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ORIGINAL ARTICLE

The role of VEGF and other parameters in tracking the clinical course in metronomic chemotherapy

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Summary

Purpose: The purpose of this study was to investigate the effect of metronomic chemotherapy on serum vascular endothelial growth factor (VEGF) levels in cancer patients.

Methods: The study included 11 metastatic cancer patients who received daily 50 mg cyclophosphamide and biweekly 5 mg methotrexate *per os* as metronomic chemotherapy. Bevacizumab together with FOLFIRI chemotherapy was administered as anti-angiogenic treatment in another group of 16 metastatic colorectal carcinoma patients. Furthermore, VEGF levels of 10 healthy individuals and 5 cord blood samples served for comparisons. VEGF levels of patients before therapy and 3 months after treatment were analyzed and compared.

Results: Serum VEGF levels prior to metronomic chemotherapy were higher compared with the healthy controls (p=0.0001). Similarly, serum VEGF levels prior to the bevacizumab-based chemoimmunotherapy were significantly higher compared with the healthy controls (p=0.005). In patients on metronomic chemotherapy VEGF levels showed non significant decrease (p=0.075). On the contary, VEGF levels decreased significantly (p=0.002) with bevacizumab treatment.

Conclusion: Serum VEGF levels may be used for assessing of the efficacy of anti-angiogenic therapies.

Key words: bevacizumab, chemotherapy, metronomic chemotherapy, VEGF

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Introduction

Currently the first and second leading causes of human deaths worldwide are characterized by lack of angiogenesis (cardiovascular diseases) and excess of angiogenesis (cancers). Conventional response parameters are not sufficient enough to assess the efficacy of the therapies targeting angiogenesis. Furthermore, the use of conventional cytotoxic drugs is restricted by their maximal tolerable dose (MTD). Although anticancer drug therapies result in the cure of a limited, and disease control in a substantial number of patients, chemotherapy given in MTD causes noticeably long and short term complications. Furthermore, with their notoriously dynamic and heterogeneous genetic instability, cancer cells have a tendency to develop resistance to multiple chemotherapeutic drugs [1]. An alternative method of administration with increased anticancer effects through continuous administration of cytotoxic drugs in low doses to avoid the onset of dose-restricting side effects and thus to eliminate the need for resting periods, is drawing increased attention. These forms of chemotherapy are called "metronomic chemotherapies". By means of targeting the activated endothelial cells that are proliferating much more slowly than tumor cells, metronomic therapies inhibit the growth of tumor cells resulting from insufficient neovascularization. It was not until recently when the beneficial effects of antiangiogenic treatment based on metronomic chemotherapy or other methods in cancer patients could be demonstrated [2,3].

In fact, a variety of protocols which were earlier called "sustained chemotherapy" in various cancers could well be described as metronomic chemotherapy: daily mercaptopurine + weekly methotrexate used successfully in leukemia; daily cyclophosphamide + weekly vincristine effective in neuroblastoma; the weekly use of vincristine in Wilms' tumor and the UFT schemes in lung adenocarcinoma are some examples [4,5].

