Bevacizumab and rapamycin inhibit tumor growth in peritoneal model of human ovarian cancer

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Abstract

Ovarian cancer is the leading cause of death from gynecologic cancer. Often, the disease has spread beyond the ovary to involve the peritoneal cavity and causes ascites. Whereas mammalian target of rapamycin (mTOR) functions to regulate protein translation, cell cycle progression, and metastasis, vascular endothelial growth factor promotes tumor angiogenesis, ascites formation, and metastasis in ovarian cancer. In this study, an i.p. model of human ovarian cancer was used to determine the antitumor activity of rapamycin, bevacizumab, and rapamycin plus bevacizumab (BEV/RAPA). We report that administration of rapamycin, bevacizumab, and BEV/RAPA in mice bearing peritoneal OV-90 ovarian carcinoma resulted in 74.6%, 82.4%, and 93.3% reduction in i.p. tumor burden, respectively. BEV/RAPA-induced reduction in microvessel density and inhibition of cell proliferation were associated with significant reduction in hypoxia-inducible factor-1α and cyclin D1 and inactivation of downstream targets of mTOR, p70S6 kinase, S6R, and 4E-binding protein 1. BEV/RAPA treatment was not only able to prolong life of i.p. mice but also more effective than rapamycin and bevacizumab to prevent the development of peritoneal carcinomatosis in adjuvant setting and reverse ascites accumulation in heavy peritoneal disease. Our data indicate that simultaneous inhibition of the vascular endothelial growth factor receptor and mTOR pathways with BEV/RAPA or their analogues may represent a novel approach for prevention of metastasis, recurrence, and treatment of ovarian cancer. [Mol Cancer Ther 2007;6(11):2959–66]

Received 4/4/07; revised 7/22/07; accepted 9/17/07.

Grant support: National Medical Research Council of Singapore grant NMRC/0887/2004 (H. Huynh).

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doi:10.1158/1535-7163.MCT-07-0237

Introduction

Peritoneal carcinomatosis is a frequent cause of death with advanced ovarian cancer (1). Often, the disease has spread beyond the ovary to involve the peritoneal cavity (2). The 5-year survival rate of patients with peritoneal carcinomatosis is only 2%, including patients with i.p. free cancer cells without peritoneal carcinomatosis (3).

The formation of malignant ascites in patients with advanced-stage cancer is an important cause of morbidity and mortality in patients with peritoneal carcinomatosis arising from colorectal, gastric, pancreatic, endometrial, and ovarian cancers (4). Ovarian carcinoma is characterized by rapid growth of solid i.p. tumors and production of large volumes of ascites. Both tumor size and the accumulation of ascites are inversely associated with survival (1, 5). Among women with stage III or IV epithelial ovarian carcinoma, development of ascites correlates with significantly decreased 5-year survival rate (5% and 45% with and without ascites, respectively; ref. 6).

During the most part of its natural course, ovarian cancer remains confined to the peritoneal cavity. This offers the possibility to administer cytotoxic agents directly into the peritoneal cavity, providing high concentration of the drug at the site of tumor for a longer period and ideally low systemic toxicity (7). The mainstay of treatment in most cases of diagnosed ovarian cancer is debulking surgery. However, disease recurrence in the peritoneal cavity is common and will eventually lead to demise of the patient. Postoperative i.p. chemotherapy with a combination of a platinum-based analogue and a taxane has been used to prevent the recurrence. Several studies have shown that cisplatin and paclitaxel had efficacy in prolonging progression-free and overall survival (8–12). Despite advances in surgical cytoreduction and cytotoxic chemotherapy, the prognosis for patients with ovarian cancer remains poor (13). Therefore, new and effective treatments for ovarian cancer are needed.

To design molecularly targeted therapy for ovarian cancer, it is important to understand the molecular mechanisms that promote the development of this disease. The possible role of vascular endothelial growth factor (VEGF) in ovarian cancer has received much attention because VEGF increases vascular permeability and enhances angiogenesis (14). Overexpression of VEGF has been reported in ovarian cancer (1, 15, 16) and several studies have indicated that VEGF-regulated angiogenesis is an important component of ovarian cancer growth (17, 18). Microvessel density and level of VEGF expression in ovarian cancer directly correlate with poor prognosis, suggesting that angiogenesis, possibly mediated at least in part by VEGF, influences disease progression (17, 18). Inhibition of VEGF production by the drug albendazole

suppressed the *in vivo* growth and metastasis of OVCAR-3 peritoneal tumor (18).

Aberrantly high phosphatidylinositol 3-kinase (PI3K)-dependent signaling has been implicated in a wide range of human cancers, and importantly, PI3K-stimulated oncogenesis is dependent on mammalian target of rapamycin (mTOR; ref. 19). The genomic region containing the gene coding for the p110 α subunit of PI3K has been identified in 40% of the cases of ovarian cancer (20). The dual function of phosphatase that negatively regulates PI3K, phosphatase and tensin homologue deleted on chromosome 10, is mutated, silenced, or deleted in several tumor types (reviewed in ref. 21). Loss of phosphatase and tensin homologue deleted on chromosome 10 activity leads to an increase in 3-phosphoinositide concentration and subsequent activation of Akt and consequently mTOR signaling (19). Activated mTOR regulates protein translation through phosphorylation of ribosomal protein S6 kinase, eukaryotic initiation factor 4E-binding protein 1 (4E-BP1), and p70S6 kinase (S6K1). It has been reported that S6K1 is overexpressed or constitutively active in the early stages of transformation in ovarian surface epithelium with BRCA1 mutations (22). Recent studies have found that the growth and proliferation of tumors displaying aberrantly high PI3K-dependent signaling, either through constitutive activation of PI3K or Akt or via inactivation of phosphatase and tensin homologue deleted on chromosome 10, displayed enhanced sensitivity to rapamycin and analogues (reviewed in ref. 23) and mTOR activation is important for the secretion of VEGF, which is a potent pro-oncogenic factor that increases the growth of blood vessels near tumors (24). Because inhibition of mTOR produces only modest effects on the rates of cell proliferation in most cell types, it is likely that treating patients with a combination of mTOR inhibitors plus other therapeutic agents may be the best approach for treating cancer in the clinic.

