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Flow cytometric analysis of circulating endothelial cells and endothelial progenitors for clinical purposes in oncology: A critical evaluation (Review)

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Abstract. Malignant tumors are characterized by uncontrolled cell growth and metastatic spread, with a pivotal importance of the phenomenon of angiogenesis. For this reason, research has focused on the development of agents targeting the vascular component of the tumor microenvironment and regulating the angiogenic switch. As a result, the therapeutic inhibition of angiogenesis has become an important component of anticancer treatment, however, its utility is partly limited by the lack of an established methodology to assess its efficacy *in vivo*. Circulating endothelial cells (CECs), which are rare in healthy subjects and significantly increased in different tumor types, represent a promising tool for monitoring the tumor clinical outcome and the treatment response. A cell population circulating into the blood also able to form endothelial colonies *in vitro* and to promote vasculogenesis is represented by endothelial progenitor cells (EPCs). The number of both of these cell types is extremely low and they cannot be identified using a single marker, therefore, in absence of a definite consensus on their phenotype, require discrimination using combinations of antigens. Multiparameter flow cytometry (FCM) is ideal for rapid processing of high numbers of cells per second and is commonly utilized to quantify CECs and EPCs, however, remains technically challenging since there is as yet no standardized protocol for the identification and enumeration of these rare events. Methodology in studies on CECs and/or EPCs as clinical biomarkers in oncology is heterogeneous and data have been obtained from different studies leading to conflicting conclusions. The present review presented a critical review of the issues that limit the comparability of results of the most significant studies employing FCM for CEC and/or EPC detection in patients with cancer.

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1. Introduction

Circulating endothelial cells (CECs) and endothelial progenitor cells (EPCs) have been proposed as non-invasive surrogate biomarkers of angiogenesis in cancer and other diseases (1-5). Their baseline number and kinetics in cancer patients has been widely investigated and several previous studies have demonstrated that they can be altered by disease status and treatments, including biological anti-angiogenic drugs and chemotherapy (CT) (1,2). However, CECs and EPCs are small, heterogeneous cell populations for which, despite extensive research, debate remains about the phenotypic definition and this makes reproducible identification and counting technically challenging (6-8).

Multicolor flow cytometry (FCM) is becoming an increasingly important technology for studies on clinical biomarkers and it is the most widely utilized method for the analysis of rare events, including CECs and EPCs, since it is a rapid, quantitative technique that has also the advantage of simultaneous determination of multiple markers (9-12). To date, several FCM-based methods to detect CECs and EPCs in patients with solid tumors have been developed. The majority of these

Table I. Selected outcomes and characteristics of eligible studies assessing CEC levels by flow cytometry in patients with cancer.

First author	Tumor type	CEC phenotype	CEC u.m.	Patients	Controls	P-value	Refs.
Goodale	Breast	CD45-, CD146+	/600 events	61	54	<0.05	(16)
Goon	Breast	CD45-, CD146+, CD3+	/ml	9.0 (5.0-12.7)	7.7 (6-10)	0.05	(17)
Kuo	Breast	CD45-, CD146+, CD31+, CD133+, Syto16+	cells/μl[a]	-0.609	n.a.		(18)
Vroling	NSCLC	CD45-, CD3bright, VEGFR2+	/ml	41	n.a.		(19)
Yuan	NSCLC	CD45-, CD31+, CD146+	/10^5 cells	299±221	117±33	<0.001	(20)
Ronzoni	mCRC	CD45-, CD146+, CD133-, CD34+	/WBC	30	20	0.09	(21)
Manzoni	mCRC	CD45-, CD146+, CD133-, CD34+	/WBC	35	18	0.01	(22)
Ramcharan	mCRC	CD45-, CD146+, CD34+	/ml	20	8	<0.05	(23)
Lin	Rectal	CD45-, CD31bright, CD133-, VEGFR2+	/10^5 events	1,000	473	<0.01	(24)
Starlinger	Pancreas	CD45-, CD146+, CD31+	/5x10^5 events	4.5	2	0.46	(25)
Yu	Gynecological	CD45-, CD146+, CD31+, CD105+	% WBC	1.36	0.18	>0.0001	(26)
Farace	RCC	CD45-, CD146+, CD31+, 7ADD-	/ml	13	n.a.		(27)
Bhatt	RCC	CD45-, CD31+, CD146+, CD133-	/μl	0.93 (0.19-11.75)	0.33 (0.12-0.99)	0.05	(28)
Blann	Prostate	CD45-, CD146+, CD34+, CD309-	/ml	25	28	0.004	(29)
Fuereder	Prostate	CD45-, CD146+, CD133-, CD31+, Syto16+	% WBC	0.22092	n.a.		(30)
DuBois	Osteosarcoma	CD45-, CD146+, CD133-, CD31+	/ml	645	1,670	0.12	(31)
Cuppini	Malignant glioma	CD45-, CD146+, CD133-, CD31+,	/ml	101	26	0.01	(32)
Brunner	Head and neck	CD5-, CD146+, CD31+	/5x10^5 events	20	17	<0.01	(33)
Mancuso	Various	CD45-, CD146+, CD133-, CD31+, Syto16+	/ml	951	140	<0.0001	(34)

