials of tyrosine kinase inhibitors, leading to the recent approval of sunitinib malate (Sutent; Pfizer, New York, NY) and sorafenib (Nexavar; Bayer Pharmaceuticals, West Haven, CT and Onyx Pharmaceuticals Inc, Emeryville, CA), whose inhibitory spectra include VEGF-receptor kinases.² But to date, no surrogate marker for antiangiogenic therapy, critical in guiding the application of these therapies, has been validated in cancer patients.

The blood concentrations of circulating endothelial cells (CECs) and progenitor cells are elevated in cancer patients³ and have been explored as biomarkers for measuring the impact of antiangiogenic treatments of cancer.^{4–9} But these populations are likely heterogeneous and may potentially be of different origin (eg, shed by tumor endothelium or mobilized from the bone marrow).^{6,10} Thus, phenotypic characterization of these blood-circulating cells in cancer patients is warranted before their use as biomarkers.

Serial analysis of gene expression in endothelial cells derived from blood vessels of normal and malignant colorectal tissues identified CD146 on endothelial cells.¹¹ In peripheral blood, the methodologies often used to detect CECs include magnetic bead selection for CD146⁺ cells¹² or polymerase-chain reaction (PCR)¹³ of CD146 mRNA. In vitro characterization of endothelial cells expanded from human blood has established that CD146 is a reliable endothelial marker.^{12,14,15} But other in vitro studies and more recent in vivo data questioned the specificity of CD146 expression for CECs.^{14,16–18} Yet, CD146 is extensively used to identify circulating cells of endothelial lineage and to distinguish them from leukocytes in preclinical and clinical studies.^{3,12,13,19,20} Therefore, the identification of CECs and progenitor cell populations by CD146 must be rigorously validated in the blood of cancer patients.

PATIENTS AND METHODS

Given its current use in defining CECs, and the increased exploration of these cells as surrogate markers in clinical trials, we investigated the expression of CD146 on peripheral-blood cells. In control experiments, we examined the expression of CD146 on primary human umbilical vascular endothelial cells (HUVECs) and human dermal microvascular endothelial cells (HDMECs; BioWhittaker, Walkersville, MD). In other control experiments, we evaluated mononuclear cells from buffy coats obtained from the peripheral blood of healthy donors and from umbilical cord blood samples (IRB Protocols No. 2003-P-000150 and #2003-P-000588). Finally, we analyzed the phenotype and number of CD146⁺ cells in the peripheral blood of rectal cancer patients (n = 8) at baseline and on days 3 and 12 following a single injection of bevacizumab (5 or 10 mg/kg) before chemoradiotherapy. Peripheral blood was obtained with informed consent from patients with locally advanced, previously untreated, adenocarcinoma of the rectum enrolled in a clinical trial of bevacizumab with chemoradiotherapy.^{5,9} Blood circulating cells were phenotyped and quantified by flow cytometric analyses of CD3, CD4, CD11b, CD31, CD34, CD45, CD56, CD133, and CD146 expression using fluorescence-labeled monoclonal antibodies (see Supplementary Table 1 for details).^{5,15} Fluorescence-labeled isotype-matched nonspecific immunoglobulin G (IgG) antibodies were used as controls. Cell concentrations were calculated as percentages of the total number of mononuclear cells after an evaluation of at least 50,000 cellular events. The quantitative analysis end point was the change in the fraction of CD146⁺ cells within the mononuclear blood cell population in patients receiving bevacizumab treatment alone. Percent values obtained at days 3 and 12 after first infusion of the drug were compared with pretreatment values in individual patients using the Wilcoxon signed rank test.

