Cancer Chemother Pharmacol (1999) 43(Suppl): S72–S77 

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# Involvement of VEGF and its receptors in ascites tumor formation

**Abstract** Vascular endothelial growth factor (VEGF) has potent endothelial cell mitotic and vascular permeability activity. Several reports have suggested that VEGF may be one of the major factors regulating ascites formation, although no quantitative and systematic analyses have been carried out. To determine the role of VEGF in ascites formation, we examined the expression of VEGF in 13 mouse ascites tumors (5 sarcomas, 3 carcinomas, and 5 hematopoietic malignancies). We found that significant amounts (6–850 ng/mL) of biologically active VEGF accumulated in the ascites fluid of all 13 tumors, particularly in tumors of sarcoma and carcinoma origin (430 ± 234 ng/mL). The microvessel densities in the peritoneal walls of tumor-bearing mice, which are significantly higher than those in healthy mice, basically correlated with but did not parallel VEGF concentrations, suggesting the existence of an additional modulator(s) of the angiogenic process. Administration of anti-mouse VEGF-neutralizing antibody to mice bearing the carcinoma-derived ascites tumor MM2 suppressed ascites accumulation, tumor growth, and tendency to bleed. These results directly demonstrate the crucial role of VEGF in carcinoma-derived ascites tumor formation in vivo.

Work presented at the 14th Bristol-Myers Squibb Nagoya International Cancer Treatment Symposium, "Challenges in Cancer Metastasis," 11-12 September 1998, Nagoya, Japan

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**Key words** Ascites tumor · Vascular endothelial growth factor · Vascular permeability · Neutralizing antiserum

## Introduction

Vascular endothelial growth factor (VEGF), also known as vascular permeability factor (VPF), has two major biological functions: growth stimulatory activity for a variety of vascular endothelial cells; and increasing microvessel permeability [2, 10, 18]. VEGF binds and stimulates the tyrosine kinase receptors Fit-1 [1, 19] and KDR/Flk-1 [9, 24]. However, the kinase activity of KDR/Flk-1 is much greater than that of Flt-1, suggesting that most positive signals are mediated by KDR/Flk-1 [3, 15–17, 20, 21].

Mouse VEGF includes at least three subtypes of 120-, 164-, and 188-amino acid due to alternative splicing. $VEGF_{164}$ and $VEGF_{188}$ carry a weak and a strong basic amino acid-enriched region, respectively. Although $VEGF_{164}$ is suggested to have the most potent biological activity, the biological significance of each subtype in in vivo angiogenesis remains to be determined.

Overexpression of VEGF has been widely demonstrated in many human and animal tumors [2, 10, 18]. It has also been reported that VEGF accumulates in several types of ascites tumors, although no systematic analysis of the roles of VEGF in ascites tumor formation has been carried out [5, 11]. To ascertain whether VEGF secretion by ascites tumor cells is responsible for the initiation and maintenance of the ascites pattern of tumor growth, we first developed a new sensitive radioreceptor binding assay for mouse VEGF. Using this mouse VEGF assay system, we found that significant amounts of VEGF accumulate in all ascites tumors. The results of therapeutic experiments using anti-mouse VEGF neutralizing antibody suggest that VEGF is the major factor for accumulation of ascites in mice bearing carcinoma/sarcoma-derived ascites tumors [6, 7].

## Materials and methods

### Cell lines and animals

BP 8 sarcoma, MH134 hepatoma, MM2 mammary tumor [22], OG/Gardner lymphoma 6C3HED [4], SR-C3H/He sarcoma, and X5563 plasmacytoma transplantable ascites-producing tumors were passaged at $4 \times 10^6$ cells/mouse weekly in the peritoneal cavities of syngeneic C3H/He mice, Ehrlich mammary tumor, sarcoma 37, and SR-DDD sarcoma ascites tumors in DDD mice (provided by Dr. A. Matsuzawa, University of Tokyo), L1210 leukemia and P815 mastocytoma in DBA/2 mice, and sarcoma 180 (S180) and RG/Gardner lymphoma 6C3HED in BALB/c and CBA mice, respectively. All mice used were 6-week-old males. Recombinant mouse $VEGF_{164}$ (rm-VEGF) and goat anti-mouse neutralizing antibody AF-493-NA were purchased from R&D Systems (Minneapolis, MN, USA).

