

**ORIGINAL****Levels of soluble vascular endothelial growth factor receptor 1 are elevated in the exudative pleural effusions**

Hideki Tomimoto, Seiji Yano, Hiroaki Mugeruma, Soji Kakiuchi, and Saburo Sone

*Department of Internal Medicine and Molecular Therapeutics, Institute of Health Biosciences, The University of Tokushima Graduate School, Tokushima, Japan***Abstract :****Purpose :** Vascular endothelial growth factor (VEGF) plays a critical role in the production of malignant pleural effusions. In the present study, we examined the levels of soluble VEGF receptor-1 (sVEGFR-1) and angiopoietin-2 (Ang-2), as possible regulators of VEGF activity, in transudative and exudative pleural effusions.**Methods :** Forty-two patients were included in this study : 4 with transudative pleural effusions due to heart failure (HF), 38 with exudative pleural effusions (lung cancer [LC], 22 ; other malignant diseases [MD], 10 ; tuberculosis [TB], 6) . The levels of VEGF, Ang-2, and sVEGFR-1 in the pleural effusions were measured by an enzyme-linked immunosorbent assay.**Results :** The levels of VEGF, Ang-2, and sVEGFR-1 in exudative effusions were higher than those in transudative effusions. Interestingly, the levels of VEGF and Ang-2 in bloody effusions were significantly higher than those in non-bloody effusions ( $p < 0.05$ ), but the level of sVEGFR-1 in bloody effusions was lower than that in non-bloody effusions. The levels of VEGF and Ang-2 were significantly higher in the malignant effusions, compared with effusion from HF and TB ( $p < 0.05$ ). In addition, sVEGFR-1 was significantly higher in the effusion from LC, MD, and TB compared with effusion from HF ( $p < 0.05$ ). In the malignant effusions, direct correlations were observed among VEGF, sVEGFR-1, and Ang-2.**Conclusions :** The sVEGFR-1 levels were elevated in exudative pleural effusions, and were lower in bloody effusions than in non-bloody effusions, thus suggesting the regulatory role of sVEGFR-1 in the exudative pleural effusions. *J. Med. Invest.* 54 : 146-153, February, 2007**Keywords :** *bloody effusions, VEGF, angiopoietin-2***INTRODUCTION**

Pleural effusion is associated with both benign and malignant diseases. It is divided into exudative effusion and transudative effusion (1). While transudative effusion is mainly seen in patients with heart

failure, renal failure, and liver cirrhosis, exudative effusion is seen in patients with various diseases, including malignant diseases, pneumonia, pulmonary tuberculosis, and collagen diseases (1-4). In particular, malignant effusion is seen frequently in patients with lung cancer and is known as a marker of poor prognosis for the patients (5). Therefore, the management of pleural effusion is clinically important.

Pleural effusion formation is multifactorial. At least, pleural effusion formation is associated with 1) impaired drainage of the pleural space due to obstruction of vessels and lymphatics of the lungs and

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Address correspondence and reprint requests to Dr. Saburo Sone, Department of Internal Medicine and Molecular Therapeutics, Institute of Health Biosciences, The University of Tokushima Graduate School, Kuramoto-cho, Tokushima 770-8503, Japan and Fax : +81-88-633-2134

pleura, and 2) increased pleural fluid formation (6-8). Considering the latter mechanism, vascular endothelial growth factor (VEGF), a 34 to 42-kDa dimeric protein, is a potent mediator (9, 10). Initially discovered because of its ability to increase vascular permeability, the molecule was first called vascular permeability factor (VPF) (11). We previously reported that exudative pleural effusions associated with lung cancer contained significantly higher amounts of VEGF than transudative pleural effusions (8). In addition, tumor-cell derived VEGF facilitated the production of bloody pleural effusions by inducing hyperpermeability in the thoracic cavity (12), indicating the critical role of VEGF in the production of bloody malignant pleural effusions. However, the presence of other cofactors or inhibitors with VEGF/VPF might regulate pleural fluid formation.

