Differential Inhibition of Fluid Accumulation and Tumor Growth in Two Mouse Ascites Tumors by an Antivascular Endothelial Growth Factor/Permeability Factor Neutralizing Antibody

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ABSTRACT

In the accompanying paper (Luo et al., Cancer Res., 58: 2652-2660, 1998), we demonstrated that vascular endothelial growth factor (VEGF), also designated vascular permeability factor (VPF), significantly accumulated in all mouse malignant ascites tested, suggesting its fundamental role in ascites tumors. Removal of VEGF may inhibit the development of ascites tumors. In this study, using a goat antimouse VEGF-neutralizing antibody, we tested this hypothesis with two well-defined syngeneic mouse ascites tumors: MM2 breast adenocarcinoma and OG/Gardner lymphoma 6C3HED (expressing moderate and low levels of VEGF, respectively). This antibody significantly inhibited MM2 and OG cell-free ascites fluid-induced hyperpermeability of mouse peritoneal microvessels and in vitro endothelial cell growth. Mice bearing tumors were administered i.p. daily with the antibody or normal goat IgG as controls for 8 days, at doses of 20-fold (for MM2-bearing mice) or 40-fold (for OG-bearing mice) the estimated amounts of VEGF that kinetically accumulated in the ascites fluid after the tumor inoculation. The average volume of ascites fluid, number of tumor cells and leaked RBCs, and the peritoneal microvessel permeability in MM2-bearing mice that received the antibody treatment were significantly lower than those in the matched controls (P < 0.01). Unexpectedly, OG-bearing mice did not show satisfactory response to the anti-VEGF treatment. This discrepancy was not likely due to inadequate doses or different host immune responses, but it was quite possibly to the different characteristics of MM2 carcinoma and OG lymphoma tumors, the latter being strongly invasive, and/or the existence of an inflammatory mediator(s), such as bradykinin or cytokine(s) other than VEGF. In summary, our results directly demonstrated, for the first time, differential roles for VEGF in ascites tumors in vivo and suggest the potential of VEGF inhibition as a specific therapy for ascites tumors of carcinoma origin, which are the major cause of the malignant ascess in adult humans.

INTRODUCTION

The accumulation of malignant ascites fluid is an important cause of morbidity and mortality in patients with intraabdominal tumors. However, the mechanisms involved are not well understood. At least three pathological features are supposed for ascites tumor formation: first, a reduced lymphatic recovery system, which was proposed from the evidence that tumor cells obstruct the draining lymphatics (Ref. 1); this finding was later confirmed by both experimental and clinical findings that diaphragmatic lymphatic drainage from the peritoneal cavity decreased in mice bearing ascites tumor; Refs. 2-4); second, angiogenesis, which was associated with the accumulation of malignant ascites based on the observations that a marked peritoneal neovascularization accompanied some ascites tumors and that angiogenesis inhibitor significantly reduced the ascites accumulation after the inhibition of vessel proliferation (5, 6); and finally, and probably the most importantly, hyperpermeability of the microvessels lining peritoneal cavity, which was thought to be essentially responsible for the malignant ascites accumulation and therefore has been extensively investigated (7-11).

The hyperpermeability of microvessels associated with tumors in vivo has been shown to be mediated by a variety of factors, including: (a) many inflammatory mediators, such as prostaglandin (12), leukotrienes (13), bradykinin (14-16), histamine (17) and nitric oxide (18); (b) several cytokines, such as tumor necrosis factor and interleukin-2 (19), transforming growth factor-β (20), and so forth (c) VEGF, also known as VPF because it was originally identified as a tumor-secreted protein that significantly increases vascular permeability (10), about 50,000 times more potent than histamine on a molar basis. As a bifunctional protein enhancing vascular permeability and stimulating endothelial growth, VEGF is thought to be responsible for fluid accumulation and angiogenesis in ascites tumors (21). Consistent with this notion, abundant VEGF activity has been found in guinea pig (10), mouse (22), and human malignant ascites fluid (23), and VEGF protein has been demonstrated to be concentrated in the leaky microvessels lining the peritoneal cavities of ascites tumor-bearing animals (24).