### Preparation of affinity-purified antibodies against mouse VEGF

Polyclonal antisera against mouse VEGF were raised in rabbits with two synthetic peptides corresponding to the termini of mouse VEGF. The peptides were covalently conjugated to a carrier protein, keyhole limpet hemocyanin (Sigma, St. Louis, MO, USA), to immunize rabbits. The antisera against mouse VEGF were affinity-purified on columns prepared by coupling 1 mg of the respective peptide to 1 g of formyl-cellulofine-activated sepharose (Biochemicals, Tokyo, Japan). The affinity-purified antibody against the N terminus of VEGF was designated pN2.

### Radioreceptor binding assay

The radioreceptor binding assay was carried out in 96-well immunoplates. Each well was coated with 100 μL of a solution containing affinity-purified anti-VEGF antibody pN2 10 μg/mL in carbonate buffer (pH 9.6) 50 mM, and the plates were incubated for 16 h at 4°C. The plates were washed twice with phosphate-buffered saline (PBS) (pH 7.4), blocked with 300 μL/well of 10% fetal bovine serum in PBS (pH 7.4) for 1 h at 26°C, and washed once with the same blocking solution. One hundred-microliter aliquots of the serial 2-fold diluted standard solution of rm-VEGF (ranging from 0 pg/mL to 3000 pg/mL, 6 measurements each) or diluted samples of ascites fluid (triplicate measurements of 3 serial dilutions) were added to each well and the plates were incubated for 3.5 h at 26°C with gentle agitation. The supernatants were then discarded and the pellets were washed 3 times with PBS (pH 7.4). One hundred microliters of detector solution containing $2 \times 10^5$ cpm of $^{125}$I-labeled human 7N Flt-1 was added to each well, followed by incubation for 2 h at 26°C with gentle agitation. 7N Flt-1, a recombinant protein of the extracellular domain of human *fms*-like tyrosine (Flt-1) kinase receptor containing seven Ig-like domains [23], has a similarly high affinity for mouse and human VEGF. 7N Flt-1 was labeled with $^{125}$I as described previously [16] and specific activity of $1.2 \times 10^5$ cpm/ng was obtained. After the supernatant was discarded, the plates were washed with 10% fetal calf serum in PBS (pH 7.4), and finally the activity in the wells was measured using a gammacounter.

### Endothelial cell growth assay

Rat liver sinusoidal endothelial cells (RLSEC) were isolated from rat liver as described previously [25] and grown in EGM-UV medium (Kurabo, Osaka, Japan) in the presence of rm-VEGF, samples of diluted ascites, or partially purified VEGF from ascites fluid by heparin column chromatography. The amounts of VEGF contained were determined by radioreceptor binding assay.

### Vascular permeability assay

The Miles assay [8] was used to determine vascular permeability. The backs of anesthetized guinea pig were shaved and the animals injected with 1 mL of 0.5% Evans Blue ic (Sigma). Thirty minutes later, 200 μL of samples or control PBS (pH 7.4) were injected sc into the shaved backs, and dye leakage was detected by the presence of blue spots surrounding the injection site after an additional 30 min.

### In vivo administration of anti-mouse VEGF neutralizing antibody to ascites tumor-bearing mice

To reduce possible interference by the humoral immune response due to the administration of goat protein, we limited the period of antibody administration to 8 days. Mice injected with MM2 cells $1.5 \times 10^6$ ip or OG cells $3 \times 10^7$ ip were administered anti-VEGF antibody AF-493-NA at doses of 20- (for MM2-bearing mice) or 40-molar fold (for OG-bearing mice) ip. VEGF gradually accumulated in the peritoneal cavity from the day of tumor transplantation (designated day 0) to day 7. Mice injected intraperitoneally with tumors were administered normal goat IgG in the same amount as the antibody or left intact to serve as controls. On day 8, mice were killed or left untreated until natural death occurred.

