Soluble FL-1 Expression Suppresses Carcinomatous Ascites in Nude Mice Bearing Ovarian Cancer

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ABSTRACT

Vascular endothelial growth factor (VEGF), a bifunctional protein enhancing vascular permeability and stimulating endothelial growth, is thought to be responsible for fluid accumulation and angiogenesis in ascites tumors. To investigate the effects of stable expression of the soluble form of Flt-1 VEGF receptor (sFlt-1), a known endogenous inhibitor of VEGF, on the malignant ascites tumors, we cotransduced RMG-1 human ovarian cancer cells with adeno-associated virus vectors carrying the sFlt-1 cDNA and Neo gene or Neo gene alone and isolated both the sFlt-1-expressing clone and the Neo-expressing clone. In vitro growth characteristics were essentially the same. As expected, conditioned medium collected from the sFlt-1-expressing cells significantly inhibited the human umbilical vein endothelial cell proliferation in the presence of recombinant VEGF. Expression of sFlt-1 significantly suppressed RMG-1 cell-induced angiogenesis in vivo in the mouse dorsal air sac assay model. We then inoculated sFlt-1- or Neo alone-expressing cells i.p. into female BALB/c nude mice. The average volume of ascites fluid, number of leaked RBCs, and number of cancer cells were significantly lower in mice injected with sFlt-1-expressing cells than in the controls. Survival time was significantly prolonged in mice injected with sFlt-1-expressing cells. These results suggest that inhibition of VEGF activity by sFlt-1 expression may provide a means to control carcinomatous ascites and angiogenesis of malignant ascites tumors.

INTRODUCTION

More than half of all patients with ovarian cancer are not diagnosed until the advanced stages of the disease (1). One common pathway of tumor progression in ovarian carcinoma is peritoneal dissemination, and a progressive accumulation of ascites is frequent with or without malignant tumor cells. Both tumor size and the accumulation of ascites are inversely associated with survival (2, 3).

Tumor angiogenesis is critical for supporting the rapid growth of solid tumors (4) and is thought to be associated with the accumulation of malignant ascites. It was shown that marked peritoneal neovascularization accompanied some ascites tumors and that an angiogenesis inhibitor significantly reduced the accumulation of ascites after the inhibition of vessel proliferation (5, 6). Also, hyperpermeability of the microvessels lining the peritoneal cavity is considered to be essentially responsible for the accumulation of malignant ascites based on clinical (7, 8) and experimental observations (9–11). The hyperpermeability of microvessels associated with tumors has been shown to be mediated by a variety of factors including inflammatory mediators such as prostaglandin (12), leukokinin (13), bradykinin (14–16), histamine (17), and cytokines (18, 19). Among others, VEGF, a bifunctional cytokine enhancing vascular permeability and stimulating endothelial growth, is thought to be responsible for fluid accumulation and angiogenesis in ascites tumors (20). VEGF exerts its functions by interacting with two different high affinity tyrosine kinase receptors, Flt-1 and/or KDR/Flk-1, which are selectively expressed in vascular endothelia (21).

It has been reported recently that human epithelial ovarian carcinomas overexpress VEGF with elevated serum VEGF levels being correlated with decreased survival (22). Given its potential role in promoting tumor angiogenesis, metastasis, and fluid accumulation, VEGF is an attractive target for therapeutic intervention. One strategy to block tumor angiogenesis and elevated vascular permeability is to perfuse the vasculature with a truncated sFlt-1. sFlt-1 is a known endogenously expressed selective inhibitor of VEGF. It is an alternatively spliced version of the Flt-1 VEGF receptor (23). The splicing alteration results in retention of an intron within the mRNA that is translated to the first in-frame stop codon. This alternatively spliced form of the Flt-1 protein retains six of seven NH2-terminal extracellular immunoglobulin-like domains fused to the unique intron-encoded 31-amino acid residue COOH-terminal sequence but is devoid of the membrane proximal immunoglobulin-like domain, the membrane-spanning polypeptide, and the entire intracellular tyrosine kinase-containing region. The product, sFlt-1, binds to VEGF with the same affinity as and an equivalent specificity to the full-length receptor and forms a receptor-ligand complex. sFlt-1 is expressed by vascular endothelial cells and can inhibit their mitogenic response to VEGF in culture by sequestering VEGF. In addition, it interacts with the VEGF receptors in a dominant-negative fashion by heterodimerizing with the extracellular ligand-binding region of the membrane spanning Flt-1 (23) and KDR/Flk-1 (24) VEGF receptors, thereby blocking the activation of downstream signal transduction.

