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Inhibition of Growth and Metastasis of Ovarian Carcinoma by Administering a Drug Capable of Interfering with Vascular Endothelial Growth Factor Activity

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The present study investigates the relationship between *in vivo* growth/metastasis of tumor cells and their capacity to produce the vascular endothelial growth factor (VEGF), as well as the regulation of tumor growth/metastasis using an angiogenesis-inhibitory drug. Two cloned tumor cell lines designated OV-LM and OV-HM were isolated from a murine ovarian carcinoma OV2944. OV-LM and OV-HM cells grew in cultures at comparable rates. However, when transplanted s.c. into syngeneic mice, OV-HM exhibited a faster growth rate and a much higher incidence of metastasis to lymph nodes and lung. Histologically, intense neovascularization was detected in sections of OV-HM but not of OV-LM tumor. OV-HM and OV-LM tumor cells obtained from *in vitro* cultures expressed high and low levels of VEGF mRNA, respectively. A difference in VEGF mRNA expression was much more clearly observed between RNAs prepared from fresh OV-HM and OV-LM tumor masses: RNA from OV-HM contained larger amounts of VEGF mRNA, whereas RNA from OV-LM exhibited only marginal levels of VEGF mRNA. An angiogenesis-inhibitory drug, FR118487 inhibited the VEGF-mediated *in vitro* growth of endothelial cells but did not affect the expression *in vitro* of VEGF mRNA by OV-HM tumor cells. Intraperitoneal injections of FR118487 into mice bearing OV-HM tumors resulted in: (i) a subsequent growth inhibition of primary tumors; (ii) a marked decrease in neovascularization inside tumor masses expressing comparable levels of VEGF mRNA to those detected in control OV-HM masses; and (iii) almost complete inhibition of metastasis to lymph nodes and lung. These results indicate that growth/metastasis of tumor cells correlates with their VEGF-producing capacity and that an angiogenesis inhibitor, FR118487, inhibits tumor growth and metastasis through mechanism(s) including the suppression of VEGF function *in vivo*.

Key words: Tumor metastasis — Neovascularization — Vascular endothelial growth factor — Anti-metastatic effect

Angiogenesis occurs not only in physiological states such as ovulation, menstruation and the development of the placenta, but also in pathological states such as solid tumor growth.[1)] Generation of new vessels in a tumor mass also increases the opportunity for tumor cells to enter the circulation and initiate metastasis.[2–4)] Considerable experimental evidence has indicated that tumor growth and metastasis are closely related to angiogenesis inside a tumor mass.[5–8)]

Tumor angiogenesis is regulated by growth factors that are produced by tumor cells.[5,9)] Several endothelial growth factors have been described as potential mediators of tumor angiogenesis. These include fibroblast growth factors (FGF),[10)] tumor necrosis factor-α (TNF-α), transforming growth factor-β (TGF-β)[11)] and platelet-derived growth factor (PDGF).[12)] However, it is unclear whether any of these agents actually mediates tumor angiogenesis that is involved in solid tumor growth and metastasis *in vivo*. Vascular endothelial growth factor (VEGF) has been found to be an endothelial cell-specific mitogen[13,14)] and is produced by a variety of tumor cells.[15,16)] Moreover, recent studies revealed that VEGF is expressed in human tumors *in situ*[17,18)] and that tumor growth *in vivo* is inhibited by neutralization of VEGF activity using an anti-VEGF monoclonal antibody (mAb).[19)] Thus, it is increasingly evident that VEGF has a critical role in inducing tumor angiogenesis and promoting solid tumor growth.

In this study, we investigated whether the regulation of VEGF expression and/or function leads to the suppression of tumor growth/metastasis in a tumor model in which VEGF has been identified as a prominent tumor

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angiogenesis factor *in vivo*. The results obtained using two cloned ovarian tumor lines, OV-LM and OV-HM, demonstrated that (i) both lines grew at comparable rates in culture, whereas OV-HM exhibited a faster growth rate of primary tumors and a much higher incidence of metastasis to lung and lymph nodes; (ii) the OV-HM tumor mass developed intense neovascularization and expressed high levels of VEGF mRNA compared to the OV-LM mass; and (iii) administration of an angiogenesis-inhibitory drug, FR118487 capable of inhibiting VEGF activity markedly reduced neovascularization, resulting in significant suppression of primary tumor growth as well as almost complete inhibition of metastasis. These results support the concept that VEGF has a critical role in tumor angiogenesis, influencing the primary tumor growth rate and incidence of metastasis, and suggest that an anti-angiogenesis drug capable of inhibiting VEGF activity might be effective for preventing tumor metastasis.

