High Levels of Circulating VEGFR2$^+$ Bone Marrow–Derived Progenitor Cells Correlate with Metastatic Disease in Patients with Pediatric Solid Malignancies

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Abstract

Purpose: Pediatric solid malignancies display important angiogenic potential, and blocking tumor angiogenesis represents a new therapeutic approach for these patients. Recent studies have evidenced rare circulating cells with endothelial features contributing to tumor neovascularization and have shown the pivotal role of bone marrow–derived (BMD) progenitor cells in metastatic disease progression. We measured these cells in patients with pediatric solid malignancies as a prerequisite to clinical trials with antiangiogenic therapy.

Patients and Methods: Peripheral blood was drawn from 45 patients with localized ($n = 23$) or metastatic ($n = 22$) disease, and 20 healthy subjects. Subsets of circulating vascular endothelial growth factor receptor (VEGFR)2$^+$-BMD progenitor cells, defined as CD45$^-$CD34$^+$VEGFR2 (KDR)$^+$7AAD$^-$ and CD45^{dim}CD34$^+$VEGFR2$^+$7AAD$^-$ events, were measured in progenitor-enriched fractions by flow cytometry. Mature circulating endothelial cells (CEC) were measured in whole blood as CD31$^+$CD146$^+$CD45$^-$7AAD$^-$ viable events. Data were correlated with VEGF and sVEGFR2 plasma levels.

Results: The CD45$^-$CD34$^+$VEGFR2(KDR)$^+$7AAD$^-$ subset represented <0.003% of circulating BMD progenitor cells (≤0.05 cells/mL). However, the median level (range) of the CD45^{dim}CD34$^+$VEGFR2$^+$7AAD$^-$ subset was higher in patients compared with healthy subjects, 1.5% (0%-10.3%) versus 0.3% (0%-1.6%) of circulating BMD progenitors ($P < 0.0001$), and differed significantly between patients with localized and metastatic disease, 0.7% (0%-8.6%) versus 2.9% (0.6%-10.3%) of circulating BMD progenitors ($P < 0.001$). Median CEC value was 7 cells/mL (0-152 cells/mL) and similar in all groups. Unlike VEGFR2$^+$-BMD progenitors, neither CECs, VEGF, or sVEGFR2 plasma levels correlated with disease status.

Conclusion: High levels of circulating VEGFR2$^+$-BMD progenitor cells correlated with metastatic disease. Our study provides novel insights for angiogenesis mechanisms in pediatric solid malignancies for which antiangiogenic targeting of VEGFR2$^+$-BMD progenitors could be of interest.

Pediatric solid malignancies have an overall better survival and outcome compared with adult cancers (1). However, prognosis remains grim for patients with metastatic or relapsed solid malignancies in spite of aggressive therapy associating surgery, radiation, and intensive chemotherapy often including high-dose regimens with autologous hematopoietic stem cell support. Novel therapeutic approaches are urgently needed to improve prognosis in this pediatric population (2). Angiogenesis, the process of new–blood vessel formation from preexisting vasculature, plays a key role in the growth, development, and metastatic dissemination of solid malignancies (3). Antiangiogenic therapy is emerging as one of the most significant advances in clinical oncology and, although most clinical studies have focused on adult malignancies (4), antiangiogenesis represents an appealing therapeutic strategy in pediatric oncology as well. Unlike adult solid malignancies, which derive mainly from epithelial tissue and have low proliferation rates and vascularity, pediatric solid malignancies display a strong angiogenic profile because they are nearly exclusively undifferentiated tumors characterized by high proliferation rates and increased vascularity. Preclinical data and early phase I clinical trials support the potential role of antiangiogenic agents in treating pediatric solid malignancies (5, 6). Subsequently, understanding the angiogenic processes involved could help guide the optimal use of antiangiogenic strategies in the clinical setting.

Tumor vascularization is dependent on the sprouting of nearby blood vessels, with migration and differentiation of

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Translational Relevance

Pediatric solid malignancies display important angiogenic potential, and blocking tumor angiogenesis represents a new therapeutic approach for these patients. This is the first report evaluating circulating endothelial cells, bone marrow – derived (BMD) endothelial progenitor cells, and angiogenic plasma proteins in the peripheral blood of patients with pediatric solid malignancies. We observed that strikingly high levels of BMD endothelial progenitors correlated with metastatic disease. These results support and extend recent preclinical findings indicating that these cells may play a pivotal role in metastatic disease progression. Our data suggest that monitoring and targeting of BMD endothelial progenitor cells could be of interest to guide the optimal use of antiangiogenic treatments in patients with pediatric solid malignancies. Finally, our findings raise important questions about clinical situations that mobilize hematopoietic, and possibly endothelial progenitors, because these cells may adversely promote tumor vasculogenesis after anticancer treatment associated with stem cell growth factors.

existing mature endothelial cells (angiogenesis), and on the recruitment of mobilized bone marrow – derived (BMD) endothelial progenitor cells (vasculogenesis; refs. 7, 8). Rare circulating BMD endothelial progenitors (commonly referred to as CEP) contribute to tumor neovessels in cancer-bearing animals (7, 9 – 13) as well as in humans (14, 15). Moreover, inhibition of circulating endothelial progenitor (CEP) recruitment prevents tumor growth in animal models (9, 11, 12), thus establishing their significance in tumor progression. Besides true CEP structurally incorporating growing vessels, several subsets of BMD progenitor cells are also involved in tumor vasculogenesis (9, 11, 16 – 19). Hematopoietic (VEGFR1$^+$) and endothelial (VEGFR2$^+$) BMD progenitors collaborate in disease progression, the first to initiate the premetastatic niche and the second to promote the vascularization of metastatic lesions (20 – 22). For these reasons, circulating BMD endothelial progenitors and mature endothelial cells have been intensively studied in cancer patients. However, whereas these rare cells have been documented in adult patients and are currently being investigated as potential biomarkers of antiangiogenic therapies (8, 23), there exists no data in a pediatric population.

The aim of the present study was to provide the first evaluation of levels of circulating VEGFR2$^+$-BMD progenitor cell subsets and circulating endothelial cells (CEC), as well as plasma levels of VEGF and sVEGFR2, in patients with various pediatric solid malignancies as a prerequisite to future clinical trials with antiangiogenic agents. Results were compared with data from healthy volunteers of similar ages. Whereas CECs, VEGF, and sVEGFR2 levels were not correlated with disease status, a subset of VEGFR2$^+$-BMD progenitors, immunophenotypically defined as CD45^{dim}CD34$^+$VEGFR2$^+$7AAD$^-$ viable events, was found in significantly higher levels in patients with metastatic disease.

