

Albendazole: a Potent Inhibitor of Vascular Endothelial Growth Factor and Malignant Ascites Formation in OVCAR-3 Tumor-Bearing Nude Mice

Mohammad Hossein Pourgholami, Zhao Yan Cai, Ying Lu, Lisa Wang, and David Lawson Morris

Abstract Purpose: Angiogenesis and vessel hyperpermeability are the two factors leading to the formation of ascites. Vascular endothelial growth factor (VEGF) plays a pivotal role in malignant ascites formation. We have recently shown that albendazole inhibits peritoneal growth of human colorectal cancer cells (HT-29). The present study was designed to find out if albendazole can suppress ascites formation in ascites-producing peritoneal carcinomatosis.

Experimental design: Female nude mice bearing peritoneal tumors of human ovarian cancer cells (OVCAR-3) were treated with albendazole. Following i.p. inoculation and ascites development, mice were given i.p. albendazole (150 mg/kg) or the vehicle \times 3 weekly for 4 weeks.

Results: Whereas vehicle-treated mice developed overt ascites requiring repeated aspiration, ascites formation in the albendazole-treated mice was markedly suppressed. As a result of this, 7 of 10 mice from the control group had to be euthanized before the course of treatment was over. Suppressed ascites production and reduced tumor vascularity observed was a result of dramatic reduction in tumor VEGF production as revealed by profoundly lower VEGF ascites fluid and plasma levels. *In vitro*, incubation of SKOV-3 cells with various concentrations of albendazole led to significant dose-dependent inhibition of VEGF secretion. Examination of floating tumor cells collected from the peritoneal wash revealed profound down-regulation of VEGF mRNA in albendazole-treated mice.

Conclusions: These findings suggest for the first time that in nude mice bearing OVCAR-3 peritoneal tumors, by inhibiting VEGF production, albendazole abolishes tumor angiogenesis and ascites formation.

The formation of malignant ascites in patients with advanced-stage cancer is a difficult problem to manage in clinical oncology (1). It is an important cause of morbidity and mortality in patients with peritoneal carcinomatosis arising from colorectal, gastric, pancreatic, endometrial, and ovarian cancers (2). Ovarian carcinoma is characterized by rapid growth of solid i.p. tumors and production of large volumes of ascites. Although malignant progression of this disease is often predominantly confined to the peritoneal cavity (3), two thirds of patients already have advanced disease when diagnosed, and rapidly accumulating ascitic fluid is associated with poor prognosis (4). Both tumor size and the accumulation of ascites are inversely associated with survival (5). Among women with stage III or IV epithelial ovarian carcinoma, development of

ascites correlates with a significantly decreased 5-year survival rate (5% with ascites versus 45% without ascites; ref. 6). Despite advances in surgical cytoreduction and cytotoxic chemotherapy, the prognosis for patients with ovarian cancer remains poor (7). Current therapeutic treatment of patients with advanced ovarian cancer associates cytoreductive surgery and chemotherapy, the combination of paclitaxel and a platinum salt being considered as the standard chemotherapy for advanced ovarian cancer (8).

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 concentrations of the drug at the site of tumor for a longer period of time and ideally low systemic toxicity (9, 10).

Albendazole, a benzimidazole carbamate anthelmintic drug, has been in clinical use for almost three decades. As an oral anthelmintic, its efficacy and safety has been well established (11). The primary mode of action of albendazole in susceptible parasites has been described as binding to β -tubulin and leading to inhibition of microtubule polymerization (12, 13). Our interest in benzimidazole carbamates dates back to the 1980s when the drug was tested for hydatid disease (14). Mechanisms described for benzimidazole carbamate anthelmintic activity rekindled our interest in these compounds as possible anticancer agents. Through our studies, we were able to show that in cell culture, several

Authors' Affiliation: Cancer Research Laboratories, Department of Surgery, St. George Hospital, University of New South Wales, Sydney, New South Wales, Australia

Received 6/2/05; revised 11/11/05; accepted 12/21/05.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

Requests for reprints: David Lawson Morris, Department of Surgery, St. George Hospital, Kogarah, Sydney, New South Wales 2217, Australia. Phone: 61-2-9350-2070; Fax: 61-2-9350-3997; E-mail: david.morris@unsw.edu.au.

©2006 American Association for Cancer Research.

doi:10.1158/1078-0432.CCR-05-1181

(but not all) benzimidazole carbamates and in particular albendazole is a highly potent inhibitor of cell proliferation. Albendazole proved to be quite a potent inhibitor of a wide variety of cancer cell lines, including a range of hepatocellular cancer cells (15), colorectal, pancreatic, ovarian, and a number of other human cancer cells.¹ We subsequently, reported from a pilot study where some patients with colorectal cancer and liver metastases had shown a decline or stabilization of tumor marker (carcinoembryonic antigen) when treated with oral albendazole (16). In 2002, Mukhopadhyay et al. (17) and Sasaki et al. (18) provided exciting evidence on the antitumor activity of mebendazole, a close analogue of albendazole and a commonly used anthelmintic drug. Potent antiproliferative activity together with poor solubility and extensive first-pass metabolism (19, 20) make albendazole very well suited for regional (i.p.) treatment of peritoneal carcinomatosis. Thus, we went on to test efficacy of i.p. administered albendazole in a nude mice model of peritoneal carcinomatosis arising from human HT-29 colorectal cancer cells (21). Results of the study provided the first evidence of antitumor activity of albendazole upon i.p. administration and lack of significant efficacy upon oral treatment in this animal model. Subsequently, the current study was designed to test efficacy of i.p. albendazole in inhibiting ascites formation in a nude mouse model of peritoneal carcinomatosis with malignant ascites production. To do this, the OVAR-3 nude mouse model was used. The model closely resembles advanced-stage ovarian epithelial carcinoma, with both extensive dissemination of ovarian carcinoma cells to peritoneal surfaces and development of massive ascites (22, 23).

Materials and Methods

Animals. For all experiments, 6- to 8-week-old female nude athymic BALB/c *nu/nu* mice (Animal Resources Centre, Perth, Western Australia) were used. They were kept under specific pathogen-free conditions and fed autoclaved pellets and water *ad libitum*. The general health status of the animals were monitored daily. All animal work was approved by the institutional ethical committee.

Drug preparation. Albendazole was suspended in 0.5% carboxymethyl cellulose and administered at a dose of 150 mg/kg to all treated animals (3 mg/mL/20 g mice).

