Maintenance Treatment with Bevacizumab Prolongs Survival in an In vivo Ovarian Cancer Model

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Abstract

Purpose: Vascular endothelial growth factor (VEGF) plays a central role in tumor angiogenesis and is regarded as a promising therapeutic target. We hypothesized that treatment with bevacizumab, a humanized recombinant anti-VEGF monoclonal antibody, could enhance antitumor response to cisplatin and prolong survival in a murine ovarian cancer model.

Experimental Design: We conducted an MTS assay to examine the effect of bevacizumab on proliferation of the VEGF producing human ovarian cancer cell lines in vitro. Next, the antiangiogenic activity of bevacizumab was investigated by in vivo angiogenesis and wound healing assays. We then determined the toxicity and antitumor response of bevacizumab and cisplatin as single agents or in combination in xenograft models of ovarian cancer. Finally, using the same xenograft model, we examined the effect of these regimens, as well as bevacizumab maintenance therapy, on survival.

Results: Bevacizumab had no effect on the proliferation of ovarian cancer cells in vitro but significantly inhibited angiogenesis and delayed wound healing in vivo. Bevacizumab inhibited i.p. tumor growth and ascites production in the nu/nu mouse xenograft model and enhanced the therapeutic efficacy of cisplatin. Combination therapy with bevacizumab and cisplatin for 3 weeks was associated with complete disappearance of all macroscopic evidence of disease. Moreover, maintenance treatment with bevacizumab after 3 weeks of induction combination therapy inhibited recurrence and significantly prolonged survival.

Conclusions: Bevacizumab has significant antitumor activity not only as a single agent or in combination with cisplatin but may also prolong survival when used as maintenance therapy after a complete response to cisplatin-based chemotherapy.

Ovarian cancer is characterized by widespread i.p. carcinomatosis and voluminous ascites. Standard primary treatment for ovarian cancer involves surgical cytoreduction (1) followed by systemic chemotherapy, typically with six cycles of a taxane combined with platinum agent (2). Although ovarian cancer is increasingly recognized as a chronic disease, most patients in complete clinical remission after primary treatment will relapse within 18 months (3). One potential strategy that may improve patient outcome is maintenance therapy targeting residual subclinical disease (4). Using conventional cytotoxic anticancer agents, several clinical studies have been conducted to evaluate the benefit of maintenance therapy for ovarian cancer patients (1, 5, 6) but no study has shown prolongation of overall survival.

There is a strong rationale for the use of angiogenesis-targeted agents in the maintenance therapy setting. Tumor growth and metastasis have been shown to be dependent on angiogenesis (7); thus, angiogenesis has been recognized as an attractive target for anticancer therapy in general (8). Because it has been reported that tumors require a vascular blood supply to grow beyond 2 to 3 mm (9), persistent subclinical ovarian tumors present after complete clinical response to first-line chemotherapy should require angiogenesis for continued proliferation, invasion, and metastasis.

A variety of proteins have been identified as potential targets of antiangiogenesis therapy (10). One of the potent mediators of angiogenesis is vascular endothelial growth factor (VEGF), which can promote proliferation, survival, and migration of endothelial cells and potentially induce microvascular permeability (11). VEGF can also affect new vessel formation in tumors by acting as a chemoattractant for bone marrow–derived progenitor cells (12). The major physiologic stimulus for VEGF...
expression is hypoxia, which commonly develops within tumors when cancer cell proliferation exceeds the rate of blood vessel formation (13).

Because it has been previously reported that VEGF is overexpressed in ovarian tumors (14–16), the VEGF pathway holds promise as a target in the therapy of ovarian cancer. Preclinical studies have shown that inhibition of VEGF activity by a monoclonal antibody to human VEGF (A4.6.1) significantly inhibited ovarian tumor growth and ascites formation and enhanced the sensitivity to paclitaxel in an athymic mouse model of ovarian cancer (17, 18). Bevacizumab is a humanized form of A4.6.1; this agent is now used for treatment of a variety of human cancers (19).

In 2004, the U.S. Food and Drug Administration approved bevacizumab for use in combination with first-line chemotherapy for advanced colorectal cancer. This followed from a randomized clinical trial demonstrating a statistically significant improvement in progression-free and overall survival (20). Bevacizumab has also been prospectively examined in the phase II trials for the patients with recurrent ovarian cancer (21, 22) and has shown significant single-agent activity.