Patients and Methods

Patients and blood sample collection. Informed parental consent and, when appropriate, child consent were obtained for all patients and healthy volunteers. The present study was approved by our Institutional Review Board and local ethics committee. Patients ages 1 to 25 y with localized or metastatic pediatric solid malignancies were included either at initial diagnosis or at relapse. Patients undergoing anticancer therapy, or with a known history of vascular disease or deep venous thrombosis, were excluded. All patients were sampled before anticancer treatment to avoid influencing results with potential therapy-induced vascular lesions. For this, patients at initial diagnosis were sampled before treatment and at least 2 wk after minor surgery (central venous catheter or tumor biopsy). Also, patients at relapse were included only if the minimum delay between the end of prior anticancer treatments and blood sampling was 2 mo. Patients with lymphoma were distinguished according to Ann Arbor staging as having localized (stage I or II) or metastatic disease (stage III or IV). Standard clinicopathologic information was recorded for all patients.

Healthy volunteer subjects of similar ages were recruited from a local general pediatric department and were free of inflammatory, infectious, autoimmune, or vascular disease. Blood sampling for the study was done during routine blood workup.

After discarding the first 2 mL after venupunction, peripheral blood samples were drawn: 2 mL of whole blood was collected in Cellsave preservative tubes (Immunicon) for CEC analysis, and 10 mL whole blood was collected in standard heparin tubes for circulating VEGFR2$^+$-BMD progenitor cell analysis. Cellsave tubes contain EDTA and a cell preservative agent that stabilizes fragile cells, such as CECs, and have been previously validated in our laboratory for CEC measurement by flow cytometry (24).

Measurement of circulating VEGFR2$^+$-BMD progenitors. Circulating subsets of VEGFR2$^+$-BMD progenitor cells were measured in 10 mL of progenitor-enriched whole blood by four-color flow cytometry as previously reported (14). Briefly, Ficoll-gradient mononuclear cells were enriched with the use of the RosetteSep antibody cocktail

Table 1. Characteristics of patients with pediatric solid malignancies (N = 45)

Patients	Localized	Metastatic
n	23	22
Male	15	17
Median age, y	9.1	12.9
Range, y	1-22.5	2.7-25
Initial diagnosis	22	12
Relapsed disease	1	10
Tumor type		
CNS	8	2
Sarcoma	8	9
Lymphoma	2	3
Other*	5	8
Metastatic site		
Lung		8
Bone		5
Bone marrow		7
Other		8

*Other: neuroblastoma, nephroblastoma, hepatoblastoma, and primitive neuroectodermal tumor.

Table 2. Results of CEC, VEGFR2$^+$-BMD progenitor, VEGF, and sVEGFR2 levels for 45 patients with localized or metastatic pediatric solid malignancies

Patient	Age/Sex	Localized disease (n = 23)	Initial diagnosis/ relapsed disease (n = 34/11)	CEC/mL	% VEGFR2$^+$-BMD*,†,‡,§ (absolute value/mL)	CD34$^+$/mL‖	VEGF (ng/mL)	sVEGFR2 (ng/mL)
CNS tumor								
P1	1.3/M	Glioneuronal tumor	I	16	0.5 (15)	3,230	841	10,313
P2	2.7/M	Brain stem pilocytic astrocytoma	I	0	0.7 (24)	3,330	988	7,977
P3	2.7/M	Low grade glioma (ventricles)	I	41	0.3 (4)	1,405	532	9,170
P4	9.4/F	Brain stem glioma	I	6	1.7 (ND)	ND	0	9,492
P5	11.5/M	Atypical teratoid rhabdoid tumor	I	17	4.1 (ND)	ND	60	9,781
P6	6.3/M	Medulloblastoma	I	14	0.1 (3)	2,366	72	11,942
P7	9.5/F	Medulloblastoma	I	0	0.7 (135)	19,957	27	12,708
P8	18/F	Medulloblastoma	I	7	2.3 (ND)	ND	28	7,784
Sarcoma								
P9	4.7/M	Rhabdomyosarcoma	I	52	0.3 (6)	1,975	0	9,045
P10	13/M	Rhabdomyosarcoma	I	6	0.7 (16)	2,550	52	7,401
P11	9/F	Undifferentiated sarcoma (liver)	I	0	0.7 (4)	514	152	11,304
P12	9.3/F	Synovialosarcoma	R	12	6.2 (78)	1,360	44	13,164
P13	17/F	Primitive neuroectodermal tumor	I	3	2.7 (16)	610	0	6,988
P14	14.3/M	Ewing's tumor	I	15	0 (0)	ND	0	11,566
P15	16.5/M	Ewing's tumor	I	0	0.4 (ND)	ND	28	10,392
P16	22.5/M	Ewing's tumor	I	3	0.1 (4)	3,910	21	10,541
Lymphoma¶								
P17	5.3/F	Hodgkin's disease (I)	I	7	2.4 (30)	1,495	15	10,352
P18	16.8/M	Burkitt lymphoma (I)	I	0	0.8 (8)	1,030	32	8,431
Other								
P19	1/F	Hepatoblastoma	I	7	0.4 (5)	1,028	726	10,958
P20	1.4/M	Hepatoblastoma	I	40	0.2 (2)	1,345	30	10,284
P21	1/M	Nephroblastoma (bilateral)	I	4	0.2 (3)	1,773	241	9,467
P22	5.8/M	Nephroblastoma	I	4	0.7 (19)	2,608	124	9,526
P23	5.7/M	Neuroblastoma (NMYC+)	I	11	8.5 (76)	1,020	110	7,602

Patient	Age/Sex	Metastatic disease (n = 22)	Initial diagnosis/ relapsed disease (n = 34/11)	Metastatic site	CEC/mL	% VEGFR2$^+$-BMD*,†,‡,§ (absolute value/mL)	CD34$^+$/mL‖	VEGF (ng/mL)	sVEGFR2 (ng/mL)
Sarcoma									
P24	11/M	Osteosarcoma	I	Bone	122	1.5 (55)	3,676	103	12,598
P25	14.9/M	Osteosarcoma	I	Lung, bone	38	7.3 (23)	380	79	11,216
P26	15/M	Osteosarcoma	I	Lung	152	6.4 (71)	1,190	95	9,817
P27	15/F	Ewing's tumor	I	Lung	30	6.1 (18)	370	0	7,309
P28	10/M	Alveolar Rhabdomyosarcoma	R	BM	15	9.4 (16)	295	59	11,209
P29	17/M	Osteosarcoma	R	Lung	40	2.8 (46)	1,780	9	9,931
P30	18.5/M	Osteosarcoma	R	Lung	8	10.3 (33)	410	4	6,813
P31	19.2/M	Osteosarcoma	R	Lung	4	0.7 (4)	645	28	7,759
P32	19.9/M	Osteosarcoma	R	Lung	7	4.3 (ND)	ND	15	7,976
Lymphoma¶									
P33	3.5/M	Anaplasic lymphoma (III)	I	Pleural	58	2.1 (ND)	ND	37	9,439
P34	11.5/F	Anaplasic lymphoma (III)	I		2	2.9 (60)	2,575	0	21,113
P35	14.3/M	Hodgkin's disease (IV Bb)	I	Lung, bone	5	0.8 (ND)	ND	240	10,064

