Relation of serum vascular endothelial growth factor as an angiogenesis biomarker with nitric oxide & urokinase-type plasminogen activator in breast cancer patients

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Background & objectives: The primary mediator of angiogenesis is vascular endothelial growth factor (VEGF). It is well documented that angiogenic activity in human cancer depends on nitric oxide (NO) levels in tissues. Additionally, urokinase type plasminogen activator (u-PA) plays a role in cell adhesion and migration. Serum VEGF and its relationship between NO and u-PA concentrations are poorly reported in breast cancer patients. The aim of this study was to investigate the relationship between serum levels of VEGF and NO and u-PA in patients with breast cancer.

Methods: Serum concentrations of VEGF, NO and u-PA were measured in groups of pre-operative breast cancer patients without metastasis (n=20), post-operative breast cancer patients without metastasis (four wk after the operation, n=20), breast cancer patients with metastasis (n=23), patients with benign breast disease (n=11) and healthy female controls (n=20).

Results: There was no difference in serum concentrations of VEGF, NO and u-PA between controls and patients with benign breast disease. Serum VEGF, NO and u-PA concentrations were significantly higher in pre-operative breast cancer patients than in controls and in patients with benign breast diseases (P<0.01). Post-operative breast cancer patients without metastasis had significantly lower serum VEGF and u-PA concentrations than the pre-operative patients (P<0.01). In breast cancer patients with metastasis, serum VEGF, and u-PA were significantly higher than post-operative non-metastatic patients (P<0.01). Serum VEGF concentrations were positively correlated with serum uPA in all of the patients groups (r=0.886, P<0.01). Serum VEGF levels were positively correlated with serum NO levels in breast cancer patients with metastasis (r= 0.386, P<0.05).

Interpretation & conclusions: Our results demonstrated that the angiogenic activity was increased in patients with breast cancer. Elevated VEGF levels as an angiogenesis marker may be associated with uPA. VEGF, NO and uPA seem to be associated with the angiogenetic and metastatic process of breast cancer.

Key words Angiogenesis - breast cancer - nitric oxide - urokinase type plasminogen activator - vascular endothelial growth factor
Enhanced angiogenesis can lead to accelerated growth of the primary tumour, and also facilitates the process of metastasis. It is well established that the primary mediator of this process in tumour is the cytokine, vascular endothelial growth factor (VEGF). VEGF, a heparin-binding growth factor, has been alternately designated as vascular permeability factor. VEGF has been shown to promote endothelial cell migration and acts as mitogen for endothelial cells. Expression of VEGF may be induced by several factors; hypoxia, nitric oxide (NO), tumour suppressor genes (mutated p53), cytokines, such as interleukin-1β, oncogenes (e.g., v-raf), growth factors such as insulin like growth factor-1, or hormones such as progestins. It has been suggested that VEGF expression correlates with the degree of angiogenesis and plays a predominant role in breast cancer prognosis.

NO is responsible for numerous functions such as neurotransmission, vascular homeostasis, immune regulation and host defense, as well as playing critical roles in the antipathogen and tumouricidal response of the immune system. Despite these beneficial effects, high concentration of NO and its derivatives produced in inflamed tissues has also been implicated as a deleterious agent in various pathophysiological conditions including cancer. NO production is also a part of the angiogenic switch in tumour development. On the other hand, tumour-associated proteolytic factors enable tumour cells to disintegrate the stroma in their immediate vicinity, intravasate into lymphatic or blood vessels, and then spread systemically. Among the key players in the proteolytic cascade leading tumour invasion and metastasis are factors of the plasminogen activation system. The urokinase type plasminogen activator (u-PA) plays a pivotal role in the regulation of cell adhesion and migration during tissue remodeling. u-PA not only specifically cleaves plasminogen and converts it into plasmin but also activates intracellular signaling upon binding to certain receptors on the cell surface.

Serum VEGF and its relationship between NO and u-PA concentration are poorly reported in breast cancer patients. We undertook this study to determine the association between serum concentrations of VEGF, NO and u-PA in patients with breast cancer.