[a]cells/ml, adjusted regression coefficient. CEC, circulating endothelial cell; WBC, white blood cell; CD, cluster differentiation; Syto16, cell-permeant green fluorescence nucleic acid stain; RCC, renal cell carcinoma; NSCLC, non-small cell lung cancer; mCRC, metastatic colorectal cancer; VEGF, vascular endothelial growth factor; n.a., not available; u.m., units of measurement.

are based on different combinations of surface markers, sample-handling and staining protocols, gating strategies and data analysis programs.

The present review analyzed some of the main published FCM analyses of CECs and EPCs in order to identify the methodological aspects most responsible for the discordant

Table II. Correlation between CECs and clinical endpoints defined in the studies analyzed.

First author	Tumor type	Clinical correlations	Refs.
Goodale	Breast	CECs correlate with disease stage	(16)
Goon	Breast	CECs positively correlate with Nottingham Prognostic Index, tumor size and invasiveness	(17)
Kuo		CECs are not surrogate biomarker of angiogenesis in patients receiving chemotherapy plus antiangiogenic agents	(18)
Vroling	NSCLC	CECs correlate with response to tyrosine kinase inhibitors	(19)
Yuan	NSCLC	CECs may potentially become biomarkers for diagnosis	(20)
Ronzoni	mCRC	CECs correlate with progression-free survival	(21)
Manzoni	mCRC	CECs are predictive biomarkers of response to chemotherapy and correlate with progression-free survival	(22)
Ramcharan	mCRC	CECs are not able to better predict the 2 year outcome in comparison with Dukes and AJCC stage	(23)
Lin	Rectal	CECs may be prognosis and morbidity biomarkers	(24)
Starlinger	Pancreas	CECs may potentially become prognostic and/or predictive biomarkers	(25)
Yu	Gynecological	Not found	(26)
Farace	RCC	CECs don't correlate with either progression-free survival and overall survival	(27)
Bhatt	RCC	Not found	(28)
Blann	Prostate	Not found	(29)
Fuereder	Prostate	Not found	(30)
DuBois	Osteosarcoma	Not found	(31)
Cuppini	Malignant glioma	Not found	(32)
Brunner	Head and neck	Not assessed	(33)
Mancuso	Various	Not assessed	(34)

CEC, circulating endothelial cell; NSCLC, non-small cell lung cancer; RCC, renal cell carcinoma; mCRC, metastatic colorectal cancer.

results observed. The aim was to establish the pre-analytical and analytical factors that must be carefully taken into consideration when CECs and EPCs are quantified for clinical purposes in oncology.

2. Flow cytometric analysis of circulating endothelial cells in patients with cancer

Despite the lack of universal consensus on phenotypic identification, CECs are accepted as cells, which circulate into the blood and express endothelial markers in the absence of progenitor and hematopoietic markers (13,14). Elevated CEC levels have been described in a range of tumor types and several studies have suggested that their number, viability and kinetics would be useful as a prognostic/predictive tool in patients with cancer (2,10,13,15).