(*Continued on the following page*)

Table 2. Results of CEC, VEGFR2$^+$-BMD progenitor, VEGF, and sVEGFR2 levels for 45 patients with localized or metastatic pediatric solid malignancies (Cont'd)

Patient	Age/Sex	Metastatic disease (n = 22)	Initial diagnosis/ relapsed disease (n = 34/11)	Metastatic site	CEC/mL	% VEGFR2$^+$-BMD*,†,‡,§ (absolute value/mL)	CD34$^+$/mL‖	VEGF (ng/mL)	sVEGFR2 (ng/mL)
CNS tumor									
P36	4/F	Glioblastoma	I	Leptomeningeal	0	1.2 (ND)	ND	72	12,295
P37	20.7/M	Medulloblastoma	R	Brain, BM	2	2.5 (63)	2,595	0	11,761
Other									
P38	2.7/M	Neuroblastoma (NMYC-)	I	BM, bone	6	0.9 (ND)	ND	69	10,133
P39	3/F	Neuroblastoma (NMYC-)	I	BM, liver	4	0.4 (15)	4,068	545	6,601
P40	3/M	Neuroblastoma (NMYC-)	I	Lymph nodes	6	2.5 (ND)	ND	521	11,067
P41	5/M	Neuroblastoma (NMYC-)	I	BM, bone	18	0.6 (88)	14,812	988	11,070
P42	7/F	Neuroblastoma (NMYC-)	R	BM	94	8.6 (50)	582	134	8,470
P43	25/M	Neuroblastoma (NMYC-)	R	BM, liver	54	5.2 (60)	1,161	76	8,695
P44	10/M	Hepatoblastoma	R	Endovascular	0	7.9 (89)	1,285	30	7,695
P45	21/M	Nephroblastoma (2nd relapse)	R	Pancreas	0	7.7 (ND)	ND	30	7,939

Abbreviations: BM, bone marrow; I, initial diagnosis; R, relapsed disease; ND, not determined.
*VEGFR2$^+$-BMD progenitor cells were defined as CD45^{dim}CD34$^+$VEGFR2$^+$7AAD$^-$ events.
†P values: all 45 patients, localized versus metastatic disease, $P < 0.001$.
‡All 45 patients, initial versus relapsed disease, $P < 0.0001$.
§Patients at initial diagnosis (n = 34), localized versus metastatic disease, $P < 0.02$.
‖CD34+ cell counts determined as CD45$^{dim/-}$CD34$^+$ events.
¶Patients with lymphoma were distinguished according to Ann Arbor staging as having localized (stage I or II) or metastatic disease (stage III or IV).

(StemCell Technologies Inc.) and then distributed into control and test tubes before treatment with FcR blocking reagent (Miltenyi Biotec). Staining was done with the following monoclonal antibodies: CD45-FITC (clone T29/33; DakoCytomation), CD34-APC (clone BIRMA-K3; DakoCytomation), KDR-PE (clone 89106; R&D Systems), and 7AAD (BD Biosciences). The viability dye 7AAD (BD Biosciences) was used to eliminate background noise occasioned by dead cells. Antibody batches were titrated rigorously to determine the optimal dosage and to be used in excess, except KDR-PE, which was used according to manufacturer's recommendations. Control tubes included isotypic and fluorescence-minus-one controls for each of the fluorochromes. A control tube, including a mouse IgG1-PE reagent (CD45-FITC/mouse IgG1-PE/CD34-APC/7AAD), was done to measure background noise accurately and to adjust the gates precisely. The IgG1-PE reagent was purchased from the same manufacturer and used in the exact same quantity as the KDR-PE. Cells were acquired on a FACSCalibur system (BD Biosciences), and data were analyzed with the use of CellQuest 3.2 software. Additionally, absolute circulating CD34$^+$ cell counts (CD45$^{dim/-}$CD34$^+$ events) were determined for each blood sample according to the BD Procount progenitor cell enumeration kit (BD Biosciences). Results were expressed as the percentage of VEGFR2$^+$ cells among circulating BMD progenitor (CD45^{dim}CD34$^+$7AAD$^-$ and CD45$^-$CD34$^+$7AAD$^-$) cells, and as the absolute values of CD45^{dim}CD34$^+$VEGFR2$^+$7AAD$^-$ and CD45$^-$CD34$^+$VEGFR2$^+$7AAD$^-$ progenitor cells per milliliter, respectively.

Measurement of CECs. CECs were measured in 1 mL whole blood by four-color flow cytometry according to a method we previously reported (24). Briefly, immunofluorescent staining was done with the following monoclonal antibodies: CD31-FITC (clone WM59; BD Pharmingen), CD146-PE (clone P1H12; BD Pharmingen), and CD45-APC (clone T29/33; DakoCytomation). Antibody batches were titrated rigorously. An IgG-PE control was done in 0.5 mL of whole blood (CD45-APC/CD31-FITC/mouseIgG1-PE/7AAD) to measure background noise and to adjust the gates precisely. Flow-Check microbeads (Beckman Coulter) were added to a tube containing isotypic controls to adjust forward scatter (FSC) voltage and to identify cell events with a size >10 μm. Cells were analyzed on a FACSCalibur system (BD Biosciences). To ensure statistical analysis, all of the cells contained in the IgG-PE control tube and in the CEC test tube were acquired, representing $\sim 2.5 \times 10^6$ and 5×10^6 events, respectively. Data were analyzed with the use of CellQuest 3.2 software.

Determination of plasma VEGF and sVEGFR2 levels. Plasma levels of VEGF and sVEGFR2 were determined with the use of commercially available ELISA kits (R&D Systems) and according to manufacturer's guidelines. All plasma samples were assayed in duplicates. The absorbance values obtained were plotted against standard curves generated on the ELISA plate with a correlation coefficient >0.99. Absorbance values were considered significant if found to be at least twice as high as background noise.

Statistical analysis. Results are expressed as medians and range. Groups were compared with the use of a two-sided nonparametric Mann-Whitney test. Data were analyzed with SPSS software (version 15.0). A P value ≤ 0.05 was considered as statistically significant. Correlations were measured with the use of the Pearson correlation coefficient.