Because VEGF plays an important role in malignant ascites formation, angiogenesis, and ovarian tumor growth (17, 18) and mTOR pathway regulates VEGF expression in cancer cells (24–26), we investigated if combined targeting of VEGF protein and expression by rapamycin plus bevacizumab (BEV/RAPA) treatment would inhibit ovarian tumor growth and prolong life of mice bearing i.p. tumors. To that end, we used the human ovarian cancer cell lines SKOV-3 and OV-90 to develop an *in vivo* model of ovarian cancer in *SCID* mice that recapitulated the i.p. carcinomatosis and ascites production seen in women with this disease. We show that BEV/RAPA treatment inhibited i.p. tumor burden and prolonged survival to a significantly greater degree than rapamycin or bevacizumab monotherapy.

Materials and Methods

Reagents

Anti-p70S6 kinase, anti–cleaved caspase-3, anti-mTOR, anti-S6R, and anti-4E-BP1 antibodies and phosphorylation-specific antibodies against p70S6 kinase at Thr^{421}/Ser^{424}, p70S6 kinase at Thr^{389}, S6R at $Ser^{235/236}$, 4E-BP1 at Thr^{70}, Akt at Ser^{473}, and extracellular signal-regulated kinase 1/2 at Thr^{202}/Tyr^{204} were obtained from Cell Signaling Technology. The antibodies against cyclin D1, extracellular signal-regulated kinase 1/2, CXCR-4, hypoxia-inducible factor-1α (HIF-1α), and α-tubulin were from Santa Cruz Biotechnology, Inc. CD31/platelet/endothelial cell adhesion molecule 1, VEGF, and Ki-67 antibodies were from Lab Vision.

Tumorigenicity in *SCID* Mice

The study received ethics board approval at the National Cancer Centre of Singapore as well as the Singapore General Hospital. Female *SCID* mice of 9 to 10 weeks of age were purchased from the Animal Resources Centre (Canning Vale, West Australia, Australia). All mice were maintained according to the ''Guide for the Care and Use of Laboratory Animals'' published by NIH. They were provided with sterilized food and water *ad libitum* and housed in negative pressure isolators with 12-h light/dark cycles.

OV-90 and OV-SK3 ovarian carcinoma cells were obtained from the American Type Culture Collection. They were maintained in MEM (Life Technologies) supplemented with 15% fetal bovine serum (growth medium). To create the i.p. tumor model of ovarian cancer, female mice were injected i.p. with 5×10^6 OV-90 or SKOV-3 cells in 200 μL PBS for all the experiments.

Dose-Response Study

Two weeks after peritoneal inoculation of OV-90 ovarian cells, mice bearing i.p. OV-90 tumors were treated with 0.5, 1, 1.5, and 2 mg/kg/d rapamycin (sirolimus, Wyeth Pharmaceuticals Co.) or 2.5, 5, 7.5, and 10 mg/kg/2 weeks bevacizumab (Avastin, Genentech, Inc.) or 5 mg/kg bevacizumab plus four doses (0.5, 1, 1.5, and 2 mg/kg/d) of rapamycin. Each treatment group was consisted of five mice. Mice were sacrificed on day 28 during the treatments. I.p. tumor burden was determined and expressed as percent of control.

To investigate the effects of bevacizumab and rapamycin on i.p. tumor burden and ascites formation, mice bearing i.p. OV-90 (or SKOV-3) tumors were divided into four treatment groups and each consisted of 14 mice. They were treated with either 100 μL saline or 5 mg/kg bevacizumab i.p. weekly or p.o. given 1 mg/kg rapamycin daily or BEV/RAPA for 4 weeks. Bevacizumab, rapamycin, and BEV/RAPA were given immediately (for adjuvant therapy) or 2 weeks after peritoneal inoculation of ovarian cells (for treatment). To monitor the extent of the development of peritoneal carcinomatosis, the body weights of the mice were routinely measured. In all the experiments, all the mice were sacrificed and necropsied when >80% of the mice in the vehicle-treated group became moribund (approximately 42 to 50 days after injection of tumor cells). The end points that were measured included the volume of ascites present and the tumor load that was quantitated by (*a*) the total number of tumor nodules, (*b*) total weight of all excised tumor nodules and omentum, and (*c*) tumor dissemination to other organs including liver.