Since CECs are rare events, their precise quantification in peripheral blood (PB) samples requires a technically rigorous analytical approach, which should take many factors into consideration (8). Several pre-analytical and analytical steps significantly affect not only the quantification of CECs, but can also result in a change in the definition of these cells, leading to problems in the interpretation of the results (Table I) and in their potential association with clinical endpoints (Table II) (16-34).

Circulating endothelial cells cannot be identified by any single surface marker and combinations of fluorochrome-conjugated monoclonal antibodies (MoAbs), which vary profoundly between studies, are utilized in the attempt to improve the analytical capability of FCM. The lack of a unified strategy is due to the extreme variety of phenotypic definitions of CECs, even between studies on the identical tumor type (13).

In many previous studies, CECs are identified as those positive for a nuclear binding fluorochrome, negative for the leukocyte marker, cluster of differentiation (CD)45, and positive for CD31 and CD146 (25,28,30-34). Previously, the expression of CD109, a cell surface glycoprotein which has been shown to be overexpressed in tumor endothelial cells, has been utilized to identify a specific subpopulation of CECs potentially useful as a prognostic marker in specific tumor types (35). Another complicating factor, reported only in certain studies, is the choice of the marker for the definition of CECs with apoptotic features (17,36). The different marker utilized can cause a significant change in the baseline count of this CEC subset, making it difficult to explore its clinical relevance (Table I) (17).

In other previous studies, the CEC phenotype is defined by similar combinations of markers, however, with different degrees of expression. This amplifies the range of combinations of MoAbs utilized and, particularly for panels made

Table III. Selected outcomes and characteristics of eligible studies assessing EPC levels by flow cytometry in cancer patients.

First author	Tumor type	EPC phe.o9notype	EPC u.m.	Patients	Controls	P-value	Refs.
Naik	Breast	CD14+, CD133+, VEGFR2+	/5x10^5 events	165	n.a.		(40)
Goon	Breast	CD34+, CD133+, CD45-	/ml	121 (81-186)	169 (106-241)	<0.05	(17)
Kuo	Breast	CD45-, CD31+, CD146+, CD133+	/10^5 eventsxWBC	0.295	n.a.		(18)
Jain	Breast	CD45dim, CD133+, VEGFR2+	/ml	21.3	n.a.		(41)
Bogos	SCLC	CD34+, VEGFR3+	/ml	1.625 (600-2.750)	455 (370-530)	<0.01	(42)
Nowak	NSCLC	CD34+, CD133+, VEGFR2+	%	11±0.007	0.025±0.018	<0.001	(43)
Morita	NSCLC	CD45-, CD34+, CD31+, CD133+	/μl	37	11	<0.05	(44)
Sakamori	NSCLC	CD31+, CD34+, CD133+, CD45-	/μl	40	4	<0.001	(45)
Pirro	NSCLC	CD34+, VEGFR2+	/ml	2.3±0.32	2.3±0.26	>0.05	(46)
Ronzoni	mCRC	CD45-, CD34+, CD133+, CD146+	xWBC/100	0.2	0.1	>0.05	(21)
Ramcharan	mCRC	CD34+, CD45-, VEGFR2+	7ml	21 (10-44)	7 (0-14)	<0.001	(23)
Lin	Rectal	CD31+, VEGFR2+, CD45dim, CD133+	/10^5 events	30	34	<0.01	(24)
Su	Ovarian	CD34+, VEGFR2+	/ml	1.260	368	<0.01	(47)
Qiu	Ovarian	CD34+, VEGFR3+	%	0.98 (0.55-1.94)	0.15 (0.10-0.23)	<0.01	(48)
Kim	Gynecological	CD45-, CD31+, CD133+, VEGFR2+	%	0.032±0.014	0.002±0.002	<0.01	(49)
Yang	RCC	CD45-, CD34+, VEGFR2+	%	0.28	0.08	<0.01	(50)
Farace	RCC	CD45dim, CD34+, VEGFR2+, 7ADD-	% CD34 cells	0.50	n.a.		(27)
Bhatt	RCC	CD34+, CD133+, CD146+, CD45-	/μl	0.97 (0.39-5.88)	0.19 (0.08-0.47)	<0.01	(28)
Blann	Prostate	CD34+, CD309+, CD45-, CD146-	/ml	38 (15-74)	32 (18-82)	>0.01	(29)
Fuereder	Prostate	CD45-, CD31+, CD146+, CD133+, 7ADD-, Syto16+bright	% WBC	0.29233	n.a.		(30)
DuBois	Osteosarcoma	CD45-, CD31+, CD146+, CD133+	/ml	126	260	0.69	(31)
Rafat	Glioblastoma	CD34+, VEGFR2+	1.23±1.09	0.08±0.04	<0.05		(51)
Corsini	Glioma	CD45dim, CD34+, CD133+	/μl	3.8±5.3	3.6±2.8	>0.05	(52)
Brunner	Head & neck	CD133+, VEGFR2+	/10^5 events	4.5 (1-41)	2 (0-7)	<0.001	(33)
Ha	Gastric	CD34+, CD133+	/ml	20±13.9	4±2.6	<0.05	(53)
Sieghart	HCC	CD34+, Cd133+, VEGFR2+	%	0.14±0.09	0.06±0.04	<0.01	(54)

