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Low-dose Oral Metronomic Chemotherapy Prevents Mobilization of Endothelial Progenitor Cells into the Blood of Cancer Patients

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Abstract. *Circulating endothelial progenitor cells (EPCs) actively supply cells that may participate in tumor angiogenesis. The differing effects of low-dose metronomic trofosfamide as opposed to conventional dose-dense chemotherapy on plasma levels of vascular endothelial growth factor (VEGF) and the numbers of circulating EPC are reported. Patients and Methods: Blood samples were obtained from cancer patients, 18 receiving oral metronomic chemotherapy of trofosfamide with or without celecoxib, and 24 receiving conventional dose-dense chemotherapy, eight of them in adjuvant intention. Mononuclear cells were analyzed by flow cytometry for CD34, CD45 and vascular endothelial growth factor-receptor 2 (VEGF-R2) coexpression, defining EPCs, and for plasma levels of VEGF by ELISA at day 0, 10 and 21 of therapy. Results: After conventional dose-dense chemotherapy, the numbers of circulating EPCs and the VEGF plasma concentrations increased sharply, doubling pretherapeutic levels at day 21. In contrast, under low-dose metronomic chemotherapy, the numbers of circulating EPCs decreased significantly and VEGF plasma concentrations remained unchanged. Conclusion: These observations provide evidence that conventional dose-dense chemotherapy leads to rebound EPC mobilization even when given with adjuvant intention, while low-dose metronomic scheduling of cytotoxic substances such as trofosfamide may sharply reduce EPC release into the circulation.*

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Tumors depend on their capacity to connect to the blood circulation for oxygenation, nutrient supply and to enable metastasis. Antiangiogeneic interventions that target and inhibit tumor vessel growth may lead to high response rates of cancer patients and to prolonged median overall survival (1, 2). Circulating endothelial progenitor cells (EPCs) that contribute to tumor neovascularisation (3-5) are defined by specific cell surface markers, such as CD34, vascular endothelial growth factor receptor-2 (VEGF-R2) and CD133 (6, 7). Cells exhibiting these markers are predominantly found in bone marrow (8). In the peripheral circulation of adults, most EPCs are CD133 negative, but express VEGFR-2 and CD34 (9) and have been described as early circulating endothelial progenitor cells (cEPCs) (10).

The precise role of EPCs in tumor neovascularisation remains controversial (11, 12). However, accumulating evidence shows that high cEPC numbers are strongly correlated with tumor progression in animals (13). Modulating effects of maximally tolerated doses of cyclophosphamide as well as low-dose metronomic cyclophosphamide on cEPCs have been described in immunodeficient mice bearing human lymphoma cells (13). Maximum tolerable dose chemotherapy led to high cEPC levels whereas metronomic scheduling resulted in low cEPC numbers. Another group of drugs that are well tolerated and that can be administered at frequent, regular intervals with potential to inhibit tumor angiogenesis are the inhibitors of cyclooxygenase 2 (COX-2), such as celecoxib. These may easily be administered together with low-dose metronomic chemotherapy (14, 15) to increase the antitumor efficacy (16) because COX-2 is overexpressed in several human malignancies and their neovasculature (17, 18). Recent reports have indicated that EPCs are directly targeted by the COX-2 inhibitors (19).

Trofosfamide along with cyclophosphamide belongs to the oxazaphosphorine drug family. The former differs from the more popular cyclophosphamide in carrying a third chlorethyl group, which makes it more lipid-soluble resulting in good enteral resorption characteristics.

Although its treatment efficacy has so far not been proven in phase III studies, trofosfamide is approved in Germany for the palliative treatment of several lymphoid malignancies and solid tumors. This drug is generally prescribed for oral application at low daily doses of 50 to 100 mg *bid*. At this level it has a very good toxicity profile, showing few side-effects among which hematotoxicity is its main long-term consequence. Recent studies have shown that chemotherapeutics, used in a low-dose metronomic schedule, may have potential anticancer efficacy not only by direct cytotoxicity, but also *via* a second mechanism, namely inhibition of tumor angiogenesis. We and others have demonstrated in preclinical studies that metronomic scheduling of cytotoxics has anti-angiogenic effects (14, 20-23). The clinical efficiency of low-dose metronomic trofosfamide has been shown in numerous phase II studies demonstrating beneficial effects in various human malignancies, including lymphoma, breast cancer and sarcoma (20, 21). We have recently shown, in an animal tumor model, that inhibition of tumor neovascularsation may be an important principle of trofosfamide action (22).

