Abstract

Ovarian cancer is characterized by i.p. carcinomatosis and massive ascites. Vascular endothelial growth factor (VEGF) plays a pivotal role in tumor angiogenesis and vascular leakage leading to ascites. We assessed the efficacy of a soluble decoy receptor (VEGF Trap) combined with paclitaxel, in a mouse model of human ovarian cancer. Tumor burden after VEGF Trap plus paclitaxel was reduced by ~98% versus controls. No measurable ascites developed in the treated group. Morphologic studies showed that most residual tumor had degenerative changes. Diaphragmatic and hepatic tumors were not found in the VEGF Trap plus paclitaxel group in contrast to controls, indicating lack of metastasis. In vivo FITC-lectin tumor vessel imaging showed sparse, short, straight vessels in treated mice as compared to controls, in which vessels were numerous, irregular, tortuous, and leaky. In a survival study, all controls underwent euthanasia between 29 and 58 days after tumor cell inoculation (cachexia, extensive ascites, and tumor masses). In the VEGF Trap plus paclitaxel group, mice were ambulating and eating normally with no signs of disease for at least 81 days after tumor cell inoculation, and survival occurred for 129.9 ± 38.88 days with no further treatment. We conclude that combination therapy with VEGF Trap plus paclitaxel may provide a novel, long-lasting therapeutic strategy for treatment of patients with ovarian cancer associated with ascites.

Somewhat uniquely, advanced epithelial ovarian cancer, the most lethal of the gynecologic malignancies, is frequently characterized by i.p. carcinomatosis and massive ascites with little evidence of distant metastases. Ovarian tumorigenesis is associated with increased tumor vessel formation and increased vascular permeability. Vascular endothelial growth factor (VEGF), also termed vascular permeability factor, plays a critical role both in stimulating capillary mitogenesis and ascites formation (1), leading to tumor growth and often massive ascites. VEGF can also stimulate production of marrow-derived circulating endothelial progenitor cells (2). Thus, VEGF inhibitors should be effective agents in treating ovarian cancer, and in preclinical models of the disease, this has been shown by us and others (3–6). Specifically, we showed that a novel, soluble VEGF decoy receptor, the VEGF Trap, comprised of fragments of VEGF receptor (VEGFR)-1 (flt1) and VEGFR2 (flk1, KDR), could markedly decrease visible ascites formation and reduce tumor burden by ~63% in a xenogeneic nude mouse model of advanced human ovarian cancer (4).

Recent studies indicate that combining antiangiogenic agents with conventional chemotherapeutics enhances the inhibition of tumor growth and metastasis (3, 7–10). As paclitaxel, a cytotoxic chemotherapeutic agent, is commonly used to treat patients with ovarian cancer, we tested it in combination with various agents believed to have antiangiogenic activity. For instance, we previously showed that combination of a monoclonal antibody to human VEGF and paclitaxel reduced tumor burden by ~83% and almost completely inhibited ascites in our mouse model (3). We have also investigated the effects of combining paclitaxel with an inhibitor of phosphatidylinositol-3 kinase, as it is involved in the signaling pathway of VEGF (11) and is present in increased copy number in approximately half of all primary ovarian cancers and cell lines (12). As with the antibody to VEGF, we found enhanced efficacy when the agents were combined and that the phosphatidylinositol-3 kinase inhibitor prolonged the tumor's sensitivity to paclitaxel (13).

Because the VEGF Trap proved to be particularly potent in our model of ovarian cancer, we felt that combination...
studies with the VEGF Trap and paclitaxel were warranted. As continuous low-dose chemotherapy coupled with an anti-VEGFR-2 blocking antibody has been a successful treatment strategy in some preclinical models of cancer (14), we chose a dosing strategy that involved prolonged treatment with a relatively low dose of paclitaxel in combination with the VEGF Trap.

We show that when this combination is used to treat athymic nude mice bearing human epithelial ovarian cancer, it inhibits measurable ascites while reducing tumor burden by a striking ~98% without visible side effects. Furthermore, in a survival study, controls were euthanized between 29 and 58 days after tumor cell inoculation because of cachexia, extensive ascites, and tumor masses. In the VEGF Trap plus paclitaxel group, mice were amputating and eating normally with no signs of disease for at least 81 days after tumor cell inoculation, and survival continued for up to 225 days with no further treatment.

Materials and Methods

Materials. Paclitaxel was from Sigma Chemical Co. (St. Louis, MO). VEGF Trap, vehicle, and human Fc control were from Regeneron Pharmaceuticals (Tarrytown, NY). The human OVCAR-3 cell line, which causes ascites earlier in the course of the disease process than in mice bearing SKOV3 cell-derived tumors that we had used previously, was kindly provided by T. Hamilton, Fox Chase Cancer Center, Philadelphia, PA. All cell culture reagents were obtained from the Cell Culture Facility, University of California, San Francisco.

Experimental animals. Sixty female athymic immunodeficient mice (Simonsen Laboratories, Gilroy, CA) were delivered to the University of California, San Francisco Laboratory Animal Resource Center, housed in isolated conditions, fed autoclaved standard pellets and water, and allowed to adapt to their new environment. All protocols involving immuno deficient mice were approved by the Committee on Animal Research, University of California, San Francisco.