In the paper immediately preceding this one (25), we reported that VEGF significantly accumulated in the malignant ascites of all mouse tumors tested, strongly suggesting that VEGF plays a fundamental role in ascites tumor formation. However, a vast difference in the levels of VEGF accumulated in the ascites fluids was observed. The ascites tumors originated from sarcoma and carcinoma expressed VEGF strongly, whereas those of lymphocytic and hematological tumor origins expressed relatively low levels of VEGF. These observations suggest that the importance of VEGF varies according to tumors origin and the exact role of VEGF in these tumors needs to be further defined. In this study, using an antibody VEGF-neutralizing antibody, we addressed this question in vivo. We selected two well-defined tumors: MM2 (derived from mammary adenocarcinoma, secreting moderate levels of VEGF) and OG (derived from Gardner lymphoma 6C3HED, secreting relatively low levels of VEGF) as models. Because the two tumors are also syngeneic, the experimental results of the anti-VEGF treatment may provide an insight into potential clinical application.

MATERIALS AND METHODS

Animals, Tumors, and Other Reagents. MM2 and OG tumors are two syngeneic transplantable mouse ascites tumors that have been well defined (26-30). MM2 tumor originated from a spontaneous mammary adenocarcinoma (26-28), whereas OG tumor is one of the three forms of Gardner lymphoma 6C3HED, which is sensitive to antitumor agent l-asparaginase (29, 30). They were maintained in the peritoneal cavities of syngeneic, 6-week-old C3H/He male mice (SLC, Hamamatsu, Japan). MM2 is not particularly invasive.

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3 The abbreviations used are: VPF, vascular permeability factor; VEGF, vascular endothelial growth factor; r.m-VEGF, recombinant mouse VEGF; pN2, affinity-purified antibodies to NH2-terminal peptide of mouse VEGF; HUVEC, human umbilical vein endothelial cell.
sive (28) and is grown in ascites form. The mice inoculated i.p. with 1.5 x 10^6 MM2 cells/mouse died, with a mean survival period of 22 ± 0.5 days accompanying peritoneal lining tissues (25). The mice inoculated i.p. with 3 x 10^7 OG cells/mouse died, with a mean survival period of 10 ± 0.5 days, without an accompanying significant peritoneal bleeding. r.m-VEGF164 and antimouse VEGF-neutralizing antibody AF-493-NA, which has the ability to neutralize the bioactivity of 10 ng/ml r.m-VEGF at the lowest dosage of 0.35 µg/ml with a low level of endotoxin, were obtained from R&D Systems (Minneapolis, MN). FITC-D (M, of 71,200) was from Sigma Chemical Co. (St. Louis, MO). When used, FITC-D was dissolved (30 mg/ml) in PBS (pH 7.4) and passed over a Sephadex 50 column (Amersham Pharmacia Biotech, Uppsala, Sweden) to remove any low molecular weight contaminants in advance. The bradykinin-quantifying kit Markit A was from Dainippon Seiyaku (Osaka, Japan).

Collection of Ascites Fluid. Immediately after a mouse was sacrificed, 2 ml of cold PBS (pH 7.4) were injected i.p., the contents of the peritoneal cavity were mixed, and the ascites fluid was harvested to the fullest extent possible. The exact volume of the ascites fluid was recorded by subtracting the 2 ml of PBS injected from the total fluid volume recovered. The total number of tumor cells and RBCs were counted. After protease inhibitors (final concentrations: phenylmethylsulfonyl fluoride, 0.35 mg/ml; aprotinin, 200 kallikrein-inactivating units/ml) were added to the ascites fluid, cell-free ascites fluid was obtained by centrifuging at 180 x g for 20 min at 4°C. For the quantitation of ascites bradykinin, instead of the above protease inhibitors, an inhibitor cocktail (16) containing 1 mM phenylmethylsulfonyl fluoride, 0.05% polybrene, 1.5% EDTA, 1 mM Captopril, and 1 mM carboxypeptidase N inhibitor was added to the ascites fluid. One-ml aliquots of the cell-free ascites fluids were centrifuged at 15,000 x g for 5 min at 4°C, and the supernatants were stored at -80°C for subsequent assays. Bradykinin was quantified according to the manufacturer’s instructions (Dainippon Seiyaku, Osaka, Japan).