Tumor tissue was obtained from rectal cancer biopsies before and then 12 days after bevacizumab infusion, as well as from archived colorectal carcinoma biopsies. Biotin-labeled CD146-specific antibody was used for tissue immunostaining following manufacturer's recommendations. CD45-specific antibody was used for immunostaining of hematopoietic cells, while human CD31 and alpha-smooth muscle actin (α -SMA)-specific antibodies (see Supplementary Table 1) were used for immunostaining of endothelial cells and pericytes, as previously described.⁵

RESULTS

Flow cytometric analysis of primary mature endothelial cells (HUVECs) detected a CD31⁺CD146⁺CD45⁻ phenotype (Fig 1A). Similar results were obtained for HDMECs (not shown). These endothelial cells were homogeneously CD31⁺CD146⁺ and negative for the lymphocyte marker CD3, confirming that cultured human endothelial cells can be identified by flow cytometric analysis of CD146 staining. In contradistinction to mature vascular endothelial cells, most of the CD146⁺ mononuclear cells in the peripheral blood of both healthy donors (Supplementary Fig 1A) and cancer patients (Fig 1B) co-expressed the panleukocyte marker CD45. We have previously reported that the blood concentrations of the CD31^{bright}CD45⁻ viable CECs and that of the CD133⁺ progenitor cells were significantly decreased 3 days after VEGF blockade with 5 or 10 mg/kg of bevacizumab in rectal cancer patients.^{5,9} The concentration of CD146⁺ cells did not significantly change at day 3 at either dose (median value, 1.60%; range, 0.11% to 2.73% at baseline, v a median value of 1.75%; range, 0.57% to 3.32% at day 3; *P* = .5). There was a trend toward increased concentrations of CD146⁺ cells at day 12 after VEGF blockade in these patients (median, 2.39%; range, 0.97% to 6.22%; *P* = .1). In rectal cancer patients, CD146⁺CD45⁺ cells were primarily a subset of the CD3⁺ lymphocytes, and were largely negative for CD56 (a natural killer cell-specific marker) and CD11b (a myeloid marker; Figs 1C through 1E). These CD146⁺CD3⁺CD45⁺ cells were also positive for CD4 (not shown).

We also investigated the possibility that the CD146⁺CD45⁺ phenotype may identify circulating progenitor/stem cells, some of which are thought to incorporate into tumor vasculature.⁸ In the blood of the rectal cancer patients, the rare CD34⁺CD133⁺ progenitor cells expressed low levels of CD45 but did not express CD146 (Fig 1F and Supplementary Fig 1B). Moreover, in umbilical cord blood—highly enriched in progenitor/stem-cell populations—CD34⁺CD133⁺CD45^{dim} precursor cells did not express detectable levels of CD146 (Figs 1G through 1I).

To confirm CD146 expression in tumor endothelial cells, we next performed immunostaining for CD146 in tumor tissue biopsies. In both colon and rectal cancer biopsies, we detected CD146⁺ vascular endothelial cells, pericytes, and rare round stromal cells that were not associated with the vessel wall. The lineage of the CD146⁺ vascular cells was confirmed by immunostaining for CD31 (highly positive for endothelial cells, and negative for pericytes), α -SMA (positive for pericytes, not shown), and CD45 (negative for both; Fig 2). In meta-static foci in adjacent lymph nodes, we found a vascular staining pattern for CD146 (Figs 2D and 2E). Among the numerous hematopoietic cells infiltrating the tumor tissue proper, a small subset of cells coexpressed CD146 and were not associated with the vessel wall (Figs 2H and 2I). These patterns of expression were confirmed in archived colorectal, lung, and brain tumor specimens (Fig 2 and Supplementary Figs 2 and 3).

