Circulating Endothelial Cells and Their Subpopulations: Role as Predictive Biomarkers in Antiangiogenic Therapy for Colorectal Cancer

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Abstract

Several anticancer therapies have been developed to block angiogenesis, a key mechanism in tumor growth and metastasis. The predominantly cytostatic action of these compounds makes an assessment of their clinical activities inadequate if based only on the reduction of the tumor dimensions, as this may not reflect their true biologic efficacy. Thus, it is crucial to identify biomarkers that permit the recognition of potentially responsive subjects and to spare toxicity in those who are unlikely to benefit from treatment. Circulating endothelial cells (CECs) have been recently indicated as potential surrogate biomarkers of angiogenesis in several types of cancer. The possibility of rapidly quantifying these cells represents a promising tool for monitoring the clinical outcome of tumors with the potential to assess response to various treatments. However, the identification and quantification of CECs is technically difficult and not well standardized. A variety of methods to detect CECs in patients with solid tumors have been used; these are based on different technical approaches, combinations of surface markers, sample handling, and staining protocols. With an expanding interest in the field of potential clinical applications for CECs in oncology, the development of standardized protocols for analysis is mandatory. The aim of this review was to critically summarize the available data concerning the clinical value of CECs and their subpopulations as biomarkers of antiangiogenic therapy in patients with metastatic colorectal cancer.

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Introduction

Angiogenesis is one of the key mechanisms in tumor growth and contributes to the spread of blood-borne metastasis, according to Folkman's original hypothesis.[1] The crucial regulator of this process is vascular endothelial growth factor (VEGF), which is overexpressed in many tumors, and in particular in 40% to 60% of colorectal cancers, where its level correlates with intratumoral vascular density and disease progression.[2] Recently, several anticancer therapies have been developed to block this factor, such as neutralizing antibodies to VEGF, low molecular weight VEGF receptor (VEGFR) tyrosyne kinase inhibitors, and soluble VEGF constructs (VEGF-Trap).[3] Bevacizumab, a recombinant humanized monoclonal IgG1 antibody neutralizing VEGF-A, has shown the most consistent clinical results in metastatic colorectal cancer (mCRC) patients treated with first- and second-line therapies.[4-8] Aflibercept, a fully humanized recombinant fusion protein consisting of the VEGF binding portion from human VEGFR-1 and -2 fused to the Fc portion of human immunoglobulin G1, has shown benefit in survival in combination with FOLFIRI.[9] Finally, regorafenib, an orally active inhibitor of angiogenic tyrosine kinases (including VEGFR1 and -3) seems to be active in mCRC patients who have experienced disease progression after standard therapy.[10]

Tumor reduction may not reflect true biologic efficacy. In addition, no definitive clinical or biologic tools are currently available to select patients who would likely benefit from VEGF pathway inhibitors or exclude those who may be prone to experience specific adverse events.[11-13] Many potential biomarkers, both

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tumoral and systemic, are under evaluation in clinical trials, but none of these studies has closely monitored the vascular structure of a particular cancer during different clinical stages and in relation to treatment prescribed.[14-16]

Circulating endothelial cells (CECs) are surrogate markers of endothelial damage.[17] They are rare but quantitatively stable in healthy individuals[18] and increase in a very wide spectrum of disorders such as vascular, autoimmune, infectious, and ischemic disease.[19,20] Increased CEC counts are also observed in cancer patients,[21] where they seem to be promising biomarkers for tumor progression and monitoring therapeutic effects.[22] In this case, the endothelial phenotype displays a variety of different features: some CECs have a phenotype compatible with terminally differentiated endothelial cells, while in other cases they are apoptotic or necrotic and thus they likely derive from the turnover of vessel walls. Other endothelial cells express progenitor-associated antigens in addition to endothelial antigens and are considered circulating endothelial progenitors (CEPs) deriving from bone marrow rather than from vessel walls.[23,24] Absolute baseline number and changes in number and viability of CECs (and CEPs) have shown predictive value for response to an antiangiogenetic therapy for breast cancer.[25-28] Relatively few data are available in patients affected by other solid tumors.[29-31] The aim of this review was to summarize the available results concerning CECs and their subpopulations as biomarkers of antiangiogenic therapy in mCRC.