Cytotoxicity Test of MM2 Cells with Neutralizing Antimouse VEGF Antibody. The fractionated MM2 cells, prepared as described before, were grown in MEM-2% PCS in a humidified atmosphere of 5% CO2 and 95% air at 37°C. To determine the effect of neutralizing antimouse VEGF antibody AF-493-NA on the proliferation of MM2 cells in vitro, 2 x 10^4 cells/well in triplicate were cultured in a 24-well microtiter plate (Sumilon, Tokyo, Japan) in 0.5 ml/well fresh MEM-2% FCS either containing or not containing 10 µg/ml AF-494-NA neutralizing antibody for 72 h. Thereafter, cell number and viability were determined by counting nonstained cells using a Coulter counter after the addition of 0.1 ml of 0.5% Trypan blue to each well.

Hyperpermeability Analysis of Microvessels Lining Mouse Peritoneal Cavity with FITC-D as Tracer. For in vivo experiments, normal or ascites tumor-bearing mice were injected i.v. with 10 mg/mouse FITC-D (M, of 71,200); 2 h thereafter, they were sacrificed with dry ice, and the ascites fluid was harvested as described above. For the transient permeability experiment of mouse peritoneal lining vessels, 2 ml of suitably diluted cell-free ascesis or r.m-VEGF diluted in 2% BSA/PBS were incubated with anti-VEGF antibody AF-494-NA or normal goat IgG of the same amount at 25°C for 30 min. The incubated solutions or the controls of 2% BSA/PBS solution were injected i.p. immediately following i.v. injection of FITC-D (10 mg/mouse) into normal mice; 2 h thereafter, mice were sacrificed, and the peritoneal fluid was recovered. The quantitation of the FITC-D in the peritoneal fluid was performed with a fluorometric assay. All samples were read at an excitation wavelength of 499 nm and emitted wavelength of 519 nm at pH 7.4-7.8. The assay accurately detects concentrations in biological fluid as low as 2 ng/ml. For the microscopic visualization of FITC-D in tissue, the excised peritoneal wall was fixed and mounted after dehydration and clearance, according to a procedure described before (22).

Endothelial Cell Growth Assay. HUVECs (Morinaga, Kanagawa, Japan) were grown in HCM (Nissui, Tokyo, Japan) containing the cell-free ascites fluids (without the addition of protease inhibitors), using an endothelial growth assay as described before (31).

Radio-Receptor Binding Assay. This assay, in which an affinity purified antimouse VEGF antibody pN2 was used as capture antibody and ^125I-labeled 7N Fit-1 as detection reagent, is described in detail in the accompanying article (25).

In Vivo Treatment with the Anti-VEGF-neutralizing Antibody. To reduce the possible interference by humoral immune response due to the administration of the goat protein, we limited the period of the antibody treatment to 7 days after the addition of 0.1 ml of 0.5% trypan blue to each well. After the ascites fluid was totally harvested, the volume of ascites fluid, the number of tumor cells, and the amount of VEGF were determined. A, VEGF; B, tumor cells; C, ascites fluid, all expressed as the average value per mouse (n = 8 for MM2-bearing mice, n = 4 for OG-bearing mice, at each time point). Data points, mean; bars, ± SD.
mice injected i.p. with tumors were treated with an amount of normal goat IgG equal to the amount of the antibody or left intact to serve as the controls. On day 8, mice were sacrificed or left untreated until natural death.

Protein G Column Filtration. Clarified ascites diluted in cold PBS (pH 7.4) were passed twice over a protein G column (Pharmacia) equilibrated with PBS, and the void volume was collected.