The goal of this study was to document the different effects of low-dose metronomic and dose-dense conventional chemotherapies on cEPCs and VEGF levels, which may both be considered important biomarkers indicative of neovascularisation. The investigators had no influence on any of the chosen treatment options but merely monitored VEGF levels and cEPCs in patients under given treatments. Our aim was not to compare the effectiveness or determine the superiority of any of the regimens.

Patients and Methods

Patients. Blood samples were drawn from 42 outpatients suffering from a variety of malignant diseases who had not received any other chemotherapy for at least the preceding three weeks (Table I). Blood was drawn according to clinical standards. Group I consisted of 14 female patients with breast cancer who received adjuvant fluorouracil (5-FU), epirubicine and cyclophosphamide (FEC). Group II consisted of four female and six male patients who received chemotherapy with cyclophosphamide, doxorubicin, vincristine, prednisolone and rituximab (CHOP-R) for malignant lymphoma. The samples from groups I and II were obtained before the application of the first cycle, at day 10 of the first cycle of chemotherapy and again before initiating the second cycle of chemotherapy (day 21). The eight patients (five females and three males) in group III received daily low-dose metronomic trofosfamide only (100-150 mg). The patients in group IV (six females and four males) received daily metronomic chemotherapy with trofosfamide (100-150 mg) in combination with celecoxib (400 mg). In groups III and IV, blood was drawn before the first dose of trofosfamide, and after 10 and 21 days of daily therapy with trofosfamide. The results from all the eligible patients were included. None of these patients received treatment with erythropoietin. The study was approved by the local Ethic's Committee.

Table I. *Patients were divided into four groups according to their therapy and cancer: Group I patients received adjuvant anthracycline-based chemotherapy for breast cancer, Group II patients received R-CHOP for malignant lymphoma, Group III received daily low-dose metronomic chemotherapy with trofosfamide without celecoxib and Group IV received a daily metronomic chemotherapy with trofosfamide with celecoxib.*

Group	Age	Gender		Diagnosis
	(years)	Male	Female	
I	59±12	0	14	Breast cancer (n=14)
II	68±9	6	4	Non-Hodgkin lymphoma (n=10)
III	59±12	3	5	Lung cancer (n=2), breast cancer (n=2), sarcoma (n=2), other (n=2)
IV	64±11	4	6	Lung cancer (n=3), colon carcinoma (n=3), other (n=4)

CD34 enrichment. To increase purity of CD34+ from the blood samples, CD34+ cells were isolated by an immunomagnetic technique using a CD34+-progenitor cell isolation kit (Miltenyi Biotec, Bergisch Gladbach, Germany) according to the manufacturer's instructions. In brief, blood samples were first incubated with monoclonal CD34 antibodies conjugated to magnetic microbeads. After the incubation, the cells were washed by a centrifugation step and diluted in MACS-buffer (500 ml PBS with 0.5% BSA and 2 mM EDTA). The cells were then loaded onto a column installed in a magnetic field. The columns were rinsed with buffer and CD34-negative cells passed through. Finally, the enriched cells were eluted from the columns and diluted in MACS buffer.

Flow cytometry. The cell densities were calculated as the total number of cEPCs per ml blood sample. The purified cells were analyzed for CD34, CD45 and VEGF-R2 expression by flow cytometry using fluorescently labelled monoclonal antibodies. Fluorescently labelled isotype-matched non-specific immunoglobulin G (IgG) antibodies were used to exclude non-specific fluorescence. Enriched cells were stained with CD34-PC7, CD45-FITC (both Beckman and Coulter, Fullerton, CA, USA) and VEGF-R2-PE (R and D- Systems, Minneapolis, MN, USA). After incubation in darkness, Versa Lyse (Beckman and Coulter) and IOTest3 Fixation (Beckman and Coulter) were added. After 30 minutes, the samples were analysed using a Cytomics FC 500 (Beckman and Coulter).

VEGF plasma concentrations. The plasma fractions of the blood samples used for purification and flow cytometry were frozen and stored at –18˚C. The VEGF plasma concentration was measured using a commercially available Elisa Kit (R&D Systems). Duplicate samples were added to individual wells. The absorption at 450 nm was measured with a Dynex Revelation microplate reader (MTX Lab Systems Inc., Vienna, VA, USA).

