Published in final edited form as:

Pediatr Blood Cancer. 2012 February ; 58(2): 181–184. doi:10.1002/pbc.23046.

Circulating Endothelial Cells and Circulating Endothelial Precursor Cells in Patients with Osteosarcoma

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Abstract

Background—Circulating endothelial cells (CECs) have been detected at increased numbers in patients with solid cancers. CECs have not been systematically evaluated in patients with osteosarcoma.

Procedure—Patients 12 months to 30 years of age with newly diagnosed high-grade osteosarcoma were eligible for this prospective cohort study. Patients provided a single blood sample at study entry for CEC quantification by flow cytometry at a single reference laboratory. CECs were defined as CD146+, CD31+, CD45-, and CD133-. CEC progenitor cells (CEPs) were defined as CD146+, CD31+, CD45-, and CD133+.

Results—Eighteen patients enrolled (11 males; median age 16 years; range 5-21 years). CEC counts did not differ between patients with osteosarcoma compared to 7 pediatric healthy controls (median 645 cells/mL, range 60-5320 cells/mL vs. 1670 cells/mL, range 330-4700 cells/mL, respectively; $p = 0.12$). CEP counts did not differ between patients compared to controls (median 126 cells/mL, range 0-5320 cells/mL vs. median 260 cells/mL, range 0-10670 cells/mL, respectively; $p = 0.69$). CEC and CEP counts did not correlate with metastatic status, tumor size, or histologic response to neoadjuvant chemotherapy.

Conclusions—CEC and CEP levels are not increased in patients with osteosarcoma compared to healthy controls. CECs and CEPs do not correlate with clinical features of osteosarcoma. Alternative novel markers of disease burden and response are needed in this disease.

Keywords

Osteosarcoma; angiogenesis; circulating endothelial cells

Introduction

Osteosarcoma is the most common primary malignant bone tumor, with a predilection for adolescent and young adult patients. Recognized adverse prognostic features in

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Conflict of Interest Statement: The authors have no conflict of interest disclosures.

osteosarcoma include presence of metastases, axial tumor location, and poor histologic response to chemotherapy [1]. Multiple lines of evidence indicate that angiogenesis plays an important role in osteosarcoma pathogenesis and prognosis. These tumors express vascular endothelial growth factor (VEGF) and platelet derived growth factor (PDGF) [2-4]. VEGF expression in osteosarcoma tumor samples has been found to be prognostic [3,5]. Patients with osteosarcoma and higher circulating VEGF levels are more likely to develop pulmonary metastases than patients with lower circulating VEGF levels [6].

Circulating endothelial cells (CECs) are believed to be mature endothelial cells that have been shed into the circulation from an area of disrupted vasculature [7,8]. In contrast, circulating endothelial precursor or progenitor cells (CEPs) are bone marrow derived cells that contribute to vasculogenesis, including tumor-associated vasculogenesis [7,9,10]. The ability to quantify CECs and CEPs in peripheral blood has provided an important biomarker of tumor vascular turnover and vasculogenesis [11]. Increased levels of CECs and CEPs have been found in the peripheral blood of adult patients with a range of carcinomas compared to controls [11-14]. In addition, the levels of these cells appear to correlate with clinical features in these patients, including disease progression and response to anti-angiogenic therapies [11].

Despite the importance of angiogenesis in osteosarcoma, levels of CECs and CEPs have not been systematically evaluated in patients with this disease. The aim of this study was to determine whether patients with newly diagnosed osteosarcoma have increased CEC and CEP levels compared to controls without cancer. In addition, correlations between levels of these cells and clinical features of the disease were evaluated.

Methods

Subject Eligibility

Patients were eligible for participation if they had histologically confirmed newly diagnosed primary high-grade osteosarcoma, were 12 months to 30 years of age, and had not yet received anticancer therapy. For all patients, surgical resection of the primary tumor was planned after a course of neo-adjuvant chemotherapy. Patients were excluded if they had a previous diagnosis of cancer or Paget's disease of the bone. Since surgery can impact CEC levels, patients were excluded if they had undergone surgery other than tumor biopsy or central line placement prior to enrollment. Seven healthy children provided blood samples as control subjects.