Immunoprecipitation Western Blot Analysis. Samples of ascites fluid were immunoprecipitated with pN2 antibody, and then protein A-Sepharose beads (Pharmacia Biotech, Uppsala, Sweden) were added. Western blot analysis was performed as described elsewhere (25).

Histology. The peritoneal wall was fixed in 4% paraformaldehyde-PBS, processed, and embedded in paraffin for routine H&E staining.

Statistics. Comparisons of the results with and without the treatment were performed using an unpaired Wilcoxon Mann-Whitney test. A value of \( P < 0.05 \) was considered significant.

RESULTS

Kinetics of VEGF Accumulation in MM2 and OG Ascites. Our preliminary tests found that i.p. inoculation with \( 1.5 \times 10^6 \) MM2 or \( 3 \times 10^7 \) OG cells for 7 days led to significant ascites accumulation, which was used as a marker for evaluating the therapeutic effects of the anti-VEGF antibody in the two tumor models. To assess the dose of the anti-VEGF antibody to be tested, we first examined the kinetics of VEGF accumulation in MM2 and OG ascites and the relationship among VEGF and tumor expansion and ascites accumulation. VEGF was found when malignant ascites fluid appeared, as early as 4 days after i.p. injection of \( 1.5 \times 10^6 \) MM2 or \( 3 \times 10^7 \) OG cells (Fig. 1A and 1C). In both tumor models, the accumulation of total VEGF in the peritoneal cavity increased progressively, accompanying the tumor expansion and ascites accumulation in a roughly parallel way (Fig. 1B). The amount of VEGF in OG ascites was relatively small, reaching a peak around day 8, approximately 2 days prior to the death of the animal, whereas that in MM2 ascites fluid was quite large, reaching a peak about day 14, approximately 1 week prior to death, and falling slightly thereafter.

Inhibitory Effects of Anti-VEGF Antibody on Cell-free Ascites Fluid-induced Peritoneal Vascular Hyperpermeability and in Vitro Endothelial Cell Growth. The effect of the antibody on endogenous VEGF action in cell-free ascites of MM2 and OG tumors was first evaluated by testing its capacity to inhibit cell-free ascites fluid-induced hyperpermeability of microvessels lining the peritoneal cavity of normal syngeneic mice, using a transient experiment with FITC-D (Fig. 2A). Prior to the i.p. injection, preincubation of these solutions with 2 \( \mu \)g of AF-493-NA antibody, which well neutralizes the activity of 20 ng of VEGF, abolished OG ascites fluid-induced extravasation of FITC-D and significantly reduced that induced by MM2 ascites fluid, from 5.64 to 2.24% (\( P < 0.001 \)). Preincubation with normal goat IgG did not significantly affect the extravasation of i.v. injected FITC-D tracer into the peritoneal cavity, respectively. In contrast, the injection of BSA solution with the same amount of protein and the same volume only led to the extravasation of 1.04% of i.v. FITC-D (Fig. 2A). Prior to the i.p. injection, preincubation of these solutions with 2 \( \mu \)g of AF-493-NA antibody, which well neutralizes the activity of 20 ng of VEGF, abolished OG ascites fluid-induced extravasation of FITC-D and significantly reduced that induced by MM2 ascites fluid, from 5.64 to 2.24% (\( P < 0.001 \)). Preincubation with normal goat IgG did not significantly affect the extravasation of i.v. FITC-D by these solutions (Fig. 2A).