Results

Patient characteristics. Levels of VEGFR2$^+$-BMD progenitor cells, CECs, plasma VEGF, and sVEGFR2 were measured in 45 patients, of whom 31 were male. Thirty-four patients were at initial diagnosis, whereas 11 patients had relapsed disease, of which 10 of 11 were at metastatic sites. At the time of evaluation, 23 patients had localized disease and 22 patients had metastatic disease. Median (range) age was 10 (1-25 y) years among patients and did not differ significantly between localized or metastatic populations (9.1 and 12.9 y, respectively; $P < 0.07$). Patients more than the age of 18 y (8 of 45) were in majority those with relapsed disease (6 of 8). Median

time between blood sampling and the end of prior anticancer treatments for relapsing patients was 5.4 (3.7-19 mo) months. The clinical and biological characteristics of the 45 patients evaluated are presented in Tables 1 and 2.

Levels of CECs and VEGFR2$^+$-BMD progenitor cells were measured in 20 healthy subjects, of whom 8 were male. Median age of healthy subjects was 8.7 (1.7-18.2 y) years and did not differ significantly from that of patients with pediatric malignancies ($P < 0.81$).

Levels of circulating VEGFR2$^+$-BMD progenitors. Subsets of VEGFR2$^+$-BMD progenitor cells were measured in progenitor-enriched whole blood and were identified as the rare fraction of circulating BMD progenitor cells (CD45^{dim}CD34$^+$7AAD$^-$ and CD45$^-$CD34$^+$7AAD$^-$) expressing the VEGFR2(KDR) receptor. VEGFR2 was not significantly expressed in the CD45$^-$CD34$^+$7AAD$^-$ subset, because the CD45$^-$CD34$^+$VEGFR2$^+$7AAD$^-$ subset represented <0.003% of circulating BMD progenitor cells (≤0.05 cell/mL) in both patient and healthy control populations. However, VEGFR2 was expressed in the CD45^{dim}CD34$^+$7AAD$^-$ subset with a median level of 0.3% (0%-1.6%) of circulating BMD progenitors in healthy subjects and a median level of 1.5% (0%-10.3%) of circulating BMD progenitors in all 45 patients (Fig. 1A). The median level of CD45^{dim}CD34$^+$-VEGFR2$^+$7AAD$^-$ cells was significantly higher in patients with pediatric solid malignancies compared with healthy subjects ($P < 0.0001$). Median absolute values of CD45^{dim}CD34$^+$VEGFR2$^+$7AAD$^-$ cells, available for 50 subjects, also differed significantly between 34 patients, 18.5 cells/mL (0-135.0 cells/mL), and 16 healthy subjects, 5.0 cells/mL (0-25.0 cells/mL; $P < 0.001$).

Among patients, the median level of CD45^{dim}CD34$^+$-VEGFR2$^+$7AAD$^-$ cells was 0.7% (0%-8.6%) of circulating BMD progenitors for those with localized disease but was 2.9% (0.6%-10.3%) of circulating BMD progenitors and was significantly higher for patients with metastatic disease ($P < 0.001$; Fig. 1A). Patient characteristics and respective CD45^{dim}CD34$^+$-VEGFR2$^+$7AAD$^-$ values are presented in Table 2. Median absolute values of CD45^{dim}CD34$^+$VEGFR2$^+$7AAD$^-$ cells also differed significantly between patients with localized ($n = 19$) and metastatic ($n = 15$) disease: 8.0 (0-135.0 cells/mL) and 50.0 cells/mL (4.0-89.0 cells/mL), respectively ($P < 0.007$). No variability in total CD34$^+$ counts was observed between localized or metastatic populations ($P < 0.27$; Table 2).

Moreover, the 11 patients with relapsed disease had significantly higher levels of CD45^{dim}CD34$^+$VEGFR2$^+$7AAD$^-$ cells compared with the 34 patients at initial diagnosis: 6.2% (0.7%-10.3%) and 0.8% (0%-8.6%) of circulating BMD