In addition, the biological activity of VEGF has been previously documented to be modified or regulated by several VEGF-related molecules. For example, VEGF binds to specific receptors, such as VEGFR-1 (Flt-1) and VEGFR-2 (Flk-1/KDR), and this binding is augmented by the presence of coreceptors (neuropilin-1 and -2) (9, 13, 14). VEGFR-2-mediated signaling is thought to be necessary for fully expressing biological function of VEGF. VEGFR-1 acts as a negative regulator of VEGF activity, as VEGF-mediated stimulation of VEGFR-1 autophosphorylation is weak in endothelial cells, while VEGFR-1 has 10 times higher affinity to VEGF than VEGFR-2 (15). Much attention has been paid to a naturally occurring, alternatively spliced soluble form of VEGFR-1 (sVEGFR-1). sVEGFR-1 binds to VEGF with high affinity and neutralizes VEGF activity (16, 17). sVEGFR-1 is reported to present in the serum and amniotic fluid of pregnant women, but it is unknown whether sVEGFR-1 exists in pleural effusion (18-20).

Angiopoietin (Ang)-1 and -2 have been identified as ligands for Tie-2, which is a receptor tyrosine kinase specifically expressed on endothelial cells, and Angs play critical roles in angiogenesis in concert with VEGF (21-23). Ang-1 binds to Tie-2 and maintains and stabilizes mature vessels by promoting the interaction between endothelial cells and surrounding extracellular matrix (23). Ang-2 competitively binds to Tie-2, and antagonizes the stabilizing action of Ang-1, which results in destabilization of vessels (22, 23). Recent studies reported that Ang-2 induced peritoneal bleeding in hepatic tumor models. In addition, elevated levels of Ang-2, but not Ang-1, have recently been reported to exist in exu-

date pleural effusion (24). However, the role of Ang-2 or relationship of Ang-2 with sVEGFR-1 in pleural effusion is not fully understood.

In the present study, we examined the levels of sVEGFR-1 and Ang-2 as possible regulators of VEGF activity in transudative and exudative pleural effusions. The significance of these VEGF-associated molecules in pleural effusion is herein discussed.

## MATERIALS AND METHODS

### *Reagents.*

Recombinant VEGF165 was obtained from R&D Systems (Inc., Minneapolis, MN), and Recombinant sVEGFR-1 from Fitzgerald Industries International (Inc., Concord, MA).

### *Patient Characteristics.*

A total of 42 pleural fluid samples, obtained from patients (34 males and 8 females) who underwent thoracentesis in Tokushima University Hospital between April 2001 and February 2005, were examined after written informed consent had been obtained (Table 1). The mean age of the patients was  $63.6 \pm 14$  years. A pleural effusion was categorized as malignant if the results of pleural fluid cytology testing or pleural biopsy were positive for malignancy or if the patient had a known metastatic malignancy without other cause for the effusion. The 32 patients in the malignant effusion group were diagnosed as follows: Primary lung carcinomas - 22 (4 small cell, 12 adenocarcinoma, 4 squamous cell, 2 large cell), Malignant mesothelioma - 2, Malignant lymphoma - 2, Adult T-cell leukemia - 1, Renal cancer - 1, Epiphyaryngeal cancer - 1, Acral myxoinflammatory fibroblastic sarcoma - 1, Skin cancer (squamous cell carcinoma) - 1, Adenocarcinoma of unknown origin - 1.

Tuberculous pleuritis (n=6) was categorized as one that was either microbiologically positive or a tuberculous lesion in pleural biopsy or purulent fluid with pulmonary tuberculosis which had no features of bacterial pneumonia and malignant. Pleural effusion with cardiac insufficiency (n=4) was categorized as a type of transudative effusion in a patient with symptoms and signs of CHF who responded to appropriate therapy.