Table III. Continued.

First author	Tumor type	EPC phenotype	EPC u.m	Patients	Controls	P-value	Refs.
Mancuso	Various	CD45-, CD31+, CD146+, CD133+, 7ADD-, Syto16+bright	/ml	429	181	0.00019	(34)
Masouleh	Various	CD45-, CD31+, CD133+	% MNCs	0.1-3.1	0.17-1.9	<0.01	(9)

EPC, circulating endothelial cell; WBC, white blood cell; MNCs, mononuclear cells; CD, cluster differentiation; VEGFR, vascular endothelial growth factor receptor; 7ADD, 7-amino-actynomicin D; Syto-16, cell-permaneant green fluorescence nucleic acid stain; RCC, renal cell carcinoma; u.m., units of measurement; HCC, hepatocellular carcinoma.

up of a large number of reagents, gives rise to an additional source of possible criticism (interferences between the various probes).

Since in the analysis of rare events, precision increases with the number of cells collected, CEC identification must be performed with a large number of acquired events, meaning an adequate sample of PB must be collected. When the steps in the pre-analytical phase of FCM protocols were compared, information on the modality of sample collection and of sample storage, and on the protocols for erythrocyte-depletion, were either lacking or significant differences emerged between the various studies, as shown in Table I.

A lack of uniformity was also revealed regarding the characteristics of patients and samples: Type of cancer treatment used, PB sample size, the presence or absence of a healthy control group, and tumor histology/subtype and disease stage (early or metastatic).

The numerous differences in FCM and experimental procedure make the clinical interpretation of the CEC numbers obtained highly difficult and affects the validity of the differences recorded between patients and controls, therefore, greatly limiting comparability of studies (Table II).

3. Flow cytometric analysis of endothelial progenitor cells in patients with cancer

Several assessment techniques have been proposed for EPCs, since they were first described by Asahara *et al* (37) with FCM being one of the most widely utilized.

Endothelial progenitor cells include numerous subtypes, which serve a variety of roles in promoting vascular growth (38) and, as yet, no universal consensus is available on the markers that require identification (7,39). Furthermore, the range of cellular markers that can be used to identify EPCs is even wider compared with that for CECs. As a consequence, wide variation, in terms of choice of MoAbs and extreme heterogeneity in their combinations, emerged across the previous studies. The focus of numerous previous studies in humans has been on the simultaneous expression of stem cell markers, including CD34 or CD133, and endothelial antigens, including CD31, type 2 vascular endothelial growth factor receptor (VEGFR-2 or kinase-insert domain, KDR) and VEGFR-1 (Table III) (9,17,18,21,23,24,27-34,40-54).

