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Original Article

Changes of Serum VEGF and b-FGF in 26 Patients with Breast Cancer after Treatment with *Hang-Am-Dan* (HAD), an Antiangiogenic Botanical Prescription

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Objectives : Recently, angiogenesis has gained an increasing interest as a prognostic factor in breast cancer. In this study we aimed to assess the antiangiogenic effects of HAD, a botanical anticancer remedy which has been prescribed in Daejeon University Oriental Hospital in Korea, on patients with breast carcinoma by measuring the serum vascular endothelial growth factor (VEGF), basic fibroblast growth factor (b-FGF) and platelets levels.

Methods : The study included 26 consecutive breast cancer patients (mean age ± standard deviation: 47.5±8.7 years) with stage II to IV disease who were treated with HAD (mean duration ± standard deviation: 264.5±121.6 days). In addition to routine laboratory and staging procedures, serum VEGF, b-FGF levels and platelet counts were determined as antiangiogenic markers. The antiangiogenic effects of HAD were evaluated by analyzing the differences between the values of the antiangiogenic markers before and after the treatment with HAD.

Results : Serum b-FGF concentrations were significantly reduced after the treatment with HAD (P=0.042). Serum VEGF concentrations were found to have a somewhat decreasing change, though the change was not statistically significant (P=0.229). Platelet counts had little changes (P=0.80).

Conclusions : It is supposed that HAD has effects on decreasing the serum b-FGF levels related with the clinical outcome of breast cancer patients.

Key Words: VEGF; b-FGF; Breast carcinoma; angiogenesis; HAD

Introduction

Angiogenesis, defined as the sprouting of new vessels from pre-existing ones, is concededly one of the key-steps in tumor growth and progression[1]. In malignant tumors, an 'angiogenic shift' has to be initiated when the tumor reaches a volume of approximately 2 mm3, due to extra oxygen and nutritional demands[2,3].

Angiogenesis has acquired importance as an independent prognostic indicator in solid tumors continuously[4,5]. Evaluation of circulating serum levels of angiogenic cytokines might be a possible indirect measurement of angiogenesis in a non-invasive and observer?independent fashion[6,7].

Among several identified peptides with angiogenic

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properties, basic fibroblast growth factor (b-FGF) and vascular endothelial growth factor (VEGF) are thought to play a major role in tumor angiogenesis[8,9]. VEGF and b-FGF are strong mediators of angiogenesis, both in vivo and in vitro, b-FGF appears to act in synergy with VEGF when both are present simultaneously[10,11].

Recently, immunosorbent assays have been developed and the possibility to measure VEGF and b-FGF in peripheral blood has led to many publications in various cancer-related journals[12,13].

Breast cancer is the most common cancer related with angiogenesis. Although localized breast cancer can be cured by surgery, it has a high mortality rate primarily due to frequent metastasis to other organs on the time point of diagnosis[14]. Importantly, tumor angiogenesis in invasive breast cancer correlates with the presence of local and distant metastasis[15,17]. Beside the importance for significant and independent prognostic indicator in breast cancer, antiangiogenesis is currently considered as a therapeutic target. Current approaches to target angiogenesis rely on inhibiting growth factors that stimulate vascular endothelial cells or blocking their receptors to breast cancer[14,18,19].

In previous studies, we reported that HAD inhibited angiogenesis through the suppression of b-FGF and VEGF *in vitro* and *in vivo*[20,21]. Here, we evaluated the antiangiogenic effects of HAD by determining the serum levels of b-FGF and VEGF and platelets counts in 26 patients with breast carcinoma before and after treatment with HAD.

Material and methods

1) Patients and Drug

Twenty-six breast cancer patients were selected for this study by the following criteria.

1) Patients should be administered with HAD between January 2001 and April 2003 at least 3 months continuously.

2) Sera were drawn from all patients before and after treatment and clinical information was extracted from the clinical records.