MATERIALS AND METHODS

Tumors An ovarian tumor (OV2944) developed in a female (C57BL/6 × C3H/He) F_1 mouse given a single whole-body neutron irradiation of 2.7 Gy from a ^{252}Cf source.[20] Two lines with high or low metastatic character were isolated from the parental OV2944 tumor.[20] A single cloned line was established from each of the high and low metastatic lines and designated as OV-HM or OV-LM, respectively. As control, CSA1M fibrosarcoma[21] was used.

Mice Female (C57BL/6 × C3H/He) F_1 mice and BALB/c nude mice were obtained from Shizuoka Experimental Animal Laboratory, Hamamatsu and used at 6–9 weeks of age.

An angiogenesis inhibitor, FR118487 A new angiogenesis inhibitor, FR118487 was synthesized by chemical modification of the fermentation products of a fungus, *Scolecobasidium arenarium* (F-2015), at Fujisawa Pharmaceutical Co., Ltd. (Tsukuba).[22] The inhibitory effect of this drug on angiogenesis in rabbit cornea was previously described.[22] For *in vitro* use, it was dissolved in methanol and diluted with PBS. For *in vivo* administration of this drug, 10 mg FR118487 was suspended in a vehicle of 1% ethanol and 5% gum arabic in saline and stirred with a vortex mixer. The mixture was given via the s.c. route.

Determination of tumor growth *in vitro* Tumor cells (1×10^3/well) were cultured in 96-well microplates in RPMI-1640 medium supplemented with 10% fetal bovine serum (FBS) for various times. Culture SNs were removed with paper towels, and crystal violet staining was performed. Color eluted with ethanol in phosphate buffer was measured at 540 n*M* with a microplate reader. Absorbance at A_{540}, representing *in vitro* tumor cell growth, was expressed as the mean of triplicate cultures/group.

Assay for growth inhibition of endothelial cells Endothelial cells (6×10^3/well) from human umbilical vein (HUVE cells) were plated on 96-well microtiter plates coated with gelatin, and cultured in 100 μl of MCDB-107 medium supplemented with 15% FBS in the presence of 5 ng/ml basic FGF (bFGF; R&D systems, Minneapolis, MN) or 25 ng/ml human vascular endothelial cell growth factor (VEGF; Pepro Tech Inc., Rocky Hill, NJ). FR118487 dissolved at various concentrations in methanol was added to the cultures, and the cells were incubated for 120 h at 37°C in 5% CO_2 incubator. At the end of culture, 10 μl of 5 mg/ml 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT, Sigma Chemicals, St. Louis, MO) in PBS was added and the plates were incubated for 6 h. To each well, 150 μl of 0.04 *N* HCl in isopropanol was added to solubilize the reduced MTT, and the absorbance was measured at 550 n*M* using a microplate reader.

cDNA probe and Northern blot analysis A 605 bp fragment of cDNA for human VEGF was kindly provided by Dr. K. Igarashi, Takeda Pharmaceutical Co., Ltd., Osaka, Japan. This fragment of human VEGF cDNA had approximately 90% homology with the corresponding portion of mouse VEGF cDNA, permitting the detection of mouse VEGF mRNA in Northern blotting. Crude total RNAs were extracted from tumor cells cultured *in vitro* or from solid tumor masses by the acid guanidinium thiocyanate-phenol-chloroform method.[23] Total RNAs (10 μg) were electrophoresed on a 1% formaldehyde-agarose gel and transferred onto a nylon membrane. Hybridization was carried out as described by Church and Gilbert[24] using VEGF probe labeled with [α-^{32}P]dCTP by the random primer labeling method.

Histological examination Tumor masses were fixed in 10% formalin, embedded in paraffin, sectioned, and stained with hematoxylin and eosin (HE).

RESULTS

Comparison of growth *in vitro* and *in vivo* as well as metastatic character between OV-LM and OV-HM tumor cells We compared the growth of OV-LM and OV-HM tumor cells when cultured *in vitro* and transplanted s.c. into syngeneic or nude mice. Cells of both tumor lines (1×10^3/well) were cultured in 0.2 ml of RPMI1640 medium supplemented with 10% FBS in 96-well microplates. At various times thereafter, culture SNs were removed, and viable cells remaining in the wells were stained with crystal violet. Ethanol-eluted color representing the number of remaining viable cells was measured (Fig. 1A). The results show that OV-LM and OV-HM cells grow in culture at comparable rates.