Statistical analysis. The SPSS software (SPSS Inc., Chicago, IL, USA) was used for statistical analysis. The results were tested for significance using the Wilcoxon signed-rank test and were considered significant if the p-values were ≤ 0.05.

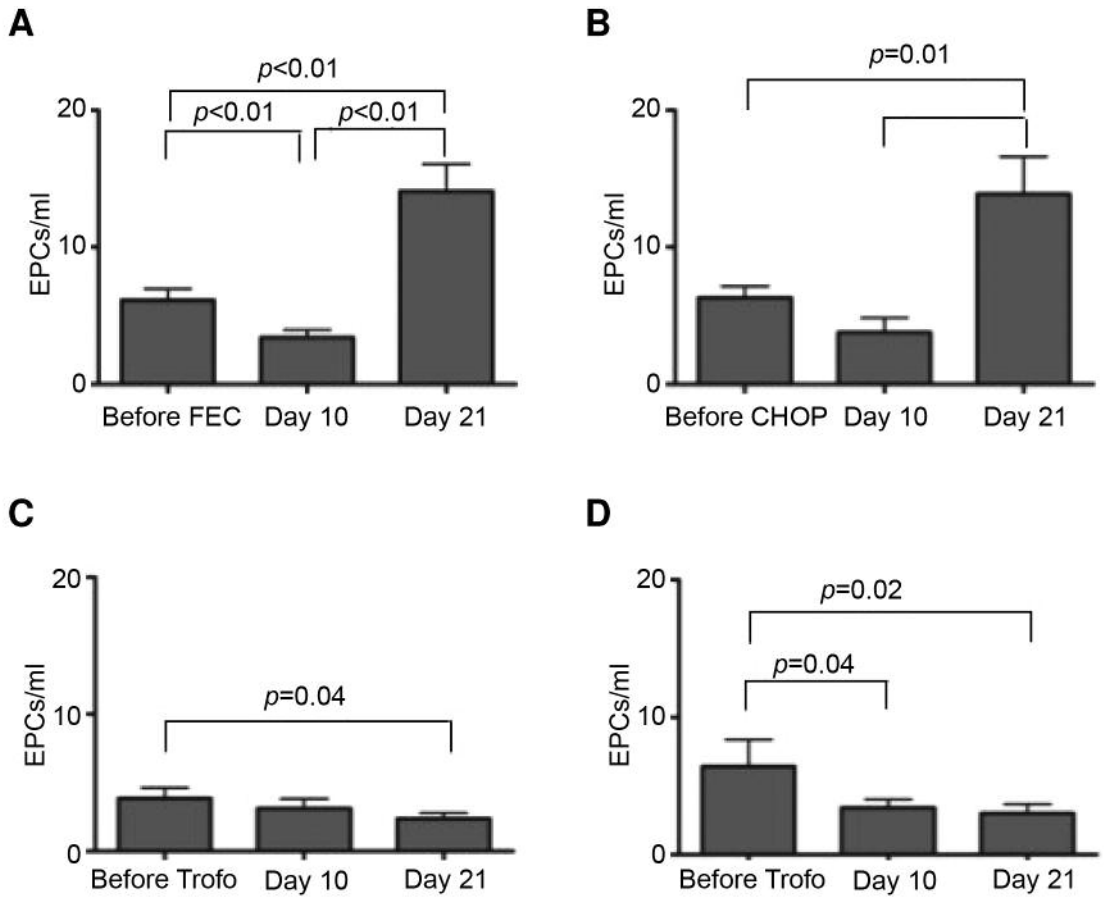

Figure 1. *Number of circulating EPCs in patients receiving chemotherapy for malignant lymphoma or solid tumors: (A) adjuvant dose-dense FEC (group I); (B) dose-dense CHOP-R (group II); (C) low-dose metronomic chemotherapy with trofosfamide (group III); (D) low-dose metronomic chemotherapy with trofosfamide and celecoxib (group IV); blood samples were taken at day 0, 10 and 21 of therapy. The data represent mean values and the standard error of mean. Trofo= trofosfamide.*

Results

Effect of chemotherapy on the cEPC number. In both the groups treated with dose-dense chemotherapy, the number of cEPCs, defined by bright positivity of the markers CD34 and VEGF-R2 as well as dim positivity of CD45, decreased on day 10, but then showed a greater than twofold increase on day 21, compared to baseline. In contrast, in both groups treated with metronomic chemotherapy, the number of cEPCs decreased under therapy and during observation (Figure 1).