VEGF is also known as the vascular permeability factor. Melder et al. [5] have shown that VEGF promotes VCAM-1 (vascular cell adhesion molecule 1) and ICAM-1 (intercellular adhesion molecule 1) in endothelial cells. This stimulation may result in the adhesion of VLA-4 (very late antigen-4) and CD 18 on the surface of the natural killer (NK) cells and the active NK cells on endothelial cells through the specific interaction between the endothelial VCAM-1 and ICAM-1. These effects may provide an explanation for the previously observed preferential adhesion of NK cells activated by IL-2 against tumor vascularization [6,7]. Gabrilovich et al. suggested that VEGF could have an inhibitory effect in the maturation of the cells that provide professional antigens such as dendritic cells [8]. These findings suggest that VEGF may facilitate tumor growth, while at the same time allowing the escape of the tumor from the stimulation of an immune response. Studies performed at various laboratories have clarified the essential role of VEGF in the arrangement of normal and abnormal angiogenesis. Particularly, the loss of even a single VEGF allele results in embryonic death and reveals the role assumed by this factor in the development and diversion of the vascular system [9,10]. The human VEGF gene is composed of 8 exons and 7 introns. Initially, the alternative portion of the exon results in the form of 4 different isoforms (VEGF121 , VEGF165, VEGF189, VEGF 206). These contain 121, 165, 189 and 206 amino acids respectively. VEGF165 is the predominant isoform and lacks the portion coded with exon 6. VEGF121 does not contain the portions coded by exon 6 and 7. The less frequent variants of the alternative portion have been described separately. These are VEGF145 and VEGF 183 [11]. Human VEGF is a 45 kDa, heparin-binding homodimeric glycoprotein. The properties of the natural VEGF match to those of VEGF165. VEGF121 is a non-heparin binding, acidic polypeptide. VEGF189 and VEGF206 are highly basic and bind heparin with high affinity. However, while VEGF121 is an easily spreading protein, VEGF189 and VEGF206 are almost exclusively present in the extracellular matrix (ECM). VEGF165 has intermediate properties. Despite secretion, a substantial portion remains on the cell surface and in the ECM. Loss of the VEGF's heparin binding region results in substantial loss of mitogenic activity [12]. Several studies have demonstrated that the combined use of anti-VEGF therapy with chemotherapy or radiotherapy resulted in anti-tumor effect higher than the one achieved by the individual use of both treatments.

Clinical trials addressing cancer patients are continued with various VEGF inhibitors that contain

humanized monoclonal antibodies (rhuMab VEGF), one anti-VEGFR-2 antibody, small molecules that inhibit VEGFR-2 signal transmission and one soluble VEGF receptor against VEGF. Phase II clinical data provide initial evidence for rhuMab VEGF. When combined with conventional chemotherapy, rhuMab VEGF increases survival in patients with metastatic colorectal cancer [13].

The purpose of this study was to investigate the effect of angiogenesis inhibitors on endothelial cells by measuring the serum VEGF levels of patients on metronomic chemotherapy and patients receiving FOLFIRI chemotherapy plus the anti-VEGF monoclonal antibody bevacizumab.

Methods

The study was approved by the local ethics committee, and written informed consent was obtained from all patients and healthy volunteers.

Patients and treatment protocols

The study included 11 metastatic cancer patients who received daily 50 mg cyclophosphamide and biweekly 5 mg methotrexate per os as metronomic chemotherapy. These patients had received many lines of chemotherapy in the past.

Metronomic chemotherapy was given continuously until unacceptable toxicity or disease progression. Patients were examined monthly with routine physical examination together with hemotological and biochemical tests for toxicity assessment.

Bevacizumab (5 mg/kg) along with conventional FOLFIRI chemotherapy (irinotecan 180 mg/m^2, 5-fluorouracil 3000 mg/m^2 , leucovorin 200 mg/m^2) were given biweekly. This anti-angiogenic therapy was used as first-line treatment to another group of 16 metastatic colorectal carcinoma patients.

In addition, VEGF levels of 10 healthy volunteers and 5 samples of cord blood were assessed. VEGF levels of patients prior to therapy and 3 months after treatment were analyzed. Then, these levels were correlated with the patient clinical outcomes.

Lab methods

Blood samples were collected prior to and 3 months after the beginning of therapy from patients receiving metronomic chemotherapy and bevacizumab plus FOLFIRI at the Medical Oncology Clinic. The serum of the collected blood samples was separated (2000 g, 7 min) and kept at -80°C.

Blood samples were also collected from 5 healthy subjects of 25±5 years of age and 5 in the 50±5 age group. The serum of the collected blood samples was separated (2000 g, 7 min) and kept at -80°C.

Despite the finding of very low VEGF level in the cord blood, 5 different cord blood serums were also used for control purposes. The patient serum samples were assayed using ELISA (Enzyme-Linked Immunosorbent Assay) for VEGF levels at the initial stage, and and 3 months after initiation of therapy. Two microwells not containing serum served as negative control. The study used Biosource brand VEGF165 ELISA kit. All serums tests were carried out in duplicate.

