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Biotechnology in Surgery

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The publication and the distribution of this volume have been supported by the Italian Society of Surgery

ISBN 978-88-470-1657-6 e-ISBN 978-88-470-1658-3

DOI 10.1007/978-88-470-1658-3

Springer Milan Dordrecht Heidelberg London New York

Library of Congress Control Number: 2010935728

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Cover design: Simona Colombo, Milan, Italy
Typesetting: Graphostudio, Milan, Italy
Printing and binding: Arti Grafiche Nidasio, Assago (MI), Italy

Printed in Italy

Springer-Verlag Italia S.r.l. – Via Decembrio 28 – I-20137 Milan
Springer is a part of Springer Science+Business Media (www.springer.com)

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effects on primary tumor growth. These effects are probably related to both coagulation and non-coagulation dependent factors [24].

In preclinical models, the use of monoclonal antibodies, anti-integrin, antiadhesion molecule (L1, JAM-C) has shown a decrease in tumor growth and adhesion.

The results of several studies and some ideas that have arisen in the field of endothelial cell and tumor interaction and hematogenous metastasis formation will probably be applied in the near future to the study of peritoneal dissemination. Moreover, the identification of metastasis-specific genes activated early in the primary tumor might make it possible to predict the tumor's behavior.

Among future developments it remains to be elucidated whether some molecules or antibodies will be used in diagnostic and therapeutic activity.

In this respect we are performing a study involving 75 enrolled patients (affected with gastric, colorectal or ovarian tumor). The primary endpoint is the analysis of some circulating molecules targeted to an early diagnosis of peritoneal carcinomatosis. A secondary endpoint is the evaluation of the molecular difference between primary tumors with high metastatic capacity and those with low metastatic capacity.

2.2 Pre-endothelial Cells and Prognosis of Intestinal Adenocarcinoma

D. Pantalone, A. Parenti, P. Cirri

2.2.1 Introduction

It is well known that the generation of new and functional vessels is one of the crucial hallmarks of cancer progression [25]. A number of ongoing studies have been published concerning the relationship between angiogenesis and colon cancer (prognosis, therapeutic targets, angiogenesis and advanced and metastatic disease). Until recently, it was generally accepted that in adults blood vessel formation is determined exclusively by completely differentiated endothelial cells (ECs). Recent studies have shown, however, that circulating bone marrow (BM) derived endothelial progenitor cells (EPCs) contribute to neovascularization and participate in new vessel formation, in a process called *postnatal vasculogenesis* [26]. Moreover, recent studies using different animal models of cancer have suggested the importance of BM-EPCs in tumor vascularization and growth [27, 28], and some evidence suggests that circulating EPCs are increased in cancer patients. EPCs are present in the peripheral blood, their levels are increased in response to certain signals/cytokines, and they dwell into the neovascular bed of malignant tissues. Therefore, on the basis of these observations, EPCs have attractive potential diagnostic and therapeutic applications for malignant diseases and the measurement of circulating EPC (C-EPC) has potential applications in colorectal cancer patients both in prognosis and therapy

2.2.2
Endothelial Progenitor Cells and Post-natal Vasculogenesis

Asahara et al. were able to show for the first time that C-EPCs exist and are recruited for tumor neovascularization [29]. It was therefore shown that new blood vessel formation in adults can also be provided by postnatal vasculogenesis. Extensive studies during the last 10 years have led to the identification and characterization of the peripheral blood (C-EPCs) and BM-EPCs as the sources of EPCs [30, 31], able to migrate through the tissue and to organize in tubules that eventually mature into vessels. These cells show the ability to repair the endothelium and to participate in the angiogenesis process, so it is conceivable that in any given moment the level of C-EPCs reflects the endothelial endogenous regenerative capacity of a patient. Thus, unlike circulating mature endothelial cells (CECs), which derive from mature endothelium and are considered a marker of vascular damage, EPCs are believed to be vasculoprotective and causally related to endothelial integrity and angiogenesis [32].