Table 1. Effects of rapamycin, bevacizumab, and BEV/RAPA on body weight, i.p. tumor burden, ascites formation, cell proliferation, microvessel density, VEGF expression, and apoptosis of OV-90 and SKOV-3 ovarian cancer cells

Ovarian cancer cells	Treatments	Body weight (g)	No. i.p. tumor/mouse	I.p. tumor burden (mg)	Ascites formation		Microvessel density	Ki-67 index (%)	Cleaved caspase-3 (%)	VEGF expression
					Incidence (%)	Volume (mL)				
OV-90	Vehicle	24.6 ± 1.3	16 ± 8[a]	2,610 ± 306[a]	10/10 (100)	5.8 ± 2.2	16.8 ± 3.2[a]	28.6 ± 3.9[a]	2.1 ± 0.9	Strong
	Bevacizumab	22.4 ± 1.1	10 ± 3[a]	459 ± 72[b]	0/10 (0)	ND	6.3 ± 2.7[b]	9.5 ± 2.1[b]	3.4 ± 0.8	Strong
	Rapamycin	24.9 ± 1.2	11 ± 4[a]	664 ± 90[b]	1/10 (10)	0.5	8.4 ± 1.9[b]	14.6 ± 3.1[b]	2.8 ± 0.8	Average
	BEV/RAPA	24.3 ± 1.3	5 ± 3[b]	176 ± 29[c]	0/10 (0)	ND	4.2 ± 1.0[b]	4.2 ± 1.8[c]	3.6 ± 0.9	Weak
SK-OV3	Vehicle	24.4 ± 0.9	15 ± 6[a]	1,863 ± 290[a]	9/9 (100)	5.6 ± 1.9	14.1 ± 2.9[a]	21.6 ± 3.8[a]	4.5 ± 0.8	Average
	Bevacizumab	22.4 ± 1.1	9 ± 3[a]	867 ± 128[b]	0/9 (0)	ND	5.1 ± 1.3[b]	9.9 ± 1.7[b]	5.7 ± 0.9	Average
	Rapamycin	23.8 ± 1.4	10 ± 3[a]	968 ± 141[b]	2/9 (22)	0.5–0.7	8.0 ± 1.5[c]	13.4 ± 2.1[b]	4.1 ± 0.7	Average
	BEV/RAPA	26.2 ± 0.8	4 ± 2[b]	139 ± 48[c]	0/9 (0)	ND	4.9 ± 1.4[b]	5.4 ± 1.8[c]	5.9 ± 1.2	Weak

NOTE: Effects of rapamycin, bevacizumab, and BEV/RAPA on body weight at sacrifice, i.p. tumor burden, microvessel density, cell proliferation, ascites formation, VEGF expression, and apoptosis of OV-90 and SK-OV3 ovarian cells. Female *SCID* mice were i.p. injected with 5×10^6 OV-90 or SKOV-3 cells in 200 μL PBS. Mice bearing i.p. tumors were randomized to one of four treatment groups and each one was consisted of 14 mice. They were treated with vehicle, bevacizumab (5 mg/kg), rapamycin (1 mg/kg), or BEV/RAPA as described in Materials and Methods. Treatment started 2 wk after inoculation of tumor cells. Ascites formation was monitored thrice weekly. The mice were sacrificed and necropsied on day 50 after injection of tumor cells. By this time, the mice in the vehicle-treated group became moribund. The presence of ascitic fluid, macroscopic peritoneal tumor dissemination, and i.p. tumor burden was recorded. Mean vessel density, VEGF expression, Ki-67 index, and apoptosis in the tumors were determined by immunohistochemical staining with antibodies against CD31, VEGF, Ki-67, and cleaved caspase-3, respectively. VEGF expression was scored as described in Materials and Methods. Differences in i.p. tumor burden, microvessel density, percentage of Ki-67–positive cells, ascites formation, and percentage of cleaved caspase-3–positive cells between vehicle-, bevacizumab-, rapamycin-, and BEV/RAPA-treated groups were analyzed by ANOVA. Treatments with different letters are significantly different from one another ($P < 0.01$).

To determine if BEV/RAPA treatment was able to prolong survival of i.p. mice, mice were injected with 5×10^6 OV-90 cells. Two weeks after peritoneal inoculation of ovarian cancer cells, mice were divided into four treatment groups and each group was consisted of 14 mice. They were treated with bevacizumab, rapamycin, and BEV/RAPA for 16 weeks as described above. Survival was evaluated by the Kaplan-Meier method and compared by log-rank test. The study was repeated at least twice.

To determine if the BEV/RAPA treatment was able to reverse ascites accumulation, the ascites was partially drained from the vehicle-treated mice on day 42 after injection of tumor cells. They were divided into two groups (14 mice per group) and treated with either vehicle or BEV/RAPA for 4 weeks as described above.

Western Blot Analysis

To determine changes in indicated proteins, three random selected tumors per treatment group were analyzed separately. Tumors were homogenized separately in buffer containing 20 mmol Tris (pH 7.5), 150 mmol NaCl, 1 mmol EDTA, 1 mmol EGTA, 1% Triton X-100, 2.5 mmol sodium pyrophosphate, 1 mmol β-glycerolphosphate, 2 mmol Na_3VO_4, 1 μg/mL leupeptin, and 1 mmol phenylmethylsulfonyl fluoride. One hundred micrograms of tumor lysate from a single mouse were subjected to Western blot analysis as described previously (27). Blots were incubated with indicated primary antibodies and 1:7,500 horseradish peroxidase–conjugated secondary antibodies. All primary antibodies were used at a final concentration of 1 μg/mL. The blots were then visualized with a chemiluminescent detection system (Amersham) as described by the manufacturer.