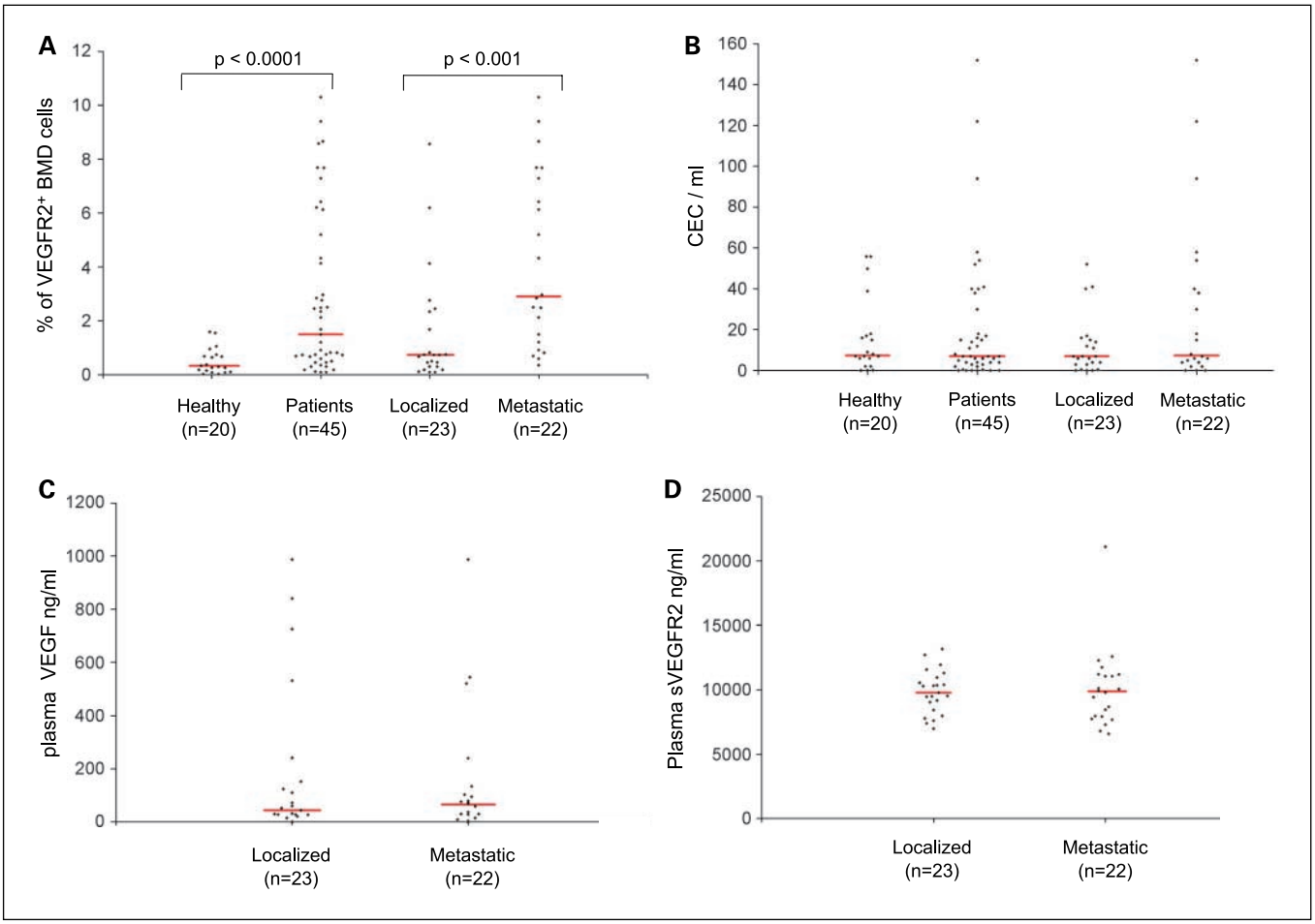

Fig. 1. Measurement of levels of CECs, VEGFR2$^+$-BMD progenitor cells, plasma VEGF, and sVEGFR2 in 45 patients with pediatric solid malignancies and 20 healthy subjects. Comparison of median levels of VEGFR2$^+$-BMD progenitor cells (*A*), CECs (*B*), plasma VEGF (*C*), and plasma sVEGFR2 (*D*) in healthy subjects and patients with localized versus metastatic disease. Horizontal line, median values.

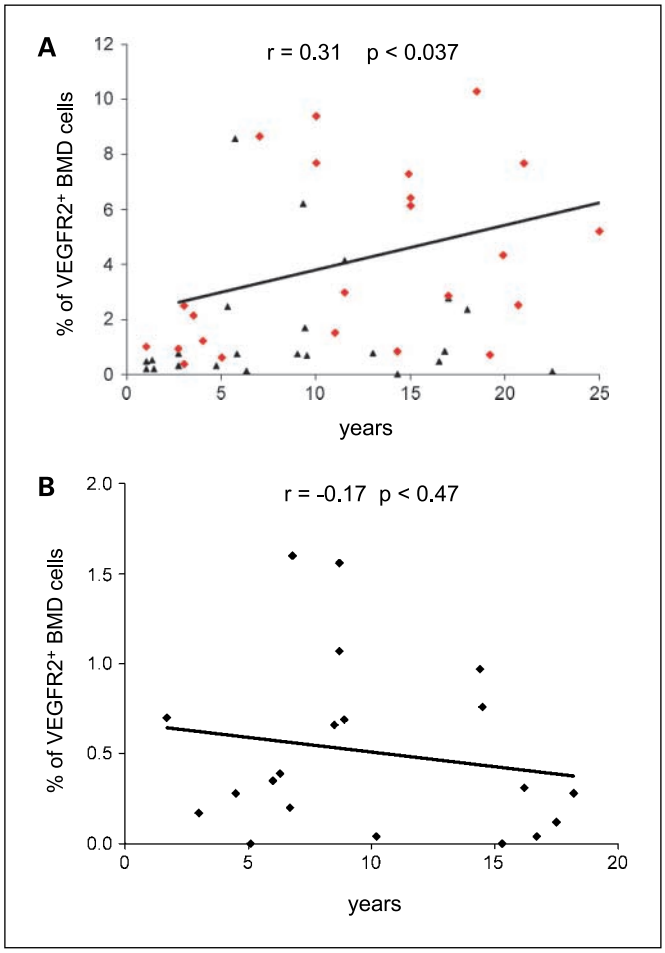

Fig. 2. Distribution of VEGFR2$^+$-BMD progenitor cells according to age in 45 patients with localized (*black triangle*) and metastatic (*red diamond*) pediatric solid malignancies (*A*) and in 20 healthy control subjects (*B*).

progenitors, respectively ($P < 0.0001$; Table 2). Because relapsing patients were older, we studied if the value of CD45^{dim}CD34$^+$-VEGFR2$^+$7AAD$^-$ cells was linked with age (Fig. 2). The correlation coefficient between levels of CD45^{dim}CD34$^+$VEGFR2$^+$7AAD$^-$ cells and age was 0.31 ($P < 0.037$) for the 45 patients with pediatric solid malignancies (Fig. 2A). However, no correlation was observed among healthy subjects ($P < 0.47$), suggesting a lack of association between age and VEGFR2$^+$-BMD progenitor cell levels (Fig. 2B). No differences were observed between VEGFR2$^+$-BMD progenitor cell levels and gender ($P < 0.66$; data not shown). Also, we excluded relapsing patients and evaluated levels of CD45^{dim}CD34$^+$VEGFR2$^+$7AAD$^-$ cells among 34 patients at initial diagnosis. In this subgroup, the median level of CD45^{dim}CD34$^+$-VEGFR2$^+$7AAD$^-$ cells was higher among 12 patients with metastatic disease compared with 22 patients with localized disease: 1.8% (0.3%-7.3%) and 0.7% (0%-8.6%) of circulating BMD progenitors, respectively ($P < 0.015$). Finally, as the biology of dissemination in lymphoma may be distinct from that of solid tumors, statistical analysis was done after excluding 5 patients with lymphoma and showed similar differences in levels of CD45^{dim}CD34$^+$-VEGFR2$^+$7AAD$^-$ cells between localized ($n = 21$) and metastatic ($n = 19$) populations: 0.6% (0%-8.6%) and 3.6% (0.3%-10.3%) of circulating BMD progenitors, respectively ($P < 0.001$).

It is noteworthy that the two patients with localized disease and displaying the highest levels of CD45^{dim}CD34$^+$VEGFR2$^+$-7AAD$^-$ cells had particular clinical features: patient P12 had locally relapsed synovialosarcoma, and patient P23 had stage III neuroblastoma with *MYCN* gene amplification. Results for a representative patient are shown in Fig. 3 according to the gating strategy used to identify circulating VEGFR2$^+$-BMD subsets by four-color flow cytometry.

Levels of CECs. CECs were identified as CD31$^+$CD146$^+$ CD45$^-$7AAD$^-$ viable events in whole blood by four-color flow cytometry (24). CEC values for the 45 patients evaluated are presented in Table 2. Median CEC levels did not differ between patients with pediatric solid malignancies and healthy subjects: 7.0 (0-152.0 cells/mL) and 7.5 cells/mL (0-56.0 cells/mL; $P < 0.71$; Fig. 1B). Also, although significant interpatient variability was observed, median circulating endothelial values were not different between patients with localized or metastatic disease: 7.0 (0-52.0 cells/mL) and 7.5 cells/mL (0-152.0 cells/mL), respectively ($P < 0.26$; Fig. 1B). CEC levels were not correlated with age (coefficient correlation, 0.015; $P < 0.91$) or with gender (data not shown). Also, no significant correlation was observed between CEC levels and initial or relapsing disease status, primary or metastatic tumor sites, or time elapsed between sampling and minor surgery (data not shown). To illustrate the gating strategy established to identify CECs by four-color flow cytometry, the results for P26, who had osteosarcoma of the leg with skin perforation and probable concurrent vascular lesions, are presented in Fig. 4.