Analytical Methods for CEC Evaluation

The quantitative evaluation of CECs and their subpopulations in peripheral blood is performed mainly using 1 of these 2 analytical techniques: multiparameter flow cytometry (FCM) or the CellSearch system.

Flow Cytometry

This analytical technique became popular in the biomedical field thanks to its peculiar advantages: the high statistical value of the approach based on the possibility of analyzing a very large number of events, and the short analysis time due to the high efficiency of the measuring principle.[32] Thanks to these characteristics, FCM was established as the method of choice to analyze thousands of cells in a small sample volume in a very short time with the possibility of evaluating many cell targets labeled with a variety of fluorescent tags.[33] More recently, this method was improved so it might more precisely enumerate the so-called rare events. One of the major strengths of FCM is its ability to perform multiple measurements on single cells within a heterogeneous mixture. However, the instruments were not developed to count cells but rather to analyze their distribution by referring to a defined (or a multiple) parameter or parameters supported by scattered light, induced fluorescence emission after specific labeling, or both.[34,35] Flow cytometry has several advantages because it is a relatively rapid approach and is based on multiparameter analysis, which enables a more specific definition of rare events such as CECs and CEPs.[36] Limitations of FCM include the following. One cells have been assessed by antibody accessible markers, no additional testing can be conducted. The sample is discarded at the end of the measurement, and related data are collected in memory bank and displayed as histograms or dot plots. In practical terms, in the case of very few analyzed cells, they appear as small clusters of points somewhere on the screen without any possibility of proving their identity. Second, standardization is difficult to achieve between different laboratories; and finally, fresh blood samples are difficult to ship.

CellSearch System

The CellSearch system (Veridex LLC, Rarital, NJ), initially designed to detect circulating tumor cells, provides a fully automated enrichment procedure that is followed by semiautomated image cytometry.[37] It allows standardized analyses of CECs in different laboratories[38,39] and shipment of blood samples in special tubes containing preservatives.[40] The generated images are evaluated for CEC content by visual inspection, in which CECs are defined as DAPI (4′,6-diamidino-2-phenylindole) positive, $CD105^{+}$, $CD146^{+}$, and $CD45^{-}$. Morphological criteria such as size and whole intact cells are also evaluated. This assay has a high yield and good reproducibility, even for low numbers of CECs as reported in healthy controls (1 to 20 CECs/mL). Drawbacks of the CellSearch system are related to the costly equipment and reagents; in addition, the assay cannot be customized. The maximum number of 8 samples that can be analyzed in a single run, combined with the relatively long duration of a complete run (approximately 4 hours), does not enable high-throughput analysis.[41]

Studies Performed With Flow Cytometry

The studies that have evaluated the prognostic/predictive value of CECs during antiangiogenic therapy in mCRC utilizing FCM are reported in Table 1.

Willet et al[42] evaluated CECs in phase 1/2 studies in which 32 patients with rectal cancer received 4 cycles of therapy consisting of bevacizumab infusion on day 1 of each cycle plus fluorouracil infusion during cycles 2 to 4 plus external-beam irradiation. Surgery was performed 7 to 10 weeks after completion of all therapies. Molecular, cellular, biochemical, and radiologic biomarkers were also measured before treatment, during bevacizumab monotherapy, and during and after combination therapy. CECs were phenotyped and enumerated as $CD31^{bright}$, $CD34^{dim}$, $CD45^{-}$, and $CD133^{-}$ cells. No correlation was found among baseline and therapy-related numerical modifications of CECs, response to treatment, and outcome of patients. Only the number of CECs before surgery significantly correlated with pathologic complete response.

Taking into account the fact that the biochemical parameters examined (such as circulating growth factors and receptors and CECs) could modify their levels in response to bevacizumab with completely different kinetics, the effort to utilize them as a combined biomarkers needs to be better defined. In particular, in the neoadjuvant setting, cellular and biochemical parameters examined should be further evaluated in larger, well-designed clinical studies as candidate biomarkers of response for the regimen used.

Ronzoni et al[43] evaluated the absolute numbers of CEPs, total CECs (tCECs), and their resting (rCECs) and activated (aCECs) subsets in 40 mCRC patients at baseline and before the administration of a third and sixth course of a bevacizumab-based first-line treatment. Fifty healthy subjects were used as controls. CEPs,