The effect of the anti-VEGF antibody on the action of native VEGF in cell-free ascites of MM2 and OG tumors was then estimated by using in vitro cultures of endothelial cells. Its effect on the action of native VEGF in mouse tumor ascites had been examined previously (25) by using rat liver sinusoidal endothelial cell. Because the effect of the antibody AF-493-NA on r.m-VEGF was originally estimated by using in vitro cultures of HUVECs (R&D Systems), in this study we used the same cells to evaluate its effect on the action of native VEGF in cell-free ascites fluids of MM2 and OG tumors. Stimulation of HUVECs with HCM culture medium including 10 ng of VEGF-containing MM2 or OG ascites for 4 days produced a significant increase in cellular DNA contents compared with the unstimulated cells. MM2 ascites fluid stimulated endothelial cells more strongly than did 10 ng of r.m-VEGF did, whereas OG ascites fluid stimulated endothelial cells at the same strength as did 10 ng of r.m-VEGF. Prior to the addition into the cultures, incubation of these solutions with 1 \( \mu \)g of the antibody significantly reduced the growth stimulation of HUVECs induced by these solutions; it completely blocked the 10 ng r.m-VEGF or OG ascites fluid-induced growth stimulation of endo-

<table>
<thead>
<tr>
<th>Tumor</th>
<th>Treatment</th>
<th>Bradykinin concentration (ng/mouse)</th>
<th>No. of samples</th>
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</thead>
<tbody>
<tr>
<td>MM2</td>
<td>NT*</td>
<td>2.3 ± 0.15*</td>
<td>7</td>
</tr>
<tr>
<td></td>
<td>nlg</td>
<td>2.5 ± 0.21</td>
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<tr>
<td></td>
<td>Nab</td>
<td>1.4 ± 0.12</td>
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</tr>
<tr>
<td>OG</td>
<td>NT</td>
<td>0.72 ± 0.12</td>
<td>4</td>
</tr>
<tr>
<td></td>
<td>nlg</td>
<td>0.66 ± 0.18</td>
<td>4</td>
</tr>
<tr>
<td></td>
<td>Nab</td>
<td>0.78 ± 0.21</td>
<td>4</td>
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* NT, no treatment; nlg, treated with normal goat IgG; Nab, treated with the anti-VEGF antibody.

\( \text{Mean} \pm \text{SD}. \)
INHIBITION OF ASCITES TUMORS BY ANTI-VEGF ANTIBODY

The inhibition of MM2 and OG ascites tumors by the anti-VEGF antibody is described. Mice i.p. inoculated with MM2 or OG tumor cells were administered i.p. daily antibody AF-493-NA or normal goat IgG from day 0 to day 7 as described in "Materials and Methods." Anti-VEGF treatment significantly inhibited MM2 tumor inoculated both in 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 effectively inhibit OG tumor (D). The values are the average of eight mice (MM2-C3H/He) or four mice [MM2-BALB/c (nu/nu) and OG-C3H/He] (bars, SD). Asterisks represent significant differences between the results of nontreatment (NT) and those of treatment with normal goat IgG (nlg) or the anti-VEGF antibody (NAb; *, P < 0.05; **, P < 0.01).

Fig. 3. Differential inhibition of MM2 and OG ascites tumors by the anti-VEGF antibody. Mice i.p. inoculated with MM2 or OG tumor cells were administered i.p. daily antibody AF-493-NA or normal goat IgG from day 0 to day 7 as described in "Materials and Methods." Anti-VEGF treatment significantly inhibited MM2 tumor inoculated both in 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 effectively inhibit OG tumor (D). The values are the average of eight mice (MM2-C3H/He) or four mice [MM2-BALB/c (nu/nu) and OG-C3H/He] (bars, SD). Asterisks represent significant differences between the results of nontreatment (NT) and those of treatment with normal goat IgG (nlg) or the anti-VEGF antibody (NAb; *, P < 0.05; **, P < 0.01).