Plasma levels of VEGF and sVEGFR2. Median levels of plasma VEGF and sVEGFR2 for the 45 patients were 52 ng/mL (0-988 ng/mL) and 9,817 ng/mL (6,601-21,113 ng/mL), respectively. Levels of plasma VEGF and sVEGFR2 did not differ between localized and metastatic disease populations (Fig. 1C and D). Also, no significant correlation was observed between plasma VEGF or sVEGFR2 levels and CECs, VEGFR2$^+$-BMD progenitor levels, age, or primary or metastatic tumor sites. Table 2 displays these data.

Discussion

We describe the first quantitative analysis of subsets of circulating VEGFR2$^+$-BMD progenitor cells and CECs in the peripheral blood of children and young adults with various pediatric solid malignancies. Due to the absence of data in healthy children, our results were compared with VEGFR2$^+$-BMD progenitor cell and CEC levels measured in a control population of 20 healthy subjects of similar ages. We found that levels of circulating VEGFR2$^+$-BMD progenitor cells, characterized as CD45^{dim}CD34$^+$VEGFR2$^+$7AAD$^-$ events, were significantly higher in patients with pediatric solid malignancies compared with healthy subjects. Moreover, among patients, strikingly elevated levels of VEGFR2$^+$-BMD progenitor cells correlated with metastatic disease. Preclinical models have provided solid evidence that circulating endothelial progenitors play an important role in tumor progression (25). However, the variable degrees of incorporation of endothelial progenitors shown in different tumor models have led to controversy about the extent of their actual involvement in tumor vascularization (10, 13, 19, 26, 27). Identification of CEP is highly complex and has been hampered by the extreme rarity of these cells as well as the overlapping antigenic similarities between hematopoietic progenitors and

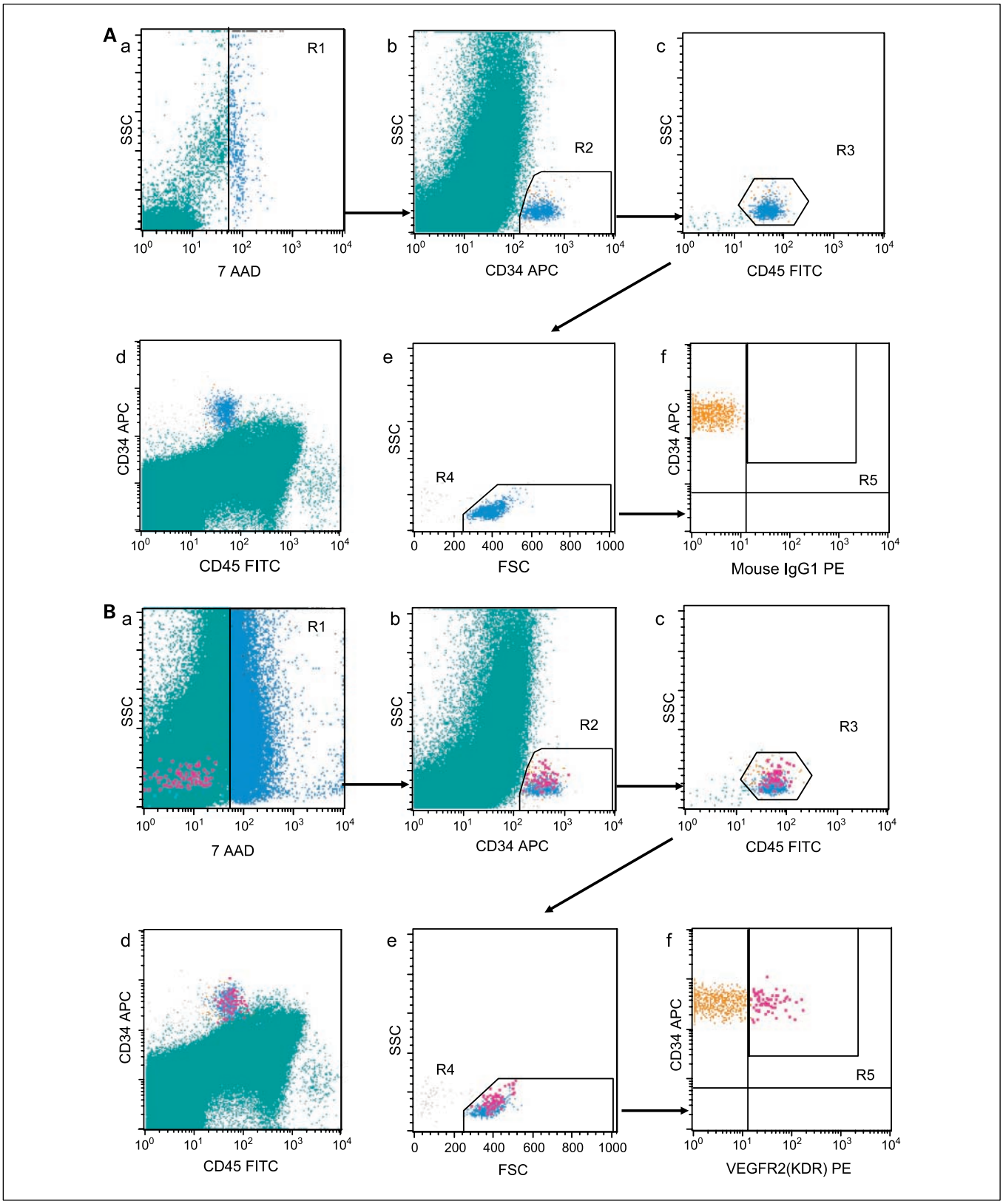

Fig. 3. control for flow cytometric detection of circulating $CD45^{dim}CD34^{+}VEGFR2^{+}7AAD^{-}$ progenitor cells. *A,* analysis of the control (CD45-FITC/mouse IgG1-PE/CD34-APC/7AAD) tube. The control tube is used to place accurately the successive gates. Only 1% of the events are shown (*a*) to select the gating of viable $7AAD^{-}$ events (R1). Other panels, 100% of events. *B,* analysis of the test (CD45-FITC/KDR-PE/CD34-APC/7AAD) tube, for which 100% of events are shown (*all panels*). *b*, the R2 gate used to select $CD34^{+}$ events; *c*, the R3 gate used to select $CD45^{dim}$ events from the R2 gate; *d*, $CD45^{dim}CD34^{+}7AAD^{-}$ viable events; *e*, the R4 gate used to eliminate debris from the R3 gate; *f*, the R5 gate resulting from the crossing of all successive gates and used to count $VEGFR2^{+}$-BMD progenitor cells. *a* to *f* are identical for both the control and test tube. $VEGFR2^{+}$-BMD progenitor cells (*bold*) are characterized as $CD45^{dim}CD34^{+}VEGFR2^{+}7AAD^{-}$ viable events.