## Results and discussion

### Radioreceptor assay for detection of mouse VEGF

No commercial assay system that is sensitive for and specific to mouse VEGF is yet available. Therefore we developed a high-titer rabbit polyclonal antiserum against the N-terminal region of mouse VEGF called pN2. We first tested the simplest assay for mouse VEGF, i.e., a competitive assay that includes a limited amount of pN2 antibody, $^{125}$I-VEGF, and samples such as cell lysates containing unlabeled VEGF. Although this competitive assay system yielded some quantitative detection of mouse VEGF, it was not sufficiently sensitive, resulting in frequent false-positive or -negative results, most likely due to the presence of bulk proteins and other materials in the samples (data not shown).

We next attempted to establish a sandwich-type assay using the pN2 antibody and the $^{125}$I-labeled ligand-binding domain of Flt-1 (7N Fit-1), as shown in Fig. 1. We and others have previously shown that the Flt-1 ligand-binding domain carries a ≥40-fold higher affinity for VEGF compared with the second VEGF receptor KDR/Flk-1 [12, 14]. This radioreceptor assay is a sensitive, stable procedure for the detection of mouse VEGF, probably due to the following two reasons. One is the rapid and efficient elimination of bulk proteins and materials. We first coated 96-well plates with a constant amount of pN2 antibody and added samples that may have contained unlabeled VEGF. After binding of this VEGF, the sample solution was removed and each well was washed with the appropriate buffer. This new procedure allowed us to retain pN2 antibody and the unlabeled VEGF bound to it without most of the unrelated materials in the sample. This purification step was found

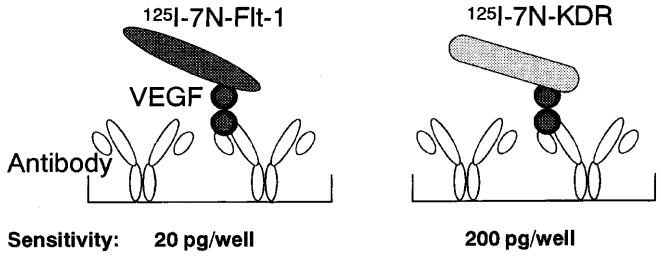


**Fig. 1** Radioreceptor assay for detection of mouse VEGF. Anti-mouse VEGF rabbit antiserum and radio-labeled 7N Flt-1 were used for a sandwich-type assay system (see Materials and Methods). 7N KDR was also found to be useful, although its sensitivity was one order lower than that of 7N Flt-1 because of the lower affinity of KDR for VEGF

to be effective in obtaining sensitive and stable measurements.

The second advantage of this procedure is that the radioisotope count obtained is basically proportional to the amount of unlabeled VEGF added to the wells with pN2 antibody, because the $^{125}$I-7N Flt-1 specifically and quantitatively binds to the VEGF in the wells. This proportionality between radioisotope count and amount of VEGF in the sample is particularly important in obtaining accurate and reproducible values for smaller amounts of VEGF.

## Significant expression of VEGF in mouse tumor ascites fluid

To examine the accumulation of VEGF in the peritoneal fluid of ascites tumor-bearing mice, we used both middle- and late-stage ascites fluid, since our preliminary results and the observations of others [26] indicated that the variation in VEGF level in ascites fluid is time dependent after intraperitoneal injection of tumor cells.

The average VEGF concentrations in the ascites fluids of mice bearing the 13 experimental tumors, as measured by our binding assay, are shown in Table 1.