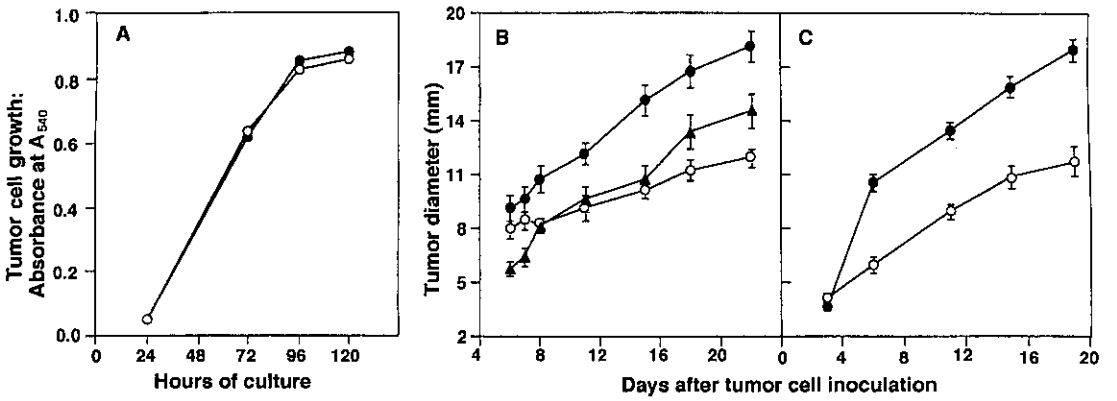

Fig. 1. Growth of OV-LM and OV-HM tumor cells *in vitro* and *in vivo*. Panel A: Cells of OV-LM (○) or OV-HM (●) (1×10^3/well) were cultured *in vitro* in 96-well microplates in 0.2 ml of RPMI1640 plus 10 % FBS. Cell growth (the mean of triplicate cultures/group) was determined as described in "Materials and Methods." Panel B: Various numbers of tumor cells (○, 10^7 OV-LM; ●, 10^7 OV-HM; ▲, 10^6 OV-HM) were inoculated s.c. into syngeneic euthymic mice. Panel C: Five millions of OV-LM (○) or OV-HM (●) tumor cells were inoculated s.c. into athymic BALB/c nude mice. Tumor growth was expressed as the mean diameter±SE of 5 mice/group.