Cell preparation and inoculation. In this investigation, we used a subpopulation of cells from the well-characterized human ovarian carcinoma cell line NIH:OVCAR-3. The cell line was originally derived from a patient refractory to combination chemotherapy with Adriamycin, cisplatin, and cyclophosphamide. The cells have the capacity to grow i.p. in female nude athymic mice. After i.p. injection of these cells, animals develop peritoneal carcinomatosis similar to that of advanced clinical ovarian cancer. Disease progression is characterized by development of massive ascites production, and the malignant ascites cells express the ovarian cancer-associated antigen CA 125. The model exhibits unique features like histopathology, embryology, and biological properties consistent with human ovarian cancer, with suitable markers for following response to treatment and also drug sensitivity profiles similar to that of human ovarian cancer (24).

Before inoculation into mice, OVCAR-3 cells obtained from the American Type Culture Collection (Manassas, VA) were precultured

using soft agar culture system (3, 25). Briefly, nude mice were injected (s.c.) with 13×10^6 OVCAR-3 cells. Eight weeks later, tumors were dissected, minced, and washed $\times 3$ in PBS. The cultured cells were suspended in 0.3% of Bacto agar in RPMI supplemented with 20% horse serum, penicillin (100 units/mL), streptomycin (2 mg/mL), insulin (3 units/mL), EGF (100 ng/mL), asparagine (0.1 mg/mL), and DEAE dextran (0.5 mg/mL) to yield a cell density of 5×10^6 /mL. 2-Mercaptoethanol was added at a concentration of 50 μ mol/L just before cells were plated. A portion (1 mL) of the resultant mixture was added onto a feeder layer that combined 0.2 mL of medium conditioned by adherent spleen cells of mineral oil-primed BALB/c mice in 0.5% Bacto agar. Cultures were incubated at 37°C in 5% CO₂. At 5-day intervals, 0.8 mL of growth medium with EGF (10 ng/mL) was layered onto the agarose surface. After 3 weeks, colonies of cells were harvested in mass and plated in 75-cm² flasks. From here, 50×10^6 cells were injected s.c. to mice. Following this, 50×10^6 tumor cells harvested from the s.c. tumors were suspended in 1 mL of medium (RPMI 1640) and injected i.p. into each carrier mice. To collect floating tumor cells, ascites fluid retrieved from these mice were washed with sterile saline and centrifuged at 300 \times g and 4°C for 5 minutes. These cells were then used immediately to inoculate the test animals ($n = 20$). Each test animal was injected i.p. with 10×10^6 viable tumor cells suspended in 1.0 mL of medium.

Drug treatment and sample collection. After the development of ascites, the peritoneal cavity was washed with 2 mL of sterile normal saline. The peritoneal contents were mixed by kneading and then completely aspirated (26). Mice were then randomly distributed into either the albendazole treatment group or the control group ($n = 10$ per group). Drug treatment was initiated immediately after aspiration of the ascites fluid.

Albendazole (150 mg/kg suspended in 1 mL of 0.5% carboxymethyl cellulose) was administered $\times 3$ per week for 4 weeks. The albendazole dose, volume, and frequency of administration were chosen based on our previous studies. In addition, institutional (University of New South Wales, Sydney, Australia) animal ethics guidelines do not allow death as an end point. At the end of the treatment period, mice were euthanized by a lethal dose of pentobarbitone. Although the intended duration of treatment was 4 weeks, animals were euthanized if either their abdominal circumference reached 9.5 cm, or if they were expected to become moribund within a short time, a requirement of institutional animal ethics committee. Survival time of each animal was calculated as the number of days elapsed between initiation of treatment and euthanasia. Before euthanasia, a blood sample was collected through cardiac puncture under anesthesia. Following euthanasia, 2 mL of physiologic saline were injected i.p., and the peritoneal cavity was completely washed and aspirated. Ascites volume and the total number of viable tumor cells present in the peritoneal cavity were recorded. These cells, together with cell-free ascites fluid, tumors dissected from the peritoneal cavity, and the plasma, were all stored at -80°C for subsequent analysis. Actual volume of ascites collected after each aspiration was calculated by subtracting the 2 mL from the total volume collected. Cumulative ascites volume was calculated by adding these values. Average volume of ascites produced per day was therefore calculated by dividing cumulative volume of ascites collected from each animal by the number of days lapsed between initiation of treatment and euthanasia for that animal.

CA 125 levels in ascites fluid. Tumor marker levels (CA 125 Ku/L) in cell-free ascites fluid were determined in the Hospital Biochemistry Laboratories.

Tumor morphology and microvessel density. The tumor tissues from vehicle-treated and albendazole-treated animals were either immediately snap frozen or fixed in 10% formalin, embedded in paraffin block, H&E stained, and examined under the microscope. Similarly, tumor vessel formation was assessed immunohistochemically by staining the tumor sections for CD31 (27). Five-micrometer frozen sections of fresh tumor samples were used to stain CD31. First, the

¹ Unpublished data.

frozen sections were thawed and air-dried. Then the sections were fixed in cold acetone for 10 minutes. After a quick air dry, the sections were washed with TBS for 5 minutes followed by overnight incubation at 4°C with rat anti mouse CD31 1:20 (BD Biosciences, PharMingen, San Jose, CA). Next day, after rinsing with TBS, sections were incubated with biotinylated rabbit anti rat IgG 1:200 (DakoCytomation, Glostrup, Denmark) for 45 minutes. Then the sections were incubated with conjugated streptavidin/horseradish peroxidase 1:200 (DakoCytomation) for another 30 minutes. Sections were developed by using diaminobenzidine (Sigma Chemical Co., St. Louis, MO). Then sections were washed with water and counterstained with hematoxylin. The brown staining indicated expression of CD31. These stained vessels were counted at $\times 200$ magnification, and average vessel counts were calculated.

Quantitative assay of vascular endothelial growth factor in ascites fluid and plasma. Before attempting to measure precise vascular endothelial growth factor (VEGF) levels in ascites fluid or plasma, Western blot analysis for the presence of VEGF protein was conducted (28) on cell-free ascites fluid collected from the peritoneal cavity of vehicle-treated and albendazole-treated mice. Following this, plasma and ascites fluid VEGF levels were measured according to manufacturer's instructions using an ELISA that detects soluble VEGF₁₆₅ (Quantikine, R&D Systems, Minneapolis, MN).