### *Measurements of VEGF, Ang-2, and sVEGFR-1.*

After thoracentesis, pleural fluid was collected and submitted to routine laboratory examinations, including the appearance (bloody or non-bloody), a protein

Table 1 Characteristics and Laboratory Findings of the Subjects

		LC (n=22)	Ma (n=10)	Tb (n=6)	HF (n=4)
Gender	Male	18	8	4	4
	Female	4	2	2	0
Age	average	65	59.6	64.6	64.3
	range	42-92	19-89	31-86	56-71
LDH (IU/L)	average	631	416	225	180
	range	108-1852	95-1775	137-365	111-209
LDH ratio	average	2.98	1.5	2.2	0.45
	range	0.5-8.87	0.74-3.29	0.74-3.29	0.31-0.56
Protein (g/dL)	average	4.02	3.13	3.45	1.48
	range	1.88-5.56	1.41-4.77	1.61-5.01	0.14-2.96
Appearance	bloody	14	6	1	1
	non-bloody	8	4	5	3
VEGF (ng/ml)	average	3.12	0.8	0.47	0.087
	range	0.016-22.5	0.025-2.66	0.005-2.73	0-0.34
Ang-2 (ng/ml)	average	39.9	41.9	19.7	10.5
	range	5.65-82.5	15.1-93.9	13.2-30.6	8.38-13.7
sVEGFR-1 (ng/ml)	average	0.722	0.967	6.76	0
	range	0-4.57	0-5.41	0.007-35.9	0

analysis, and a lactate dehydrogenase analysis.

For the measurement of VEGF, sVEGFR-1, and Ang-2, the pleural fluids were immediately centrifuged at 1200 rotations/min for 7 min at 4°C, and the supernatant was stored at -80°C until analysis. The levels of VEGF, sVEGFR-1 and Ang-2 were determined using sandwich ELISA kits (R&D Systems, Inc., Minneapolis, MN) according to the manufacturers instructions.

#### Statistical Analysis.

Mann-Whitney tests were used throughout the study to compare individual groups. The correlation was analyzed with the Spearman correlation test. The criterion of statistical significance was  $p < 0.05$ . Statistical significance was analyzed using GraphPad Prism software program Ver.4.01.

## RESULTS

### Measurement of sVEGFR-1 bound to VEGF by ELISA.

In the first set of experiments, we determined whether VEGF-sVEGFR-1 complex was measured by ELISA for VEGF and sVEGFR-1. Recombinant VEGF165 and recombinant sVEGFR-1 were incubated in medium with 10% FBS for 1 hour at 37°C, and the resultant solutions were measured by ELISA for VEGF and sVEGFR-1, respectively. As shown in Fig. 1A, B, the addition of VEGF did not affect the

level of sVEGFR-1, thus suggesting that sVEGFR-1 values determined by this ELISA contain free sVEGFR-1 and sVEGFR-1 bound to VEGF. On the

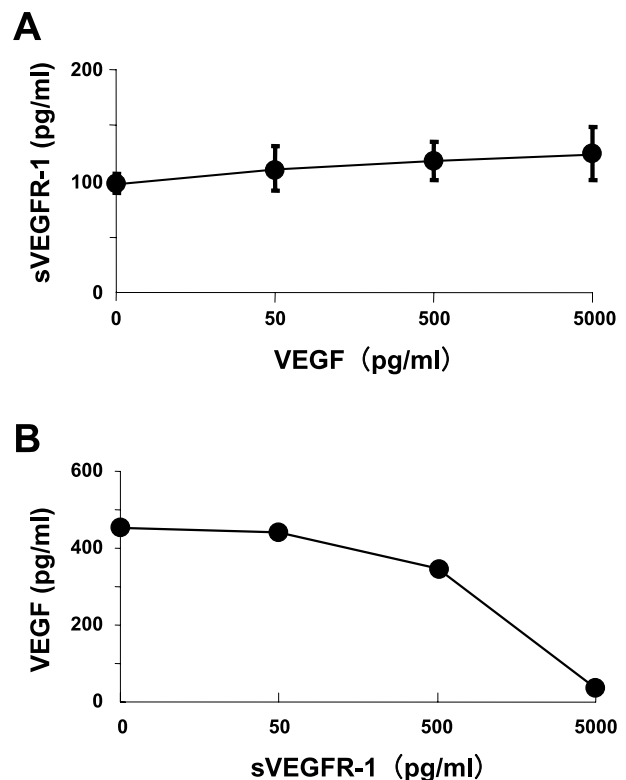


Fig. 1. Measurement of sVEGFR-1 bound to VEGF by ELISA. Recombinant VEGF 165 and recombinant sVEGFR-1 were incubated in medium with 10% FBS for 1 hour at 37°C, and the resultant solutions were measured by ELISA for VEGF and sVEGFR-1, respectively.

other hand, the addition of sVEGFR-1 resulted in a decrease in the level of VEGF, thus suggesting that VEGF values determined by this ELISA indicate free VEGF.