DISCUSSION

In peripheral blood samples from localized rectal cancer patients, CD146 marked primarily mature hematopoietic cells. More specifically, using a panel of lineage-specific markers, we found that CD146 marks a subset of T cells. This is consistent with previous results that identified CD146 expression on activated T cell subsets in healthy individuals.^{14,17,18,21} Moreover, the pretreatment blood concentration of these cells was in the same range as that of CD31^{bright}CD45[−] cells (20 to 100 cells/ μ L of blood⁹), but their concentration during anti-VEGF treatment showed no significant changes, in contrast to the significant decrease of CD31^{bright}CD45[−] cells.^{5,9} The detectable expression of CD146 in endothelial cells derived from buffy coats and expanded in culture¹⁵ may be functionally associated with cell proliferation, differentiation, formation of cell-cell junctions, and/or cytoskeletal rearrangement.^{22–24} Thus, whereas the CD146 expression may become detectable on cultured endothelial cells in the setting of ex vivo colony formation assays,^{15,25} it does not define bona fide endothelial or progenitor cells in primary blood samples from cancer patients. Our results demonstrate that similar to CD31 or CD34, multiple blood circulating cell populations are included in the analysis when using CD146 as selection marker. Moreover, changes in concentration and viability of these cell populations may independently vary with treatment or in different tumor types.⁶

In addition to exploring the viability of CECs,⁵ establishing novel and specific markers for the identification of populations of CECs and progenitor cells in the peripheral blood will be essential for optimizing the design and evaluating the efficacy of antiangiogenic treatment regimens for cancer. In localized rectal cancer patients, CD146 did not define CD34⁺CD133⁺ progenitor populations and was not detectable on CD31^{bright}CD45[−] viable CECs. It is conceivable that with increased tumor burden or in different tumor types, a larger fraction of CECs are tumor endothelium-derived and that they may differ in surface phenotype and viability. Our tissue immunophenotyping analyses confirmed that CD146 marks endothelial cells in normal and neoplastic tissues not only in the colon and rectum but also in the lungs and brain. Phenotypic analyses of blood circulating cells in patients with these tumor types will provide critical insight. Of interest, in all of the tissues we frequently detected CD146 expression on pericytes. Moreover, the massive hematopoietic cell infiltration in all these tumors occasionally contained CD146⁺CD45⁺ cells.

Given this phenotypic variation for circulating versus tissue endothelial cells and the lack of specificity of CD146 expression, our results indicate that until a highly specific marker is discovered and validated, the quantitative evaluation of CECs or progenitor cells in the blood of cancer patients should be analyzed in conjunction with hematopoietic markers by multicolor flow cytometry. Moreover, the extremely low frequency of some of the cell populations analyzed (eg, progenitor cells⁹) raises additional concerns regarding the quantification of their changes during treatment.

As blood cell evaluation is integrated into larger phase II-III clinical trials for its validation as a surrogate marker for antiangiogenic therapy,² characterizing the phenotype of CECs and progenitor cell populations of interest and improving the analytic methodology will be critical for the clinical development and optimal use of this emerging biomarker.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Fig 1.

Flow cytometry. (A) Human umbilical vascular endothelial cells (HUVECs) are homogeneously $CD146^+CD45^-CD3^-$. In red, non-specific immunoglobulin-G. (B) CD146 identifies $CD45^+$ peripheral blood cells in cancer patients. The $CD146^+CD45^+$ cells are a subset of $CD3^+$ cells (C), and are largely negative for CD56, CD11b, and CD34 (D-F). Most of the $CD133^+$ cells in umbilical cord blood are $CD146^-CD34^+CD45^{dim}$ (G-I).

Fig 2.

Immunohistochemistry. (A) In colorectal carcinoma, CD146 (brown) is expressed on endothelial cells (ECs); ECs are CD31⁺CD146⁺ within tumor and lymph node metastases proper (CD31: B, D; CD146: C, E). In adjacent tissue, CD146 expression is detectable in ECs and pericytes (F), whereas CD31 marks specific ECs (G). Vascular cells are CD146⁺ (brown, H) and CD45⁻ (red in H, brown in I; arrows indicate vessels); rare perivascular cells are CD146⁺CD45⁺ (H). The bar is 50 μ m (A), 500 μ m (B-G), 250 μ m (H), and 125 μ m (I).