Material & Methods

The study was conducted at the Department of Surgery and Clinical Oncology in Cerrahpasa Medical Faculty, Istanbul University, Turkey. During 2002 and 2003, 20 consecutive women with breast cancer with operable early-stage breast tumour (pre-operative non-metastatic group; mean age: 58.3 ± 11.5 yr); 20 patients who were considered to be free of metastatic disease after definitive surgery for breast cancer (post-operative non-metastatic group, mean age: 51.5 ± 8.5 yr); and 23 patients with recurrent breast cancer after definitive surgery and chemotherapy (metastatic group, mean age: 51.5 ± 11.1 yr) were included in the study. Twenty healthy females (controls; mean age: 50.8 ± 8.9 yr) and 11 female patients with benign breast disease as fibrocystic disease (mean age: 51.6 ± 6.03 yr) were also included. All the subjects provided written and verbal informed consent prior to the study.

Exclusion criteria included cardiovascular disease, renal or hormonal disease, smoking habits, alcohol abuse, or receiving any drug therapy such as lipid lowering therapy, vitamins, or antioxidants. None of our patients was suffering from malnutrition or neoplastic cachexia.

A single pathologist studied the tumour specimens using the American Joint Committee on Cancer tumor-node metastasis classification (AJCC-TNM) with grade determined by the modified Bloom-Richardson criteria according to Elston and Ellis. The histopathological diagnosis was invasive ductal carcinoma in all patients. Steroid hormone receptor status [estrogen receptor (ER) and progesterone receptor (PR)] was determined by immunohistochemical staining and classified as "positive" or "negative". Immunohistochemical staining for ER and PR was performed using a standard streptavidin-biotin-peroxidase detection system. A positive control was included in all runs, and for each case omission of the primary antibody was used as a negative control. At least 10 per cent of tumour cell nuclei were required to be positive for the tumour to be accepted as ER- or PR-positive, with cytoplasmic staining being disregarded.

Menopausal status was confirmed by the absence of menstruation for at least 6 months and serum concentration of follicle stimulating hormone (FSH) of > 40IU/ml (FSH IRMA, Biocline, Australia) and estradiol of < 20IU/ml (EIA gene Estradiol, Biochem Immune Systems, France).

The blood samples were taken from pre-operative group one day before their operation. In post-operative group blood samples were taken before initiation of any adjuvant therapies (after 4 wk). Blood samples were collected from metastasis group when metastasis was
detected during routine follow up examination. The patients’ medical history was documented, a physical examination was performed, and haematological tests were carried out every 3-6 months during 5 yr of follow up and once a year thereafter. Isotopic bone scanning, chest X-ray and liver ultrasonography were used for the diagnosis of metastases. In metastatic group 13 patients had bone metastases, and the remaining had visceral metastases. Duration of mean remission in metastatic patients was 48±12 month.

Peripheral venous blood samples (5ml) from all subjects in various groups were collected between 0800 and 1200 after overnight fasting. To minimize serum protease activity and, therefore possible proteolytic degradation of serum VEGF, venous samples were collected in sterile tubes, containing the protease inhibitor aprotinin at 1,000 KIU per 4ml sample. The samples were allowed to coagulate at +4°C, centrifuged at 2,000 g for 10 min, and stored in aliquots at -80°C. After thawing, each serum aliquot was assayed only once.

Serum VEGF concentration was determined using the quantitative sandwich enzyme immunoassay technique (Biosource International, Camarillo, CA, USA). A monoclonal antibody specific for VEGF was used to capture the VEGF, and an enzyme-linked polyclonal antibody specific for VEGF was used for quantification. Each standard and sample was assayed in duplicate. The minimum detectable level of VEGF was < 5pg/ml. The intra- and inter-assay coefficients of variation were 4.7-6.5 per cent and 6.1- 8.5 per cent, respectively.

Serum NO level was determined as the concentration of nitrite plus nitrate (NO₂⁻ + NO₃⁻). Nitrate was reduced to nitrite by nitrate reductase and the concentration of nitrite was measured spectrophotometrically (Jasco V-530 UV/VIS, Japan) at 430 nm using the Griess reagent. The intra- and inter-assay coefficients of variation for NO were 4.8 and 5.1 per cent, respectively.