With regards to reported EPC numbers, another source of disparity between studies is that EPC count data are presented in two different forms: Number of EPCs in the PB sample volume and frequencies for a defined number of mononuclear cells (MNCs). In addition, the numerical values of EPCs are often reported with an error-approximation, which may affect the significance of the differences between patients and controls. The units of measurement and the algorithms utilized to obtain the absolute number of EPCs were also extremely heterogeneous. Finally, the cell populations to whom EPCs are associated often include not only WBCs and MNCs, but also cell subtypes, including CD34+ and VEGFR3+cells.

Another consideration to be made is that an ideal clinical biomarker must be highly biologically informative, and also easy and rapid to obtain and show a strong statistical association with the clinical course of the disease. While complex antigen phenotypes may be more specific, they are difficult to reproduce and the complexity of antigenic combination does not necessarily improve the performance of EPCs as clinical biomarkers. Hence, instead of widening the antigenic profile of EPCs to increase specificity, research should be aimed at making their identification and quantification more simple, reproducible and easy to obtain in clinical practice.

In addition to the impact of biological and procedural issues, from a technical point of view, the use of FCM has to deal with problems associated with background noise, which may lead to false positive results. Consequently, signal enhancement and noise reduction are crucial. In their review, Van Craenenbroeck *et al* (55) also listed the various steps that should be taken into consideration for this type of analysis. Pre-analytical factors included the choice of the sample material, modality of blood collection, handling temperature and certain subject-associated confounding factors. Numerous other problems associated with data acquisition, mentioned by Van Craenenbroeck *et al* (55), were the protocols for erythrocyte-depletion, the wash/no wash approaches. The authors suggested steps that must be followed to reduce the sources of error in FCM results. The importance of standardizing an appropriate gating strategy and multiple data analysis methods are highlighted in detail in one previous study (56).

To summarize, the rapidity of the expansion of this field is partly inhibited by an incomplete understanding of the

Table IV. Correlation between EPCs and clinical endpoints defined in the studies analyzed.

First author	Tumor type	Clinical correlations	Refs.
Naik	Breast	EPCs correlate with disease stage and with response to chemotherapy	(40)
Goon	Breast	EPCs do not correlate with Nottingham Prognostic Index, tumor size, invasiveness	(17)
Kuo	Breast	EPCs change dinamicly during antiangiogenic chemotherapy, they are candidate markers of angiogenesis	(18)
Jain	Breast	EPCs correlate with risk of relapse and disease progression	(41)
Bogos	SCLC	EPCs are significantly increased and correlate with lymphatic involvement and prognosis	(42)
Nowak	NSCLC	EPCs correlate with disease stage and with risk of disease progression	(43)
Morita	NSCLC	EPCs correlate with clinical response not with progression-free survival	(44)
Sakamori	NSCLC	EPCs correlate with response to chemotherapy and with risk of disease progression	(45)
Pirro	NSCLC	EPCs correlate with risk of disease recurrence	(46)
Ronzoni	mCRC	Not assessed	(21)
Ramcharan	mCRC	EPCs do not predict 2 year outcome in CRC in comparison with Dukes' and AJCC stage	(23)
Lin	Rectal	Not assessed	(24)
Su	Ovarian	EPCs correlate with response to chemotherapy and with risk of disease progression	(47)
Qiu	Ovarian	EPCs correlate with lymph node metastasis	(48)
Kim	Gynecological	EPCs may be useful surrogate marker to monitor treatment response	(49)
Yang	RCC	Not assessed	(50)
Farace	RCC	EPCs correlate with progression-free survival and overall survival	(27)
Bhatt	RCC	Not found	(28)
Blann	Prostate	Not found	(29)
Fuereder	Prostate	Not found	(30)
DuBois	Osteosarcoma	Not found	(31)
Rafat	Glioblastoma	Not found	(51)
Corsini	Glioma	Not found	(52)
Brunner	Head & neck	EPCs surrogate marker of response to chemotherapy	(33)
Ha	Gastric	EPCs correlate with lymph node metastasis and histological differentiation	(53)
Sieghart	HCC	Not assessed	(54)
Mancuso	Various	Not assessed	(34)
Masouleh	Various	Not found	(9)