*Levels of VEGF, sVEGFR-1, and Ang-2 in exudative and transudative effusion.*

We next measured the levels of VEGF, sVEGFR-1, and Ang-2 in the pleural effusions from 42 patients, and compared the levels in exudative effusions and transudative effusions. Since the ratio of LDH in the pleural effusion/LDH in the serum is commonly used as a marker to distinguish exudative effusion from transudative effusion, we considered such effusion to be exudative when a ratio of more than 0.6 was observed. According to the results of previous reports, the levels of VEGF and Ang-2 in exudative effusion were higher than those in transudative effusion (Fig. 2 A, B). Moreover, the levels of sVEGFR-1 were also higher in the exudative effusions compared with transudative effusions (Fig. 2 C).

Bloody effusion is frequently associated with malignant diseases. Interestingly, the levels of VEGF and Ang-2 in bloody effusion were significantly higher than those in non-bloody effusion ( $p < 0.05$ ) (Fig. 3 A, B). In contrast, the level of sVEGFR-1 in bloody effusion was lower than that in non-bloody effusion (Fig. 3C).

*Comparison of the levels of VEGF, Ang-2, and sVEGFR-1 in the pleural effusions between the disease groups.*

We next compared the levels of VEGF, Ang-2, and sVEGFR-1 in the pleural effusion among various diseases. As shown in Fig. 4A~C and Table 1, the levels of VEGF and Ang-2 were significantly higher in the effusion from LC and MD, compared with effusion from HF and TB ( $p < 0.05$ ). In addition, sVEGFR-1 was significantly higher in the effusion from LC, MD, and TB compared with effusion from HF ( $p < 0.05$ ).

*Correlation of the levels of VEGF, Ang-2, and/or sVEGFR-1 in malignant pleural effusions.*

Since the high levels of VEGF, Ang-2, and sVEGFR-1 were detected in malignant effusions, we further evaluated the correlation of these three factors in malignant effusions by the Spearman correlation test analyses. There are direct correlations between VEGF and Ang-2, VEGF and sVEGFR-1, and sVEGFR-1 and Ang-2 (Fig. 5A~C).

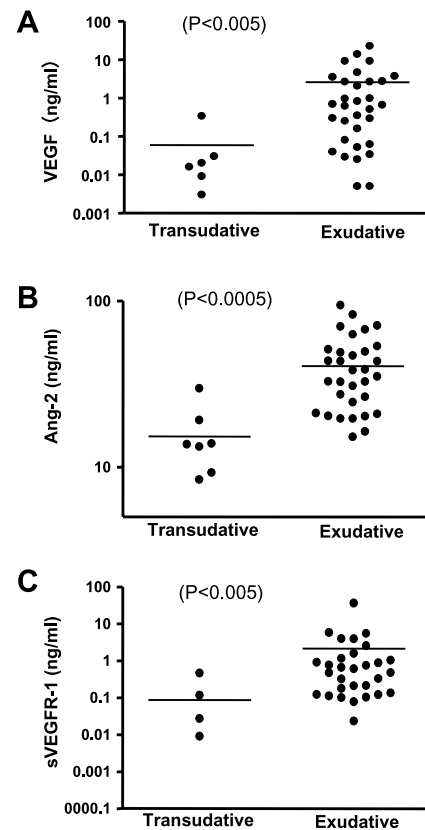


Fig. 2. Levels of VEGF, sVEGFR-1, and Ang-2 in exudative and transudative effusions. Horizontal lines represent the median values.