Experimental design. Four groups of female athymic nude mice (5-7 weeks, n = 40) were inoculated i.p. with OVCAR-3 cells. Two weeks after inoculation, one group (n = 10) was treated with VEGF Trap (10 mg/kg body weight) thrice weekly plus paclitaxel (10 mg/kg body weight) thrice weekly, on alternate days for 4 weeks. A second group (n = 10) was treated with VEGF Trap alone (10 mg/kg body weight) thrice weekly. The third group (n = 10) was treated with 10 mg/kg body weight paclitaxel alone. The remainder (n = 10) received vehicle alone. The dose of paclitaxel and VEGF Trap were chosen based on preliminary dose-response studies.

In a subsequent survival study, four groups of mice (n = 20) received VEGF Trap and paclitaxel or vehicle for 4 weeks as above but then were observed after tumor cell inoculation until undergoing euthanasia according to IACUC guidelines. Two other groups (n = 10) were treated with VEGF Trap alone or paclitaxel alone.

Methods. To prepare cells for inoculation, they were collected from ascites fluid of athymic mice inoculated with OVCAR-3 cells. Ascites fluid was collected and placed in a 4°C refrigerator for 1 to 2 hours. The supernatant was discarded and the cells were diluted with RPMI 1640 supplemented with 2.0 g/L glucose and 0.3 g/L L-glutamine that had been prewarmed in a 37°C incubator. Athymic mice (5-7 weeks) were inoculated i.p. with OVCAR-3 cells (n = 40, 2 x 10⁶ cells per mouse in 500 µL RPMI 1640). Abdominal circumference and body weight were measured weekly. At the end of the experiment, mice underwent euthanasia with CO₂. The volume of ascites was measured, tumor tissue was excised, weighed, and fixated in 4% paraformaldehyde (pH 7.4), at 4°C for 24 hours and embedded in paraffin. Paraffin sections (5 µm) were used for histochemical analysis.

Light microscopy and analysis. Tissue sections of ovarian cancer from OVCAR-3 cell–inoculated mice treated with VEGF Trap and paclitaxel, singly and in combination, were examined with a Leica DMRB or Leica Ortholux II photomicroscope at low and high magnifications. Images were collected with a Photonics DEI-470 CCD camera and a RasterOps 24XLTV frame grabber, imported directly into Adobe Photoshop, and stored on a ZIP external 100 MB drive (Iomega). Photomicrographic plates were composed from the original data in Photoshop, without alteration or manipulation, and annotated with rub-on letters and symbols.

Apoptosis assessment. Paraffin sections (5 µm) of ovarian cancer tissue from OVCAR-3 cell–inoculated mice were used to assess apoptosis. DNA labeling with digoxigenin dUTP and terminal transferase, followed by immunocytochemical staining with peroxidase-coupled antidigoxigenin antibody and diaminobenzidine, was carried out with the reagents supplied in the Apoptag kit (Intergen, Purchase, NY) according to the manufacturer’s instructions, except that TRIS was substituted for phosphate in the wash buffer. After light counterstaining with hematoxylin, nuclei that stained brown were scored as positive for apoptosis and those that stained blue were scored as negative. At least five 150× microscopic fields were scored, and the apoptotic index was calculated as the percentage of cells that were scored positive.

Tumor vessel imaging. OVCAR-3 cell–inoculated mice with and without treatment with VEGF Trap plus paclitaxel were anesthetized and injected i.v. with 100 µL FITC-labeled lycopericon lectin (Jackson Immunology Research, West Grove, PA). Ten minutes later, mice were perfused through the ascending aorta with 4% paraformaldehyde in PBS for 2 minutes. Tumors were removed and placed in fixative for 2 hours followed by immersion in 30% sucrose. Blood vessels were visualized by their uptake of fluorescent lycopericon lectin.

Statistics. Results are presented as mean ± SE. Data were analyzed using one-way ANOVA followed by unpaired Student’s t test for comparison between groups. Differences between groups were considered statistically significant at P < 0.05. Experiments were done in duplicate.

Results

Control of tumor growth. Mean tumor burden in the VEGF Trap plus paclitaxel–treated group (0.069 ± 0.02 g) was significantly reduced by 97.67%, compared with controls (2.970 ± 0.682 g). The tumor burdens in the VEGF Trap-alone (1.314 ± 0.31 g), and paclitaxel-alone (1.339 ± 0.22 g) groups were reduced by 55.68% and 54.84%, respectively, compared with controls (Fig. 1).

Control of ascites formation. Mean volume of ascites in the controls was 2.30 ± 0.54 mL. In contrast, no ascites was detectable in the VEGF Trap plus paclitaxel–treated group. Mean volume of ascites in the VEGF Trap alone group (0.082 ± 0.08 mL) and paclitaxel alone group (0.334 ± 0.15 mL) were significantly reduced by 96.43% and 85.49%, respectively, compared with controls (Fig. 2).