Statistics

Statistical analyses were performed using SPSS version 15 for Windows (SPSS Inc., Chicago, IL, USA). Comparisons between 3 groups were performed using the Kruskal-Wallis Variance analysis, while the dual group comparisons were performed by the Mann-Whitney U test with Bonferonni correction. The VEGF levels of the groups at different times were compared using the Wilcoxon test. A p value below 0.05 was considered as significant. The values in Tables and Figures were given as average, SD (standard deviation), and range.

Results

The study included 11 patients (7 females, 4 males, average age 55 years, range 42-69) receiving metronomic chemotherapy, 16 patients (4 females and 12 males, average age 55.1 years, range 28-70) receiving bevacizumab plus FOLFIRI therapy, 5 healthy individuals in the 25±5 years age group (2 females and 3 males, 27.6 years on average), and 5 healthy subjects (5 males, 51 years of age on average) in the 50±5 years age group as controls. Furthermore, 5 cord blood samples from different individuals with low VEGF levels were used for controlling purposes for the reliability of the ELISA analysis.

The age, diagnosis, pre- and post-metronomic chemotherapy patient serum VEGF levels are shown in Table 1.

The age and diagnosis of serum VEGF levels of the patients receiving bevacizumab plus FOLFIRI therapy are shown in Table 2.

The age and serum VEGF levels of the healthy controls are shown in Table 3.

The VEGF serum levels in the 5 cord blood of individuals with low VEGF levels and who served for the reliability of the ELISA analysis were 39, 44, 43.2, 38.6, and 33.4 pg/ml respectively (average 39.64 pg/ml).

The average, SD and range of VEGF of the patients

Table 1. Age, diagnosis, pre- and post-metronomic chemotherapy serum VEGF levels of patients receiving metronomic chemotherapy

Patients	*Age (years)*	*Diagnosis*	*VEGF level before metronomic chemotherapy (pg/ml)*	*VEGF level after 3 months metronomic chemotherapy (pg/ml)*
GB	48	Breast Ca	450	676
FA	69	Breast Ca	194	299
HT	64	Breast Ca	280	128.5
CC	42	Breast Ca	660	410
MS	60	Breast Ca	521.5	518.5
NK	62	Ovarian Ca	600	96
HU	54	Lung Ca	202	160.4
RY	56	Breast Ca	300	350
FD	49	Breast Ca	596	240
FS	52	Lung Ca	898	197.5
HO	49	Nasopharynx Ca	1200	368

Table 2. Age, diagnosis, and serum VEGF levels of patients receiving bevacizumab plus FOLFIRI therapy

Patients	*Age (years)*	*Diagnosis*	*VEGF level before bevacizumab therapy (pg/ml)*	*VEGF level after 3 months bevacizumab therapy (pg/ml)*
M.AK	61	Colon Ca	206	62
AY	60	Colon Ca	207	78.04
CY	53	Colon Ca	121	119.1
NK	65	Colon Ca	81	121
HK	28	Colon Ca	46	130
MC	52	Colon Ca	597	118
HK	40	Colon Ca	602	117
MS	62	Colon Ca	129	89
HE	45	Colon Ca	446	115
AK	53	Colon Ca	450	130
BO	56	Colon Ca	187	117
HK	66	Colon Ca	216	117
UK	64	Colon Ca	325	127
SC	58	Colon Ca	295	115
MK	70	Colon Ca	178	119
M.AT	50	Colon Ca	600	116

are shown in Table 4.

The average VEGF serum level of healthy individuals was 119.5 pg/ml, SD was 35.1, and range 47-195 pg/ml.

The difference of the average VEGF levels was statistically significant when VEGF levels of the patients receiving metronomic chemotherapy, those receiving bevacizumab plus FOLFIRI and healthy subjects were compared. Kruskal–Wallis analysis showed that the VEGF levels of healthy individuals were significantly lower (p=0.0001) vs. those of the patients. Serum VEGF levels of both age groups in the control population of which half was approximately 25 and the other half around 50 years old were similar. On the contrary, VEGF values in the control cord blood corresponded to approximately half of the VEGF (pg/ml) value in both groups.