Fig. 4. Reduced microvascular hyperpermeability of the peritoneal cavities in MM2-bearing mice after the anti-VEGF treatment. Normal mice and tumor bearing mice that received 8-day treatment were injected i.v. with 10 mg/mouse FITC-D; 2 h later, they were killed, and the leaked FITC-D in the peritoneal cavity was measured (see "Materials and Methods"). The values are the average of 8 mice (MM2) or 4 mice (OG, normal controls; bars, SD). Asterisks represent significant differences between the results of nontreatment (NT) and those of treatment with normal goat IgG (nlg) or the anti-VEGF antibody (NAb; *, P < 0.05; **, P < 0.01).
tumors. Such as bradykinin play a role, to some extent, in these two ascites change significantly after the antibody treatment (Table 1). This contained some amounts of bradykinin, the level of which did not concentration in the two tumor ascites fluids by using an enzyme immunoassay. We found that both MM2 and OG tumor ascites fluid present in the two tumor ascites fluids. Because bradykinin, one of the vascular permeability mediators, has been shown to express in the ascites of both mouse and human tumors (14-17), we measured its vascular permeability and endothelial cell growth in vitro stimulated by OG lymphoma. Previously, we found that mouse ascites tumors of sarcoma or carcinoma origin expressed VEGF at high levels; in contrast, those of lymphocytic or hematological tumor origins expressed VEGF at relatively low levels, indicating that the role of VEGF may differ in the two tumor types. This was confirmed by the present study.

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Although the antibody completely abolished the transient vascular permeability and endothelial cell growth in vitro stimulated by OG cell-free ascites fluid, unexpectedly it could not significantly inhibit OG tumor in vivo (Fig. 3). At least three possibilities may, separately or together, account for this contradiction. Two are associated with highly invasive characteristics of OG lymphoma. First, as a result of its invasion, the VEGF secreted by tumor cells may become too easily accessible to the blood vessels in the peritoneal tissues to be effectively blocked by the anti-VEGF antibody. Second, the invaded tumor cells may also lead to the accumulation of ascites fluid by causing the obstruction of draining lymphatic vessels.

The third possibility is that inflammatory mediators, such as bradykinin and/or non-VEGF cytokines, are involved in the development of ascites tumor, especially after the removal of VEGF by the antibody. This is supported by the finding of some amounts of bradykinin accumulated in OG ascites, because such a level of bradykinin may have biological function in vivo (15). However, considering that VEGF is about 40 times more potent than bradykinin at the molar

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level and that the concentration of VEGF in OG ascites is about 18 ng/ml, nearly 30-fold higher than that of bradykinin (0.62 ng/ml), bradykinin may only play a minor role, if any, in the ascites formation of OG tumor.

The significant inhibition of tumor by the anti-VEGF treatment directly suggests a causal role of VEGF for MM2 ascites tumor in vivo. Because angiogenesis of the peritoneal wall had not been statistically significant until day 10 after the i.p. injection of 1.5 × 10⁶ MM2 cells, this inhibitory effect can be mainly attributed to the blockage of the vascular permeability-enhancing activity of endogenous VEGF secreted by the tumor cells. In addition, as shown in Fig. 5, the treatment delayed the subsequent angiogenesis that may accelerate the process of the disease.

On the other hand, as discussed in the accompanying article (25), high-level VEGF may cause bleeding secondary to the abnormal coagulation. In this experiment, we found that the anti-VEGF treatment markedly reduced the RBC leakage, which is lethal for mouse in some ascites tumors (21). Interestingly, the treatment indeed prolonged the life span of MM2-bearing mice compared to the matched controls. Because a little free VEGF seemingly remained in the ascites fluid of MM2-bearing mice that received the anti-VEGF treatment, increasing the antibody dose may improve the therapeutic effects. However, anti-VEGF treatment may not completely inhibit MM2 tumor, because the anti-VEGF antibody could not abolish the stimulatory effects of the vascular endothelial cells induced by the cell-free MM2 ascites (Fig. 2) and also because a substantial level of bradykinin was found in the MM2 ascites.

In summary, our findings directly prove, for the first time, that VEGF plays an important role in one ascites tumor of carcinoma origin, suggesting that the inhibition of VEGF activity can be used as a specific therapy for this type of tumor, the major cause of adult malignant ascites. Our results also emphasize that the roles of non-VEGF pathological factors, such as the characteristics of tumors, inflammatory mediators and cytokines other than VEGF, and so forth, need to be determined, especially for the malignant ascites of certain lymphoma and hematological tumor origin.

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