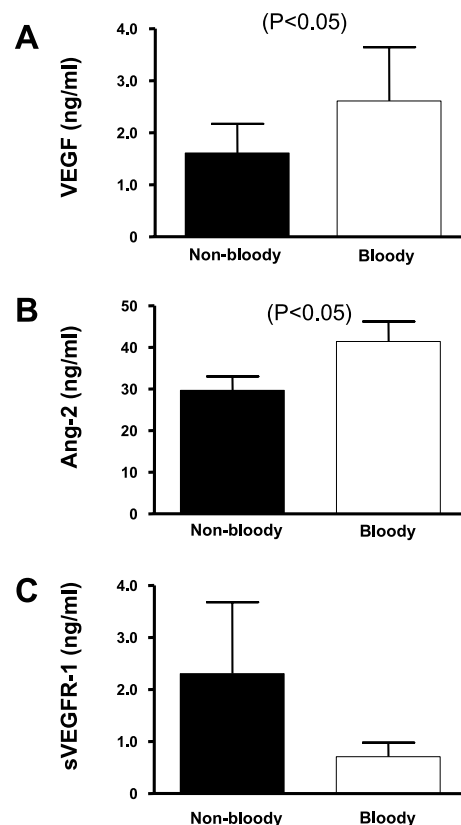


Fig. 3. Levels of VEGF, sVEGFR-1, and Ang-2 in bloody- and non-bloody effusions. Horizontal lines represent the median values.

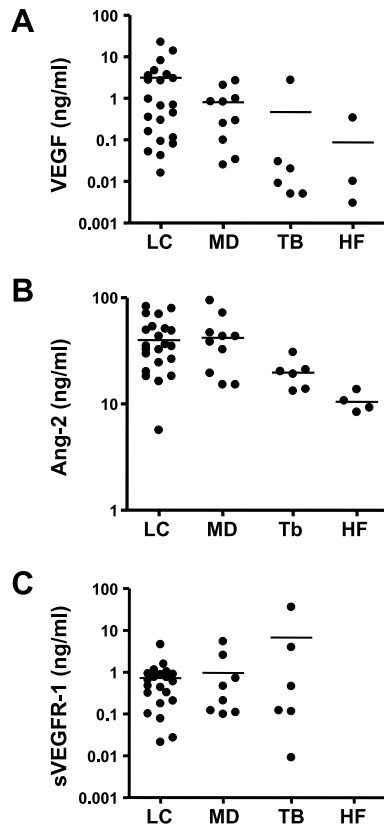


Fig. 4. The levels of VEGF, Ang-2, and sVEGFR-1 in the pleural effusions due to lung cancer (LC), other malignant diseases (MD), tuberculosis (TB), and heart failure (HF). Horizontal lines represent the median values.

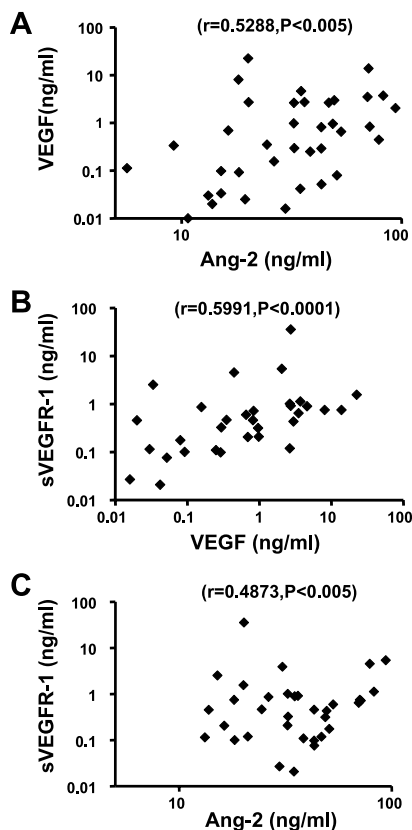


Fig. 5. Correlation of the levels of VEGF, Ang-2, and/or sVEGFR-1 in malignant pleural effusions.