Immunohistochemical Studies

Tumor tissues from organ nodules were fixed and paraffin embedded. Sections (5 μm) were cut, dewaxed, rehydrated, and subjected to antigen retrieval. After blocking endogenous peroxidase activity and reducing nonspecific background staining, the sections were incubated with the primary antibodies against CD31/platelet/endothelial cell adhesion molecule 1, VEGF, cleaved caspase-3, and Ki-67 (overnight at 4°C). Immunohistochemistry was done using the streptavidin-biotin peroxidase complex method according to the manufacturer's instructions (Lab Vision). Sections known to stain positively were incubated in each batch and negative controls were also prepared by replacing the primary antibody with preimmune sera. For Ki-67, only nuclear immunoreactivity was considered positive. Ki-67 index and caspase-3–positive cells were the number of labeled cells among at least 500 cells per region and then expressing them as percentage values. For the quantification of mean vessel density in sections stained for CD31, 10 random 0.159 mm^2 fields at ×100 magnification were captured for each tumor and microvessels were quantified.

For VEGF expression, the immunostaining was scored using the formula IS = (i + 1) * PI, where i = intensity staining varying between 1+ and 3+ and PI = % of positive cells. At least 20 high-power fields were chosen randomly, and 2,000 cells were counted. Weak, average, and strong staining of VEGF expression in carcinoma cells was considered when the IS was in between 20 and 100, 104 and 240, and 244 and 400, respectively.

To study tumor hypoxia, mice bearing i.p. OV-90 tumors (two vehicle-treated, two bevacizumab-treated, two rapamycin-treated, and two BEV/RAPA-treated mice) were i.p.

injected with 60 mg/kg body weight Hypoxyprobe-1 (Chemicon International). Ninety minutes after the injection of Hypoxyprobe-1, tumors were excised and fixed in formalin. Immunostaining of Hypoxyprobe-1 adducts in formalin-fixed tumors was carried out using Hypoxyprobe-1MAb1 as described by the manufacturer.

Determination of VEGF and Interleukin-8

Ascites was centrifuged at 3000 × *g* for 10 min at 4°C and stored at −80°C until analysis. The concentration of VEGF and interleukin-8 (IL-8) in ascites was measured using Endogen Human VEGF (Pierce Biotechnology) and IL-8 (Hycult Biotechnology) ELISA kits as described by the manufacturer. The absorbance at 450 nm was measured using an ELISA plate reader (Benchmark Plus microplate spectrophotometer, Bio-Rad). The sensitivity for VEGF and IL-8 kits was <8.0 and <5 pg/mL, respectively.

Statistical Analysis

Tumor burden, VEGF expression, IL-8 concentration, Ki-67 index, mean vessel density, and caspase-3–positive cells were compared by ANOVA. Survival analysis was computed by the Kaplan-Meier method and compared by log-rank test.

Results

On i.p. inoculation of *SCID* mice, the OV-90 and SK-OV3 cells efficiently produced peritoneal carcinomatosis. We took advantage of this model and tested the effect of bevacizumab, rapamycin, and BEV/RAPA on the development of experimental peritoneal carcinomatosis in mice. Table 1 shows that 100% i.p. mice receiving vehicle treatment developed ascites. A swollen abdomen, indicative of ascites formation and heavily peritoneal carcinomatosis, was observed within 6 to 8 weeks of OV-90 or SK-OV3 cell inoculation. All vehicle-treated animals were killed at 10 weeks because of severe cachexia associated with the developing ascites in accordance with the animal care protocol. Six to eight milliliters of ascitic fluid could be collected per vehicle-treated mouse. One rapamycin-treated OV-90 and two SK-OV3 i.p. mice exhibited mild ascites at 10 and 12 weeks, respectively. None of the bevacizumab- or BEV/RAPA-treated mice showed signs of ascites formation or cachexia (Table 1). We next determined the levels of VEGF and IL-8 in ascites by ELISA. As shown in Supplementary Fig. S1,[3] high levels of VEGF and IL-8 were detected in ascites collected from vehicle-treated mice. VEGF, but not IL-8, secretion was significantly reduced by rapamycin therapy ($P < 0.01$).

Postmortem examination revealed a widespread dissemination of tumor mass in the peritoneal cavity of vehicle-treated mice. Tumors were found on the surface of the peritoneum, diaphragm, intestines, uterus, associated fat, and stomach. Average number of i.p. tumors per mouse per treatment group was shown in Table 1. A significant decrease in the tumor mass was observed in rapamycin-, bevacizumab-, and BEV/RAPA-treated groups compared with the control ($P < 0.01$; Table 1). The best effect was observed in BEV/RAPA group (Table 1). Interestingly, bevacizumab also inhibited the growth of small tumors at the site of OV-90 and SKOV-3 cell injection that developed in some mice (data not shown). Our dose-response data revealed that 5 mg/kg/2 weeks bevacizumab and 1 mg/d rapamycin inhibited tumor growth by approximately 48 ± 12% and 45 ± 13%, respectively (Supplementary Table S1).[3] We used a graph of equally effective dose pairs for a single effect level (isobologram) as described by Tallarida (28) to assess whether BEV/RAPA was additive or synergistic. As shown in Supplementary Fig. S2,[3] the dose pair, such as point A (5 mg bevacizumab plus 0.5 mg rapamycin) or point B (5 mg bevacizumab plus 0.25 mg rapamycin), attained 50% effect with greater quantities and was therefore subadditive.