Given these properties of sFlt-1, it is of interest to see whether tumor cells engineered to express sFlt-1 exhibit diminished biological potentiality as tumors. In this study, we cotransduced RMG-1 human ovarian cancer cells with AAV vectors carrying sFlt-1 cDNA and the Neo gene, and isolated clones expressing sFlt-1. Here we demonstrated that an enhanced expression of sFlt-1 resulted in diminished endothelial cell-proliferating activity in vitro and suppression of angiogenesis in vivo. In addition, nude mice bearing the sFlt-1-expressing cancer cells exhibited a reduced accumulation of carcinomatous ascites and extended survival.

MATERIALS AND METHODS

Cell Lines. The 293 human embryonic kidney cell line (25) was maintained in DMEM-F12 plus 10% FBS, 2 mM glutamine, and 1% penicillin/streptomycin. RMG-1 human ovarian carcinoma cell line (26) was a gift from

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2 The abbreviations used are: VEGF, vascular endothelial growth factor; sFlt-1, soluble form of the Flt-1 VEGF receptor; AAV, adeno-associated virus; FBS, fetal bovine serum; HUVEC, human umbilical vein endothelial cell.

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Dr. S. Nozawa (Keio University, Tokyo, Japan) and maintained in RPMI 1640 plus 10% FBS and 1% penicillin/streptomycin. RMG-1 is invasive to the peritoneal lining tissue accompanying ascites accumulation. HUVECs were prepared as described previously (27) and maintained in EGM-2 Bulletkit EBM medium (BioWhittaker) according to the manufacturer’s instructions.

**Plasmids.** The AAV vector plasmids used in this study were derived from the parent vector plasmid pW1 (28), harboring a lacZ expression cassette flanked by two 145-bp inverted terminal repeats of AAV. The psFlt-1 and pNeo vector plasmids were constructed by excising the lacZ reporter gene from the parent vector plasmid and replacing it with the 2.4-kb mouse sFlt-1 cDNA (29) and 0.8-kb neomycin-resistance gene, respectively. pM45 is a helper plasmid containing the AAV rep and cap genes, which are required for replication and capsid formation of AAV vectors, and has no overlapping AAV sequence flanked with the vector plasmid, pladen-1, a plasmid containing the E2A, E4, and VA genes of the adenovirus genome, was used in place of helper adenovirus for AAV vector production. *Escherichia coli* DH5-α was used for plasmid DNA amplification.

**In Vitro Transduction.** AAV vectors were produced by cotransfection of 293 cells by the calcium phosphate coprecipitation method with the vector plasmid (psFlt-1 or pNeo), the helper plasmid (pM45), and the pladen-1 plasmid (30). The vector titer was determined by quantitative DNA dot-blot hybridization. RMG-1 cells were transduced with 1 × 10⁶ particles/cell of AAV-sFlt-1 and AAV-Neo or AAV-Neo alone, and propagated in complete medium containing 100 µg/ml G418 antibiotic to select stable clones. Analyzing cell lysates for sFlt-1 expression isolated sFlt-1-expressing clones by Western blot analysis.