VEGF plasma concentration. To further characterize the different responses to metronomic and conventional chemotherapy, VEGF plasma concentrations were measured. As shown in Figure 2, in groups I and II, the VEGF plasma concentrations increased during conventional chemotherapy, whereas under metronomic chemotherapy, the VEGF plasma concentrations remained stable.

Discussion

Significantly reduced numbers of cEPCs compared with the baseline values were formed with low-dose metronomic chemotherapy on day 21. In the patients who received both trofosfamide and celecoxib, this effect was more pronounced, and even statistically significant on day 10. This suggested that combining low-dose metronomic drugs with COX-2 inhibitors may lead to additional effects on cEPC suppression. In contrast, increased levels of cEPCs were observed three weeks after the first cycle of dose-dense chemotherapy. Similar observations have been reported (13, 24) by other groups, but this is the first report showing this effect in patients receiving adjuvant chemotherapy, with no measurable presence of tumor cells.

Elevated VEGF plasma concentrations have been recognized as the most important pro-angiogenic factors during tumor angiogenesis and ischemic injury (25). Low-

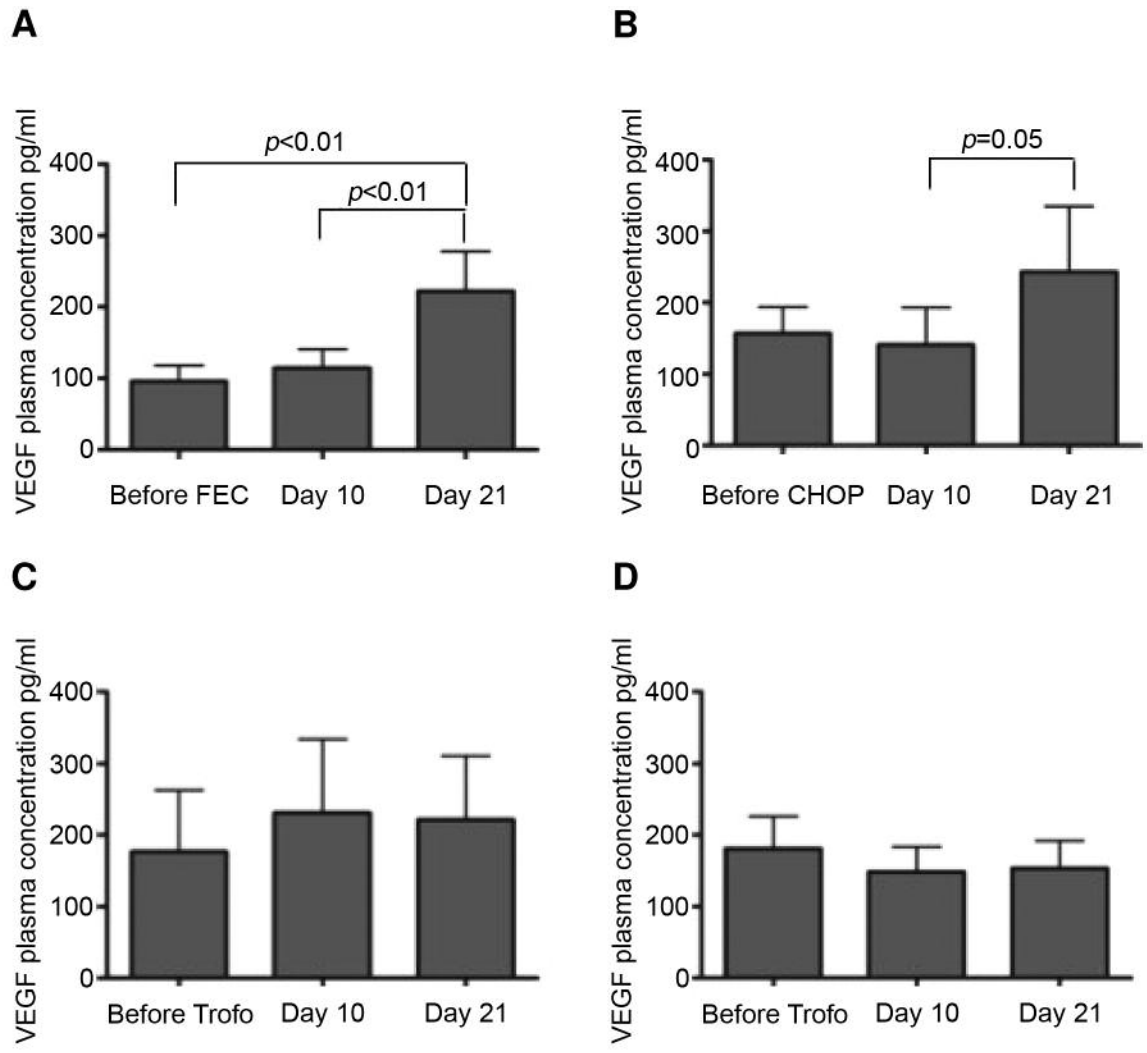

Figure 2. *VEGF plasma concentrations (pg/ml) in patients receiving chemotherapy for malignant lymphoma or solid tumors: (A) adjuvant dose-dense FEC (group I); (B) CHOP-R (group II); (C) low-dose metronomic chemotherapy with trofosfamide (group III); (D) trofosfamide and celecoxib (group IV); blood samples were taken before therapy and at days 10 and 21 of therapy. The values represent the mean VEGF plasma concentrations and the standard error. Abreviation: Trofo= trofosfamide.*

dose metronomic chemotherapy has previously been reported to decrease VEGF plasma concentrations (26). In the present study, the VEGF plasma concentrations remained unchanged under low-dose metronomic chemotherapy. In contrast, three weeks after the first cycle of dose-dense chemotherapy, the VEGF plasma concentrations increased significantly. Again, this effect was also observed in patients treated in an adjuvant setting. It remains unclear if this mobilization of VEGF and cEPCs is of biological relevance or critical to the patient's outcome. Nevertheless, there is good evidence that VEGF plays an important role in initiating micrometastasis by promoting lymphangiogenesis and regulating cell migration and adhesion (27, 28). Thus, the cytotoxic effects exerted by high drug levels may be counteracted by a stimulating effect on tumor vessel formation.