In part, to address the importance of maintenance therapy using bevacizumab, the Gynecologic Oncology Group is conducting a phase III clinical trial (23), of which the results will not be available for several years. To date, however, no preclinical study has examined the effect of bevacizumab in the maintenance therapy setting to prevent or delay the recurrence of ovarian cancer. Moreover, no preclinical study has examined whether the anti-VEGF antibody can enhance the efficacy of cisplatin-based chemotherapy in an in vivo ovarian cancer model.

In the current study, we have examined whether treatment with bevacizumab enhances antitumor response to cisplatin in vivo and whether bevacizumab treatment after a clinical complete response to cisplatin can prolong survival in an in vivo ovarian cancer model.

Materials and Methods

Reagents/antibodies. Cisplatin was obtained from Bristol Myers K.K. Bevacizumab was purchased from Roche. Enhanced chemiluminescence Western blotting detection reagents were from Perkin-Elmer. Anti-VEGF antibody was obtained from Santa Cruz Biotechnology. Anti–β-actin antibody was obtained from Sigma. The cell titer 96-well proliferation assay kit was obtained from Promega.

Drug preparation. For animal studies, 5 mg/kg bevacizumab were diluted in 200 μL of PBS just before administration. For in vitro analysis, bevacizumab was diluted to the appropriate concentration in PBS before addition to cell cultures.

Cell culture. The human ovarian cancer cell lines A2780 and Caov-3 were obtained from American Type Culture Collection. A2780 and Caov-3 cells were cultured in DMEM with 10% fetal bovine serum, as described previously (24). To expose cells to hypoxia, cells were placed in an incubator, AMP-36 (ASTEC), which was infused with a mixture of 1% O₂, 5% CO₂, and 94% N₂ and incubated at 37°C.

Cell proliferation assay. An MTS [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt] assay was used to analyze the effect of bevacizumab on cell viability as described (25). Cells were cultured overnight in 96-well plates (1 × 10⁴ cells per well). Cell viability was assessed after 72 h of exposure to bevacizumab at the indicated concentrations. The number of surviving cells was assessed by the determination of A₄₉₀ nm of the dissolved formazan product after addition of MTS for 1 h, as described by the manufacturer (Promega). Cell viability is expressed as follows:

\[
\text{A}_{\text{exp}} \div \text{A}_{\text{control}} \times 100
\]

Western blot analysis. Cells were incubated under nonhypoxic or hypoxic conditions for 16 h. After incubation, cells were washed twice with ice-cold PBS and lysed in lysis buffer [20 mmol/L Tris-HCl, 150 mmol/L NaCl, 1 mmol/L EDTA, 1 mmol/L EGTA, 1 mmol/L Na₂VO₄, 1 mmol/L β-glycerophosphate, 2.5 mmol/L sodium pyrophosphate, 1 mmol/L 4-(2-aminoethyl)benzenesulfonyl fluoride hydrochloride, 10 μg/mL aprotinin, 1 μg/mL leupeptin, and 1% Triton X-100] for 10 min at 4°C. Lysates were centrifuged at 12,000 × g at 4°C for 15 min, and protein concentrations of the supernatants were determined using Bio-Rad protein assay reagent. Equal amounts of proteins were separated by SDS-PAGE and transferred to nitrocellulose membranes. Blocking was done in 5% nonfat milk in 1 × TBS. Western blot analyses were done with various specific primary antibodies. Immunoblots were visualized with horseradish peroxidase–coupled goat anti-rabbit or anti-mouse immunoglobulin by using the enhanced chemiluminescence Western blotting system (Perkin-Elmer).