We next examined the expression of HIF-1α, cyclin D1, CXCR-4, Akt, extracellular signal-regulated kinase 1/2, and downstream targets of mTOR, p70S6 kinase, S6R, and 4E-BP1 in vehicle- and drug-treated tumors. Figure 1A shows that bevacizumab monotherapy slightly increased Akt phosphorylation ($P < 0.05$). Rapamycin and BEV/RAPA, but not bevacizumab, slightly reduced extracellular signal-regulated kinase 1/2 phosphorylation (Fig. 1A). Whereas CXCR-4 expression was not altered by any treatments, expression of cyclin D1 and HIF-1α was significantly inhibited by rapamycin and BEV/RAPA ($P < 0.01$). The levels of phosphorylated p70S6 kinase at Thr^{421}/Ser^{424} and Thr^{389}, phosphorylated S6R at $Ser^{235/236}$, and phosphorylated 4E-BP1 at Thr^{70} in tumors derived from mice treated with rapamycin and BEV/RAPA, but not bevacizumab, were significantly reduced ($P < 0.01$). Phosphorylation of p70S6 at Thr^{389} and 4E-BP1 at Thr^{70} was inhibited by the BEV/RAPA treatment to a significantly greater degree than rapamycin monotherapy. Figure 1B shows that whereas all mice in the vehicle-, bevacizumab-, and rapamycin-treated groups were moribund at days 74, 108, and 98, respectively, all BEV/RAPA-treated mice were still alive at day 124. These results indicate that the BEV/RAPA treatment is able to prolong the survival of i.p. mice.

To examine the effects of rapamycin, bevacizumab, and BEV/RAPA on cellular proliferation and apoptosis *in vivo*, sections from drug-treated tumors were stained with Ki-67 and cleaved caspase-3 antibodies. The Ki-67 labeling index in rapamycin- and BEV-treated OV-90 and SK-OV3 tumors was significantly decreased compared with vehicle-treated tumors ($P < 0.01$; Table 1). Further decrease in the number of cells stained with Ki-67 antibody was observed in tumors treated with BEV/RAPA ($P < 0.01$; Table 1). The percentage of cells stained for cleaved caspase-3 was not significantly increased in bevacizumab- and BEV/RAPA-treated tumors (Table 1), suggesting that these treatments also caused mild apoptosis. These results support the view that the antitumor effects of bevacizumab, rapamycin, and BEV/RAPA are associated with inhibition of cell proliferation.

[3] Supplementary materials for this article are available at Molecular Cancer Therapeutics Online (http://mct.aacrjournals.org/).