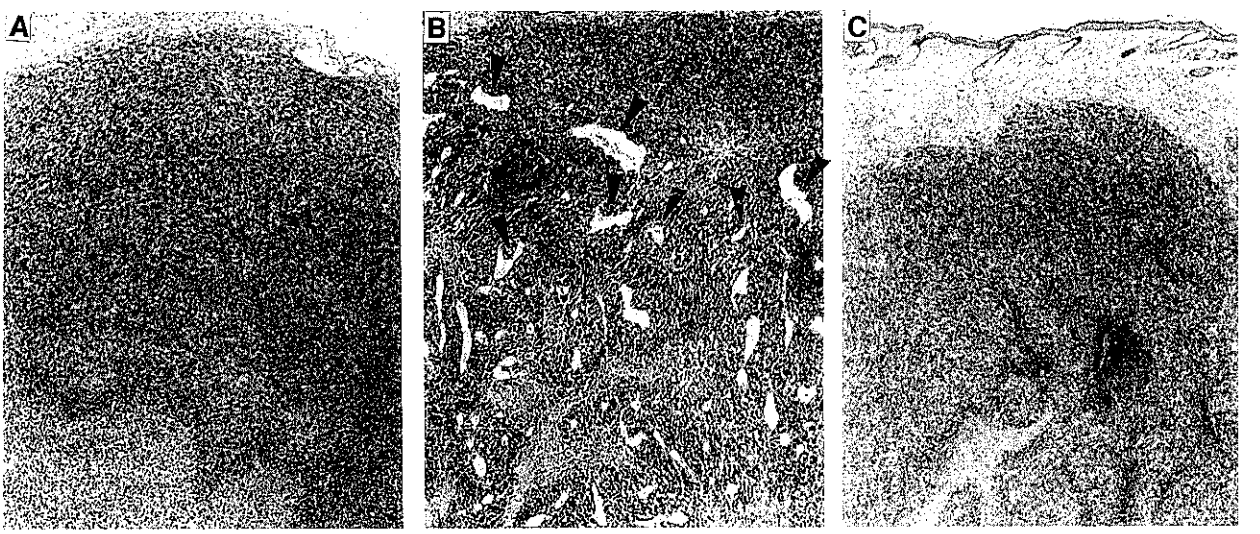

Fig. 2. Light micrographs of OV-LM and OV-HM tumors. B6C3F_1 mice were inoculated with 10^7 OV-LM or 10^6 OV-HM tumor cells. Subcutaneous OV-LM (panel A) and OV-HM tumors (panel B) were removed from the corresponding tumor-bearing mice 3 weeks after tumor implantation. Some sinus-like capillaries are shown by arrows. Panel C: FR118487 (7.5 mg/kg) in vehicle was administered 5 times at 2- to 3-day intervals into OV-HM-bearing mice. Tumor masses were removed 2 days after the fifth injection of FR118487 (3 weeks after tumor implantation).

OV-LM cells (10^7/mouse) or OV-HM cells (10^7 or 10^6/mouse) were inoculated into syngeneic F_1 mice (Fig. 2B). When the same numbers of tumor cells (10^7/mouse) were inoculated, there was a striking difference in the mean tumor size between the two tumor lines. The difference was apparent, but not significant, even at the initial stages of tumor growth. However, it became greater thereafter. In fact, at later stages the size of tumors formed by inoculation of 10^7 OV-LM cells was significantly smaller than that of tumors obtained by inoculation of 10^6 OV-HM cells. Differential growth rates were also observed when the same numbers (5×10^6/mouse) of tumor cells from both lines were inoculated into nude mice (Fig. 1C).

Table I. Comparison of Metastases in OV-LM and OV-HM Tumors

Tumor[a]	Mouse No.	Lymph node metastasis[b]		Lung metastasis[c]
		No. of sites	weight (g)	No. of nodules
OV-HM	1	4	0.4–3.9	16
	2	4	0.5–2.0	12
	3	4	0.4–1.7	5
	4	3	0.3–1.6	3
	5	3	0.4–0.9	0
OV-LM	1	1	1.7	0
	2	1	0.6	0
	3	1	0.3	0
	4	0	0	0
	5	0	0	0

a) Nude mice were inoculated with 5×10^6 OV-HM or OV-LM tumor cells. Tumor resection was performed 20 days after tumor implantation. Mice were killed one month after tumor resection.
b) Metastasis was determined by palpation of lymph nodes at 4 sites (bilateral axillary and inguinal). Palpable lymph nodes were removed and individually weighed. One of the smallest lymph nodes removed from each mouse was subjected to histological examination to confirm that enlargement was due to tumor cell infiltration.
c) Metastatic nodules in both lobes were calculated.

Previous papers have described high and low incidences of metastases from OV-HM and OV-LM tumors, respectively, in syngeneic mice.[20, 25] After confirming this (data not shown), we examined whether differential incidences and/or levels of metastases are also observed in athymic nude recipient mice (Table I). Both lines of tumor cells (5×10^6/mouse) were inoculated s.c. into nude mice. Twenty days after tumor implantation, the primary tumor masses were removed by surgical resection. Upon resection, no palpable lymph node was observed. Metastases to lymph nodes and lung were determined one month later. The results (Table I) show that much higher levels of metastases occur in OV-HM tumors, which is compatible with the findings in syngeneic euthymic mice.[20, 25] Thus, compared to OV-LM, the OV-HM tumor line is characterized by (a) comparable *in vitro* growth, (b) faster growth of the primary tumor and (c) a higher incidence of metastases both to lymph nodes and lung.

Differential capacities of the two tumor lines to induce neovascularization *in vivo* The difference in the size of OV-LM and OV-HM tumors produced even in nude mice (Fig. 1C) implies that this difference is not related to anti-tumor T cell-mediated immunity of the host. To determine the possible involvement of non-immune-mediated mechanisms, histological examination was performed using sections from OV-LM and OV-HM tumor masses. HE-stained tumor sections revealed that intense neovascularization involving the formation of sinus-like capillaries was induced in the OV-HM tumor mass, while only marginal vessels were seen in the OV-LM mass (Fig. 2). These observations are compatible with the concept that the growth rate of a solid tumor mass correlates with the capacity of the tumor cells to induce neovascularization.[5–8]