Directed in vivo angiogenesis assay. All procedures involving animals and their care were approved by the Institutional Animal Care and Usage Committee of Osaka University in accordance with Japanese government guidelines for animal experiments. The procedure for the directed in vivo angiogenesis assay has been described previously (26, 27). Angiogenic response to either VEGF or bevacizumab was quantified using a directed in vivo angiogenesis assay kit ( Trevigen, Inc.) according to the manufacturer’s instructions. Briefly, basement membrane extract with or without 500 ng/mL VEGF was placed into angioreactors and incubated at 37°C for 1 h to allow gelling. Then, the angioreactors were implanted into the dorsal flank of 5-wk-old to 7-wk-old nude mice (n = 8). The mice were randomized into two treatment groups receiving PBS or 5 mg/kg bevacizumab i.p. twice weekly for 2 wk. After the mice were maintained for 2 wk, the angioreactors were implanted into the dorsal flank of 5-wk-old to 7-wk-old nude mice (n = 8). The mice were randomized into two treatment groups receiving PBS or 5 mg/kg bevacizumab i.p. twice weekly for 2 wk. After the mice were maintained for 2 wk, the angioreactors were implanted into the dorsal flank of 5-wk-old to 7-wk-old nude mice (n = 8). The mice were randomized into two treatment groups receiving PBS or 5 mg/kg bevacizumab i.p. twice weekly for 2 wk. After the mice were maintained for 2 wk, the angioreactors were implanted into the dorsal flank of 5-wk-old to 7-wk-old nude mice (n = 8). The mice were randomized into two treatment groups receiving PBS or 5 mg/kg bevacizumab i.p. twice weekly for 2 wk. After the mice were maintained for 2 wk, the angioreactors were implanted into the dorsal flank of 5-wk-old to 7-wk-old nude mice (n = 8). The mice were randomized into two treatment groups receiving PBS or 5 mg/kg bevacizumab i.p. twice weekly for 2 wk. After the mice were maintained for 2 wk, the angioreactors were implanted into the dorsal flank of 5-wk-old to 7-wk-old nude mice (n = 8). The mice were randomized into two treatment groups receiving PBS or 5 mg/kg bevacizumab i.p. twice weekly for 2 wk. After the mice were maintained for 2 wk, the angioreactors were implanted into the dorsal flank of 5-wk-old to 7-wk-old nude mice (n = 8). The mice were randomized into two treatment groups receiving PBS or 5 mg/kg bevacizumab i.p. twice weekly for 2 wk. After the mice were maintained for 2 wk, the angioreactors were implanted into the dorsal flank of 5-wk-old to 7-wk-old nude mice (n = 8). The mice were randomized into two treatment groups receiving PBS or 5 mg/kg bevacizumab i.p. twice weekly for 2 wk. After the mice were maintained for 2 wk, the angioreactors were implanted into the dorsal flank of 5-wk-old to 7-wk-old nude mice (n = 8). The mice were randomized into two treatment groups receiving PBS or 5 mg/kg bevacizumab i.p. twice weekly for 2 wk. After the mice were maintained for 2 wk, the angioreactors were implanted into the dorsal flank of 5-wk-old to 7-wk-old nude mice (n = 8). The mice were randomized into two treatment groups receiving PBS or 5 mg/kg bevacizumab i.p. twice weekly for 2 wk. After the mice were maintained for 2 wk, the angioreactors were implanted into the dorsal flank of 5-wk-old to 7-wk-old nude mice (n = 8). The mice were randomized into two treatment groups receiving PBS or 5 mg/kg bevacizumab i.p. twice weekly for 2 wk. After the mice were maintained for 2 wk, the angioreactors were implanted into the dorsal flank of 5-wk-old to 7-wk-old nude mice (n = 8). The mice were randomized into two treatment groups receiving PBS or 5 mg/kg bevacizumab i.p. twice weekly for 2 wk. After the mice were maintained for 2 wk, the angioreactors were implanted into the dorsal flank of 5-wk-old to 7-wk-old nude mice (n = 8). The mice were randomized into two treatment groups receiving PBS or 5 mg/kg bevacizumab i.p. twice weekly for 2 wk. After the mice were maintained for 2 wk, the angioreactors were implanted into the dorsal flank of 5-wk-old to 7-wk-old nude mice (n = 8). The mice were randomized into two treatment groups receiving PBS or 5 mg/kg bevacizumab i.p. twice weekly for 2 wk. After the mice were maintained for 2 wk, the angioreactors were implanted into the dorsal flank of 5-wk-old to 7-wk-old nude mice (n = 8). The mice were randomized into two treatment groups receiving PBS or 5 mg/kg bevacizumab i.p. twice weekly for 2 wk. After the mice were maintained for 2 wk, the angioreactors were implanted into the dorsal flank of 5-wk-old to 7-wk-old nude mice (n = 8). The mice were randomized into two treatment groups receiving PBS or 5 mg/kg bevacizumab i.p. twice weekly for 2 wk. After the mice were maintained for 2 wk, the angioreactors were implanted into the dorsal flank of 5-wk-old to 7-wk-old nude mice (n = 8).
were removed. Basement membrane extract was collected from angioreactors, digested, and centrifuged. The pellets containing the invaded endothelial cells were washed and then labeled with FITC-lectin at 4°C overnight. After washing, the fluorescence was measured in 96-well plates using a microplate spectrophluorometer (excitation 485 nm, emission 510 nm).