**Western Blot Analysis.** Conditioned media were generated by culturing 3 × 10⁵ Neo-expressing cells or sFlt-1-expressing cells in 7 ml of serum-free RPMI 1640 for 72 h. Cells were lysed in a lysis buffer [10 mM Tris-HCl, 150 mM NaCl, and 1% NP40 (pH 7.6)] supplemented with 1 mM phenylmethylsulfonyl fluoride and 500 units/ml of aprotinin. Cell lysate (10 µl) or 10 µl of conditioned medium was electrophoretically separated on a 7.5% SDS-PAGE gel, transferred to a polyvinylidene difluoride membrane (Millipore), probed by rabbit polyclonal antibody to the NH₂-terminal sequence of sFlt-1, and then reacted with horseradish peroxidase-labeled antirabbit mouse antibody. Bindings were visualized with the enhanced chemiluminescence system (Amersham).

**VEGF Quantitation.** Conditioned medium were generated by culturing 3 × 10⁵ RMG-1 cells in 7 ml of RPMI 1640 plus 1% FBS for 96 h. Measurement of VEGF was performed using an ELISA kit for human VEGF (Amersham) according to the manufacturer’s instructions.

**In Vitro Proliferation of the sFlt-1-expressing Cells.** Neo-expressing cells (1 × 10⁵) or sFlt-1-expressing cells were plated in three sets of triplett wells. The cells were enumerated at 48, 72, and 96 h after plating.

**Endothelial Cell Proliferation Assay.** Conditioned medium were generated by culturing 3 × 10⁵ Neo-expressing cells or sFlt-1-expressing cells in 7 ml of serum-free RPMI 1640 for 72 h. HUVECs (2 × 10⁵/well) were plated in triplicate wells in EBM-2 medium (BioWhittaker) plus 5% FBS containing 25% of either conditioned medium with or without 20 ng/ml VEGF (Pepro Tech). The cells were counted 5 days after plating.

**Dorsal Air Sac Assay.** The dorsal air sac assay was done according to a method described previously (31). A Millipore chamber (filter pore size 0.22 µm; Millipore Co.) was filled with 0.15 ml (1 × 10⁵ cells) of cell suspension of Neo-expressing or sFlt-1-expressing cells and implanted s.c. in the dorsum of 4-week-old female BALB/c nude mice (CLEA, Tokyo, Japan). On day 5, the implanted chambers were removed from the animals. Angiogenic response was assessed under a dissecting microscope by determining the number of newly formed blood vessels longer than 3 mm with the characteristic zigzagging pattern of tumor cell-induced new vasculature in the s.c. side of the skin area that had been in contact with the chamber.

**RMG-1 i.p. Xenografts.** Four-week-old female BALB/c nude mice (CLEA) were inoculated i.p. with Neo-expressing or sFlt-1-expressing cells (2 × 10⁵ cells/mouse). Mice were sacrificed 5 weeks after the inoculation. After a mouse was sacrificed, 2 ml of PBS was injected i.p., and the peritoneal fluid was totally recovered. The volume of ascites fluid was calculated by subtracting the 2 ml of PBS injected from the total fluid volume recovered. The number of tumor cells, the number of RBCs in the ascites fluid, and the number and diameter of peritoneal disseminations were determined. Tumors 2 mm in diameter were fixed in sucrose buffered 4% paraformaldehyde, and processed for 10 µm cryostat sections and air dried. The sections were stained for alkaline phosphatase, revealing the enzyme within the capillary endothelium (32). Staining was carried out for 30 min at room temperature, using an incubation medium of the following composition: 2 mg naphtol AS-BI phosphate, 0.2 ml N,N-dimethyl-formamide, 9.8 ml 0.1 M Tris-HCl buffer, 10 mg Fast red violet LB. Slides were dehydrated in tetrachloroethylene and mounted. Another set of 4-week-old female BALB/c nude mice were inoculated i.p. (2 × 10⁵ cells/mouse) with Neo-expressing or sFlt-1-expressing cells. Mice were monitored daily.