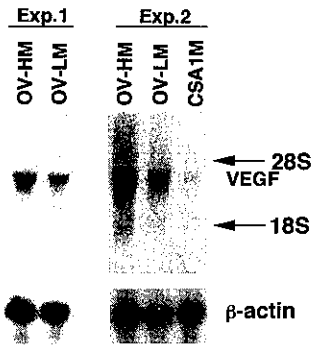

Fig. 3. Expression of VEGF mRNA by OV-LM, OV-HM or CSA1M (control) tumor cells cultured *in vitro*. Total RNA was isolated from tumor cells during *in vitro* cultures and subjected to Northern blot analyses. Northern blots were hybridized with the VEGF probe. Then the probe was removed, and the filter was rehybridized with β-actin cDNA as a probe to confirm that equivalent amounts of RNA had been loaded on the gel.

Differential capacities of the two tumor lines to express VEGF *in vitro* and *in vivo* Recent studies[13–19] have indicated the critical involvement of VEGF in tumor angiogenesis *in vivo*. Therefore, we compared the expression of VEGF in the two tumor lines. Total RNAs were isolated from OV-LM and OV-HM tumor cells during culture *in vitro* and analyzed for the expression of VEGF mRNA. The results (Fig. 3) show that high levels of VEGF mRNA were expressed by OV-HM cells. In contrast, the levels of VEGF mRNA from OV-LM cells were very low and comparable to those from another tumor cell line, CSA1M (BALB/c-derived fibrosarcoma), which has no metastatic character.

To determine the levels of VEGF mRNA expression *in vivo*, total RNAs were isolated from OV-HM and OV-LM tumor masses of similar size (Fig. 4). High levels of VEGF mRNA expression were observed in OV-HM tumor masses from mice one or two weeks after implantation with OV-HM cells. In contrast, the expression of VEGF mRNA was quite weak in OV-LM tumors. The level of VEGF mRNA seen in other non-metastatic tumors, such as CSA1M, was similar to that of OV-LM (data not shown). These results indicate that OV-HM and OV-LM cells have the capacity to express high and low levels of VEGF mRNA, respectively, both when they

are cultured *in vitro* and when they form a tumor mass in syngeneic mice.

The effect of FR118487 on the activity and mRNA expression of VEGF FR118487 has been developed as a new angiogenesis-inhibitory drug.[21] We first examined the effect of FR118487 on the VEGF-induced growth of endothelial cells in comparison with that observed on the bFGF-induced growth. Endothelial cells from HUVE were cultured with either 25 ng/ml VEGF or 5 ng/ml bFGF in the presence of various concentrations of FR118487. As shown in Fig. 5, FR118487 inhibited the growth of HUVE cells induced by bFGF, as well as VEGF, in a dose-dependent manner.

We next determined whether this drug modulates the expression of VEGF mRNA by OV-HM cells *in vitro* and *in vivo*. Various concentrations of FR118487 were added to cultures of OV-HM tumor cells. This drug at concentrations of less than 10 μg/ml had no effect on the growth of tumor cells as determined by microscopic observations and ^{3}H-TdR uptake (data not shown). The *in vitro* expression of VEGF mRNA was not inhibited by addition of FR118487 (Fig. 6A).

FR118487 contained in the vehicle was administered to OV-HM-bearing mice 3 times at 2-day intervals. RNAs were prepared from tumor masses of mice 2 days after the final inoculation of the drug. The results (Fig. 6B) show that *in vivo* administration of FR118487 did not affect *in vivo* VEGF expression in the tumor masses. Thus, an angiogenesis-inhibitory drug, FR118487, modulates the activity of endothelial cell growth factors, including VEGF, but does not inhibit the expression of VEGF.

The effect of FR118487 on tumor growth and tumor metastasis We finally investigated whether *in vivo* administration of FR118487 can inhibit the growth of the

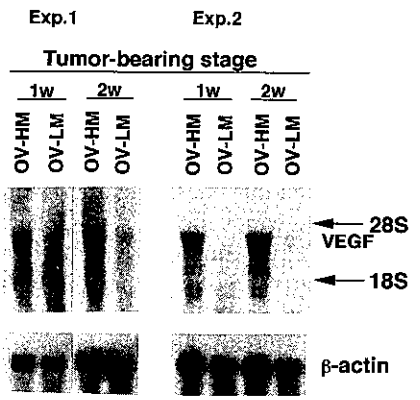

Fig. 4. Expression of VEGF mRNA in OV-LM and OV-HM tumor masses. B6C3F$_1$ mice were inoculated with 10^7 OV-LM or 10^6 OV-HM tumor cells. Tumor masses were removed 1 or 2 weeks after tumor implantation.