3) Clinical staging should be performed according to the criteria of the Union Internationale Contre le Cancer (UICC) from 1997[22].

4) Performance should be performed according to the criteria of the Eastern Cooperative Oncology Group (ECOG) from 1982.

5) Patients continued at the 1500mg dose with no changes. The composition of HAD was listed in Table 1 and voucher specimens have been deposited at the Institute of Traditional Medicine and Bioscience in Daejeon University.

6) Any prior hormonal therapy or chemotherapy must have been discontinued at least 4 weeks before study entry.

7) All patients must have recovered from any prior chemotherapy, radiotherapy or surgery before study entry.

8) Follow-ups were possible for limited patients (VEGF(26), b-FGF20), Plt(23)).

Table 1. Prescription of HAD. HAD was Offered from Daejeon University Oriental Medical Hospital.

Scientific name	Relative amount (mg)	Voucher specimen
Coicis Semen	129.5	CL-2003-01-Se
Pseudoginseng Radix	43.0	PN-2003-01-Ra
Hippocampus	13.0	HK-2003-01
Cordyceps Militaris	13.0	CM-2003-01
Santsigu Tuber	13.0	CA-2003-01-Tu
Ginseng Radix	13.0	PG-2003-01-Ra
Bovis Calculus	8.5	CB-2003-01-Ca
Margarita	8.5	PM-2003-01-Ma
Moschus	8.5	MO-2003-01
Total amount (1 capsule)	250.0	

Table 2. Characteristics of Patients

Characteristics		Number of patients
Gender	Male	0
	Female	26
Age	20-29	1
	30-39	2
	40-49	15
	50-59	6
	60-69	2
Performance (ECOG*)	1	18
	2	7
	3	1
Stage (UICC)	II	9
	III	6
	IV	11

* ECOG = Eastern Cooperative Oncology Group status

2) Measurement of VEGF and b-FGF

Sera were collected from clotted blood followed by centrifugation (3,000 rpm, 10min), aliquoted and stored at -70℃ until used. Serum concentrations of VEGF, b-FGF were measured with Quantikine human immunoassay kits (Quantikine TM human VEGF and Quantikine TM HS human FGF basic, R&D Systems, Minneapolis, MN, USA).

3) Statistical analysis

Serum VEGF, b-FGF concentrations and platelets counts were presented as mean ± standard deviation and changes of these values after treatment with HAD were analyzed using paired *t*-test. A *p*-value less than 0.05 was considered to indicate a statistically significant change.

Results

1) Demographic and clinical characteristics

Serum samples from 26 patients with breast cancer were collected. There were 26 females and 0 male in patients. The mean age ± standard deviation was 47.5 ±8.7 years (range 27-68 age) in tumor patients. Patients presented as having a performance status of 1 in 18 (69.2%) patients, status of 2 in 7 (26.9%) patients and status of 3 in 1 (3.9%) patients. Stage distribution was stage II in 9 (34.6%) patients, stage III in 6 (23.1%) patients and stage IV in 11 (42.3%) patients. The exact clinical and pathological stage distribution of all tumor patients is shown in Table 2.

2) Changes of VEGF, b-FGF levels and platelet counts

Twenty-six patients had serum VEGF, b-FGF levels and platelet counts measured. The mean VEGF level ± standard deviation for the group of 26 patients decreased with treatment from 338.1±485.5 pg/ml to 228.6±169.52 pg/ml. There were found to have a

Table 3. Serum Modifications of VEGF, b-FGF and Plt according to Treatment Response on 26 Patients

Serum	Treatment	Value	Number of patients	*p* value
VEGF (pg/ml)	Pre-treatment	338.1±485.5†	26	0.229
	Post-treatment	228.6±169.5	26	
b-FGF (pg/ml)	Pre-treatment	14.1±20.78	20	0.042*
	Post-treatment	4.1±5.4	20	
Plt (104/μl)	Pre-treatment	21.0±6.6	23	0.799
	Post-treatment	21.4±6.4	23	

†Values are represented as Mean±SD. *$p < 0.05$, **$p < 0.01$.