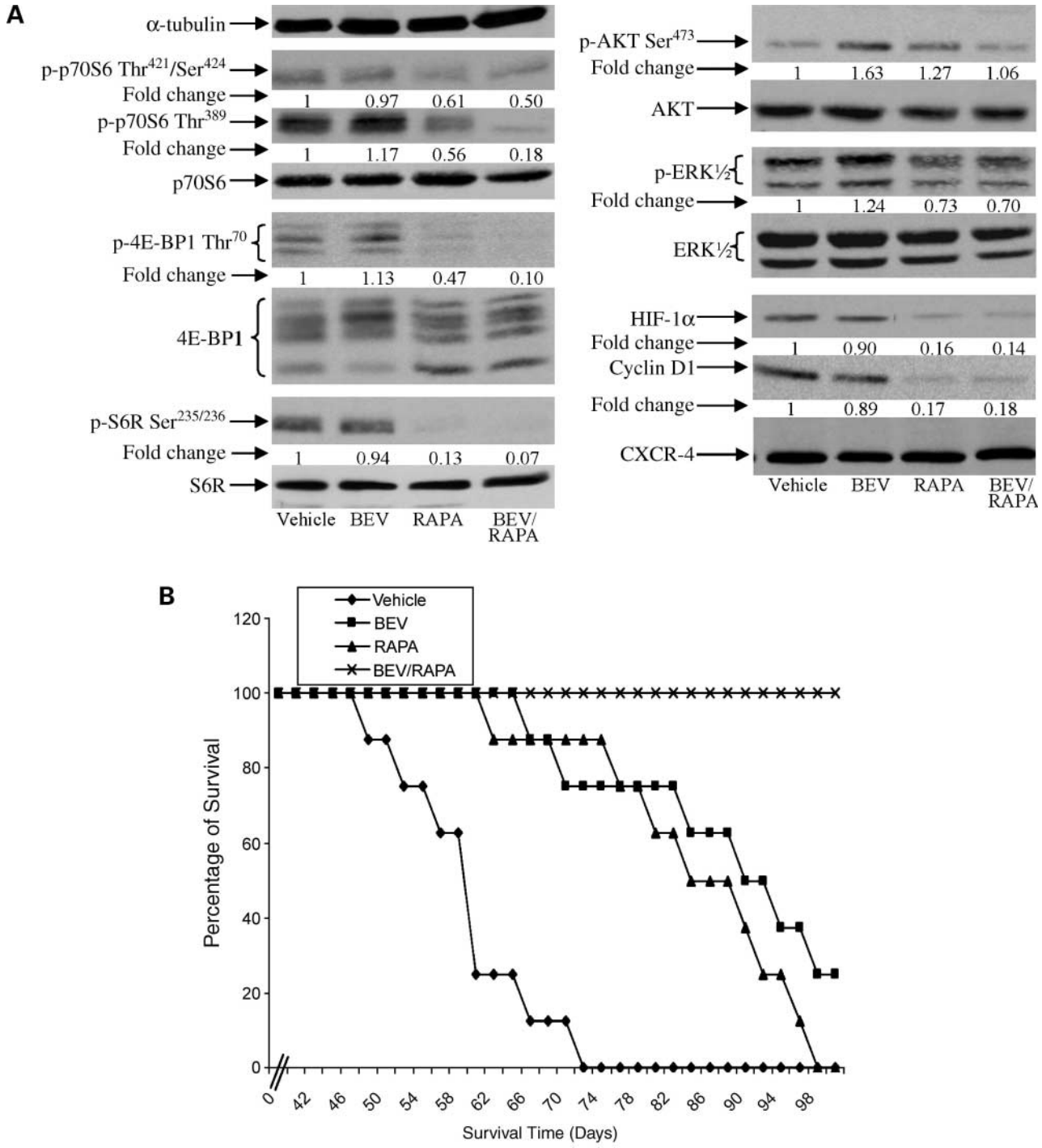

Figure 1. Effects of rapamycin (*RAPA*), bevacizumab (*BEV*), and BEV/RAPA on expression of HIF-1α, cyclin D1, and CXCR-4, phosphorylation of Akt and extracellular signal-regulated kinase 1/2 (*ERK1/2*), activation of downstream targets of mTOR, and survival of i.p. mice. Female *SCID* mice were i.p. injected with 5×10^6 OV-90 or SKOV-3 cells in 200 μL PBS. Mice bearing i.p. tumors were randomized to one of four treatment groups and each group was consisted of 14 mice. They were treated with vehicle, bevacizumab (5 mg/kg), rapamycin (1 mg/kg), or BEV/RAPA as described in Materials and Methods. Treatment started 2 weeks after inoculation of tumor cells and lasted for 4 wk. Survival was monitored thrice weekly. The mice were sacrificed and necropsied on day 50 when vehicle-treated mice became moribund. To determine changes in indicated proteins, three random selected tumors per treatment group were analyzed separately. Each lane represents tumor lysate from a single mouse. Western blot analysis was done as described in Materials and Methods. **A,** representative blots and changes in the expression of indicated proteins. Differences in the expression of indicated proteins between vehicle-, bevacizumab-, rapamycin-, and BEV/RAPA-treated groups were analyzed by ANOVA ($P < 0.01$). Phosphorylation of p70S6 at Thr^{389} and 4E-BP1 at Thr^{70} was inhibited by the BEV/RAPA treatment to a significantly greater degree than rapamycin monotherapy. For survival experiments, mice were treated for 16 wk as described above. **B,** survival was evaluated by the Kaplan-Meier method. Note that bevacizumab, rapamycin, and BEV/RAPA significantly prolong the survival of i.p. mice as determined by log-rank test ($P < 0.01$). Experiments were repeated at least twice with similar results.

We next examined the level of tissue hypoxia in the residual tumors after treatment. The association between antitumor activity of rapamycin, bevacizumab, and BEV/RAPA and their ability to inhibit VEGF expression and blood vessel formation in OV-90 and OV-SK3 tumors was also investigated. As shown in Supplementary Fig. S3,[3] the hypoxic cells in the center of vehicle-treated OV-90 tumors were positively stained for Hypoxyprobe-1. Less hypoxic cells were observed in drug-treated tumors. VEGF expression was decreased by BEV/RAPA and to a lesser extent by rapamycin (Table 1; Supplementary Fig. S4).[3] The median number of blood vessels from bevacizumab-, rapamycin-, and BEV/RAPA-treated tumors was significantly reduced compared with vehicle-treated ones ($P < 0.01$; Table 1), suggesting that rapamycin also inhibits angiogenesis. BEV/RAPA treatment had the lowest number of blood vessels per unit area.

To determine whether combined BEV/RAPA was also effective in adjuvant setting, mice were i.p. injected with OV-90 cells. Treatments started on the same day of tumor cell injection. As shown in Table 2, all the control mice developed peritoneal disease and ascites as expected. No ascites was found in all the treated mice, although peritoneal disease was detected in 100% of bevacizumab-treated and 80% of rapamycin-treated mice, respectively. Only 40% of mice treated with the combination of both drugs did not develop any peritoneal disease, and in the ones who did, the tumor burden was significantly reduced when compared with the other treatment groups (Table 2).

To determine if the combined BEV/RAPA treatment was able to reverse ascites accumulation, the ascites was partially drained from mice bearing i.p. tumor on day 42 after inoculation of OV-90 cells (Fig. 2A). They were then divided into two groups: vehicle treated and BEV/RAPA treated. As expected, the control i.p. mice rapidly developed ascites and became cachectic within 4 to 6 days after draining of ascites (Fig. 2B). All of these animals had high i.p. tumor burden when sacrificed 1 week later. All mice treated with a combination of BEV/RAPA recovered after 2 weeks of treatment and seemed normal at the time of postmortem examination (6 weeks after treatment) with no ascites detected. I.p. tumor burden was significantly reduced compared with that of vehicle-treated animals (killed 5 weeks ago; Fig. 2C, vehicle versus BEV/RAPA). The number of tumor nodules and total i.p. tumor burden between vehicle- and BEV/RAPA-treated groups was statistically significant at $P < 0.01$ as determined by ANOVA.