It is interesting that the medical history of the individual with the lowest VEGF level in the control group (his VEGF was 46.9 pg/ml vs. healthy control group average 120.7 pg/ml) revealed that both, the individual and his family were suffering of atherosclerosis. The subject with the highest level of VEGF in the control group (his VEGF was 195 pg/ml vs. healthy control group average 120.7 pg/ml) was found to be a carrier of sickle cell anemia while he had no family history of atherosclerosis. A comparison between the initial VEGF levels of patients receiving metronomic chemotherapy and bevacizumab plus FOLFIRI therapy showed that the VEGF levels of metronomic chemotherapy patients were significantly higher (p=0.030) according to Mann-Whitney U test analysis. Mann-Whitney U test analysis showed that the initial VEGF levels of patients receiving metronomic chemotherapy were significantly higher (p=0.0001) than those of healthy individuals. Also, Mann-Whitney U test analysis showed that the initial VEGF levels of patients receiving bevacizumab plus FOLFIRI were significantly higher (p=0.005) than those in healthy subjects. A comparison between the VEGF levels after 3 months of patients receiving metronomic chemotherapy and bevacizumab plus FOLFIRI treatment showed that the VEGF levels of the metronomic chemotherapy patients were significantly higher (p=0.0001) according to Mann-Whitney U test analysis. Despite the fall in the initial and 3-month average VEGF levels of patients receiving metronomic chemotherapy, this decrease was not significant according to Wilcoxon analysis (p=0.075; Figure 1). However, the decrease in VEGF levels 3 months later of patients receiving bevacizumab plus FOLFIRI was significant (p=0.002) according to Wilcoxon analysis.

Table 3. Age and serum VEGF levels of the healthy controls

Healthy controls	*Age (years)*	*VEGF level (pg/ml)*
DE	28	129
AD	30	126
KO	30	118
SU	22	119
DB	28	125
TK	49	46.9
BS	49	118.6
EY	49	195
ME	54	120
OA	54	113

Discussion

VEGF is the most important target of anti-angiogenic therapy and is secreted by approximately 60% of human tumors. Long life expectation of cancer patients bears the risk of the production of high amounts of angiogenic proteins associated with tumor cells' mutations. For example, while most breast cancers secrete only VEGF at the time of diagnosis, relapsed cases of breast cancers secrete approximately 5 different types of angiogenic proteins. Angiogenetic inhibitors prevent tumor progression by affecting the function and/or proliferation of the endothelial cells. The purpose of this study was to ensure proper observation of the results achieved through the effects of the angiogenetic inhibitor bevacizumab on endothelial cells through estimations of serum VEGF levels in patients receiving metronomic chemotherapy and bevacizumab plus FOLFIRI treatment [9-14].

Bevacizumab is an anti-VEGF monoclonal antibody that binds and neutralizes all human VEGF-A isoforms and bioactive proteolytic fragments. In two of our recent studies we observed changes in sTRAIL levels associated with outcome in response to beva-

cizumab therapy; serum IL8 levels were decreased in all patients, however, this change was not correlated with disease outcome [15,16].

Factors such as the lack of frequent resistance to drugs that target directly the tumor cells, tumor endothelial cell proliferation 50-100 times faster than normal endothelial cells, the presence of marks which are available on active endothelial cells but not on the inactive (non-proliferating, silent) ones, the highly rare occasions of anti-angiogenic drug-related side effects in adults due to limited angiogenesis, easy access to the endothelial cells through blood circulation, and the killing of tumor cells in large amounts through damage to several micro vessels are the advantages which are expected to bolster anti-angiogenic therapy along with conventional methods of treatment in the near future. The most important issue, however, would be the inability to track therapeutic activity with conventional response parameters (Response Evaluation Criteria In Solid Tumors [RECIST]) since the RECIST parameters are based on the shrinkage of the tumor tissue. However, this does not occur always in line with tumor vascularization.