## DISCUSSION

We recently reported that VEGF presented within exudative pleural effusion, and it facilitated the production of bloody pleural effusion by inducing vascular hyperpermeability in the thoracic cavity (12). In addition, Ang-2 was shown to present in the exudative effusion (24). In the present study, we confirmed that higher levels of VEGF and Ang-2 existed in the exudative effusion compared with transudative effusion, and further demonstrated that an endogenous inhibitor of VEGF, sVEGFR-1, also existed in the exudative effusion. Moreover, higher levels of VEGF and Ang-2 were detected in the bloody effusion than in the non-bloody effusion. In contrast, sVEGFR-1 levels in bloody effusion were lower than that in non-bloody effusion, suggesting the regulatory role of sVEGFR-1 in the exudative pleural effusion.

sVEGFR-1, found in trophoblasts and endothelial cells of normal placental tissue, was abnormally detected in the serum of preeclamptic patients (25). Clinical symptoms in preeclamptic patients are similar with the side effects of VEGF neutralizing antibody in cancer patients (26, 27), thus suggesting that the preeclamptic symptoms on the maternal side are due to an abnormal suppression of endogenous VEGF by sVEGFR-1. Therefore, sVEGFR-1 is thought to regulate or modulate VEGF activity as an endogenous inhibitor. Recent clinical trials showed therapeutic potential of VEGF/VEGFR inhibitors, including anti-VEGF antibody (bevacizumab), against various solid tumors, including renal cell carcinoma and non-small cell lung cancer (17, 28, 29).

It is thought that bloody pleural effusions result from vascular wall rupture or leakage of blood cells. The bloody pleural effusions are observed frequently and associated with both of benign and malignant disease. We found that the level of VEGF in the bloody effusions was higher than that in the non-bloody effusions. On the other hand, the level of sVEGFR-1 in the bloody effusions was lower than that in the non-bloody effusions. Since sVEGFR-1 is an endogenous inhibitor of VEGF (16, 17), sVEGFR-1 might be produced by host cells in response to suppression of VEGF activity. If the production level of sVEGFR-1 is insufficient, then bloody effusion might be produced because of the high VEGF activity. Further studies are warranted to examine this hypothesis.

Ang-2 has been shown to be involved in the pathogenesis of a variety of human diseases including can-

cer, diabetic retinopathy, pulmonary hypertension, and coronary artery disease (30-33). Concerning malignant diseases, Ang-2 induces the instability of the blood vessels and facilitates the angiogenesis in the presence of VEGF, and it is one of the poor prognostic factors of non-small cell lung cancer (34). Moreover, a recent report showed that Ang-2 was supposed to be produced locally in the pleural space, and that elevated levels of Ang-2, as well as VEGF, in exudative pleural effusions were detected. However, the functional significance of Ang-2 in pleural disease is poorly understood. We confirmed that a high level of VEGF and Ang-2 coexisted in the exudative effusion, especially bloody effusion. Moreover, there was a direct correlation between the levels of VEGF and Ang-2. Since Ang-2 overexpression in hepatocellular carcinoma caused the abdominal bleeding in orthotopic animal models (35), VEGF and Ang-2 might thus synergistically induce not only angiogenesis, but also vascular hyperpermeability.

In the present study, the cellular origins of the VEGF, Ang-2, and sVEGFR-1 were not determined because of the lack of the corresponding tissue samples and techniques to detect these factors appropriately. However, several lines of evidence show that VEGF is produced by various types of cells, including tumor cells, muscle cells, pericytes, glia cell, and fibroblasts (9). Ang-2 is mainly produced by endothelial cells and cancer cells (22). sVEGFR-1 is mainly produced by trophoblasts (18-20), but it might be produced by endothelial cells and macrophages because these cells express sVEGFR-1 (36). Lung cancer is the leading cause of malignant pleural effusion (37). At least 25% of all patients with lung cancer tend to develop pleural effusion at some time during the course of the disease. The standard treatment for malignant pleural effusion is drainage followed by the instillation of sclerosing agents, but the clinical efficacy of this treatment varies (38). VEGF/VPF is reported to be responsible for malignant pleural effusion formation and targeting the production of VEGF/VPF and/or blocking the VEGF/VPF receptor may be a way to control malignant pleural effusion. In addition to the anti-VEGF antibody (bevacizumab) which has been shown to have a therapeutic potential against non-small cell lung cancer, an endogenous VEGF inhibitor, sVEGFR-1, may be useful for controlling the malignant pleural effusion associated with VEGF-induced vascular hyperpermeability.

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