Serum u-PA concentrations were measured by the quantitative sandwich enzyme immunoassay technique (American Diagnostica u-PA ELISA, USA). Each standard and sample was assayed in duplicate. The intra- and inter-assay coefficients of variation were 5.8-7.5 per cent and 7.3- 9.3 per cent, respectively.

Statistical analysis: Statistical analysis of data was performed with SPSS software for Windows. ANOVA was applied to determine the significance of various changes in different groups of the patients. Spearman’s rank test was used to determine the relationship between VEGF and NO and u-PA levels in breast cancer patients. P<0.05 was considered significant.

Results

Characteristics of patients in pre- post-operative non metastatic and metastatic groups were given in Table I. There was no significant difference in serum concentrations of VEGF, NO and u-PA between controls and patients with benign breast disease. Serum VEGF, NO and u-PA concentrations were significantly higher in pre-operative patients than in controls and patients with benign breast disease (P<0.01). Serum VEGF and u-PA concentrations were significantly higher in pre-operative group than post-operative non metastatic group (P<0.01). There was a significant difference in serum VEGF and u-PA concentrations between metastatic group and post-operative non-metastatic group (P<0.01). In metastatic group, patients with visceral metastasis

<table>
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<tr>
<th>Prognostic factors</th>
<th>Pre-operative (n=20)</th>
<th>Post-operative (n=20)</th>
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<tr>
<td></td>
<td>&gt; 2</td>
<td>10</td>
<td>21</td>
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had significantly higher serum VEGF and u-PA concentrations than the patients with bone metastasis ($P<0.01$) (Table II).

Serum VEGF concentrations were positively correlated with serum u-PA concentrations in the breast cancer patients ($r=0.886, P<0.01$). VEGF concentrations were positively correlated with serum NO concentrations in metastatic group only ($r=0.386, P<0.05$).

**Discussion**

It has been hypothesised that serum VEGF concentrations may reflect the extent of active angiogenesis in patients with breast cancer\(^1\). Since post-operative patients had higher VEGF levels than the healthy controls, it was presumed that VEGF has been secreted by cancer cells and tumour infiltrating inflammatory cells into circulation and this process continued after the removal of the primary breast tumour. Consistent with the findings of Nishimura *et al*\(^1\) we obtained elevated serum VEGF levels in patient with recurrent tumours in our study. Linderholm *et al*\(^1\) showed that patients with bone metastasis had lower VEGF expression than those with visceral metastasis. Our observations were also the same. However, Gasparini *et al*\(^1\) found no association between angiogenesis and site of recurrence in breast cancer patients. Eppenberger *et al*\(^1\) showed that tissue VEGF concentrations were positively correlated with recurrence.

NO is an important bioactive agent involved in the multisteped process of carcinogenesis\(^1\). The roles of NO in tumour biology remain poorly understood. Our study has shown that serum NO concentrations were significantly elevated in patients with primary breast tumour composed with controls and remained at high levels in both metastatic and post-operative non metastatic breast cancer patients similar to those reported by Coskun *et al*\(^1\). We found no difference in serum NO concentrations between breast cancer patients with or without metastasis. It is possible that NO could be secreted by cancer cells, tumour infiltrating inflammatory cells and non immune cell types. The relation between NO and VEGF is controversial\(^2\). We found a positive correlation between serum VEGF and NO concentrations only in the breast cancer patients with metastasis.

Cancer invasion and metastasis formation is a multifactorial process and requires the co-ordinated action of cell- secreted proteolytic enzymes and their inhibitors. Elevated u-PA has been implicated in this invasive process\(^2\). Serum u-PA concentrations were higher in our breast cancer patients compared to controls. We found a positive correlation between serum VEGF and u-PA in our study groups. It has been suggested that VEGF increased the endothelial uPA expression and the degradation of extracellular matrix by plasminogen-activator system is essential for angiogenesis\(^3\). Therefore, high levels of VEGF and u-PA may synergistically contribute to angiogenesis and metastasis of breast cancer.

In conclusion, our findings suggest a positive association between elevated VEGF and uPA levels. Lower serum VEGF and u-PA concentrations in patients with bone metastasis may be related to less aggressive metastatic pattern of bone metastasis than that of visceral metastasis in breast cancer patients. Therefore,
determination of serum VEGF and u-PA together may help in better management of breast cancer patients follow up.

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References


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