This study was conducted on 11 patients (7 females, 4 males, 55 years of age on average) receiving metronomic chemotherapy, and on 16 patients (4 females, 12 males, 55.1 years on average) receiving bevacizumab plus FOLFIRI treatment. Of the patients receiving metronomic chemotherapy, 6, 2, 2 and 1 had breast, ovarian, lung, and nasopharyngeal cancer, respectively. All 16 patients receiving bevacizumab therapy had colon cancer.

A comparison of the initial VEGF levels between the patients receiving metronomic chemotherapy

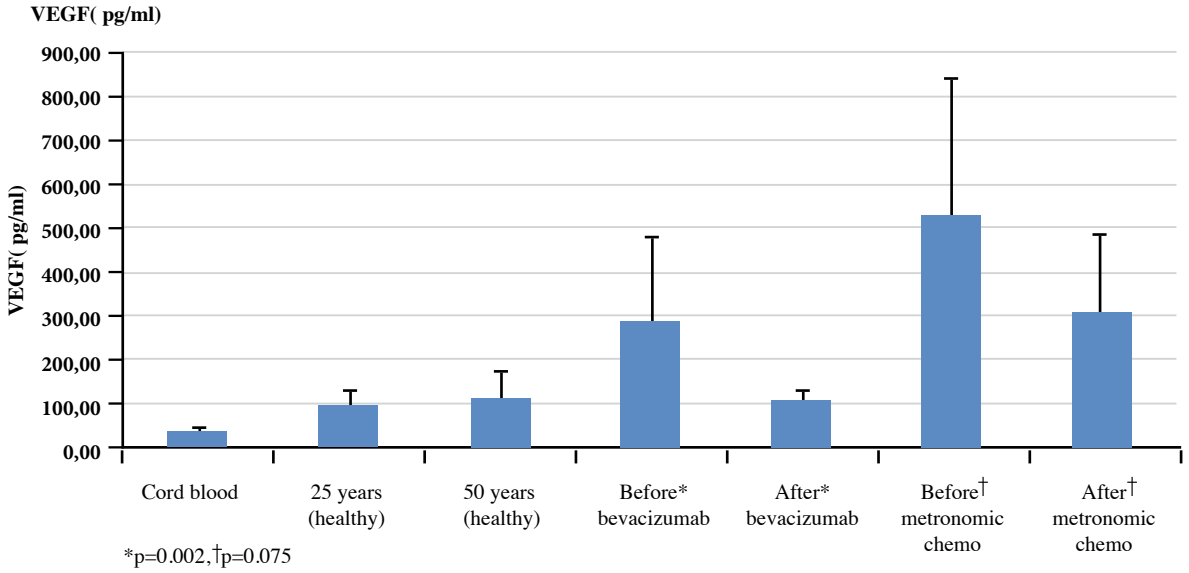

Figure 1. The average VEGF levels of healthy individuals and patients.

Table 4. Average, standard deviation (SD) and range of patient VEGF data

	VEGF levels before therapy (pg/ml)			*VEGF levels after 3 months (pg/ml)*			*p -value (before vs after)*
	Mean	*SD*	*Range*	*Mean*	*SD*	*Range*	
Metronomic chemotherapy (N=11)	520	308	194-1200	299	176	96-676	0.075
Bevacizumab plus FOLFIRI chemotherapy (N=16)	211	189	46-602	117	18.9	62-130	0.002

and those with bevacizumab treatment resulted in significantly higher VEGF levels (p=0.030) of those with metronomic chemotherapy. Patients with metronomic chemotherapy were those who received a large number of chemotherapies at the earlier and later stages. Conventional chemotherapy might stimulate angiogenesis because it is usually given every 3-4 weeks which includes a resting period of 2-3 weeks. Endothelial cells are killed during the time of chemotherapy, however they reproduce themselves during these resting periods. On the contrary, bevacizumab patients had not received prior therapy. Another reason for the difference in the initial VEGF levels in the metronomic treatment group might be the multiple organ involvement (brain, lung) and particularly bone metastases, and liver metastasis in those receiving bevacizumab treatment.