Table 4. Distribution of Mean b-FGF, VEGF and Plt Levels±Standard Deviation in Clinical Stages II-IV before and after Treatment

Clinical stage	Pre Treatment			Post treatment		
	b-FGF (pg/ml)	VEGF (pg/ml)	Plt (104/μl)	b-FGF (pg/ml)	VEGF (pg/ml)	Plt (104/μl)
II	6.5±4.9 (7)	348.4±229.4 (9)	24.3±4.2 (7)	4.1±5.4 (7)	268.1±187.0 (9)	19.7±4.6 (7) *
III	6.6±5.8 (4)	196.8±96.7 (6)	17.8±6.9 (6)	0.6±0.5 (4)	137.0±76.1 (6)	17.2±5.6 (6)
IV	23.3±28.7 (9)	406.6±724.8 (11)	20.6±7.3 (10)	6.3±6.5 (9)	246.3±184.8 (11)	25.1±6.2 (10)

( ) : number of patients. †Values are represented as Mean±SD

*$p < 0.05$, **$p < 0.01$

somewhat decreasing change, though the change was not statistically significant (p=0.229).

The mean b-FGF level ± standard deviation for the group of 20 patients decreased with treatment from 14.1 ±20.78 pg/ml to 4.1±5.4 pg/ml. Serum b-FGF concentrations were significantly reduced after the treatment with HAD (p=0.042). The mean platelet count ± standard deviation for the group of 23 patients had little changes with treatment from 21.0±6.6 ×10⁴/μl to 21.4±6.4×10⁴/μl (p=0.799). (Table 3)

3) Changes of VEGF, b-FGF levels and platelet counts according to the clinical stages

We distributed mean b-FGF, VEGF and plt levels± standard deviation in clinical stages II-IV before and after treatment. In stage II, the mean VEGF level ± standard deviation for the group of 9 patients decreased with treatment from 348.4±229.4 pg/ml to 268.1± 187.0 pg/ml (p=0.312). In stage III, the mean VEGF level±standard deviation for the group of 6 patients decreased with treatment from 196.8±96.7 pg/ml to 137.0±76.1 pg/ml (p=0.284). In stage IV, the mean VEGF level±standard deviation for the group of 6 patients decreased with treatment from 406.6±724.8 pg/ml to 246.3±184.8 pg/ml (p=0.451).

In stage II, the mean b-FGF level±standard deviation for the group of 7 patients decreased with treatment from 6.5±4.9 pg/ml to 4.1±5.4 pg/ml (p=0.412). In stage III, the mean b-FGF level ± standard deviation for the group of 4 patients decreased with treatment from 6.6±5.8 pg/ml to 0.6±0.5 pg/ml (p=0.284). In stage IV, the mean b-FGF level± standard deviation for the group of 9 patients decreased with treatment from 23.3±28.7 pg/ml to 6.3±6.5 pg/ml (p=0.107).

In stage II, the mean platelet count±standard deviation for the group of 7 patients decreased with treatment from 24.3±4.2×10⁴/μl to 19.7±4.6 ×10⁴/ μl (p=0.026). In stage III, the mean platelet count ± standard deviation for the group of 6 patients decreased with treatment from 17.8±6.9×10⁴/μl to 17.2±5.6× 10⁴/μl (p=0.848). In stage IV, the mean platelet count ± standard deviation for the group of 10 patients increased with treatment from 20.6±7.3×10⁴/μl to 25.1±6.2×104/μl (p=0.142).

The mean levels±standard deviation of b-FGF, VEGF and platelet count in the different clinical stages are shown in Tables 4.

4) Changes of VEGF, b-FGF levels and platelet counts according to the perf-ormance status

We distributed mean b-FGF, VEGF and plt levels ± standard deviation in ECOG before and after treatment.