Similar cEPC and VEGF plasma concentration kinetics were observed under different chemotherapy settings and it was assumed that chemotherapy influences the VEGF level and thereby the cEPCs for the following reasons. Systemic administration of several cytokines and growth factors, such as granulocyte monocyte colony stimulating factor (GM-CSF) and VEGF, has been shown to augment the EPC fraction (29, 30). Several preclinical studies have shown that chemotherapeutics, especially anthracyclines, can induce apoptosis in endothelial cells (31, 32) and

consequently, the repair processes may result in overexpression of VEGF. However, the exact mechanism behind the self-contained increase in VEGF levels under chemotherapy remains unclear and this area requires further research. An influence of prior chemotherapy, dating back more than 3 weeks, on attenuating therapy-induced peaks of EPCs is a possibility but not probable considering preclinical and clinical studies that suggest that baseline EPC and or plasma VEGF levels recover after chemotherapy (13, 33).

The identification of surrogate markers for angiogenesis to monitor the success of treatment remains a major challenge in the validation of chemotherapy. Using cEPCs as a marker has been suggested previously (13, 16, 30). Buckstein *et al*. (16) observed patients affected by a non-Hodgkin lymphoma, receiving a low-dose continuous cyclophosphamide and celecoxib chemotherapy. In their study, significant declines in the number of cEPCs were only observed in the responding patients. Thus it may be important to measure the levels of cEPCs and VEGF plasma concentrations regularly during chemotherapy and to compare the cEPC and VEGF levels with tumor progression and overall survival in a greater number of patients.

In summary, dose-dense conventional and low-dose metronomic chemotherapy may have opposite effects on the number of cEPCs and VEGF in human adult cancer patients. Notably, increased levels of cEPCs were also observed in patients who received adjuvant dose-dense chemotherapy in the absence of tumors. The addition of VEGF inhibitors and low-dose metronomic scheduling of agents may prevent increased VEGF plasma concentrations after conventional chemotherapy.

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