2.2.3
EPCs, Tumor Angiogenesis and Metastasis

Primary tumors, as well as metastatic neoplasms, need to undergo the *angiogenic switch* in order to grow beyond the microscopic dimension. The angiogenic switch is induced by soluble factors produced by tumor cells as well as other kinds of mesenchymal cells, nontumor cells (fibroblasts, macrophages) which constitute the tumor microenvironment. This process is a key step in the relapse from the tumor dormancy which allows the metastasis outgrowth. Experimental tumor models have shown a tumor-type dependent involvement of EPCs in tumor vascularization [33-35]. However, conflicting results exist. In some recent studies it has been shown that BM-EPCs contribute to the generation of peri-endothelial cells rather than endothelial cells and to new vessel formation [35, 36]. In mice transplantation studies, donor BM-derived EPCs integrated into newly formed blood vessels, sometimes by as much as 50% [35, 37], whereas other studies have reported significantly lower levels [38]. Recent published in vivo data suggest that small amounts of EPCs (12%) are sufficient to promote the vascularization of metastatic lesions [25, 29]. These conflicting results are mainly due to the still not exactly standardized methods for EPC phenotypic characterization via detection of cell surface markers (see Section 2.4) and to the non consideration of their functional interaction with other cell types able to contribute to the angiogenesis process [35, 39]. The involvement of EPCs in tumor angiogenesis has also been demonstrated in clinical studies. Increased EPC values have been detected in the circulation of cancer patients [40]. In one clinical study, tumors from six patients who developed cancers after BM transplantation were found to contain an average of 5% of EPCs from the donor, most likely generated by C-EPCs [41].

The contribution of EPCs to metastasis vascularization and to metastasis support

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is largely confirmed [42-44]. Kaplan et al. [44] have indeed found that VEGFR-1 positive hematopoietic cells (HPCs) derived from bone marrow act as a guide to drive circulating tumor cells to their metastatic sites. In these, HPCs train the soil for the subsequent arrival of cancer cells in the so called *metastatic niche*. In this context, EPCs participate in the construction of the metastatic niche arriving at this site concomitantly with tumor cells and hence, before vascularization occurs.

2.2.4 EPCs as a Potential Biomarker for Tumor Angiogenesis

Colon carcinoma is included in the tumors which are angiogenesis-dependent so colon cancer patients are also treated with the bevacizumab, an anti-VEGF antibody. Antiangiogenic treatments for cancer could be improved if reliable surrogate markers of drug biological activity were available. Many preclinical angiogenesis assays rely on the growth-factor-induced generation and quantification of neovessels at sites such as the cornea, skin or dorsal sac. Such surrogates are not adaptable to patients. The measurement of changes in tumor angiogenesis as a means of predicting and/or assessing the efficacy of antiangiogenic therapies has mainly been based on the evaluation of microvessel density in tumor biopsy samples [45]. Another approach is the measurement of circulating levels of angiogenic growth factors, such as VEGF, FGF-2, HGF, IL8 and others. In some types of cancer, these growth factor levels could predict survival. However, no growth factor has so far been validated in predicting the response to antiangiogenic therapies [46]. A promising strategy is the measurement of C-EPCs [47]. However, a lack of consensus about the definition of these precursor cells, using different cell surface markers, provides difficulty in the interpretation of these and other data [47]. These cells are phenotypically characterized by flow cytometry due to the expression of at least 2 hematopoietic stem cell markers ($CD133^+$or $CD34^+$), the endothelial marker VEGF-receptor 2 (VEGFR-2) or CD31. Thus, $CD34^+$ $VEGFR\text{-}2^+$, $CD133^+$ $VEGFR\text{-}2^+$, $CD34^+CD133^+$ $VEGFR\text{-}2^+$, $CD34^+CD31^+$, $CD133^+CD31^+$ and $CD34^+CD133^+CD31^+$ are all theoretically possible EPC phenotypes. In vitro studies have suggested that also $CD34^+/CD45^-$ cells have a higher capacity to acquire an endothelial phenotype, whereas $CD34^+CD133^+$ $VEGFR\text{-}2^+$ have recently been described to be primitive HPCs instead of EPCs [48]. Moreover, it should be noted that C-EPCs share membrane markers with CECs and HPCs and, in some case, with platelets and microparticles [32]. As further summarized in Table 2.1 there are no antigen-specific antibodies which, when used alone, can discriminate between C-EPCs, CEC, platelets and HPCs. For example, CD146 was an endothelial-specific marker, but more recent evidence indicates that it is also expressed by some mesenchymal cells, by a subpopulation of activated lymphocytes and most importantly by EPCs [47, 49]. At present, only CD133 is the sole antigen expressed in C-EPCs and subsequently downregulated in mature CECs, even though in humans CD133 is also expressed by HPCs. It seems that a panel of antibodies for different antigens should be used to