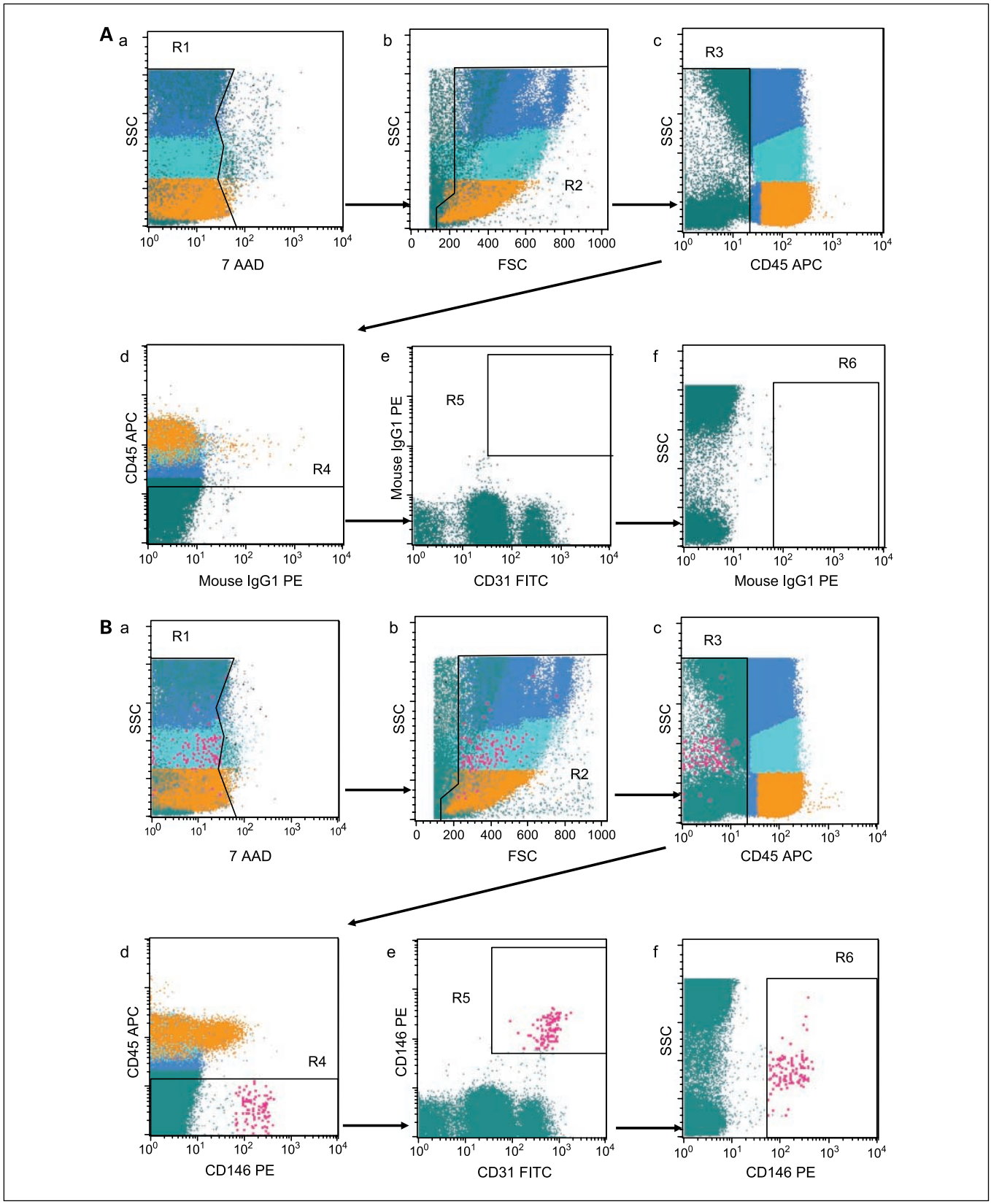

Fig. 4. Gating strategy for flow cytometric detection of CECs. *A,* analysis of the control (CD45-APC/CD31-FITC/mouse IgG1-PE/7AAD) tube. *B,* analysis of the test (CD45-APC/CD31-FITC/CD146-PE/7AAD) tube. Histograms, all stored events (2.5×10^6 events for the control and 5×10^6 events for the CEC test). *a* to *f* are identical between the control and test tube. *a*, gating of viable $7AAD^-$ events (R1); *b*, the large gate R2 used to exclude most debris and granulocytes without excluding very large cells; *c* and *d*, the R3 and R4 gates used to exclude $CD45^+$ events; *e*, $CD31^+CD146^+7AAD^-$ CECs in the $CD45^-$ gates; *f*, the R6 gate resulting from crossing all of the successive gates and used to count CECs. CECs (*bold*) are characterized as $CD31^+CD146^+CD45^-7AAD^-$ viable events.

true CEP (28, 29) that are involved in tumor vasculogenesis (11, 16, 17, 19, 26). In cancer patients, the importance of the involvement of these cells remains controversial, in part because of the lack of consensus on their phenotypic definition (30, 31). Circulating BMD endothelial progenitors are typically identified and enumerated by flow cytometry as cells coexpressing CD34, CD133, and VEGFR2 markers (8, 32, 33). Several studies have analyzed CEP levels in 50 to 100 μL of peripheral blood with the use of flow cytometry in cancer patients (34–36) but have reported discordant results. Specialists in the field agree on the extreme rarity of these cells, which represent 0.0001% to 0.003% of circulating mononuclear cells (30, 31, 33). This rarity should incite careful interpretation of CEP measurements done in small volumes of blood (100 μL). In accordance with recommendations for the identification of extremely rare events, we developed a rigorous four-color flow cytometry assay to identify subsets of circulating VEGFR2$^+$-BMD progenitors in 10 mL of progenitor-enriched whole blood. The methodologic characteristics presented here that are used to detect this rare population include: (*a*) sampling of an important volume of blood, (*b*) a pre-enrichment step, (*c*) use of a viability marker, and (*d*) a multiple gating strategy (14).