Effect of albendazole on in vitro VEGF secretion. SKOV-3 human cystadenocarcinoma cells, obtained from the American Type Culture Collection, were grown in McCoy's 5a medium with 1.5 mmol/L l-glutamine, 100 units/mL penicillin, and 100 µg/mL streptomycin, supplemented with 10% FCS. Cells were grown to confluence and harvested by trypsinization with 0.25 mg/mL trypsin/EDTA and suspended in the medium before plating. These were then seeded (2×10^5) on plastic six-well Corning culture plates. Cultures were maintained in a 37°C incubator in a humidified atmosphere of 95% O₂/5% CO₂. Twenty-four hours later, the medium was removed. Subconfluent cultures were washed thrice with phosphate buffer followed by incubation for 6 hours with culture medium containing various concentrations of albendazole (0, 0.1, 0.25, and 1.0 µmol/L) dissolved in 1% ethanol. After completion of the treatment period, media from the wells were individually collected and analyzed for their VEGF content using ELISA. At the end of the incubation period, viable cells in each well were counted using the trypan blue method, and the final VEGF concentration was normalized per 10^6 cells.

In a parallel experiment, matrix metalloproteinase 9 (MMP-9) was added to the cell culture medium (final concentration, 6.0 ng/mL) to stimulate release of soluble VEGF by SKOV-3 cells. There is evidence that MMP-9 induces the release of biologically active VEGF in the culture medium of ovarian tumor cells and in ascites of ovarian tumor-bearing mice (29). Cells were then tested for VEGF secretion in the presence of various concentrations of albendazole as described above. In addition to correcting VEGF values for cell number, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide cytotoxicity assay (30) done on identically treated cells confirmed no decrease in SKOV-3 cellular metabolic activity over the 6-hour course of the assay.

VEGF mRNA determinations. Tumor cells collected from ascites fluid (peritoneal wash) were examined for the expression of various isoforms (121, 165, 189, and 206) of VEGF mRNA by reverse transcription-PCR (31). This was only possible in only three of the albendazole-treated mice as the rest had extremely low cell numbers. Total RNA was isolated from the cells using the High Pure RNA Isolation kit (Invitrogen, Sydney, Australia) according to the manufacturer's protocol. Primers for the amplification of VEGF were constructed based on the following sequence: VEGF sense, 5'-CACATAGGAGAGATGAGCTTC-3'; VEGF antisense, 5'-CCGCCTC-GGCTTGTCACAT-3'. These primers amplify the following products: 100 bp for VEGF₁₂₁, 230 bp for VEGF₁₆₅, 300 bp for VEGF₁₈₉, and 320 bp for VEGF₂₀₆.

The β -actin gene was used as an internal control (202 bp; β -actin sense, 5'-CTTCCTGGGCATGGAGTCTCT-3'; β -actin antisense, 5'-GGAGCAAT-GATCTTGATCTT-3'); 250 ng/50 ng of total RNA was used to amplify VEGF/ β -actin using the SuperScript One Step reverse transcription-PCR with Platinum Taq (Invitrogen, Sydney, Australia). The amplification was carried out in a Palm Cycler after an initial cDNA synthesis at 54°C for 30 minutes and 5 minutes at 94°C for denaturation. This was followed by 27 cycles (denaturation at 94°C for 1 minute, primer annealing at 60°C for 1 minute, and primer extension at 72°C for 45 seconds) and a final extension of 72°C for 10 minutes. The reverse transcription-PCR products were visualized by electrophoresis (25 minutes at 120 V) on 2% agarose gel in $1 \times$ TAE buffer containing ethidium bromide. To quantify the size of the products, a 100-bp DNA ladder was run with the samples.

Statistical analysis. All data are reported as the mean \pm SE. Student's *t* test was done to compare the albendazole-treated versus vehicle-treated samples. The level of significance was set at $P < 0.05$. All statistical calculations were done using the Graph Pad Prism software package (version 3.0, San Diego, CA).

Results

Albendazole treatment inhibits ascites production. Following development of ascites and initial aspiration, vehicle-treated mice continued to produce ascites at a more frequent rate and had to be aspirated repeatedly during the course of the treatment (28 days). In contrast, mice treated with albendazole at the predetermined dose of 150 mg/kg ceased to produce macroscopically visible ascites during the entire 28 days of the treatment period (Fig. 1). Cumulative and the average volume of ascites fluid produced per animal per day are presented in Fig. 2. In these mice, albendazole treatment profoundly ($P < 0.001$) suppressed ascites production. Similarly, albendazole treatment led to significant ($P < 0.001$) reduction in the number of viable floating tumor cells present within the peritoneal cavity. Depicted in Fig. 2C is the total number of tumor cells recovered from the peritoneal cavity of each of the vehicle-treated or albendazole-treated mice.

Albendazole reduces tumor marker levels. Consistent with the reduction in floating tumor cells was the reduction in

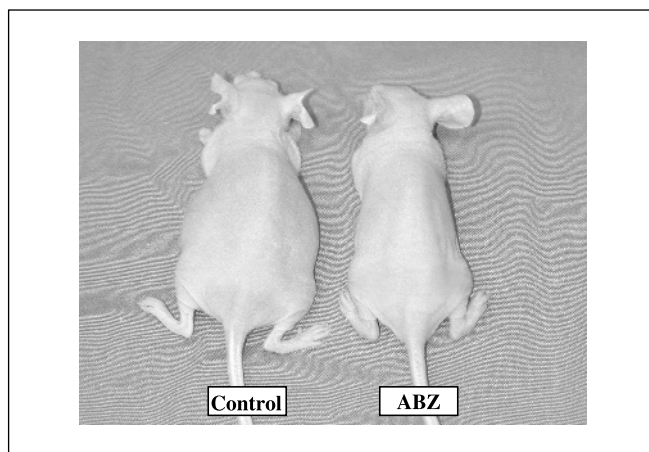


Fig. 1. Effect of albendazole (ABZ) on ascites development. Nude mice inoculated i.p. with OVCA9-3 cells were left to develop ascites and then randomly assigned to one of control or albendazole treatment groups ($n = 10$ per group). Whereas all mice in the control group developed overt ascites, there were no macroscopic signs of ascites formation in albendazole-treated mice.