**Wound healing assay.** Surgical wounds were placed on the dorsal flanks of each athymic mouse (n = 8) 2 weeks after the inoculation of 5 × 10⁵ A2780 cells, as reported previously (28, 29). Briefly, after sterile preparation of the skin with 70% ethanol, full-thickness radial excisional skin wounds of 6-mm diameter were made aseptically on both sides of the dorsal flank. Two wounds were created on the same animal. Then, the mice were randomized into two treatment groups receiving PBS or 5mg/kg bevacizumab i.p. twice weekly. Wound size (in two dimensions) was recorded daily.

**I.p. xenograft model.** Initial experiments were conducted to examine the antiangiogenic activity of bevacizumab and the toxicity of both bevacizumab and cisplatin. To examine toxicity, 24 nude mice were assigned into six treatment groups, each consisting of four mice as shown in Fig. 3. The first group was treated with PBS twice weekly for 6 wk. The second group was treated with bevacizumab twice weekly for 6 wk. The third group was treated with cisplatin weekly for 3 wk followed by treatment with PBS twice weekly for 3 wk. The fourth group was treated with cisplatin weekly for 3 wk followed by treatment with bevacizumab twice weekly for 3 wk. The fifth group was treated with bevacizumab twice weekly plus cisplatin weekly for 3 wk followed by the treatment with PBS twice weekly for 3 wk. The sixth group was treated with bevacizumab twice weekly plus cisplatin weekly for 3 wk followed by treatment with bevacizumab twice weekly for 3 wk. Bevacizumab and cisplatin were given i.p. The dose of cisplatin used in this experiment was 3 mg/kg, because we have previously reported that treatment with 3 mg/kg cisplatin did not result in a complete response but showed significant antitumor effect in an in vivo ovarian cancer model (30). The dose of bevacizumab used was 5 mg/kg. Body weight was measured weekly until 6 wk after initial treatment. Six weeks after initial treatment, all mice were sacrificed to assess the potential toxicity of drug treatment.

The second set of experiments was conducted to examine the antitumor effect of bevacizumab as a single agent, in combination with cisplatin, and when used for maintenance therapy (Figs. 4 and 5A and B). A2780 cells were suspended in PBS. Then 5 × 10⁵ cells were injected i.p. into each of the 36 female, 5-wk-old to 7-wk-old nu/nu nude mice, as described previously (30). One week after inoculation, mice were assigned to six treatment groups, as shown in Fig. 4A. The first group was scheduled to be treated with PBS twice weekly. The second group was scheduled to be treated with cisplatin weekly for 3 wk followed by treatment with PBS twice weekly for 3 wk. The third group was scheduled to be treated with bevacizumab twice weekly for 3 wk followed by PBS twice weekly treatment with PBS twice weekly. The fourth group was scheduled to be treated with cisplatin weekly for 3 wk followed by treatment with bevacizumab twice weekly. The fifth group was scheduled to be treated with bevacizumab twice weekly plus cisplatin weekly for 3 wk followed by treatment with PBS twice weekly for 3 wk. The sixth group was scheduled to be treated with bevacizumab twice weekly plus cisplatin weekly for 3 wk followed by treatment with bevacizumab twice weekly. The dose of cisplatin used in this experiment was 3 mg/kg, and the dose of bevacizumab used was 5 mg/kg. Bevacizumab and cisplatin were given i.p., and each treatment was continued until death. Abdominal circumference was measured weekly until 3 wk after initial treatment. Three weeks after initiating treatment, to assess antitumor efficacy of treatment, a representative mouse from each group was sacrificed, and the presence of macroscopic disease and the ascites volume were examined. In the remaining 30 animals, survival was calculated from the first day of treatment until death. Seventy days after initiating treatment, the experiment was terminated, and the surviving mice were sacrificed. All mice were autopsied at death to examine the antitumor efficacy of each treatment regimen.