EPC, endothelial progenitor cell; NSCLC, non-small cell lung cancer; SCLC, small cell lung cancer; RCC, renal cell carcinoma; mCRC, metastatic colorectal cancer; HCC, hepatocellular carcinoma; AJCC, American Joint Committee on Cancer.

biology, and the consequent lack of a universal definition of EPCs, as well as the lack of a standardized FCM assay procedure for their identification and characterization. Overcoming these particular obstacles can provide further insights into their possible clinical implications in oncology (Table IV).

4. Discussion

The role of angiogenesis in tumor growth is well-established and it is clear that this phenomenon is essential for the dissemination of metastases, as well as for the aggressive recurrence and refractoriness of the tumor (57-60). Several

effective anti-angiogenic drugs are now available and several are under development and, in order to improve the individualization of cancer treatment, blood-based biomarkers that accurately reflect their effects are urgently required (61-63). Numerous reports suggest that serial counting of CECs and/or EPCs can be successfully used to this end, however, this interesting prospect needs to be fully corroborated in the clinical setting, first of all by overcoming the areas of controversy that persist in the study of CEC and EPC biology.

Elevated CEC counts are associated with certain malignancies, however, conflicting results concerning their actual prognostic or predictive value during chemotherapy with or without anti-angiogenic therapy have been reported. The clinical utility of CEC counts can be limited, in part, by the lack of specificity for tumor vasculature and the possible variety of non-malignant causes, which can impact their number. In this regard, it has recently been hypothesized that specific antigens, tumor endothelial markers (TEM), enriched in tumor, vs. non-malignant endothelia, may be detectable on CEC surface and that these circulating TEM+ endothelial cells may constitute more specific blood-based biomarkers (64).

For EPCs, it must be also emphasized that it is now clear that the EPC phenotype is dynamic and a definite EPC identity may become elusive. Indeed, the endothelial differentiation potential can vary according to local environmental conditions and change over time. For these reasons, as long as clinical applications are concerned, a detailed functional characterization of these cells may be even more relevant compared with their antigenic phenotype (65-67).

On the other hand, all studies on clinical biomarkers would be required to be performed utilizing an highly efficient, specific and reproducible assay (68). Multi-color flow cytometric techniques are widely utilized in clinical studies to detect and quantify CECs and EPCs in whole blood, however, they remain technically challenging. The number of these cells is extremely low and they cannot be identified by a single marker, but only by a combination of antigens. As for other types of flow cytometric analysis of rare events, frequent sources of error include the contamination of cell populations with false positive events and the fluorescence associated with non-specific events. Such limitations can only be overcome through the optimization of MoAb panels, proper compensation for the staining with each individual fluorescently conjugated MoAb to maximize signal to noise ratio, appropriate selection of the regions of interest on the graphic display, the utilization of the linear scale for the low intensity staining regions (reserving the log scale for brightly staining markers), the utilization of hardware that allows high data rate collection and the utilization of dedicated data analysis programs. The majority of published protocols fail to properly address the majority of these issues.

In conclusion, the lack of a consensus on a consistent CEC and EPC phenotypic definition and the multitude of flow cytometric methods applied, which are not always sufficiently detailed, has resulted in a great heterogeneity in the reported blood levels of CECs and EPCs. These aspects, together with the heterogeneity of the patients series in the various studies, limit their potential to guide therapeutic strategies in clinical practice (69). In spite of these shortfalls, steps forward in the definition of the potential utility of CECs and EPCs for clinical purposes have been achieved, although reliable quantification of these cells is a work in progress and the interpretation of results must be made cautiously (35,10,70,71). In order to validate future reports that indicate, within well-designed trials, a true clinical value for both CECs and EPCs, unambiguous phenotypic definition of these cells together with careful inter-laboratory standardization of the quantitative techniques of analysis, including FCM, are mandatory.

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