**Statistics.** Comparisons of the results of the endothelial cell proliferation assay and dorsal air sac assay were performed using Student’s t test. Comparisons of the results of the *in vivo* assays were performed using the Wilcoxon signed-rank test. The statistical significance of differences in survival times among groups was determined using a Kaplan-Meier survival analysis Wilcoxon test. A value of P < 0.05 was considered significant.

**RESULTS**

**Expression of sFlt-1 in RMG-1 Cells Transduced with AAV-sFlt-1.** We first examined whether RMG-1 cells produce VEGF by performing an enzyme immunonasssay using the conditioned medium of RMG-1 cells. The mean VEGF level in the conditioned medium was 458 pg/ml. To determine the expression of sFlt-1 protein in RMG-1 cells transduced with AAV-Neo alone or cotransduced with AAV-sFlt-1 and AAV-Neo, stable clones were isolated and characterized for protein expression. As shown by the Western blotting (Fig. 1), the clone transduced with AAV-Neo alone (Neo clone) did not express sFlt-1 protein, whereas the clone cotransduced with AAV-sFlt-1 and AAV-Neo (sFlt-1 clone) expressed sFlt-1 protein in both the cell lysate and conditioned medium.

**Growth Characteristics of sFlt-1-expressing RMG-1 Cells in Vitro.** These clones were microscopically indistinguishable and had similar growth rates in *vitro* (Fig. 2). These results demonstrate that sFlt-1 did not affect tumor cell mitogenesis in *vitro*.

**Inhibitory Effects of sFlt-1 on In Vitro Endothelial Cell Growth.** The effect of the sFlt-1 expression of sFlt-1 clone cells on the action of VEGF was estimated using *in vitro* cultures of endothelial cells. Stimulation of HUVECs with EBM culture medium including 20 ng/ml recombinant VEGF plus 25% conditioned medium from Neo clone cells for 5 days produced a 70% increase in the cell number (P < 0.05). This mitogenic effect of VEGF on endothelial cells was completely abrogated by the addition of conditioned medium from sFlt-1 clone cells instead of that from Neo clone cells (P < 0.05; Fig. 3A).

**Inhibitory Effects of sFlt-1 on In Vivo Angiogenesis.** The effect of sFlt-1 expression on tumor angiogenesis was examined using mouse dorsal air sac assay. Millipore chambers filled with Neo-expressing (n = 5) or sFlt-1-expressing (n = 5) cells were implanted into nude mice. As shown in Fig. 3B, the average number of newly formed vessels in mice implanted Millipore chambers containing sFlt-1-expressing cells (2.6/mouse) was significantly lower than in
mice implanted Millipore chambers containing Neo-expressing cells (4.2/mouse; \( P < 0.05 \)). This result demonstrates that persistent expression of sFlt-1 suppressed VEGF-induced angiogenesis in vivo.

**Suppression of Ascites Accumulation in Vivo and Capillary Density of the Tumors.** To determine the effect of persistent sFlt-1 expression on ascites accumulation and tumorigenicity in vivo, either Neo-expressing (\( n = 5 \)) or sFlt-1-expressing (\( n = 5 \)) cells (\( 2 \times 10^7 \)) were injected i.p. into nude mice. As shown in Table 1, the average volume of ascites fluid, number of tumor cells, and number of leaked RBCs in mice injected with sFlt-1 clone cells (0.07 ml; 3.83 \( \times 10^5 \) tumor cells and 7.70 \( \times 10^5 \) RBCs/mouse) were significantly lower than those in mice injected with Neo clone cells (1.80 ml; 3.50 \( \times 10^5 \) tumor cells and 8.28 \( \times 10^5 \) RBCs/mouse; \( P < 0.05 \)). There was no significant difference in the number of peritoneal disseminations (85.6 \( \pm \) 54.6 versus 121.4 \( \pm \) 54.1). Histochemical staining of tumor sections for alkaline phosphatase showed that capillary density of the tumors. There was not a significant difference in vascularity between these groups (data not shown). The number of peritoneal disseminations >2 mm in diameter was significantly smaller in mice i.p. injected with sFlt-1 clone cells than with Neo clone cells (\( \chi^2 \)-statistics, \( P < 0.05 \)). These results demonstrate that persistent expression of sFlt-1 resulted in suppression of ascites accumulation, RBC leakage, and tumor growth.