**Table 1** Characteristics and concentration of VEGF in tumor-induced mouse ascitic fluid

| Ascites sample | Characteristic | VEGF (ng/mL) |
|---|---|---|
| BP-8 (sarcoma) | Bloody | 850 |
| Ehrlich mammary carcinoma | Bloody | 600 |
| SRC57B1 (sarcoma) | Bloody | 560 |
| SRDDD (sarcoma) | Bloody | 520 |
| MH134 (hepatoma) | Bloody | 350 |
| S37 (sarcoma) | Bloody | 310 |
| S180 (sarcoma) | Bloody | 168 |
| MM2 (mammary carcinoma) | Bloody | 83 |
| X5563 (plasmacytoma) | Bloody | 38 |
| L1210 (leukemia) | Bloody | 23 |
| OG (lymphoma) | Nonbloody | 20 |
| P815 (mastocytoma) | Nonbloody | 18 |
| RG (lymphoma) | Nonbloody | 14 |
| Normal mouse serum | – | 2 |

VEGF was readily detected in the ascites fluid of all mice bearing the 13 tumors and ranged from 6–850 ng/mL. High VEGF levels (168–850 ng/mL) were found in 5 sarcomas and 2 carcinomas; moderate levels (38–83 ng/mL) in one carcinoma and one plasmacytoma; and low levels (6–23 ng/mL) in 2 lymphomas, one leukemia, and one mastocytoma. VEGF levels in the ascites fluid of sarcoma and carcinoma origin (430.4 ± 234.2 ng/mL) were higher than those of lymphoma and hematological tumor origin (19.20 ± 10.45 ng/mL). The presence of VEGF in the tumor ascites was easily visualized by immunoprecipitation-Western blotting.

Interestingly, we found a strong correlation between hemorrhagic tendency and the amount of VEGF in ascites fluid. These results suggest that abnormally high levels of VEGF trigger bleeding through disturbance of the blood coagulation system or generation of fragile, newly formed blood vessels.

## Biological activities of VEGF in mouse tumor ascites fluid

An important question is whether VEGF in ascites fluid is biologically active, and whether its accumulation accounts for most of the vascular permeability activity of ascites fluid. To answer these questions, we carried out several experiments including determination of the subtype profiles of VEGF in ascites fluid, in vitro endothelial cell culture, and the Miles assay. The results can be summarized as follows:

1) The major subtypes of VEGF accumulated were the 164-amino acid subtype, which is thought to the most potent.
2) Degradation of VEGF was unexpectedly minor, and most VEGF accumulated in ascites fluid was biologically active in terms of growth-promoting activity for endothelial cells and vascular permeability activity in the Miles assay.
3) Anti-mouse VEGF neutralizing antibody suppressed 80% to 90% of cell free ascites-induced vascular permeability and endothelial cell mitotic activity. These findings indicate that most of the biological activity associated with crude ascites fluid is derived from VEGF itself.
4) A high level of expression of VEGF in ascites tumors appears to be constitutive but not induced by hypoxic conditions in the abdominal cavity, since ascites tumor cell lines such as MM2, Ehrlich mammary tumor, and S180 in the normoxic condition were found to secrete VEGF in amounts similar to those in the ascites tumor condition. This constitutive expression of VEGF by S180 tumor cells was previously reported by Rosenthal et al. [13]. It may be important to examine whether such constitutive up-regulation of VEGF is a critical step for the establishment of ascites tumors from the original solid tumors.