The initial VEGF levels of patients receiving metronomic chemotherapy were significantly higher (p=0.0001) when compared to healthy individuals. Likewise, the initial VEGF levels of patients receiving bevacizumab plus FOLFIRI therapy were also significantly higher (p=0.005) compared with healthy subjects.

These observations support the idea that VEGF secretion and the angiogenetic process are high in metastatic cancers. A comparison between the VEGF levels after 3 months of patients receiving metronomic chemotherapy and bevacizumab treatment showed that the VEGF levels of the metronomic chemotherapy patients were significantly higher (p=0.0001), while the bevacizumab therapy yielded an average decrease of 94.5 pg/ml in VEGF levels; the average decrease of VEGF levels achieved with metronomic chemotherapy was 221 pg/ml. Although there was a decreasing trend in the initial and 3-month later VEGF levels in patients subjected to metronomic chemotherapy, this decrease was not statistically significant (p= 0.075). A study by Artac et al. performed in our country showed a relation between the decrease of serum VEGF levels and non-progression survival in 8 patients with metastatic breast cancer who received metronomic cyclophosphamide plus etoposide chemotherapy [17]. The average serum VEGF levels of the metastatic patients in this non-controlled study, that was 495 pg/ml prior to therapy, dropped to 346 pg/ml 2 months after treatment. The serum VEGF values in our study, however, were 520 pg/ml prior to treatment and 299 pg/ml 3 months after the metronomic chemotherapy application.

The statistical analysis did not find significant differences, most likely due to the small number of patients that could be included in the study, as well as their heterogeneous distribution. However, the decrease in the average initial VEGF levels and those 3 months after in some patients receiving metronomic chemotherapy appears clinically significant. For example, the VEGF levels in C.C, a patient with breast cancer (stable disease) who responded to metronomic chemotherapy dropped from 660 to 410 pg/ml; VEGF level of H.T, a breast cancer patient who responded partially to metronomic chemotherapy, decreased from 280.2 to 128.5 pg/ml, and VEGF level in F.S, a small cell lung cancer patient, decreased from 898 to 197.5 pg/ml. However, the VEGF level in M.S, a breast cancer patient who did not respond to metronomic chemotherapy remained stable between 521.5 and 518.5 pg/ml, while the VEGF level of another breast cancer patient (G.B.), who progressed under metronomic chemotherapy rose from 450.5 to 676 pg/ml. A certain regression from 202 to 160.4 pg/ml was observed in H.U, a small cell lung cancer patient in progress under metronomic chemotherapy. There seems to be a trend of association between the patients' clinical data receiving metronomic chemotherapy and their VEGF levels. This aspect supports the idea that the activity of metronomic chemotherapy could be traceable prior to and after treatment, while it also appears to be worth a research with larger number of patients.

The decrease in the initial and 3-month later levels of VEGF in patients who received bevacizumab was significant (p=0.002). Likewise, a decrease in VEGF levels was also observed in patients who responded to bevacizumab therapy. Like with metronomic chemotherapy, the traceability of the bevacizumab activity using the pre- and post-therapy VEGF parameters seems possible. However, administration of the costly anti-VEGF therapy to patients with normal VEGF levels (150 ± 50 pg/ml) is questionable. These problems may possibly be overcome by means of a more strict

selection of patient subgroups where the anti-VEGF monoclonal antibody therapy would not be effective.

The development and formulation of new anti-VEGF therapies is continued. For example, anti –VEGF monoclonal antibody, soluble Flt-1, anti-KDR kinase (SU 5416 and SU 6668) and anti-KDR or anti-Flt-1 antibody are the anti-VEGF agents that have been used up until now [10]. The results of the present study suggest that the angiogenetic process in metastatic tumors could be traceable by simple and cost-effective serum VEGF level measurements. These measurements should be tried in all treatments and activity follow up studies that have an effect on the angiogenetic process including anti-VEGF monoclonal antibodies, metronomic therapies, thalidomide, lenalidomide and even some alternative/complementary methods said to target angiogenesis.

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