**Survival Kinetics of Nude Mice Injected i.p. with sFlt-1-expressing Tumor Cells.** A total of \( 2 \times 10^7 \) Neo-expressing cells (\( n = 6 \)) or sFlt-1-expressing cells (\( n = 6 \)) were injected i.p. into nude mice to evaluate the effect of sFlt-1 on the mortality rate. i.p. inoculation of the parent RMG-1 cells into nude mice leads to death with reproducible kinetics (data not shown). As depicted in Fig. 4, mice injected with sFlt-1 clone cells had a median survival time of 70 days. This was significantly longer than the median survival time of 55 days of mice injected with Neo clone cells (\( P < 0.05 \)).

**DISCUSSION**

In the present study, we have established an animal model of human ovarian cancer in which cancer cells persistently express sFlt-1, a selective inhibitor of VEGF. Using this model, we clearly demonstrated that nude mice inoculated i.p. with the sFlt-1-expressing cancer cells display longer survival times associated with reduced volumes of ascites.

### Table 1

<table>
<thead>
<tr>
<th>Tumor cells</th>
<th>Ascites volume (ml)</th>
<th>RBC in ascites fluid (1 ( \times 10^3 ) /mouse)</th>
<th>No. of tumor cells</th>
<th>No. of peritoneal disseminations</th>
</tr>
</thead>
<tbody>
<tr>
<td>Neo</td>
<td>1.80 ( \pm ) 1.74</td>
<td>828 ( \pm ) 534</td>
<td>35.0 ( \pm ) 20.2</td>
<td>121.4 ( \pm ) 54.1</td>
</tr>
<tr>
<td>sFlt-1</td>
<td>0.07 ( \pm ) 0.03b</td>
<td>0.8 ( \pm ) 0.6b</td>
<td>0.4 ( \pm ) 0.2c</td>
<td>85.6 ( \pm ) 54.6</td>
</tr>
</tbody>
</table>

**A. Characteristics of ascites**

**B. Number of peritoneal disseminations classified by size**

<table>
<thead>
<tr>
<th>Tumor cells</th>
<th>Diameter of tumors</th>
<th>Total no. of mice</th>
</tr>
</thead>
<tbody>
<tr>
<td>Neo</td>
<td>&gt;2 mm</td>
<td>5</td>
</tr>
<tr>
<td>sFlt-1</td>
<td>19^b</td>
<td>5</td>
</tr>
</tbody>
</table>

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^ The ascites volume and the number of tumor cells, RBCs in the ascites fluid, and peritoneal disseminations were measured. Data shown represent mean \( \pm \) SD. The statistical difference between groups was computed using Wilcoxon signed-rank test. sFlt-1 expression reduced the ascites volume, leakage of RBCs, and the number of tumor cells in the peritoneal fluid but not the number of peritoneal disseminations.

^b \( P < 0.05 \).

^ The peritoneal disseminations were counted by size. The number of peritoneal disseminations >2 mm in diameter was significantly smaller in mice i.p. injected with sFlt-1 clone cells than in mice injected with Neo clone cells (\( P < 0.05 \), \( \chi^2 \)-statistics).
sFlt-1 is an endogenously expressed selective inhibitor of VEGF. It is an alternatively spliced version of the Flt-1 VEGF receptor. Inhibition of VEGF activity by sFlt-1 is mediated both by the sequestering of VEGF and by the forming of inactive heterodimers with VEGF receptors in a dominant negative fashion (23, 24). Anti-VEGF antibodies were reported to inhibit tumor growth, metastasis, and fluid accumulation of ascites tumors (33, 34). It has been additionally demonstrated that interference with VEGF-mediated angiogenesis by sFlt-1 via gene transfer inhibited tumor growth and metastasis (35–37). This approach targets the angiogenic vasculature directly, and does not require transfer of genes to each tumor cell.