All subjects (or legal guardians, as appropriate) provided informed consent for participation. The study was approved by the Institutional Review Board of each participating institution.

Study Procedures

Patients with osteosarcoma were prospectively recruited from three participating centers: UCSF School of Medicine, the Hospital for Sick Children, and Dana-Farber Cancer Institute / Children's Hospital Boston. At the time of enrollment, patients provided a single 5 mL peripheral blood sample in an ethylenediaminetetraacetic acid (EDTA) tube. The sample was shipped overnight at 4°C to the study reference laboratory at the Hospital for Sick Children.

Samples were processed for analysis within 24 hours of collection. Enumeration of CECs and CEPs was carried out using four-color flow cytometry. A panel of monoclonal antibodies, including anti-CD45 (BD Bioscience; San Jose, CA), anti-CD31 (BD Bioscience), anti-CD146 (Chemicon; Temecula, CA), and anti-CD133 (Miltenyi Biotec; Auburn, CA) were used to enumerate CECs and CEPs while excluding hematopoietic cells.

Nuclear staining (Procount, BD Biosciences) was used to exclude the potential interference of platelets and cellular debris with CEC and CEP enumeration. After RBC lysis, cell suspensions were evaluated by a FACS Calibur cell analyzer and CellQuest Pro software (BD Biosciences) using analysis gates designed to exclude dead cells, platelets and debris. A minimum of 100,000 events per sample was acquired to obtain the percentage of CECs or CEPs per sample. The absolute number of CECs or CEPs was derived using a single platform flow cytometric analysis with the addition of Stem Count beads (Beckman Coulter; Brea, CA). To assess counting accuracy, the total white blood cell count from the flow cytometer was compared to clinical results for each subject's white blood cell count. Stained cells were determined and compared with appropriate negative controls and positive staining was defined as being greater than nonspecific background staining.

CECs were primarily defined as CD146+CD31+CD45-CD133- and CPCs were primarily defined as CD146+CD31+CD45-CD133+. Recognizing that other groups have incorporated other markers into the definitions of these cells, testing was also performed using each of the following markers in place of CD146: VEGFR2 (R&D Systems; Minneapolis, MN); VEGFR3 (R&D Systems); CD105 (endoglin; Invitrogen; Carlsbad, CA); CD143 (AbD Serotec; Raleigh, NC); and CD144 (BD Biosciences) expression [7,8,11,15,16]. Quantification of apoptotic subsets involved evaluation of 7-amino-actinomycin D (7-AAD) staining. Clinical patient data were collected on a standardized data form at study entry and again after resection of the primary tumor.

Statistical Methods

The initial goal sample size was 20 patients. With this sample size, comparisons of circulating endothelial cell counts between 2 groups with 10 patients each would have 80% power to detect a 2-fold difference between groups with a p-value of 0.05. Group comparisons were made using the Wilcoxon rank sum test. All statistical analyses were performed using STATA, version 10.1 (College Station, TX).

Results

Eighteen patients enrolled and provided a baseline blood sample for analysis. Patient characteristics were typical for patients with osteosarcoma (Table I). All patients received initial chemotherapy that included methotrexate, doxorubicin, and cisplatin. Seventeen of 18 patients underwent surgical resection, with 8 of those patients showing > 90% histologic necrosis at the time of resection.

CEC counts did not differ between patients with osteosarcoma compared to 7 pediatric healthy controls (median 645 cells/mL, range 60-5320 cells/mL vs. 1670 cells/mL, range 330-4700 cells/mL, respectively; $p = 0.12$; Figure 1A). CEP counts did not differ between patients compared to controls (median 126 cells/mL, range 0-5320 cells/mL vs. median 260 cells/mL, range 0-10670 cells/mL, respectively; $p = 0.69$; Figure 1B).