Table 5. Distribution of Mean b-FGF, VEGF and Plt Levels ± Standard Deviation in ECOG 1-3 pre and post Treatment

ECOG	Pre Treatment			Post treatment		
	b-FGF (pg/ml)	VEGF (pg/ml)	Plt ($10^4/\mu l$)	b-FGF (pg/ml)	VEGF (pg/ml)	Plt ($10^4/\mu l$)
1	12.3±22.4 (14)	396.1±567.5 (18)	22.2±6.0 (16)	3.0±4.5 (14)	249.7±191.6 (18)	19.9±7.0 (16)
≥2	18.2±17.5 (14)	207.6±181.3 (8)	18.1±7.6 (7)	7.6±11.7 (14)	181.3±98.7 (8)	24.8±2.8 (7)

( ) : number of patients. †Values are represented as Mean±SD
*$p < 0.05$, **$p < 0.01$

There was only 1 patient in 3 performances. So we included her into 2 performance groups and divided 1 and ≥2 performance group. In 1 performance, the mean VEGF level±standard deviation for the group of 18 patients decreased with treatment from 396.1±567.5 pg/ml to 249.7±191.6 pg/ml (p=0.266). In ≥2 performance, the mean VEGF level± standard deviation for the group of 8 patients decreased with treatment from 207.6±181.3 pg/ml to 181.3±98.7 pg/ml (p=0.532).

In 1 performance, the mean b-FGF level±standard deviation for the group of 14 patients decreased with treatment from 12.3±22.4 pg/ml to 3.0±4.5 pg/ml (p=0.153). In ≥2 performance, the mean b-FGF level ±standard deviation for the group of 14 patients decreased with treatment from 18.2±17.5 pg/ml to 7.6 ±11.7 pg/ml (p=0.077).

In 1 performance, the mean platelet count ± standard deviation for the group of 16 patients decreased with treatment from $22.2\pm6.0\times10^4/\mu l$ to $19.9\pm7.0\times10^4/\mu l$ (p=0.157). In ≥2 performance, the mean platelet count ± standard deviation for the group of 7 patients increased with treatment from $18.1\pm7.6\times10^4/\mu l$ to $24.8\pm2.8\times10^4/\mu l$ (p=0.068). The mean levels± standard deviation of b-FGF, VEGF and platelet count in the different ECOG are shown in Tables 5.

DISCUSSION

Angiogenesis is the process of new blood vessel formation from existing blood vessels and a natural response of tissues to ischemia[23]. Furthermore, angiogenesis has been established as a basic feature in tumor development, growth and spread beyond regional borders[3]. Normally, angiogenesis is under tight regulatory control, consisting of both angiogenic and antiangiogenic factors. In malignant tumors, this control is lost and the production of angiogenic molecules exceeds that of endogenous angiogenic inhibitors.† Sustained angiogenesis is essential for tumor growth beyond a tumor size of 1-2mm^2 [24]. Of the known angiogenic factors, b-FGF and VEGF are most commonly expressed[25,26]. b-FGF, VEGF can be detected in human serum by ELISA, and elevated serum concentrations have been reported in patients with various types of tumor, implying a significant role for these factors in tumor growth and progression[27,28]. In animal models, treatment with angiogenesis inhibitors has a proven anti-tumor effect in vivo, and can both reduce metastasis and lead to regression of primary growth by necrosis following capillary retraction[29,30].

For clinical applications of tumor angiogenesis, various approaches have been widely considered. Anti-angiogenesis might be a new strategy for anti-tumor therapy. Some clinical trials assessing the anti-tumor activity of the angiogenesis inhibitors are going on. Angiogenesis may be triggered by different pathways in different tumors. Among the factors, VEGF has been characterized as the most potent regulator of

angiogenesis in human carcinogenesis. Various therapeutic approaches aimed at inhibiting the function of VEGF are currently under investigation[31]. FGF, especially the b-FGF is one of the best?characterized and most potent angiogenic factors.