Discussion

Ovarian cancer has often spread beyond the ovary to involve the peritoneal cavity at the time of diagnosis (2) and associated with malignant ascites formation (29–31). Debulking surgery followed by i.v. chemotherapy may allow clinical remission of the disease but disease recurrence in the peritoneal cavity is common and will eventually lead to demise of the patient. In the present study, we report that ovarian cancer xenografts growing in *SCID* mice are inhibited by bevacizumab and rapamycin. Both rapamycin and bevacizumab as a single treatment have a defined effect on the inhibition of tumor growth, tumor cell dissemination, and prolongation of survival. When combined with rapamycin, however, bevacizumab reduces tumor growth by ~94% and significantly prolongs survival. Indeed, the combination of BEV/RAPA induces a significant decrease in the number of proliferating cells, the mean of Ki-67–positive cells, the mean vessel density, and ascites formation and inhibited tumor cells from disseminating to internal organs to a greater degree than single-agent therapy; all these are associated with prolongation of survival compared with control and monotherapy. The BEV/RAPA combined protocol not only prevented tumor development in 40% of mice but also induced an additive effect, including inhibition of phosphorylation of S6R at $Ser^{235/236}$ and p70S6 at Thr^{389} (Fig. 1A) and reduction in VEGF expression (Supplementary Fig. S4).[3] In addition, hypoxic cells and expression of hypoxic inducible genes, such as *VEGF* and *HIF-1α*, are significantly reduced in both rapamycin- and BEV/RAPA-treated mice ($P < 0.01$). These observations agree with a previous study (32) showing that rapamycin enhanced proteolytic degradation of HIF-1α, a transcription that drives VEGF expression.

Table 2. Effects of bevacizumab, rapamycin, and BEV/RAPA on body weight, tumor incidence, i.p. tumor burden, and ascites formation on prevention model of OV-90 ovarian cancer in *SCID* mice

Treatments	Body weight (g)	Tumor incidence (%)	I.p. tumor burden (mg)	Ascites formation	
				Incidence (%)	Volume (mL)
Vehicle ($n = 5$)	23.6 ± 1.4	5/5 (100)	2,304 ± 360[a]	5/5 (100)	6.7 ± 1.4
Bevacizumab, 5 mg/kg ($n = 5$)	21.7 ± 0.9	5/5 (100)	902 ± 147[b]	0/5 (0)	ND
Rapamycin, 1 mg/kg ($n = 5$)	22.5 ± 0.9	4/5 (80)	850 ± 168[b]	0/5 (0)	ND
BEV/RAPA ($n = 5$)	22.8 ± 1.1	3/5 (60)	216 ± 75[c]	0/5 (0)	ND

NOTE: Effects of rapamycin, bevacizumab, and BEV/RAPA on body weight at sacrifice, tumor incidence, i.p. tumor burden, and ascites formation on adjuvant model of OV-90 ovarian cancer in *SCID* mice. Female *SCID* mice were i.p. injected with 5×10^6 OV-90 cells in 200 μL PBS. Mice bearing i.p. tumors were randomized to one of four treatment groups and each treatment group was consisted of five mice. They were treated with vehicle, bevacizumab, rapamycin, or BEV/RAPA as described in Fig. 1. Treatment started 12 h after peritoneal inoculation of ovarian cells. Survival and ascites formation were monitored thrice weekly. The mice were sacrificed and necropsied when they became moribund. The presence of ascitic fluid, macroscopic peritoneal tumor dissemination, and i.p. tumor burden was recorded. Treatments with different letters are significantly different from one another ($P < 0.01$) as analyzed by ANOVA.