CECs and CEPs were also quantified using markers other than CD146 (VEGFR2, VEGFR3, CD105, CD143, CD144). Levels of these cell populations were evaluated for potential differences between patients and controls (Table II). Only VEGFR2+ CECs showed a significant difference between groups, with patients showing lower levels compared to controls (median 725 cells/mL, range 0 – 6040 cells/mL vs. median 2230 cells/mL, range 490 - 5880 cells/mL, respectively; $p = 0.027$).

CEC and CEP levels were evaluated for potential associations with clinical features of osteosarcoma (Table III). None of the CEC or CEP cell populations obtained using markers other than CD146 differed significantly based on clinical features of the disease.

Discussion

This study is the first to systematically evaluate CECs and CEPs in patients with osteosarcoma. The definition of CECs and CEPs has differed between studies and a consensus definition has not been established [17]. The current study therefore evaluated a range of markers that have been used to define these cells in previous studies. Despite investigating CECs and CEPs across a range of definitions, cell levels generally did not correlate with osteosarcoma diagnosis or clinical features. Only VEGFR2+ CECs showed differential cell levels between patients and controls, with patients having lower levels than controls. Other groups have also correlated levels of CEC activation and viability with clinical features in patients with cancer [11,13]. Evaluation of these same subsets in patients with osteosarcoma in the current study yielded no meaningful associations.

These negative results raise the possibility of methodologic flaws in the current study, though this possibility seems less likely for several reasons. First, the cell counts obtained are within the ranges reported in previous studies [13,18]. Second, CEC and CEP counts demonstrated large interpatient variability in both cases and controls, as has been reported by other groups [14,18,19]. Third, the reference laboratory used in the current study serves as the reference laboratory for evaluation of CECs and CEPs as biomarkers in Children's Oncology Group studies of antiangiogenic agents. This laboratory therefore routinely performs this assay on samples obtained from patients across North America. Fourth, while the samples sizes are small and inadequate power may account for these negative results, CEC and CEP levels completely overlapped between each evaluated clinical category. As such, enrollment of additional patients was thought to be unlikely to yield additional meaningful information. Finally, other groups have incorporated CD34 expression into the definition of CEPs [7,15,20]. Our flow cytometry panel did not include CD34, but utilized CD133 to differentiate CECs from CEPs [11]. This difference from other published reports evaluating CEPs may have also contributed to our negative results.

Another explanation for these negative results may reflect a fundamental difference in the process of angiogenesis between patients with sarcoma and carcinoma. For example, the pathways that regulate VEGF expression appear to differ between *ras*-transformed fibroblasts and epithelial cells [21]. Moreover, microvessels appear to be more diffusely distributed in sarcoma, while more clustered in carcinoma [22]. In addition, osteosarcoma typically arises from trabecular bone, which has a unique and rich vascular supply.

While most studies of CECs and CEPs have been performed in patients with carcinoma, three reports have included patients with sarcoma. CEC levels were approximately two-fold higher among 15 patients with gastrointestinal stromal tumor compared to healthy controls [23]. CEP levels appear to be increased in patients with classic Kaposi's sarcoma compared to healthy controls [24]. Another report evaluated 45 children with a range of solid cancers, including 17 patients with sarcoma (7 with osteosarcoma) [19]. While CEC levels did not differ between patients and controls, VEGFR2+ CEP levels were increased in patients compared with controls and in patients with metastatic disease compared with patients with localized disease. No additional subset analyses were performed based on histology.

In summary, the current results add to a growing body of literature evaluating CECs and CEPs in patients with cancer. While CEC levels have typically been shown to be increased in patients with cancer, the current study indicates that this finding is not universal across cancer histologies. Additional evaluation of CECs and CEPs in patients with sarcoma will be necessary to determine if these biomarkers are useful in sarcoma histologies.