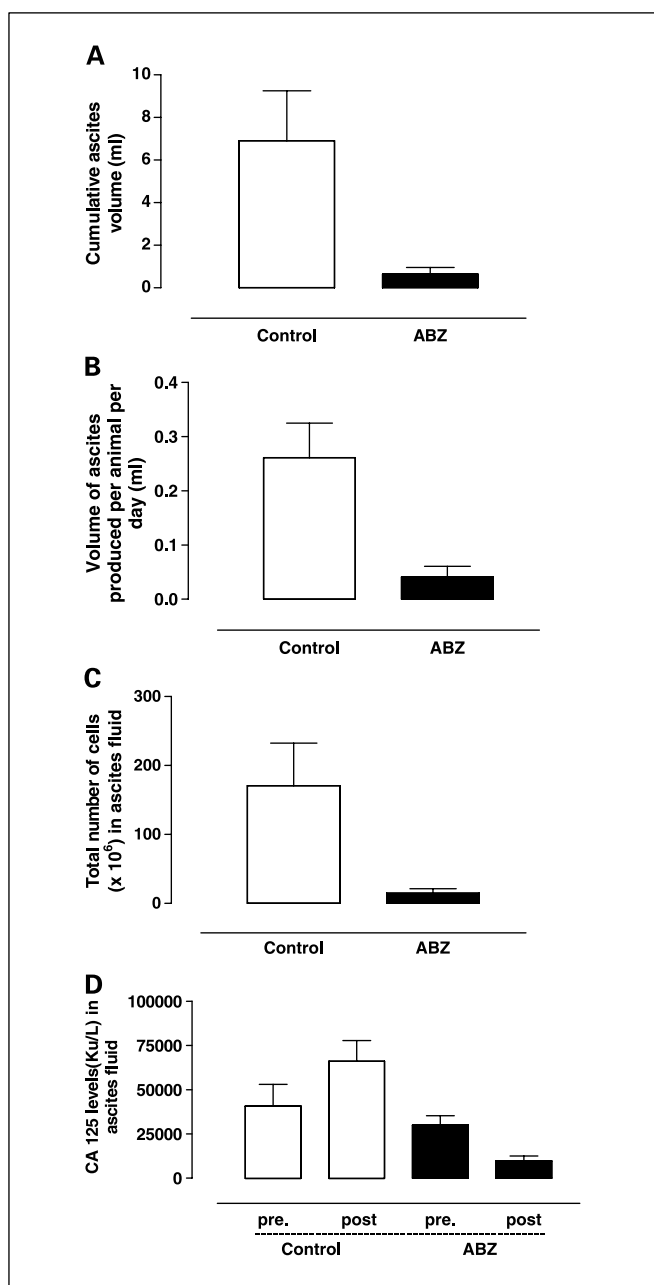


Fig. 2. Following euthanasia, peritoneal cavity of each mouse was washed with 2 mL of normal saline, and after aspiration, the volume of ascites present was recorded. Volume of ascites collected from each animal from initiation of therapy [vehicle or albendazole (ABZ)] was added up to give the cumulative ascites volume for that animal (A). Volume of ascites produced per animal per day (B) was calculated by dividing the cumulative ascites volume by the number of days lapsed between initiation of therapy to euthanasia. Total number of viable OVCAR-3 cells (in million) present in the ascites fluid (peritoneal wash) collected at euthanasia (C). Effect of albendazole on suppressing the rise in tumor marker level (CA 125 Ku/L; $K = 1,000$) in the ascites fluid (peritoneal wash) immediately before initiation of drug or vehicle therapy and at euthanasia (E). Columns, mean; bars, SE.

cell-free ascites fluid CA 125 levels. Changes in mean CA 125 levels for both vehicle-treated and albendazole-treated mice immediately before initiation of drug treatment and at euthanasia are presented in Fig. 2D. It is evident that albendazole treatment has significantly ($P = 0.003$) blocked

the increase in tumor marker levels seen in the vehicle-treated group. At euthanasia, compared with control values of 27,050 to 123,300 Ku/L, the tumor marker range in albendazole-treated mice were 968 to 30,580 Ku/L.

Albendazole treatment increases survival. Whereas all mice in the albendazole treatment group survived the full intended length of the study (28 days), 7 of 10 mice from the control group had to be euthanized due to ill health resulting from overt ascites production, before the treatment period was over (Fig. 3).

The survival advantage brought about by albendazole treatment was probably a consequence of the profound reduction of ascites production in albendazole-treated animals.

Albendazole changes tumor morphology. In this study, albendazole administration was initiated at an advanced stage of the disease with considerable tumor tissue present within the peritoneal cavity. Treatment of the mice with albendazole starting at this stage of the disease did not lead to significant reductions in tumor weight (range of 2.3-11.0 g with a median of 4.3 g in controls compared with 2.7-6.8 and a median of 3.2 g in drug-treated animals). However, morphologic examination of the tumors revealed that albendazole treatment, while not significantly reducing tumor burden, had led to morphologic changes in the tumor tissue (Fig. 4A). This revealed the existence of clear differences in the tumor tissues excised from the peritoneal cavity of these animals. High mitotic activity in control tumors compared with necrosis and lymphocyte infiltration in albendazole-treated tumors was quite evident.

Albendazole reduces tumor vascular density. Sections prepared from excised tumors of control and albendazole-treated mice and stained with CD31 antibody are presented in Fig. 4B. It is evident from these sections that tumor exposure to albendazole has led to marked reduction in the number of vessels (53.9 ± 16.25 in control compared with 21.4 ± 4.6 in albendazole treated). These values (mean \pm SD) clearly show the antiangiogenic activity of albendazole. Inhibition of both ascites formation and angiogenesis is an almost unique feature of VEGF inhibition.

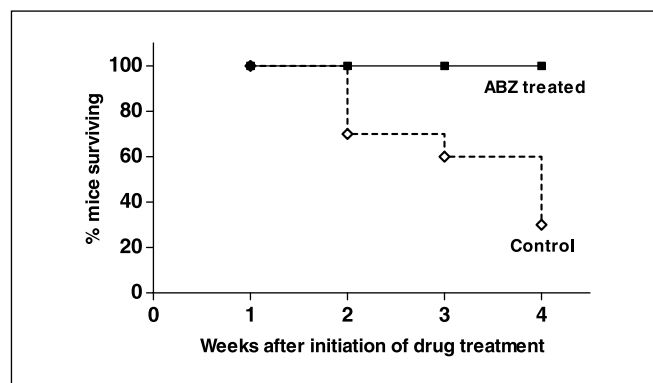


Fig. 3. Survival curve showing the effect of albendazole (ABZ) on survival. Whereas for all animals the intended duration of treatment was 4 weeks, mice (10 per group) were euthanized if due to ill health, they were expected to become moribund within a short time. Survival was calculated as the number of days lapsed between initiation of treatment and euthanasia, and % mice surviving was the number of animals remaining in each group ($\times 10$) at the end of each week following initiation of treatment.