A third set of experiments was conducted using Caov-3 cells (Fig. 5C). 5 × 10⁶ Caov-3 cells in PBS were injected i.p. into each of 18 female, 5-wk-old to 7-wk-old nu/nu nude mice. One week after inoculation, mice were assigned to three treatment groups. The first group was scheduled to be treated with PBS twice weekly. The second group was scheduled to be treated with bevacizumab twice weekly plus cisplatin weekly for 3 wk followed by treatment with PBS twice weekly. The third group was scheduled to be treated with bevacizumab twice weekly plus cisplatin weekly for 3 wk followed by treatment with bevacizumab twice weekly. Seventy days after initiating treatment, the experiment was terminated, and the surviving mice were sacrificed. All mice were autopsied at death to examine the antitumor efficacy of each treatment regimen.

**Statistical analysis.** Differential cell proliferation was analyzed by the Wilcoxon exact test. Body weight, tumor volume, ascites volume, and survival were compared among PBS-treated, bevacizumab-treated, cisplatin-treated, and cisplatin plus bevacizumab-treated groups. Abdominal circumference, ascites volume, and body weight were analyzed by Wilcoxon exact test. Survival was analyzed by the log-rank test. For all tests, a P value of <0.05 was considered statistically significant.
Results

Effect of bevacizumab on the proliferation of A2780 cells. We first examined whether A2780 ovarian cancer cells, which express wild-type p53 and are sensitive to cisplatin (31), express VEGF in vitro. It is generally accepted that hypoxia commonly develops within tumors and HIF-1α-mediated VEGF expression promotes angiogenesis to meet the metabolic requirements for sustained tumor growth (32). Therefore, we examined the expression of VEGF under both hypoxic and nonhypoxic conditions. As shown in Fig. 1A, A2780 cells expressed VEGF, and the expression of VEGF was up-regulated in response to hypoxia. MTS assays were done to examine the effect of bevacizumab on proliferation of A2780 in vitro. As shown in Fig. 1B, treatment with either VEGF, bevacizumab, or the combination for 72 hours had no effect on the proliferation of A2780 cells; this is consistent with the tumor-suppressive effect of bevacizumab being mediated through inhibition of neovascularization.

Antiangiogenic activity of bevacizumab in vivo. We next investigated the antiangiogenic activity of bevacizumab in vivo. Angiogenesis is involved in a variety of physiologic and pathologic conditions, including embryonic development, tumor growth, metastasis, and chronic inflammation (33). Moreover, it is accepted that the wound healing process is highly dependent on angiogenesis (34). Therefore, we first conducted wound-healing assays as described in Materials and Methods. Full thickness skin wounds were surgically created on the back of nude mice, and wound size was then measured. Wounds were considered healed when no macroscopically visible epithelial defect or adherent scab was present. In general, 1 day after wounding, wounds were covered with a dry scab. Seven days after wounding, wound area was reduced by >60% in both PBS-treated and bevacizumab-treated mice.
PBS-treated mice showed earlier healing, with some wounds completely healed after 9 days, and with 100% of the wounds healed by 15 days after wounding. In contrast, none of the wounds in bevacizumab-treated mice were healed at 14 days, and 21 days were required for all wounds to be healed. As shown in Fig. 2A(i, ii), 14 days after wounding, there were significant differences in between the wound areas of the two treatment groups. These results indicate that treatment with bevacizumab significantly delayed wound healing as a result of inhibition of angiogenesis.

To investigate further the antiangiogenic activity of bevacizumab, we also conducted directed in vivo angiogenesis assays, as described in Materials and Methods. As shown in Fig. 2B(i), red fluorescence, indicating the new vessel formation, was observed in the angioreactors containing 500 ng/mL VEGF. VEGF-induced new vessel formation was almost completely inhibited by treatment with bevacizumab (Fig. 2B, i and ii) These results suggest that i.p. treatment with 5 mg/kg bevacizumab can inhibit VEGF-induced angiogenesis in vivo.