It is to be noted that nude mice bearing sFlt-1-expressing cells have reduced amounts of ascites fluid despite showing no significant change in the number of peritoneal disseminations. In the light of the decreased number of RBCs in ascites fluid, we reasoned that sFlt-1 suppresses cancer-associated vascular hyperpermeability by acting on the microvasculature and thereby decreasing fluid from plasma to the peritoneal cavity (20, 33).

In this study, although VEGF-induced vascular endothelial cell mitogenesis in culture was efficiently inhibited and the number of tumor cells in the ascites fluid in mice i.p. injected with sFlt-1 clone cells was significantly low compared with the control group, there was no significant difference in the number of peritoneal disseminations. It may be that the local expression of sFlt-1 was not high enough or that the tumor cells eventually acquired the ability to increase proteolytic degradation of sFlt-1 (35). The possibility that inflammatory mediators and/or other angiogenic cytokines are involved in tumorigenicity also remains. Several human ovarian carcinomas were reported to express multiple genes that regulate angiogenesis, which was associated with the pattern of the disease and its progression (38).

It is interesting to note that the number of peritoneal disseminations >2 mm in diameter was significantly smaller in mice bearing sFlt-1-expressing cancer cells than in the control, a finding consistent with the fact that tumors cannot grow to >1–2 mm3 unless they acquire their own blood supply (4). Formation and growth of thin layers and small solid tumors of i.p. carcinomatosis may be maintained by the preexisting vasculature, whereas larger solid tumors require neovascularization for continued growth (4). Our histochemical analysis of the tumor vasculature did not reveal significant difference in vascularity of each tumor between these groups. This also reflects the fact that the decreased number of tumors, which correlated well with the result of vascular formation in dorsal air sac assay, has more biological meaning, and if there is a tumor larger than a certain size, there are some levels of vascularity. The tumor vasculature in the sFlt-1-transduced group may be induced by the other tumor angiogenesis factors besides VEGF. On the other hand, we speculate that the VEGF activity was efficiently inhibited by the transgene-derived sFlt-1, because the ascitic tumors, which is known to be caused by VEGF-mediated angiogenesis, was almost completely suppressed. The combination of sFlt-1 gene and the other type of angiogenesis-inhibiting gene would additionally suppress the i.p. ovarian tumor formation.

Analysis of the survival kinetics of nude mice i.p. injected with tumor cells showed that persistent expression of sFlt-1 significantly prolonged survival time in tumor-bearing mice compared with the control. It is conceivable that inhibition of both ascites fluid accumulation and smaller sized disseminations may be responsible for prolongation of survival. This finding is interesting considering a previous report that pretreatment VEGF levels could be predictive of the outcome of ovarian cancers (39).

In view of the efficient inhibition of VEGF activity requiring the continuous presence of its inhibitor, sFlt-1 gene therapy could be a viable approach. AAV vectors can efficiently transduce epithelial cancer cells (40) as well as quiescent fibroblasts and muscle cells (41, 42). Given that many malignant ascites tumors are epithelial in origin, AAV vectors are suitable for this. Another advantage is that a relatively long transgene expression is expected after a single administration of recombinant AAV in vivo (43). In this study, transfer of the sFlt-1 cDNA to ovarian cancer cells resulted in the expression of functional sFlt-1 protein. We are currently evaluating the efficacy of in vivo injection of AAV-sFlt-1.

In summary, we demonstrated that stable sFlt-1 expression resulted in a reduction in the amount of ascites fluid and lengthening of survival in an ovarian cancer model, presumably by inhibiting vascular formation and permeability, and ultimately tumor growth through a mechanism of antagonizing VEGF activity.

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