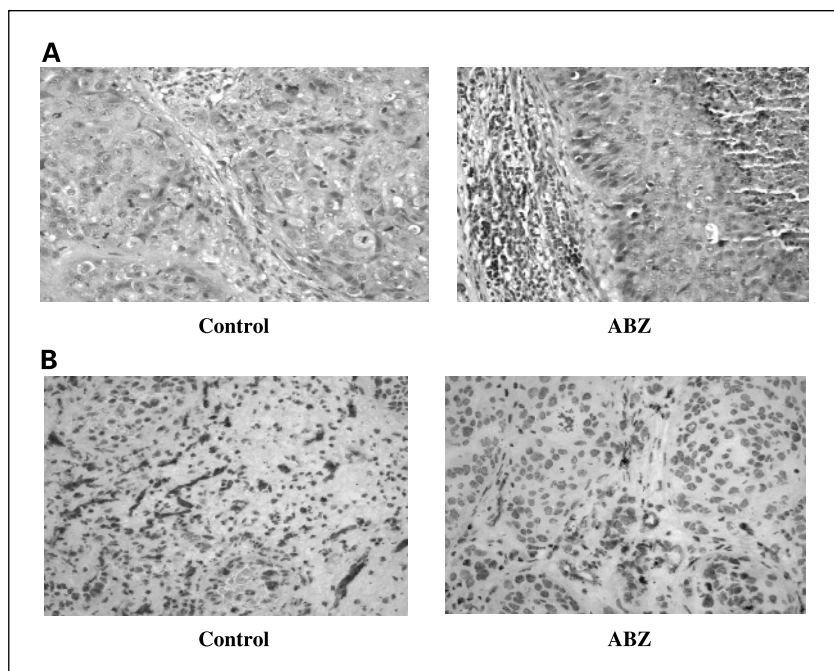


Fig. 4. H&E-stained sections of tumors collected from control or albendazole (ABZ)-treated mice showing marked morphological changes (A). Immunohistochemical staining of tumor vessels for CD31, where brown stains indicate the expression of the CD31-stained vessels (B). Vessel counts done (as described in Materials and Methods) revealed marked reduction in tumor vascular density of albendazole-treated mice. Magnification, $\times 200$.

Albendazole reduces VEGF levels. Results revealing inhibition of ascites production and reduction in vascularity of tumors prompted us to investigate VEGF status in the ascites fluid of control and albendazole-treated mice. Using the standard ELISA kit (described in Materials and Methods), concentrations in the ascites fluid and plasma were determined. The kit measures VEGF₁₆₅, the predominant and probably the most important VEGF isoform (32). Results showed profoundly lower VEGF levels in both plasma (Fig. 5A) and ascites fluid (Fig. 5B) of albendazole-treated mice ($P < 0.01$ and $P < 0.001$, respectively).

Albendazole reduces in vitro VEGF secretion. To further validate the VEGF effect, SKOV-3 cells were used, which constitutively release high levels of VEGF compared with OVCAR-3 cells. These cells were treated in culture with various concentrations of albendazole. Also run in parallel were the MMP-9-stimulated cells. Results obtained are presented in Fig. 5C and D, respectively. It is clear from these results that albendazole reduces VEGF concentrations in a dose-dependent fashion in both normal and MMP-9-stimulated SKOV-3 cells. Trypan blue exclusion method was used to count the number of viable cells present at the end of the drug treatment period. Corrected VEGF concentrations are expressed as picograms per 10^5 cells. At the highest albendazole concentration used ($1.0 \mu\text{mol/L}$), VEGF levels were reduced by 64%. The 0.25 and the $0.1 \mu\text{mol/L}$ albendazole concentrations were also effective ($P < 0.05$) in reducing VEGF levels and, similarly, in blocking the excess VEGF secretion induced by MMP-9 stimulation.

Stimulation of cells with MMP-9 led to a 44.8% increase in VEGF concentration during the 6-hour incubation period. 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay showed no decrease in cell number over the 6-hour time course of the assay (data not shown). For albendazole ($1.0 \mu\text{mol/L}$)-treated cells, the value for cells treated with the highest concentration of albendazole used was 96.3 ± 5.3

(mean \pm SD) of the untreated control value. These results further verify the inhibitory effect of albendazole on VEGF production.

Albendazole down-regulates VEGF mRNA levels. To explore the mechanism by which albendazole lowers VEGF levels, VEGF mRNA was examined. Using reverse transcription-PCR, VEGF mRNA extracted from ascites cells were analyzed. Three different bands corresponding to VEGF₁₂₁, VEGF₁₆₅, and VEGF₁₈₉ were picked up by the method employed (Fig. 6). Bands marked C represent mRNA extracted from vehicle-treated cells, whereas those marked ABZ were extracted from albendazole-treated cells. Reduced density of the VEGF bands in albendazole-treated samples reveals VEGF mRNA down-regulation in OVCAR-3 ascites cells collected through peritoneal wash from albendazole-treated mice.

Discussion

These results reveal for the first time the capacity of albendazole to profoundly suppress VEGF and ascites formation. Accumulation of malignant ascites is an important cause of morbidity and mortality in patients with peritoneal carcinomatosis. Colon, gastric, pancreatic, endometrial, and ovarian cancers are all causes of malignant ascites formation (2).

High concentrations of biologically active VEGF have been detected in pleural and ascites fluids of cancer patients (1, 33, 34). Several studies have indicated that VEGF plays a pivotal role during malignant ascites formation by increasing vascular permeability to plasma proteins (5, 35–37). Ascites is linked to peritoneal, as well as tumor microvascular hyperpermeability. In addition, whereas it has been suggested that tumor secretion of VEGF is both essential and sufficient to promote ascites formation and accumulation (38), tumor-associated stroma has also been nominated to represent an important site of VEGF production, possibly in a tumor

type-dependent fashion (32). In animal experimental models, a correlation between ascites volume and VEGF levels has been reported, and VEGF blockade has been shown to lead to dramatic reduction in ascites formation (33, 34, 37, 39, 40).