Bevacizumab inhibits intraabdominal dissemination of ovarian cancer cells and enhances efficacy of cisplatin in an i.p. ovarian cancer model. Before examining the growth inhibitory effect of bevacizumab on ovarian cancer cells in vivo, we first investigated the tolerability of the proposed treatment schedule (Fig. 3A). Athymic mice were randomized into six treatment groups, as described in Materials and Methods. Although cisplatin-treated mice tend to show a ~5% decrease in body weight compared with PBS-treated mice, the difference did not reach statistical significance (Fig. 3B). Overall, drug treatment was well tolerated, with no apparent toxicity throughout the study, and organ macroscopic examinations were normal at sacrifice.

We then examined the growth inhibitory effect of bevacizumab in vivo. Peritoneal dissemination is the primary route of progression in human ovarian cancer, and the amount of ascites and disseminated tumor burden correlates with patient prognosis in humans (35). Therefore, we used an i.p. xenograft model. Athymic mice were inoculated i.p. with A2780 cells to examine the effect of bevacizumab on intraabdominal dissemination of ovarian cancer, ascites formation, and tumor growth. One week after inoculation, as shown in Fig. 4A, mice were randomized into six treatment groups (groups A-F) receiving PBS, bevacizumab, cisplatin, or bevacizumab plus cisplatin, as described in Materials and Methods. The treatment schedules are shown in Fig. 4A.

As shown in Fig. 4B, the mean abdominal circumferences 3 weeks after initiating treatment in the mice treated with bevacizumab (group B) and cisplatin plus bevacizumab (group E) were significantly lower than in mice treated with PBS (group A) or cisplatin alone (group C), suggesting inhibition of ascites production by treatment with bevacizumab. To examine antitumor efficacy, 3 weeks after initiating treatment, a representative mouse from each group was sacrificed, and ascites volume, size of disseminated tumors, and extent of dissemination were examined at autopsy. Large abdominal tumors, bloody ascites, and peritoneal dissemination were observed in PBS-treated mice (Fig. 4C, I, II, and IV-VIII).

Histologically, these abdominal tumors were poorly differentiated serous adenocarcinomas, reflecting the most common histologic type of human epithelial ovarian cancer (Fig. 4C, III). Tumors detected in a representative mouse treated either with cisplatin or bevacizumab were 172.6 mm$^3$ and 336.55 mm$^3$, respectively, which were smaller than that in PBS-treated mice (470.3 mm$^3$). Surprisingly, no macroscopic tumor implants were detected in a mouse treated with cisplatin plus bevacizumab. Striking effects were also seen with regard to tumor ascites formation (Fig. 4C, II). Abundant ascites was detected in a representative PBS-treated mouse. Ascites volume in a mouse treated either with bevacizumab or cisplatin was 3.6 and 4.3 mL, respectively, each lower than in a PBS-treated mouse (6.1 mL). Most importantly, ascites was undetectable in a mouse treated with cisplatin plus bevacizumab. These results indicate the achievement of a clinical complete response by the 3-week treatment with bevacizumab-cisplatin combination. Because all mice treated with cisplatin for 3 weeks (groups C and D) showed enlarged abdomens with ascites and palpable tumors (indicating the existence of clinical disease), we did not treat these mice in group D with bevacizumab as a maintenance therapy but treated with PBS after a 3-week treatment with cisplatin.