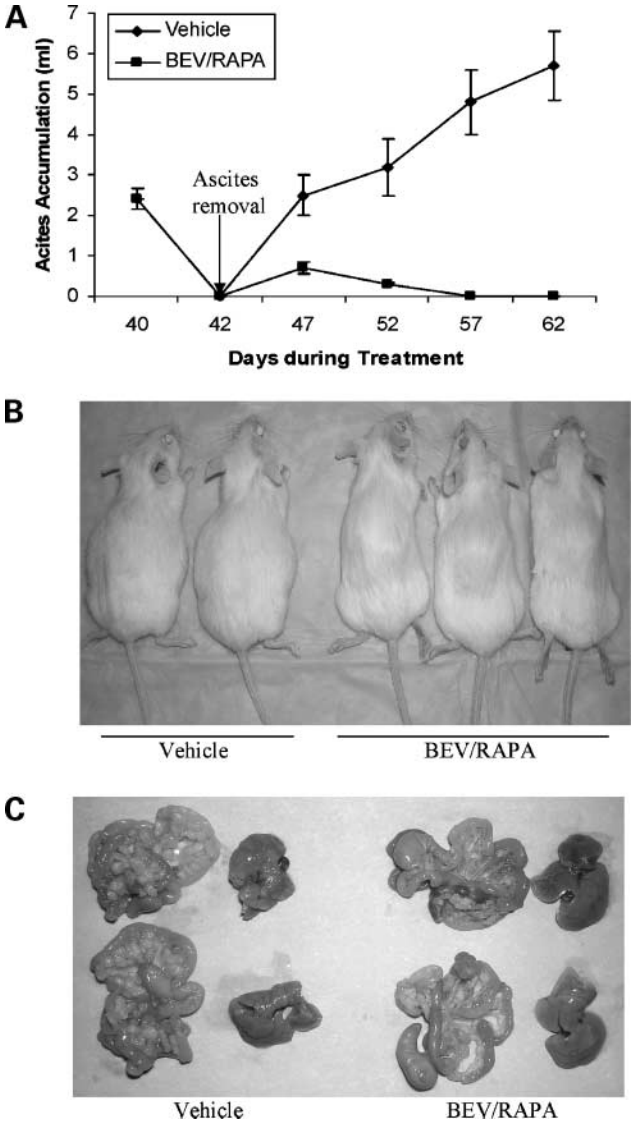

Figure 2. BEV/RAPA therapy prevented ascites accumulation and caused tumor regression in mice bearing i.p. OV-90 tumors. Female *SCID* mice were i.p. injected with 5×10^6 OV-90 cells as described above. For therapeutic experiments, the ascites was partially drained from the i.p. mice on day 42. They were divided into two groups and each group was consisted of 14 mice. They were treated with either vehicle or BEV/RAPA for 4 wk. **A** and **B,** note that the control i.p. mice rapidly developed ascites and became cachectic on day 67. **C,** representative omental tumors in the peritoneal cavity of vehicle- and BEV/RAPA-treated mice. All mice treated with a combination of BEV/RAPA recovered after 2 weeks of treatment and seemed normal at the time of postmortem examination (4 wk after treatment) with no ascites detected. Number of tumors and i.p. tumor burden were significantly reduced compared with that of vehicle-treated animals as determined by ANOVA ($P < 0.01$).

Our present study shows that neither bevacizumab nor rapamycin alone is able to inhibit the formation of tumor sheets on the peritoneal surfaces or the formation of small solid tumor foci as seen in both OV-90 and SKOV-3 i.p. models when given 2 weeks after i.p. inoculation of cells. I.p. administration of BEV/RAPA is effective to shrink the established tumors but not able to eradicate them. Few small tumors are still seen in BEV/RAPA-treated mice. It is possible that these small tumors survive by diffusion of nutrients from underlying host vasculature and the surrounding peritoneal fluid. Finally, i.p. administration of BEV/RAPA has some efficacy effective in inhibiting the peritoneal disease when given early on in the disease process, suggesting that this drug combination has a definite role in the adjuvant setting. This is promising as it can potentially be used to reducing disease recurrence in the peritoneal cavity after debulking surgery. Clinical trial is needed to assess whether BEV/RAPA is more efficacious in prolonging progression-free and overall survival than golden standard i.p. chemotherapy administration of cisplatin and paclitaxel (11).

The mechanisms by which BEV/RAPA suppresses the development of peritoneal carcinomatosis and ascites formation in xenograft models of ovarian cancer in *SCID* mice remain to be illustrated. It has been reported that tumor-derived CXCL12 synergizes VEGF-mediated neovascularization of multifocal i.p. dissemination in human ovarian carcinomas, and VEGF also up-regulates CXCR-4 expression on vascular endothelial cells and tumor cells (33). The interaction between VEGF, CXCR-4, and CXCL12 has been reported to be relevant to the development of peritoneal metastasis (34, 35). Hypoxia synchronously induces CXCL12 and VEGF production by ovarian cancers (35) and also induces CXCR-4 expression in cancer cells (36). In the present study, we do not detect any changes in CXCR-4 (Fig. 1A) and CXCL-12 (data not shown) expression. Apart from inhibition of cyclin D1 and inactivation of downstream targets of mTOR, such as p70S6, S6R, and 4E-BP1, BEV/RAPA may also mediate its effect through antiangiogenic activity related to impaired VEGF activity and VEGF production as reported previously (37, 38). Indeed, tumors from BEV/RAPA-treated mice express lowest levels of VEGF (Supplementary Fig. S4)[3] and have the lowest number of blood vessels per unit area (Table 1).

The formation of malignant ascites, as part of the continuum of the malignant process, represents a poor prognosis in advanced peritoneal carcinomatosis (29–31). With the progression of cancer to the stage of ascites formation, quality of life and survival become limited (39). One approach to the control of malignant ascites is to limit the formation of ascites by affecting the causative malignant cells in the peritoneal cavity. Various chemotherapeutic agents have had limited success in reducing malignant ascites when used systematically or i.p. (40). In the present study, inhibition of tumor-derived VEGF activity by bevacizumab and VEGF production by rapamycin effectively inhibit the development of ascites in an ovarian model of peritoneal carcinomatosis. The observation that BEV/RAPA is able to reverse ascites accumulation when given to mice exhibiting abdominal swelling has clinical implications, as it suggests that inhibition of VEGF and mTOR activity may reverse the accumulation of ascitic fluid in women with ovarian cancer, which could significantly contribute to treatment of the disease. The absence of ascites formation and low remnant tumor burden

after giving this drug combination to mice with peritoneal disease suggest that BEV/RAPA may have a role in reducing tumor burden in a clinical setting. This is promising as it can potentially be used to downstage peritoneal disease in patients who present at the initial setting with heavy peritoneal disease to a situation that may be amenable to surgery. In mice with obvious advanced peritoneal disease, the instillation of the BEV/RAPA is effective in the prevention of further ascites formation after initial drainage. It would be attractive to consider therapy with this combination of drugs in a palliative setting, where absence of ascites and the problems associated with it (e.g., abdominal distension and discomfort, bowel symptoms, and dyspnea) may contribute to a better quality of life in the terminal stages of advanced ovarian cancer.

In summary, we have shown that simultaneous inhibition of angiogenesis and mTOR pathways with bevacizumab and rapamycin significantly prolongs life and suppresses the development of peritoneal carcinomatosis and ascites formation in xenograft models of ovarian cancer in mice. This combined therapy may be useful for the treatment of peritoneal carcinomatosis and may also be a novel, efficient strategy for reducing recurrence of ovarian cancers.

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Mol Cancer Ther 2007;6:2959-2966.