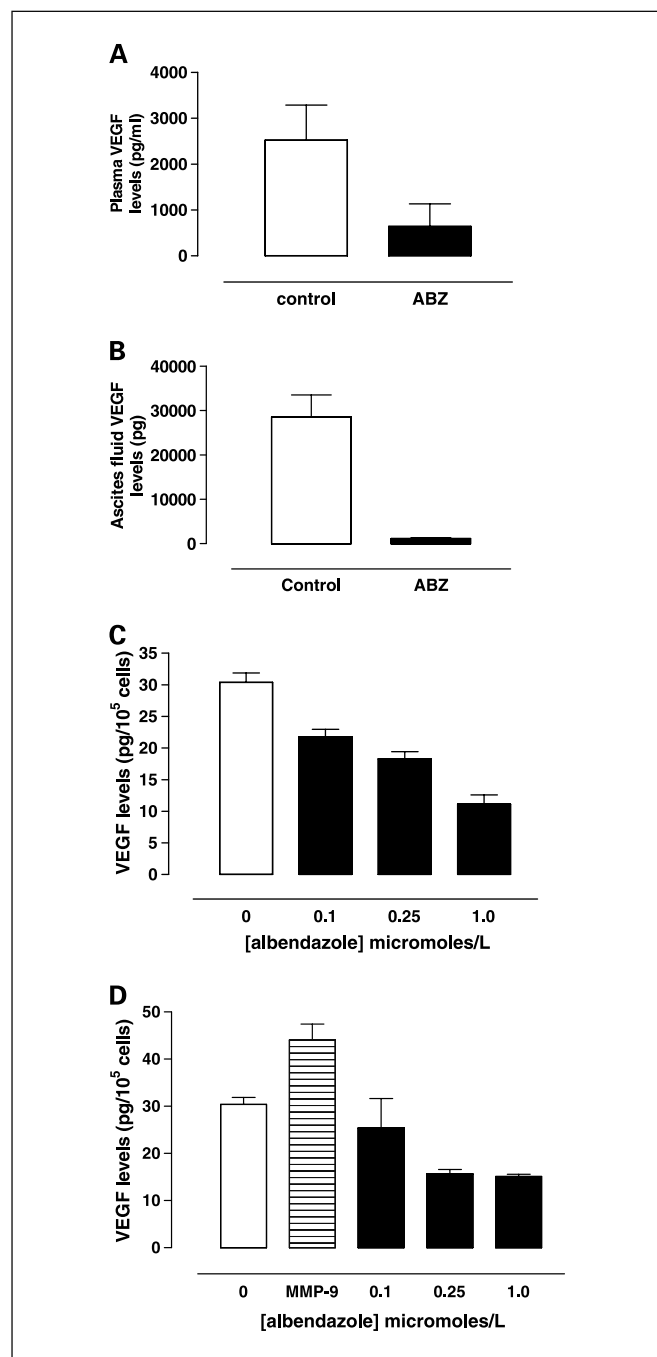


Fig. 5. ELISA kit was used to measure VEGF levels (picograms of VEGF per 100,000 cells) in plasma (A) and cell-free ascites fluid (B) of vehicle and albendazole (ABZ) – treated (150 mg/kg, i.p. × 3 weekly for 4 weeks) mice. *In vitro*, the effect of albendazole on VEGF production was examined by incubating SKOV-3 cells for 6 hours with various concentrations of albendazole (0.1-1.0 μmol/L) in culture medium bathing the cells with either albendazole alone (C) or with medium containing albendazole plus 6 ng/mL of MMP-9 as a VEGF stimulator (D). Columns, mean; bars, SD.

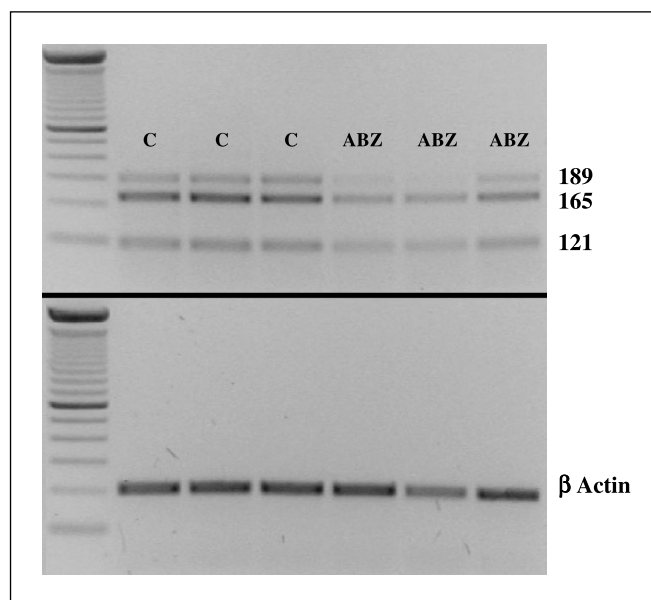


Fig. 6. RT-PCR showing VEGF₁₂₁, VEGF₁₆₅, and VEGF₁₈₉ in cells collected from three vehicle-treated (C) and three albendazole-treated (ABZ) mice. β-Actin gene was used as internal control. Products were visualized by electrophoresis on 2% agarose gel in 1 × TAE buffer containing ethidium bromide.

A highly conserved, disulfide-bonded dimeric glycoprotein VEGF is expressed in various forms of monomers containing amino acid residues (VEGF₁₂₁, VEGF₁₄₅, VEGF₁₆₅, VEGF₁₈₉, and VEGF₂₀₆; ref. 29). VEGF₁₆₅, a potent multifunctional cytokine exerts several important and possibly independent actions on vascular endothelium (41). VEGF binds two related receptor tyrosine kinases, VEGFR-1 and VEGFR-2, expressed principally but not exclusively on vascular endothelial cells (42–44). There is now consensus that VEGFR-2 is the major mediator of the mitogenic and permeability-enhancing effects of VEGF (32). The vascular permeabilizing activity of VEGF has been reported to be some 50,000 times more potent than histamine. By some as yet unknown mechanism, VEGF causes the stomata to open, providing a pathway for plasma and plasma protein extravasation (45). The expression of VEGF and its receptors in ovarian carcinoma has been associated with growth and invasion (46). High levels of VEGF have been found in serum or plasma and in ascites fluid of ovarian cancer patients (38, 47, 48). It has been well documented that inhibitors of VEGF activity reduce the formation of malignant ascites in human ovarian carcinoma xenograft models (1, 22, 39, 40). In this study, following the first administration of albendazole to mice with advanced peritoneal disease, ascites production was markedly reduced. Also evident was the reduced tumor vascularity resulting from albendazole treatment. Inhibition of ascites fluid production and reduction in vascularity of the tumor tissue strongly implicated involvement of a VEGF-mediated pathway. This is because VEGF is the only angiogenic cytokine identified thus far that renders microvessels hyperpermeable to circulating macromolecules, a characteristic feature of angiogenic blood vessels (49). Monoclonal antibodies against VEGF, inhibitors of the VEGFR tyrosine kinase or soluble receptors have all been shown to reduce ascites volume in mice bearing ovarian carcinomas (5, 41, 50).