Both primary and metastasis tumors in the breast are dependent on angiogenesis and primary malignant breast tumors are among those human neoplasms that exhibit the greatest angiogenic activity[16,32].

Antiangiogenic therapy appears to be improved when administered over a long period of time without treatment interruptions. Therefore, oral therapy in doses, which would be tolerable in the long run, should be considered. Among orally available drugs for the treatment of breast cancer, significant bioavailability was demonstrated for both CTX and MTX[33,34].

Breast tumor growth and metastasization are both hormone-sensitive and angiogenesis-dependent. A single angiogenic inhibitor is not capable to inhibit angiogenesis. Therefore, we need a balanced combination of angiogenesis inhibitors. At present, anti?angiogenesis therapies require long-term and continuous administration of the agents. Their use is appropriate in the earlier stages of disease, especially for improving survival in patients with a poor prognosis if toxic effects can be made tolerable. Looking at the future, multidisciplinary approaches involving anti-angiogenic strategies should improve patients' prognosis and their quality of life.

In this study, to investigate the effect of HAD on anti-angiogenic activity, we analyzed the changes of serum VEGF, b-FGF levels and platelet counts in 26 breast cancer patients before and after treatment with HAD.

At the start, we have observed changes between serum VEGF and b-FGF levels and various variances (clinical stage and performance status). No significant associations were observed between levels of serum VEGF and b-FGF levels and each factor. In general, previous studies showed no statistically significant differences in histology, gender, age, pathological stage with respect to serum VEGF and b-FGF. Our findings did not show any correlation with each factor. In the change of serum b-FGF level after HAD treatment, it was reduced significantly. Even though it wasn't statistically significant, serum VEGF concentrations decreased, too. It is reported that serum VEGF levels increase during clotting as a result of its release from platelets, and plasma sample instead of serum was recommended for measuring the circulating VEGF more accurately. However, platelets have been implicated in tumor metastasis since circulating tumor cells forming aggregates with platelets were observed. Hence, to exclude the change of serum VEGF levels are affected by blood platelets, we examined the change of serum platelet counts. Our findings did not show statistically significant differences in the change of serum platelet counts. Therefore, we supposed that it can be excluded the change of serum VEGF levels are affected by blood platelets from our findings.

From above the results, we can conclude that HAD may present the anticancer effects by anti-angiogenic response specific to breast cancer. However, some methodological limitations of our study should be exhibited[1]. These results are out of place as for being generalized because the sample under investigation is too small, and results might apply only to Korea[2]. Various cancer patients in our study represent a heterogeneous group. Moreover, our study is a small one of retrospective series, thus making conclusion can be a jump in the logic. This study is needed for more accurate estimation with various variances and the administration period should be controlled more strictly.

In conclusion, oral HAD demonstrated significant efficacy to reduce serum b-FGF levels of breast cancer patients. These results do not preclude activity of HAD in other settings, such as in patients other types of

malignancies. Moreover, they do not preclude possible activity of the drug in combination with other classically active agents, such as hormone therapy or chemotherapy. Likewise, HAD might be active with other biologic therapies, such as other inhibitors of angiogenesis or immunomodulators. If such studies are performed, our results suggest that HAD might be used at the lower dose levels. The lower dose was better tolerated, and the one near response that was observed was at this dose. Theoretically, treatments aimed at inhibiting angiogenesis should be chronically administered for a prolonged period. These treatments might have particular usefulness for subsets of patients with limited tumor burden, such as early breast cancer[35]. Moreover, combinations of therapy that inhibit angiogenesis plus cytotoxic therapy may be more effective than either type of therapy alone. Use of oral HAD should be investigated further as a strategy against tumor progression after standard chemotherapy in the adjuvant setting.

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