Table 2.1 Phenotypic differences of endothelial progenitor cells, circulating endothelial cells and hematopoietic progenitor cells

Marker	C-EPCs	CECs	HPCs	platelets	EC micro-particles
CD34	++	+/-	++	+/-	+/-
CD133	++	-	++	-	-
CD117	+	-	++	-	-
CD31	+	++	+	++	++
CD45	+/-	-	+	++	-
CD144	++	++	+/-	-	++
CD146	++	++	+/-	-	++
VEGF-R2	++	+	+/-	-	-/+
VWf	+	++	-	+	++

CECs, circulating endothelial cells; *EPCs*, endothelial progenitor cells; *HPCs*, hematopoietic progenitor cells

discriminate both C-EPC and the above mentioned cell population. In an attempt to develop a robust clinically useful FACS protocol to study C-EPCs and/or CEC, it is conceivable to define CECs as cells expressing $CD31^{bright}$ $CD45^{-}$ $CD34^{dim}$ $CD133^{-}$ and C-EPCs as $CD31^{+}$ $CD45^{dim}$ $CD34^{bright}$ $CD133^{+}$ [50].

The clinical relevance C- EPCs in tumor patients is under investigation. Recent studies reported an EPC increase in cancer patients, suggesting that these precursors contribute to postnatal vessel formation [35, 47]. C-EPCs might correlate with invasive breast cancer [51], with lung [52], head and neck cancer patients [53] and with the levels of circulating VEGF in lymphoma patients [54]. Elevated circulating endothelial progenitor marker CD133 mRNA levels have been detected in colon cancer patients and predict malignancy recurrence [55], although larger prospective studies are needed. The number of C-EPCs in chemotherapy-treated patients has also been correlated with survival [56]. The characterization of C-EPCs in relation to the clinical setting represents an attractive surrogate marker for monitoring tumor responses to conventional and to antiangiogenic therapy [57]. It has been demonstrated, indeed, that endostatin reduced C-EPC number along with tumor regression [58]. Moreover, clinical studies have been undertaken in patients treated with antiangiogenic drugs. Particularly encouraging in this regard is a trial (now Phase II) with the anti-VEGF antibody bevacizumab which has demonstrated its ability to reduce the tumor vascular density and the number of EPCs in rectal carcinoma patients [59]. This is the reason why there is an urgent need of clinical trials and standardized methods to characterize C-EPCs in cancer patients, in order to not only assess antiangiogenic therapy, but also to help define the optimal dose range and the establishment of the appropriate tumor response criteria.

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2.2.5
Molecular Markers of Tumor Angiogenesis and Antiangiogenic Drug Activity

Molecular markers can certainly improve our understanding of prognosis and the treatment of cancer. This is true also for colorectal cancer (CRC). CRC is a heterogeneous disease resulting from the co-occurrence of oncogenic mutations in a multistep process. Studying molecular classification and molecular correlates can provide clues to the pathogenesis and provide surrogate markers in clinical or research studies [60]. Many attempts are currently being made to obtain a precise molecular fingerprinting of both EC and EPCs, by searching for proteins such as tumor angiogenesis biomarkers. In addition, the possible definition of these new biomarkers will be helpful in clinical situations such as relapse after surgery or chemotherapy. Hence, EPC biomarkers will be useful to monitor either chemotherapeutic and/or antiangiogenic drug activity. In addition to the enumeration of C-EPC through FACS analysis (flow cytometry) of their surface markers, an overexpression of mRNA levels in EPC derived from tumor specimens compared to EPCs of normal tissues has been found [61]. The measurement of cancer-related EC and EPC populations looks promising, but standardized protocols are needed in view of this possible clinical use.