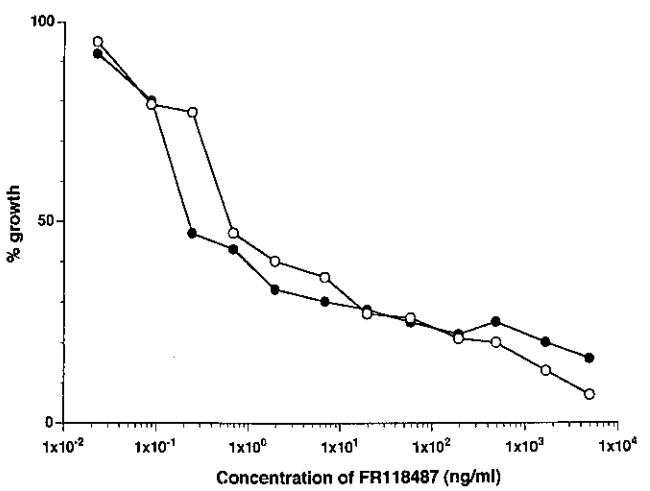

Fig. 5. Effect of FR118487 on the VEGF- or bFGF-induced growth of HUVE cells. HUVE cells (6×10^3 cells/well) were cultured with 25 ng/ml of VEGF (○) or 5 ng/ml of bFGF (●) in the presence of the indicated concentrations of FR118487 for 120 h. The growth of HUVE cells was determined by MTT assay ("Materials and Methods") and expressed as % growth using the mean value of triplicate cultures/group. % growth = [the mean value in the presence of FR118487/ the mean value in the absence of FR118487] × 100.

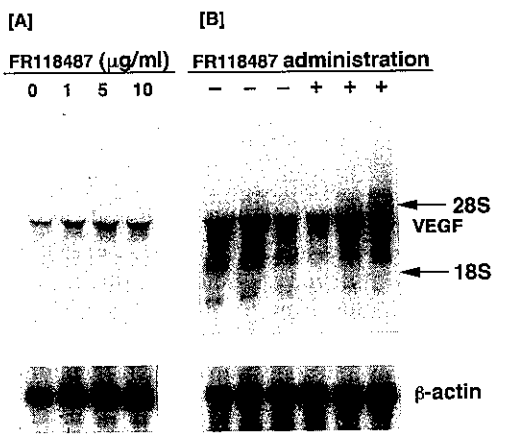

Fig. 6. Effect of FR118487 on the expression *in vitro* and *in vivo* of VEGF mRNA by OV-HM cells. OV-HM cells were cultured with various doses of FR118487 for 24 h (panel A). Starting 7 days after OV-HM cell inoculation, three i.p. injections of FR118487 in vehicle were given to OV-HM-bearing mice at 2-day intervals. RNA was individually prepared from each tumor mass of 3 untreated or 3 treated mice 2 days after the final FR118487 injection (panel B).

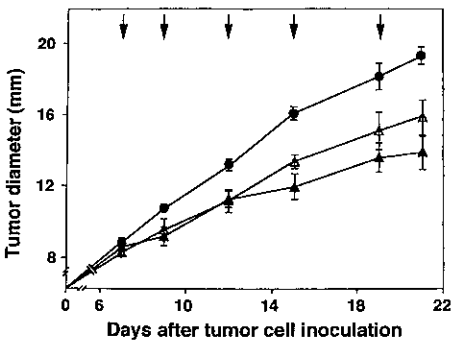

Fig. 7. Effect of FR118487 on the growth of a primary tumor. FR118487 (△, 5.0 mg/kg; ▲, 7.5 mg/kg) in vehicle was given 5 times at 2- to 3-day intervals (as indicated) to OV-HM-bearing mice. ● indicates vehicle alone. Tumor growth was expressed as the mean tumor diameter±SE of 5 mice/group.