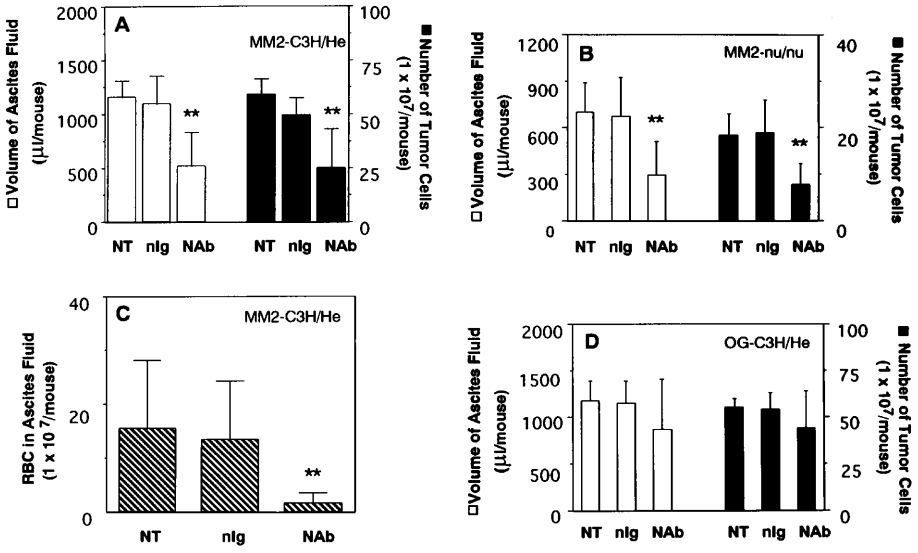


**Fig. 2** Differential inhibition of MM2 and OG ascites tumors by the anti-mouse VEGF neutralizing antibody. Mice intraperitoneally inoculated with MM2 or OG tumor cells were intraperitoneally administered neutralizing antibody AF-493-NA or normal goat IgG from day 0 to day 7. Anti-VEGF administration significantly inhibited MM2 tumor growth in both C3H/He (**A**) and BALB/c (nu/nu) mice (**B**), and also reduced the leakage of RBCs in the peritoneal fluid (**C**). In contrast, it did not inhibit the growth of lymphoma-derived OG tumor (**D**). The values are averages of 8 mice (MM2-C3H/He), or 4 mice (MM2BALB/c [nu/nu], OG-C3H/H) (bars, ± SD). Asterisks represent significant differences between no treatment (NT) and treatment with normal goat IgG (nIg) or the anti-VEGF antibody (NAb) (*$P < 0.05$; **$P < 0.01$). Reproduced, with permission, from Luo et al. [6]

## Inhibition of ascites accumulation and tumor growth in vivo using anti-mouse VEGF neutralizing antibody (AF-493-NA)

For therapeutic experiments in vivo, we chose two types of ascites tumor lines: MM2 of carcinomia origin, expressing a moderate amount of VEGF; and OG tumor of lymphoma origin, expressing a relatively low amount of VEGF. OG cells have a strong tendency to infiltrate peritoneal wall tissues and to dissociate muscular fibers.

No cytotoxicity of the anti-VEGF antibody AF-493-NA on MM2 and OG cells was found in the in vitro growth assay at the dose used in this study (data not shown). AF-493-NA antibody was administered intraperitoneally daily for 8 days at doses of 20- (for MM2 tumor-bearing mice) or 40-molar fold (for OG tumor-bearing mice) the amount of VEGF which kinetically accumulated in the peritoneal cavity. Af-493-NA administration markedly inhibited MM2 tumor growth, but unexpectedly did not significantly inhibit OG tumor growth (Figs. 2 and 3).