In the present study, we confirmed the presence of VEGF and its profound suppression by albendazole in plasma and ascites fluid of OVCAR-3 tumor-bearing mice. To elucidate how this reduction in protein VEGF levels is brought about, VEGF mRNA status of the cells were examined. Results revealed down-regulation of VEGF mRNA in cells collected from the peritoneal cavity of albendazole-treated mice. Presence of VEGF mRNA expression has been shown in many human tumors, including lung, breast, gastrointestinal tract, renal, and ovarian carcinomas (32).

In summary, given its potential role in promoting tumor angiogenesis, metastases, and fluid accumulation VEGF is an attractive target for therapeutic intervention. Results of the present study showing inhibition of ascites production is accompanied by dramatic drop in ascites fluid and plasma

VEGF levels reconfirms the central role played by VEGF and substantiates the importance of VEGF inhibition in abolishing ascites production. Additionally, the present study reveals for the first time a novel mode of action for albendazole and its potential usefulness in treating malignant ascites and possibly other VEGF-related conditions. Further work on the development of albendazole as an anti-VEGF agent is currently ongoing in our laboratories.

Acknowledgments

We thank Katherine Reise for her technical assistance with the reverse transcription-PCR work and Dr. Mathew Links (Cancer Care Centre, St. George Hospital) for useful discussions.

References

- Verheul HM, Hoekman K, Jorna AS, Smit EF, Pinedo HM. Targeting vascular endothelial growth factor blockade: ascites and pleural effusion formation. *Oncologist* 2000;5:45–50.
- Smith EM, Jayson GC. The current and future management of malignant ascites. *Clin Oncol (Royal College of Radiologists)* 2003;15:59–72.
- Ozols RF, Wilson JK, Weltz MD, Grotzinger KR, Myers CE, Young RC. Inhibition of human ovarian cancer colony formation by Adriamycin and its major metabolites. *Cancer Res* 1980;40:4109–12.
- Boocock CA, Charnock-Jones DS, Sharkey AM, et al. Expression of vascular endothelial growth factor and its receptors flt and KDR in ovarian carcinoma. *J Natl Cancer Inst* 1995;87:506–16.
- Hasumi Y, Mizukami H, Urabe M, et al. Soluble FLT-1 expression suppresses carcinomatous ascites in nude mice bearing ovarian cancer. *Cancer Res* 2002;62:2019–23.
- Graves LE, Ariztia EV, Navari JR, Matzel HJ, Stack MS, Fishman DA. Proinvasive properties of ovarian cancer ascites-derived membrane vesicles. *Cancer Res* 2004;64:7045–9.
- Liu J, Yang G, Thompson-Lanza, JA, et al. A genetically defined model for human ovarian cancer. *Cancer Res* 2004;64:1655–63.
- Johnston SRD. Ovarian cancer: review of the National Institute for Clinical Excellence (NICE) guidance recommendations. *Cancer Invest* 2004;22:730–42.
- Willemsse PH, de Vries EG. Intraperitoneal chemotherapy for ovarian cancer: a question of feasibility? *Drug Resist Update* 2003;6:165–7.
- Mohamed F, Stuart OA, Sugarbaker PH. Pharmacokinetics and tissue distribution of intraperitoneal docetaxel with different carrier solutions. *J Surg Res* 2003;113:114–20.
- Horton J. Albendazole: a review of anthelmintic efficacy and safety in humans. *Parasitology* 2000;121:S113–32.
- Barrowman MM, Marriner SE, Bogan JA. The binding and subsequent inhibition of tubulin polymerization in *Ascaris suum* (*in vitro*) by benzimidazole anthelmintics. *Biochem Pharmacol* 1984;33:3037–40.
- Lacey E. Mode of action of benzimidazoles. *Parasitol Today* 1990;6:112–5.
- Morris DL, Dykes PW, Dickson B, Marriner SE, Bogan JA, Burrows FG. Albendazole in hydatid disease. *BMJ* 1983;286:103–4.
- Pourgholami MH, Woon L, Almajd R, Akhter J, Bowers P, Morris DL. *In vitro* and *in vivo* suppression of growth of hepatocellular carcinoma cells by albendazole. *Cancer Lett* 2001;165:43–9.
- Morris DL, Jourdan JL, Pourgholami MH. Pilot study of albendazole in patients with advanced malignancy. Effect on serum tumor markers/high incidence of neutropenia. *Oncology* 2001;61:42–6.
- Mukhopadhyay T, Sasaki J, Ramesh R, Roth JA. Mebendazole elicits a potent antitumor effect on human cancer cell lines both *in vitro* and *in vivo*. *Clin Cancer Res* 2002;8:2963–9.
- Sasaki J, Ramesh R, Chada S, Gomyo Y, Roth JA, Mukhopadhyay T. The anthelmintic drug mebendazole induces mitotic arrest and apoptosis by depolymerizing tubulin in non-small cell lung cancer cells. *Mol Cancer Ther* 2002;1:1201–9.
- De Silva N, Guyatt H, Bundy D. Anthelmintics. A comparative review of their clinical pharmacology. *Drugs* 1997;53:769–88.
- Marriner SE, Morris DL, Dickson B, Bogan JA. Pharmacokinetics of albendazole in man. *Eur J Clin Pharmacol* 1986;30:705–8.
- Pourgholami MH, Akhter J, Wang L, Lu Y, Morris DL. Antitumor activity of albendazole against the human colorectal cancer cell line HT-29: *in vitro* and in a xenograft model of peritoneal carcinomatosis. *Cancer Chemother Pharmacol* 2005;55:425–32.
- Hu L, Zaloudek C, Mills GB, Gray J, Jaffe RB. *In vivo* and *in vitro* ovarian carcinoma growth inhibition by a phosphatidylinositol 3-kinase inhibitor (LY294002). *Clin Cancer Res* 2000;6:880–6.
- Byrne AT, Ross L, Holash J, et al. Vascular endothelial growth factor-trap decreases tumor burden, inhibits ascites, and causes dramatic vascular remodeling in an ovarian cancer model. *Clin Cancer Res* 2003;9:5721–8.
- Hamilton TC, Young RC, Louie KG, et al. Characterization of a xenograft model of human ovarian carcinoma which produces ascites and intraabdominal carcinomatosis in mice. *Cancer Res* 1984;44:5286–90.
- Hamburger AW, Salmon SE, Kim MB, et al. Direct cloning of human ovarian carcinoma cells in agar. *Cancer Res* 1978;38:3438–44.
- Hu L, Hofmann J, Lu Y, Mills GB, Jaffe RB. Inhibition of phosphatidylinositol 3'-kinase increases efficacy of paclitaxel *in vitro* and *in vivo* ovarian cancer models. *Cancer Res* 2002;62:1087–92.
- Stoeltzing O, Ahmad SA, Liu W, et al. Angiopoietin-1 inhibits tumour growth and ascites formation in a murine model of peritoneal carcinomatosis. *Br J Cancer* 2002;87:1182–7.
- Kobayashi H, Yagyu T, Inagaki K, et al. Bikunin plus paclitaxel markedly reduces tumor burden and ascites in mouse model of ovarian cancer. *Int J Cancer* 2004;110:134–9.
- Belotti D, Paganoni P, Manenti L, et al. Matrix metalloproteinases (MMP9 and MMP2) induce the release of vascular endothelial growth factor (VEGF) by ovarian carcinoma cells: implications for ascites formation. *Cancer Res* 2003;63:5224–9.
- Vistica DT, Skehan P, Scudiero D, Monks A, Pittman A, Boyd MR. Tetrazolium-based assays for cellular viability: a critical examination of selected parameters affecting formazan production. *Cancer Res* 1991;51:2515–20.
- Pellizzaro C, Coradini D, Daidone MG. Modulation of angiogenesis-related proteins synthesis by sodium butyrate in colon cancer cell line HT29. *Carcinogenesis* 2002;23:735–40.
- Ferrara N, Hillan KJ, Gerber HP, Novotny W. Discovery and development of bevacizumab, an anti-VEGF antibody for treating cancer. *Nat Rev Drug Discov* 2004;3:391–400.
- Zebrowski BK, Liu W, Ramirez K, Akagi Y, Mills GB, Ellis LM. Markedly elevated levels of vascular endothelial growth factor in malignant ascites. *Ann Surg Oncol* 1999;6:373–8.
- Zebrowski BK, Yano S, Liu W, et al. Vascular endothelial growth factor levels and induction of permeability in malignant pleural effusions. *Clin Cancer Res* 1999;5:3364–8.
- Senger DR, Galli SJ, Dvorak AM, Perruzzi CA, Harvey VS, Dvorak HF. Tumor cells secrete a vascular permeability factor that promotes accumulation of ascites fluid. *Science* 1983;219:983–5.
- Senger DR, Perruzzi CA, Feder J, Dvorak HF. A highly conserved vascular permeability factor secreted by a variety of human and rodent tumor cell lines. *Cancer Res* 1986;46:5629–32.
- Nagy JA, Masse EM, Herzberg KT, et al. Pathogenesis of ascites tumor growth: vascular permeability factor, vascular hyperpermeability, and ascites fluid accumulation. *Cancer Res* 1995;55:360–8.
- Kraft A, Weindel K, Ochs A, et al. Vascular endothelial growth factor in the sera and effusions of patients with malignant and nonmalignant disease. *Cancer* 1999;85:178–87.
- Mu J, Abe Y, Tsutsui T, et al. Inhibition of growth and metastasis of ovarian carcinoma by administering a drug capable of interfering with vascular endothelial growth factor activity. *Jpn J Cancer Res* 1996;87:963–71.
- Luo JC, Toyoda M, Shibuya M. Differential inhibition of fluid accumulation and tumor growth in two mouse ascites tumors by an antivascular endothelial growth factor/permeability factor neutralizing antibody. *Cancer Res* 1998;58:2594–600.
- Hu L, Hofmann J, Zaloudek C, Ferrara N, Hamilton T, Jaffe RB. Vascular endothelial growth factor immunoneutralization plus paclitaxel markedly reduces tumor burden and ascites in athymic mouse model of ovarian cancer. *Am J Pathol* 2002;161:1917–24.
- Dvorak HF. Vascular permeability factor/vascular