Table II. Inhibitory Effect of FR118487 on Tumor Metastasis and Intraperitoneal Invasion

Exp.	Dose (mg/kg) of FR118487[a)]	Metastasis[b)] to		Intraperitoneal invasion[b)]
		lymph node	lung	
1	0.0	4/5	3/5	3/5
	5.0	2/5	0/5	0/5
	7.5	1/5	0/5	0/5
2	0.0	8/8	5/8	8/8
	5.0	3/8	0/8	0/8
	7.5	1/8	0/8	0/8

a) Mice were inoculated s.c. with 10^6 OV-HM tumor cells. Administration of FR118487 was started on day 8 after tumor implantation and performed 6 (Exp. 1) or 10 times (Exp. 2) at 2- to 3-day intervals.
b) Incidence of metastasis and intraperitoneal invasion was determined 44 days after tumor implantation.

primary tumor, as well as the development of tumor metastasis. FR118487 was administered to OV-HM-bearing mice 5 times at 2- to 3-day intervals (Fig. 7). This treatment induced significant, albeit incomplete, inhibition of tumor growth. Two days after the final treatment, tumor masses from some of the mice in each group were removed and examined histologically to evaluate the degree of neovascularization. Sections of tumor masses from FR118487-untreated mice again revealed intense neovascularization (data not shown), which was essentially the same as that in Fig. 2B. In contrast, the tumor masses from mice treated with FR118487 (7.5 mg/kg, 5 times) exhibited strikingly reduced neovascularization (Fig. 2C).

We also determined the incidence of tumor metastasis in mice which received the *in vivo* treatment with FR118487 (Table II). The results show that metastasis to lymph nodes was markedly inhibited by the FR118487 treatment, although the inhibition was not complete. This treatment, however, induced complete inhibition of lung metastasis and intraperitoneal invasion. Taken collectively, these results indicate that FR118487 is capable of inhibiting the activity of endothelial growth factors; consequently it suppresses neovascularization in tumor masses, and strikingly reduces the incidence of tumor metastasis especially to the lungs.

DISCUSSION

Experimental evidence has indicated that the growth of a tumor beyond a certain size is dependent on angiogenesis.[1,5] Tumor cell metastasis occurs through a series of steps, the first of which involves angiogenesis inside the tumor mass.[5-8] Thus, tumor neovascularization is the crucial process for survival of a primary tumor, as well as for initiation of metastasis, and the control of this critical process could be a key to effective therapy for malignancies.

This study was designed to investigate whether the expression of a particular type of angiogenesis factor by two tumor sublines correlates with the growth of the primary tumor and with metastasis, and if so, whether controlling the expression and/or function of this factor *in vivo* could lead to a reduction of primary tumor growth, as well as a decrease in the incidence of metastasis. The results demonstrated that OV-HM and OV-LM sublines derived from the same parental ovarian tumor line exhibited comparable rates of *in vitro* growth, whereas these sublines exhibited rapid and slow growth *in vivo* as well as high and low incidence of metastasis, respectively. This property of OV-HM was found to be associated with potent expression *in vitro* and *in vivo* of VEGF mRNA and intense neovascularization in tumor masses. The results also showed that a recently developed anti-angiogenesis drug, FR118487, with the capacity to inhibit VEGF activity, induced moderate inhibition of primary tumor growth and almost complete suppression of metastasis, especially to the lungs.

Several potential mediators of tumor angiogenesis have been described, including basic and acidic FGF,[10] TNF-α, TGF-β,[11] PDGF[12] and VEGF.[13,14] Among these, VEGF has been recognized as an actual mediator of angiogenesis and tumor growth *in vivo*, because (i) its mitogenic activity is limited to endothelial cells;[13,14] (ii) VEGF mRNA is found in primary tumors;[17,18] and (iii) neutralization of VEGF by *in vivo* administration of anti-VEGF mAb results in the suppression of tumor growth *in vivo*.[19] These observations imply that VEGF critically contributes to tumor angiogenesis and tumor growth/metastasis *in vivo*. This concept was strengthened

by experiments which demonstrated that the regulation of VEGF expression and/or function leads to the simultaneous inhibition of tumor angiogenesis and metastasis.

The role of VEGF in promoting tumor angiogenesis, primary tumor growth and tumor metastasis was investigated here using two clones (OV-HM and OV-LM) derived from the same parental ovarian tumor line. These tumor clones were originally separated based on the capacity to metastasize mainly to lung when inoculated s.c.[20] However, their differential growth *in vitro* and *in vivo* was not examined in the original study.[20] In this report, we showed that they had comparable growth rates in *in vitro* cultures containing 10% FBS, whereas their *in vivo* growth greatly differed. This suggested that the difference in tumor growth *in vivo* is produced by some mechanisms related to the host-tumor interaction. Tumor growth *in vivo* is, in general, influenced by the host anti-tumor immune response. Therefore, the differential *in vivo* tumor growth may be explained by a difference in the magnitude of the immune response against OV-HM and OV-LM. In the present system, however, this is unlikely because the difference in primary tumor growth was also seen in nude mice. The induction of neovascularization mediated by tumor-derived factors is required for promotion of tumor growth[5-8] and this represents another mechanism of host-tumor interaction. In fact, histological analyses of OV-HM and OV-LM tumor masses revealed a striking difference in the intensity of neovascularization. Moreover, the OV-HM tumor mass, forming intense neovascularization, was found to express high levels of VEGF mRNA, indicating a close correlation between tumor growth and intratumoral VEGF expression in the present system.