Acknowledgments

Supported by the Campini Foundation, Friends of Dana-Farber, and NIH/NCRR/OD UCSF-CTSI Grant Number KL2 RR024130.

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Figure 1. CD146+ CEC (A) and CD146+ CEP (B) levels in patients with osteosarcoma and controls without osteosarcoma.

Table I

Patient characteristics (n = 18).

Clinical Feature	Number (%), unless otherwise indicated
Age (median; range)	16.4 years (5.1 – 21.7 years)
Male	11 (61.1%)
White	11 (61.1%)
Primary Tumor Site	
Femur	9 (50%)
Humerus	4 (22.2%)
Tibia	3 (16.6%)
Fibula	1 (5.6%)
Iliac	1 (5.6%)
Metastatic Disease	5 (27.8%)
Isolated lung metastasis	3 (16.6%)
Lung and bone metastasis	2 (11.1%)
Isolated bone metastasis	1 (5.6%)
Osteosarcoma Histology	
Conventional, osteoblastic	12 (66.7%)
Chondroblastic	3 (16.6%)
Telangiectatic	2 (11.1%)
Unclassified	1 (5.6%)
Maximal Tumor Size (median; range)	12.4 cm (3.6 – 19.5 cm)
Neoadjuvant Chemotherapy	18 (100%)
MAP*	17 (94.4%)
MAP + zoledronic acid	1 (5.6%)
Surgical Resection of Primary Tumor	17 (94.4%)
Tumor Necrosis > 90%	8 / 17 (47%)

* MAP = Methotrexate, doxorubicin, and cisplatin.

Table II

CEC and CEP levels in 18 patients with osteosarcoma and 7 healthy controls.

Parameter	Median cells/mL (range)	
	Patients	Controls
CD146+ CECs	645 (60 – 5320)	1670 (330 – 4700)
VEGFR2+ CECs*	725 (0 – 6040)	2230 (490 – 5880)
VEGFR3+ CECs	550 (0 – 11750)	1990 (490 – 5610)
Apoptotic CECs	301 (0 – 8430)	1090 (130 – 3450)
Endoglin+ CECs	703 (0 – 7700)	770 (0 – 3170)
CD143+ CECs	1470 (50 – 67030)	6240 (1550 – 32990)
CD144+ CECs	360 (0 – 8010)	420 (330 – 3590)
CD146+ CEPs	126 (0 – 5320)	260 (0 – 10670)
VEGFR2+ CEPs	498 (0 – 5420)	720 (0 – 16870)
VEGFR3+ CEPs	398 (0 – 8290)	340 (0 – 11090)
Endoglin+ CEPs	545 (0 – 7460)	0 (0 – 10650)
CD143+ CEPs	1012 (0 – 64140)	4970 (0 – 21380)
CD144+ CEPs	298 (0 – 4930)	870 (0 – 8550)

* p = 0.027 for comparison between patients and controls

Table III

Correlation of CEC and CEP levels with clinical features in 18 patients with osteosarcoma.