As shown in Fig. 5A and Table 1, the median overall survival was 22 days in the PBS-treated mice (group A) versus 33 days, 29.5 days, and 37 days in groups treated with bevacizumab (group B), cisplatin (group C), or the combination of bevacizumab and cisplatin (group E), respectively. The median survival was significantly lower in PBS, bevacizumab, and cisplatin, compared with PBS, bevacizumab, and cisplatin, respectively, which were smaller than that in PBS-treated mice (470.3 mm$^3$). Surprisingly, no macroscopic tumor implants were detected in a mouse treated with cisplatin plus bevacizumab. Striking effects were also seen with regard to tumor ascites formation (Fig. 4C, II). Abundant ascites was detected in a representative PBS-treated mouse. Ascites volume in a mouse treated either with bevacizumab or cisplatin was 3.6 and 4.3 mL, respectively, each lower than in a PBS-treated mouse (6.1 mL). Most importantly, ascites was undetectable in a mouse treated with cisplatin plus bevacizumab. These results indicate the achievement of a clinical complete response by the 3-week treatment with bevacizumab-cisplatin combination. Because all mice treated with cisplatin for 3 weeks (groups C and D) showed enlarged abdomens with ascites and palpable tumors (indicating the existence of clinical disease), we did not treat these mice in group D with bevacizumab as a maintenance therapy but treated with PBS after a 3-week treatment with cisplatin.
Fig. 4. Effect of bevacizumab on tumor growth in vivo. A, treatment schedule. Athymic nude mice were inoculated i.p. with A2780 cells. One week after inoculation, mice were treated with PBS, bevacizumab, cisplatin, or cisplatin plus bevacizumab, as described in Materials and Methods. B, graph depicting weekly abdominal circumference for treatment groups A, B, C, and E. Points, mean; bars, SD. Statistically significant differences are indicated by asterisks: *, $P < 0.05$ significantly different from placebo-treated mice; **, $P < 0.05$ significantly different from bevacizumab-treated or cisplatin-treated mice. C, representative images 3 wk after the initial treatment. Athymic mice were inoculated i.p. with A2780 cells. Three weeks from the first day of treatment, a representative mouse in treatment groups A, B, C, and E was sacrificed. I, II, physical appearances of representative mice; IV-VIII, magnified views of intraabdominal dissemination pattern of the same mice. Intraabdominal organs (IV and V), diaphragm (VI), liver (VII), and genital tract (VIII). Arrows and brackets indicate tumor implants. III, histologic features of ovarian tumors. Tissue sections were stained with H&E, and representative fields are shown.
overall survival in group D was 28 days (data not shown), which is almost the same as that in group C. Interestingly, as shown in Table 1, at the time of death, abdominal circumferences and ascites volumes in the mice treated with bevacizumab were significantly lower than those in PBS-treated mice and lowest in mice treated with bevacizumab plus cisplatin. Collectively, these findings indicate that bevacizumab has significant antitumor effects as a single agent, with the ability not only to inhibit ovarian tumor growth and metastasis, decrease the production of ascites, and prolong survival but also to enhance the antitumor effect of cisplatin in vivo.

**Maintenance therapy with bevacizumab prolongs survival in an i.p. ovarian cancer model.** We next examined the efficacy of bevacizumab in the setting of maintenance therapy. Because mice treated with cisplatin plus bevacizumab for 3 weeks showed clinical complete responses, they were randomized to treatment with either PBS or bevacizumab. As shown in Fig. 5A, all mice randomized to treatment with PBS died with abdominal tumor and ascites, and the median overall survival was 37 days. On the other hand, although 40% of mice randomized to maintenance bevacizumab died, 60% survived until the end of the study without demonstrating any obvious evidence of recurrence (Fig. 5B). To further confirm the efficacy of bevacizumab maintenance therapy, we did an in vivo experiment using another VEGF-producing ovarian cancer cell line, Caov-3. As shown in Fig. 5C, maintenance treatment with bevacizumab after 3 weeks of treatment with cisplatin plus bevacizumab significantly prolonged survival of the mice. These results indicate that maintenance treatment with bevacizumab after a complete response to cisplatin plus bevacizumab significantly delays or inhibits the regrowth of ovarian cancer cells and produces a survival benefit.

**Discussion**

Ovarian cancer is characterized by the spread of solid i.p. tumors and the formation of ascites and is the most lethal of all gynecologic malignancies. Usually, after achieving complete responses to standard primary treatment, including surgical debulking and frontline chemotherapy, patients are monitored without further therapy until disease progression is documented. Although salvage chemotherapy for recurrent ovarian cancer may result in meaningful palliation, the 5-year survival of patients with advanced ovarian cancer remains <20% (3). Therefore, it is important to develop strategies to enhance or extend the response to frontline treatment to prevent or delay recurrence. Thus, we believe the concept of maintenance therapy after standard primary treatment is of potential benefit for patients with advanced stage ovarian cancer.