2.2.6
EPC as Cellular Vehicles for Anticancer Therapy

It has also been suggested that EPCs could be used as a vehicle for anticancer therapy. This might be achieved by ex vivo manipulation of EPCs in order to deliver suicide genes, toxins, or antiangiogenic drugs. This novel approach has been applied in mice with promising results [62].

2.2.7
Conclusions and Future Perspectives

Our Institution is carrying out a study on EPCs and colon cancer by means of the collection of blood samples from patients with CRC and investigating each patient's matched normal colon mucosa and CRC tissue, resected from the surgical specimen.

The immunohistochemical evaluation and flow cytometry analysis on tumor and draining lymph nodes specimens is performed by using the following mAb: CEA, MHC class I and II molecules, CD4 and CD8, CD16 and CD56.

Blood samples are taken prior to surgery and follow-up samples are taken after 24, 48, 72 h and 1 week after surgery, during chemotherapy and after 3 months of chemotherapy.

EPCs contribute to tumor angiogenesis and seem to play a crucial role in the guidepost of tumor cells TP to the metastatic niche. However, it is not currently clear what is their relative contribution to post-natal vasculogenesis when compared

to that of in situ angiogenesis (i.e. endothelial sprouting). Moreover, it can be assumed that new antiangiogenic drugs might also have significant side effects on the cardiovascular system. Surrogate markers capable of predicting not only the clinical anticancer benefit, but also the side effects will be needed. Short- and long-term studies of CEC and EPC kinetics, phenotype, activation and viability, both in response to antiangiogenic drugs will become increasingly important in helping to answer these questions.

2.3
Microarray Gene Expression of Pancreatic Carcinoma

D. Pantalone, I. Giotti, V. Ceccherini

2.3.1
Introduction

Pancreatic cancer is still a highly lethal disease if not the most lethal disease of all the solid malignancies [63]. It has been estimated that worldwide, approximately 213,000 people were diagnosed with a pancreatic cancer in 2005 [64]. In Italy, it is the tenth malignancy in women in terms of new diagnoses and the eleventh in men with about 9,000 new cases each year. It is the sixth cause of death from cancer in women and the seventh in men. It is also the second cause of death from gastrointestinal cancer. The high mortality of pancreatic adenocarcinomas can be attributed to the fact that the overwhelming majority of patients present locally advanced or distant metastatic disease, which therefore is inoperable. However, even among patients presenting resectable pancreatic cancers, currently only approximately 15% have the earliest stage cancers (T1, or T2, N0). Those cancers have a better survival outcome, but only 2-3% of all patients presents at an early stage. At present, the detection of pancreatic cancer at an early and therefore potentially curable stage remains the best possibility of improving the patient survival [65].

Unfortunately, the screening tests capable of detecting pancreatic cancer at an early stage are not available. Appropriate cancer screening should lead to the early asymptomatic or unrecognized cancer detection by means of acceptable and inexpensive widely applicable tests.

A better knowledge of genetic modification may be an important step for both cancer diagnosis and treatment and could provide evidence for the identification of early markers. Recent studies demonstrate that a high risk of developing pancreatic cancer can be identified in a patient's family history. An improvement in the identification of a high risk subject for pancreatic cancer will be provided by the identification of genetic alterations that predispose the inherited forms of pancreatic cancer. While it is important to target a high-risk family, this strategy does not facilitate the early detection of sporadic forms of pancreatic cancer.