Parameter	Clinical Variable Median cells/mL (range)	
	Metastatic Disease	Localized Disease
CD146+ CECs	330 (130 – 2590)	690 (60 – 5320)
VEGFR2+ CECs	490 (0 – 4320)	820 (0 – 6040)
VEGFR3+ CECs	490 (0 – 11000)	650 (0 – 11750)
Apoptotic CECs	1090 (253 – 8430)	280 (0 – 4400)
Endoglin+ CECs	150 (0 – 2720)	705 (0 – 7700)
CD143+ CECs	650 (85 – 67030)	2160 (50 – 29840)
CD144+ CECs	240 (25 – 8010)	515 (0 – 3840)
CD146+ CEPs	140 (122 – 1490)	85 (0 – 5320)
VEGFR2+ CEPs	500 (0 – 3970)	495 (0 – 5420)
VEGFR3+ CEPs	470 (0 – 7510)	325 (0 – 8290)
Endoglin+ CEPs	100 (0 – 2520)	560 (0 – 7460)
CD143+ CEPs	440 (85 – 64140)	1295 (0 – 26370)
CD144+ CEPs	170 (0 – 4930)	345 (0 – 3560)
	Osteoblastic Histology	Other Histology
CD146+ CECs	722 (130 – 5320)	600 (60 – 2590)
VEGFR2+ CECs	790 (0 – 6040)	690 (150 – 4320)
VEGFR3+ CECs	570 (0 – 11750)	610 (160 – 11000)
Apoptotic CECs	700 (0 – 4400)	280 (60 – 8430)
Endoglin+ CECs	440 (0 – 7700)	1390 (30 – 2720)
CD143+ CECs	2340 (85 – 29840)	650 (50 – 67030)
CD144+ CECs	843 (0 – 3840)	240 (30 – 8010)
CD146+ CEPs	185 (0 – 5320)	30 (0 – 1490)
VEGFR2+ CEPs	498 (0 – 5420)	560 (120 – 3970)
VEGFR3+ CEPs	413 (0 – 8290)	470 (130 – 7510)
Endoglin+ CEPs	348 (0 – 7460)	860 (30 – 2520)
CD143+ CEPs	1558 (0 – 26370)	520 (0 – 64140)
CD144+ CEPs	490 (0 – 3560)	210 (30 – 4930)
	Tumor Size > 8 cm	Tumor Size ≤ 8 cm
CD146+ CECs	330 (60 – 5320)	678 (140 – 2590)
VEGFR2+ CECs	490 (50 – 6040)	825 (0 – 4320)
VEGFR3+ CECs	300 (0 – 11750)	655 (0 – 11000)
Apoptotic CECs	310 (0 – 4400)	226 (0 – 8430)
Endoglin+ CECs	320 (0 – 7700)	893 (0 – 2720)
CD143+ CECs	2350 (50 – 11930)	1370 (85 – 67030)
CD144+ CECs	360 (0 – 3840)	438 (0 – 8010)
CD146+ CEPs	130 (0 – 5320)	104 (0 – 1620)

Parameter	Clinical Variable Median cells/mL (range)	
	Metastatic Disease	Localized Disease
VEGFR2+ CEPs	500 (0 – 5420)	248 (0 – 3970)
VEGFR3+ CEPs	300 (0 – 8290)	453 (0 – 7510)
Endoglin+ CEPs	240 (0 – 7460)	685 (0 – 2520)
CD143+ CEPs	1820 (0 – 11290)	938 (85 – 64140)
CD144+ CEPs	370 (0 – 3560)	298 (0 – 4930)
	≤ 90% Tumor Necrosis	> 90% Tumor Necrosis
CD146+ CECs	755 (130 – 5320)	690 (60 – 2770)
VEGFR2+ CECs	820 (50 – 6040)	490 (0 – 4320)
VEGFR3+ CECs	610 (0 – 11750)	575 (0 – 11000)
Apoptotic CECs	310 (0 – 4400)	645 (0 – 8430)
Endoglin+ CECs	1080 (0 – 7700)	705 (0 – 2720)
CD143+ CECs	2160 (440 – 29840)	1550 (50 – 67030)
CD144+ CECs	515 (0 – 3840)	360 (0 – 8010)
CD146+ CEPs	130 (30 – 5320)	240 (0 – 1490)
VEGFR2+ CEPs	495 (0 – 5420)	500 (0 – 3970)
VEGFR3+ CEPs	325 (0 – 8290)	540 (0 – 7510)
Endoglin+ CEPs	665 (0 – 7460)	455 (0 – 2520)
CD143+ CEPs	1295 (300 – 26370)	1200 (0 – 64140)
CD144+ CEPs	345 (0 – 3560)	310 (0 – 4930)