Because the expression level of CD45 (either "dim" staining or negative) on circulating BMD progenitors with endothelial features is still matter of debate, we analyzed VEGFR2$^+$ expression in both the CD45^{dim}CD34$^+$7AAD$^-$ and the CD45$^-$CD34$^+$7AAD$^-$ subsets. VEGFR2$^+$ expression was not significantly present in the CD45$^-$CD34$^+$7AAD$^-$ fraction. However, we observed that the rare CD45^{dim}CD34$^+$VEGFR2$^+$7AAD$^-$ subset was the only population identified by flow cytometry to significantly express the VEGFR2 receptor. This finding is consistent with the fact that hematopoietic and endothelial progenitors derive from a common precursor, the hemangioblast (28), and thus share the CD45 hematopoietic stem cell marker. In preliminary experiments, we showed that the vast majority of these cells were also positive for the CD133 marker (data not shown). Importantly, although there is no consensus, the CD45^{dim}CD34$^+$VEGFR2$^+$7AAD$^-$ immunophenotype coincides with current definitions of BMD progenitors showing typical endothelial features described by others (8, 32, 37).

The lack of consensus on the appropriate method for CEC measurement has yielded conflicting data and hampered the understanding of their role in cancer patients (38). CECs have been detected with the use of flow cytometry (39, 40) but have been observed in very high numbers in cancer patients (1-39,000 cells/mL) and in healthy individuals (1-7,900 cells/mL) compared with the values reported with the immunomagnetic separation method of reference (<10 cells/mL; ref. 41). These findings have raised considerable questioning of the reliability of the flow cytometry method for CEC measurement (38, 42, 43). Recently, in 2007, Strijbos et al. argued that cells identified as CECs by flow cytometry in these studies were, in fact, large platelets (42). In a pediatric phase I trial with the VEGF neutralizing antibody bevacizumab (5), "mature CEC levels" were monitored in six children undergoing antiangiogenic therapy for refractory solid tumors and were found to range from 0 to 12,000 cells/mL with the use of flow cytometry. Previously, we have shown that median CEC levels were 6.5 cells/mL (0-15.0 cells/mL) in healthy adults and 15.0 cells/mL (0-179.0 cells/mL) in patients with metastatic carcinoma ($P < 0.001$; ref. 24). In the present study, the median CEC values observed in healthy subjects [7.0 cells/mL (0-56.0 cells/mL)] and in patients with localized [7.0 cells/mL (0-52.0 cells/mL)] or metastatic [7.5 cells/mL (0-152.0 cells/mL)] pediatric solid malignancies were within a similar range as the values reported in healthy adults with the use of the immunomagnetic separation method (<10 cells/mL; refs. 38, 41).

VEGF and sVEGFR2 have been evaluated as markers of endothelial cell mobilization (7, 11, 18, 44) and have been shown previously to correlate with tumor burden in cancer-bearing animals (45, 46) as well as in humans (47). In this study, we observed no correlation between angiogenic factor levels and CECs, circulating VEGFR2$^+$-BMD progenitor cells, or clinical status. Our study population did not allow for comparison of tumor burden between patients and could explain the absence of association between angiogenic factors and disease status.

We observed significant interpatient variability in both CEC and VEGFR2$^+$-BMD progenitor cell levels, which may be inherent to the heterogeneity of our population. However, we recently published a study on CEC levels in 125 adult patients with various metastatic solid malignancies, in which CEC levels also displayed significant interpatient variability despite the similarity of tumor types and stage (24). These observations highlight the biological complexity of variables influencing CEC and VEGFR2$^+$-BMD progenitor levels in cancer patients as well as the difficulties in understanding the significance of these rare cells in tumor angiogenesis.

Interestingly, we found that patients with metastatic or relapsing disease had high levels of circulating VEGFR2$^+$-BMD progenitor cells. Recent studies have shown that circulating VEGFR2$^+$-BMD endothelial progenitors play a major and catalytic role in tumor progression, which could be maximal in metastatic (20) and relapsing disease (25) by promoting the progression of avascular micrometastases to vascularized macrometastases (20). In the present study, differences in levels of VEGFR2$^+$-BMD endothelial progenitors were independent of age, gender, therapeutic intervention, or therapy-induced vascular lesions, suggesting that the results observed might be associated with the clinical behavior of pediatric solid malignancies. Mobilization of VEGFR2$^+$-BMD progenitors could result from two possible mechanisms. First, bone marrow involvement or proximity of bone metastases could locally stimulate the release of VEGFR2$^+$-BMD progenitors into the circulation via inflammation or tumor-derived cytokines. Second, VEGFR2$^+$-BMD progenitors could be actively recruited by proangiogenic characteristics that underline metastatic disease. Because metastatic lesions are almost never resected, we could not do immunohistochemistry to analyze if VEGFR2$^+$-BMD progenitors incorporated metastatic neoendothelium. Our data are not sufficient to affirm that VEGFR2$^+$-BMD progenitor cell elevations are independently associated with metastatic progression because the initial design of the study and population size did not allow for multivariate assessment of the clinical and biological factors that may also influence CECs and VEGFR2$^+$-BMD progenitor cell levels in patients. However, the significantly higher levels of VEGFR2$^+$-BMD progenitors found in numbers paralleling clinical severity, despite the diversity of tumor types investigated, raise great interest about the general relevance of these cells in metastatic disease progression. Further investigations in larger and more homogenous study populations will be necessary to analyze other covariates that may be responsible for the association with VEGFR2$^+$-BMD progenitor cell levels.

Finally, our data may raise concern about clinical situations that mobilize hematopoietic, and possibly endothelial, progenitors because these cells might induce unintended adverse effects. Fast tumor regrowth has been observed with increased levels of circulating BMD progenitors induced by stem cell growth factor (48, 49) as well as by vascular trauma resulting from radiation (16) or from vascular disrupting agents in tumor-bearing animals (25). Recently, we showed for the first time that vascular disrupting agent–induced VEGFR2$^+$-BMD progenitor mobilization is also present in human patients (14). Consequently, "vasculogenic rebounds" induced by anticancer therapies and possibly sustained by stem cell growth factor could adversely promote BMD progenitor mobilization and tumor angiogenesis, and, therefore, could increase the risk of relapse, perhaps at metastatic sites (50).

Pediatric solid malignancies present strong angiogenic potential and hopes to improve prognosis in these patients rely on antiangiogenic strategies. This is the first study to show evidence of increased levels of circulating VEGFR2$^+$-BMD progenitor cells in patients with metastatic pediatric solid malignancies. This finding may offer new insights to angiogenesis mechanisms in pediatric malignancies, in which monitoring and targeting of circulating VEGFR2$^+$-BMD progenitor cell levels could be of interest to guide the optimal use of antiangiogenic treatments in patients.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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