It is possible that endothelial growth factors other than VEGF are also involved in angiogenesis or growth promotion of endothelial cells in the present tumor models. For example, we have found that culture SNs from OV-HM and OV-LM tumor cells contained activities capable of slightly supporting the growth of both an EGF-dependent endothelial-like cell line (MSS-31)[26] and a bFGF-dependent cell line (Sc-3)[27] (our unpublished observations). Although the activities of EGF and bFGF detected in the OV-HM SN were significantly higher than those in the OV-LM SN, the magnitude of the difference in activities was less than 2-fold. Moreover, we failed to detect mRNA expression for these factors in the tumor masses (our unpublished observations). Therefore, it is difficult to explain the difference in neovascularization between OV-HM and OV-LM tumor masses mainly in terms of these growth factors, although partial involvement is not excluded.

A more important aspect of the present study concerns the analyses of regulation of tumor angiogenesis by an anti-angiogenesis drug. Fumagillin is known for its potent anti-angiogenesis activity in a chorioallantoic membrane model, as well as for its ability to inhibit tumor neovascularization. However, difficulty in its clinical use has been reported due to severe complications.[28,29] Therefore, much effort has been made to develop synthetic analogs of fumagillin with less toxicity. Recently, a synthetic analog termed TNP-470 was prepared,[30,31] and the inhibitory effect of this drug on tumor angiogenesis and growth/metastasis has been reported.[32-34] Another fumagillin analog, FR118487, was more recently developed and shown to have potent anti-angiogenesis activity.[22] The results of a previous study[35] and ours demonstrated that both synthetic fumagillin analogs are capable of inhibiting the activity of bFGF, as well as VEGF, which is considered to be critical for tumor angiogenesis *in vivo*. Further studies will be required to investigate whether FR118487 inhibits the activity and/or production of endothelial growth factors other than VEGF and FGF.

Various tumor cell lines, including the OV-HM cells used here, express VEGF.[16,36,37] However, the cell type(s) responsible for the production of VEGF is not restricted to tumor cells. VEGF is also produced by macrophages.[17,38] Therefore, it is possible that high levels of VEGF expression *in vivo* observed for the OV-HM line are due not only to OV-HM tumor cells, but also to macrophages infiltrating the tumor masses, if any. Nevertheless, it is conceivable that FR118487 suppresses VEGF activity *in vivo*, irrespective of which types of cells produce VEGF.

Although administration of FR118487 resulted in growth inhibition of primary OV-HM, the inhibition was not potent. The rate of OV-HM growth in mice receiving this drug was comparable to or slightly higher than that of OV-LM in mice not receiving the drug. OV-LM cells grow *in vivo* in association with barely detectable levels of neovascularization. Thus, inhibition of neovascularization in the OV-HM tumor mass by FR118487 did not induce complete growth suppression of primary OV-HM masses, but allowed them to grow at comparable rates to those observed in OV-LM without drug. The most beneficial effect of the drug was the inhibition of metastasis, especially to the lungs, and intraperitoneal invasion. This may be interpreted according to the concept that tumor metastasis to the lungs proceeds through invasion of tumor cells into blood vessels and the fact that FR118487 induces a striking inhibition of new vessel formation at the tumor site.

Our results illustrate that in two ovarian carcinoma sublines, higher levels of VEGF expression are found in the more intensely vascularized tumor and that such a tumor exhibits a higher growth rate as well as a higher incidence of metastasis. This correlation is supported by

the fact that an anti-angiogenesis drug can strikingly reduce the VEGF function and neovascularization, and thereby inhibit not only the growth of the primary tumor, but also tumor metastasis. Thus, the treatment of hosts with an anti-angiogenesis drug, in combination with immunotherapy aimed at enhanced anti-tumor immunity, could provide an integrated anti-tumor/anti-metastatic approach.

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