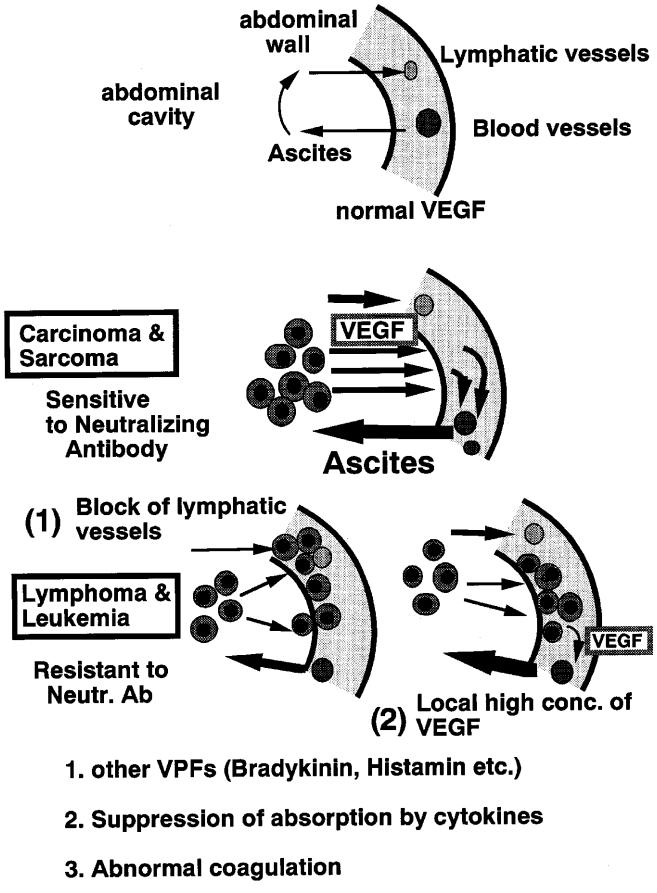


**Fig. 3** Model for ascites formation in mouse malignant ascites tumors. *Upper*: Normal abdominal wall was found to express basal levels of VEGF. Therefore a balance between the secretion of a small amount of ascites fluid and the absorption of the fluid by lymphatic vessels may exist under normal conditions. *Middle*: Carcinoma/sarcoma-derived ascites tumors such as MM2 secrete relatively large amounts of VEGF into the abdominal cavity, which results in the formation of a large volume of ascites fluid. Since tumor cells do not significantly affect the abdominal wall, blocking of VEGF with neutralizing antibody is effective in suppressing ascites formation. *Lower*: Leukemia/lymphoma-derived ascites tumors such as OG tend to infiltrate into the abdominal wall. These infiltrating tumor cells may block the absorption of ascites by lymphatic vessels or locally stimulate vascular permeability with VEGF or other molecules. This type of ascites formation is resistant to mouse VEGF-neutralizing antibody

The average volume of ascites fluid, number of tumor cells, and red blood cells (RBCs) in MM2-bearing mice that received the anti-VEGF antibody (0.51 mL, $2.50 \times 10^8$ tumor cells and $1.61 \times 10^7$ RBC per mouse, respectively) were significantly lower than those in untreated mice or mice administered the same doses of normal goat IgG (1. 16 mL, $5.90 \times 10^8$ tumor cells and $1.55 \times 10^8$ RBC per mouse, respectively) ($P < 0.01$) (Fig. 2). Such differences were not observed among OG tumor-bearing mice that received the same treatment and their matched controls ($P > 0.05$).

Consistent with the above results, the vascular permeability of microvessels lining the peritoneal cavity of MM2 tumor-bearing mice decreased significantly in those administered anti-VEGF antibody compared with the matched controls ($P < 0.01$), as assessed using the quantitative assay of the extravasated FITC-D tracer from the circulation into the peritoneal cavity. These therapeutic effects on MM2 tumors were expected from in vitro studies, in which the anti-VEGF neutralizing antibody efficiently suppressed the vascular permeability activity and endothelial cell mitotic activity associated with crude cell-free ascites fluid.

Surprisingly, the therapeutic effects of neutralizing antibody were very weak in OG tumor ascites fluid. Several possibilities for this resistance of OG tumors to the neutralizing antibody treatment could be considered (Fig. 3). An important characteristic of the OG tumor cell line is massive infiltration of peritoneal tissues. Therefore OG cells may mechanically or biologically block the lymphatic vessels which are crucial for absorption of ascites fluid. Another possibility is that OG cells that infiltrate tissues secrete relatively small amounts of VEGF, but the local VEGF concentration is sufficient to stimulate the vascular permeability of blood vessels in the abdominal walls. Such local stimulation by VEGF may be difficult to block using neutralizing antibody. Vascular permeability factor(s) other than VEGF, such as bradykinin, should be considered. However, it seems unlikely that bradykinin has a significant role since the amount of bradykinin in OG ascites fluid was only 0.7–0.9 ng/mouse ascites fluid and did not change after neutralizing antibody treatment.

In conclusion, we found that differential inhibition of two different ascites tumors occurred with VEGF neutralizing antibody. Since the major types of human ascites tumors are of carcinoma/sarcoma origin, we expect that anti-VEGF therapy may be effective in suppressing ascites tumors and improving the quality of life of cancer patients.

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