Molecular-targeted therapeutics may potentially yield improvements in long-term disease control, and many such agents are better tolerated than traditional cytotoxic drugs. Among the wide variety of molecular targets, VEGF and its signaling pathway are reported to be a promising target in the therapy of ovarian cancer (21–23). VEGF (also called VEGF-A) is a dimeric protein, which has been shown to stimulate angiogenesis (11, 12). It also enhances vascular permeability and was recognized as vascular permeability factor (36). In addition, VEGF has also been shown to regulate the invasiveness of cancer cells by altering the expression of matrix metalloproteinase-2 (37). Among ovarian cancer specimens, ~90% express VEGF (14–16). Moreover, high levels of VEGF have been found in serum, plasma, and ascites of ovarian cancer patients and are reportedly associated with poor patient...
prognosis (17, 38, 39). Several approaches have been tried to target VEGF signaling pathway, targeting VEGF itself, targeting VEGF receptors (40), targeting the upstream signaling, including nuclear factor-κB (41), HIF-1α (42), and mTOR (43). A monoclonal antibody against VEGF has shown antitumor activity in preclinical studies in several tumor models (40). Single-agent therapy with bevacizumab or its murine equivalent (A4.6.1) resulted in tumor growth inhibition (17, 18). Studies with in vivo cell lines did not reveal a direct effect of bevacizumab on the growth of tumor cells, suggesting that tumor suppression is likely mediated through inhibition of neovascularization, which should be essential for subclinical ovarian tumors after complete response to front-line chemotherapy to proliferate, invade, or disseminate. Moreover, because bevacizumab is a cytostatic rather than a cytotoxic agent, investigation of this compound in the maintenance therapy setting is attractive.

Our results show the antitumor efficacy of bevacizumab in ovarian cancer control within a previously characterized xenograft model. The dose of bevacizumab used (5 mg/kg) is consistent with the previous preclinical reports (19, 44) and a clinical study (20). The dose of cisplatin used in our experiment (3 mg/kg) is also roughly equivalent to the standard clinical dose (50–75 mg/mm²) used in patients (45). I.p. given bevacizumab and cisplatin were well tolerated (Figs. 3–5) at a dose level, and schedule was associated with the inhibition of angiogenesis peripherally (Fig. 2). Treatment with bevacizumab as a single agent significantly inhibited tumor growth and dissemination. Furthermore, bevacizumab significantly enhanced sensitivity to cisplatin. Bevacizumab is believed to normalize intratumoral blood vessels, which are markedly different from their counterparts in normal tissue (46). Intratumoral, neoangiogenic vessels are hyperpermeable, leading to interstitial hypertension and impaired perfusion in tumors. Normalization of tumor vasculature results in a decrease in interstitial pressures and improved delivery of oxygen, nutrients, and cytotoxic agents (47). Vascular normalization can potentially explain why bevacizumab is efficacious in combination with chemotherapy. Furthermore, such treatment led to the control of ascites production (Fig. 4 and Table 1), as reported previously (18). Because advanced or recurrent ovarian cancer frequently suffer from malignant ascites and require paracentesis for symptomatic relief, we believe that the ability of bevacizumab to inhibit ascites formation is an attractive property for patients with ovarian cancer.

When used in maintenance therapy in this murine xenograft model, bevacizumab significantly inhibited the regrowth of ovarian cancer and prolonged survival (Fig. 5). Surprisingly, 50% to 60% of mice treated with bevacizumab after induction therapy with cisplatin plus bevacizumab survived until the end of study without demonstrating any evidence of recurrence. To our knowledge, this is the first preclinical report of a survival benefit for bevacizumab in the setting of maintenance therapy.

Bevacizumab is currently being evaluated by the Gynecologic Oncology Group in protocol GOG 218 (23). This trial is designed to evaluate the benefit of bevacizumab in combination with first-line chemotherapy, as well as the benefit from this agent given as maintenance therapy. We believe that our data support the scientific justification for this and future clinical trials with bevacizumab in patients with ovarian cancer.

References

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

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Table 1. Effect of bevacizumab in vivo

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Survival (d), median</th>
<th>Ascites volume (mL), mean</th>
<th>Abdominal circumference (cm), mean</th>
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<tr>
<td>PBS</td>
<td>22</td>
<td>5.55</td>
<td>10.1</td>
</tr>
<tr>
<td>Bevacizumab</td>
<td>33*</td>
<td>3.18*</td>
<td>9.5*</td>
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<td>Cisplatin</td>
<td>29.5</td>
<td>4.76</td>
<td>9.9</td>
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<tr>
<td>Cisplatin + bevacizumab</td>
<td>37*</td>
<td>2.37*</td>
<td>8.8*</td>
</tr>
</tbody>
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*Significantly different from PBS group (P < 0.01).
†Significantly different from PBS group (P < 0.05).
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