- endothelial growth factor: a critical cytokine in tumor angiogenesis and a potential target for diagnosis and therapy. *J Clin Oncol* 2002;20:4368–80.
43. Ferrara N, Gerber HP, LeCouter J. The biology of VEGF and its receptors. *Nat Med* 2003;9:669–76.
44. Mukhopadhyay D, Nagy JA, Manseau EJ, Dvorak HF. Vascular permeability factor/vascular endothelial growth factor-mediated signaling in mouse mesentery vascular endothelium. *Cancer Res* 1998;58:1278–84.
45. Dvorak HF. Rous-Whipple Award Lecture. How tumors make bad blood vessels and stroma. *Am J Pathol* 2003;162:1747–57.
46. Fujimoto J, Sakaguchi H, Hirose R, Ichigo S, Tamaya T. Biologic implications of the expression of vascular endothelial growth factor subtypes in ovarian carcinoma. *Cancer* 1998;83:2528–33.
47. Yamamoto S, Konishi I, Mandai M, et al. Expression of vascular endothelial growth factor (VEGF) in epithelial ovarian neoplasms: correlation with clinicopathology and patient survival, and analysis of serum VEGF levels. *Br J Cancer* 1997;76:1221–7.
48. Manenti L, Paganoni P, Floriani I, et al. Expression levels of vascular endothelial growth factor, matrix metalloproteinases 2 and 9 and tissue inhibitor of metalloproteinases 1 and 2 in the plasma of patients with ovarian carcinoma. *Eur J Cancer* 2003;39:1948–56.
49. Mukhopadhyay D, Zeng H, Bhattacharya R. Complexity in the vascular permeability factor/vascular endothelial growth factor (VPF/VEGF)-receptors signaling. *Mol Cell Biochem* 2004;264:51–61.
50. Mesiano S, Ferrara N, Jaffe RB. Role of vascular endothelial growth factor in ovarian cancer: inhibition of ascites formation by immunoneutralization. *Am J Pathol* 1998;153:1249–56.