Vascular architecture. Both the VEGF Trap alone and, to an even greater extent, the VEGF Trap with paclitaxel, remodeled the tumor vasculature (Fig. 3A-D). As seen in Fig. 3A, vessels in the untreated mice were numerous, irregular, tortuous, and formed irregular loops. Vessels in mice treated with the VEGF Trap alone were straighter and less numerous than those in the controls (Fig. 3B), similar to those we reported previously (4). Vessels in mice treated with paclitaxel alone were irregular in size and shape, less numerous than in the untreated mice, with some evidence of vascular leakage.
(Fig. 3C). Vessels in the VEGF Trap plus paclitaxel mice were the least numerous of all of the groups, and were short and straight (Fig. 3D).

**Apoptosis.** Figure 4 illustrates apoptosis in ovarian cancer tissue from OVCAR-3-inoculated mice. Brown nuclei (indicated by arrows) indicate cells that underwent apoptosis. Apoptosis in OVCAR-3 cells in the VEGF Trap–treated group and paclitaxel-treated group was ~10% and 30%, respectively. Over 90% of tissue in Fig. 3D from the VEGF Trap plus paclitaxel group was necrotic or had undergone apoptosis with cytoplasmic debris and calcification; the small amount of residual tumor cells showed degenerative changes, including decreased nuclear size, hyperchromasia and smudging of nuclear chromatin. In some cells, cytoplasm was dense and decreased in amount, whereas a few cells were swollen. There were no significant changes in either control group. At least three 150× microscopic fields were scored.

**Metastasis.** Ninety percent of mice in the control and VEGF Trap–alone groups and 80% of the mice in the paclitaxel-alone groups had tumors on the diaphragm. Ninety percent of the mice in the control and 60% of the mice in both VEGF Trap-alone and paclitaxel-alone groups had tumors in the hilus of the liver. However, these diaphragmatic and hepatic tumors were not found in the combined VEGF Trap plus paclitaxel groups, indicating lack of metastasis.

**Mouse appearance and behavior.** There was no observable difference in behavior (degree of activity and eating) between mice treated with the VEGF Trap + paclitaxel and normal, non-tumor-inoculated mice (mouse video: http://obgyn.ucsf.edu/page.cfm?id=363). As seen in Fig. 4A and B, there was extensive increase in abdominal girth, indicative of ascites and tumor burden and extent of i.p. tumor dissemination in the non-treated tumor-inoculated mice, reduction in both abdominal girth and extent of i.p. tumor dissemination in both the paclitaxel-only and VEGF Trap–only treated mice, and marked decrease in girth and tumor in the VEGF Trap + paclitaxel–treated mice.

**Survival study.** In both replicates of the study, control mice underwent euthanasia between 29 and 58 days after tumor cell inoculation (mean, 47.8 ± 7.4 days) in accordance with IACUC guidelines. Conversely, mice in the VEGF Trap plus paclitaxel group survived for at least 81 days after tumor cell inoculation (129.9 ± 38.88 days), and several were eating and ambulating normally for up to 120 days after tumor cell inoculation (Fig. 5). When mice in the VEGF Trap plus paclitaxel group were sacrificed, some had significant ascites, hepatic enlargement, jaundice, bile duct and/or bowel obstruction, indicative of advanced disease. The survival duration of the paclitaxel-only group was 70.6 ± 23.5 days (SD) following tumor cell inoculation. In the VEGF Trap–only group, the survival duration after tumor cell inoculation was 49.4 ± 4.48 days.

**Discussion**

We show in this study that the combination of the VEGF Trap and paclitaxel causes a striking reduction in tumor burden of ~98% and virtually complete inhibition of ascites formation and tumor metastasis in an athymic xenograft mouse model of human ovarian cancer, and that the effect is markedly prolonged after discontinuing treatment. The changes during treatment occurred in the absence of observable side effects: eating behavior and mobility of VEGF Trap plus paclitaxel–treated mice and noninoculated controls showed no visible differences. Although we (3) and others (7–10) have shown that the combination of antiangiogenic and chemotherapeutic agents inhibit tumor growth and metastasis, the magnitude of ovarian tumor reduction and lack of observable side effects in the present study have not been reported previously, to the best of our knowledge. Both the potency of the VEGF Trap, as well as the relatively low extended dose of paclitaxel that was employed, are likely to have contributed to the success of this drug combination.

One of the most striking effects that occurred as a consequence of combination therapy was the dramatic enhancement of tumor apoptosis, which subsequently led to tumor necrosis. In tumors from the combined therapy group, even the small number of residual tumor cells showed degenerative changes, and this profound effect of the combined treatment was reflected in the survival study, in which mice receiving combined therapy lived for protracted periods after treatment was discontinued. In contrast to the dramatic tumor cell death observed in the combined therapy group, tumor cell
apoptosis was limited in mice receiving either agent alone, and this is reflected in the survival study. Mice receiving only VEGF Trap did not live longer than control mice, whereas those receiving paclitaxel as a single agent had only a modest survival benefit following cessation of drug treatment. Understanding the factors underlying the differences in survival in each of the treatment groups may provide insight into the mechanisms by which the potency of combined therapy is achieved and consequently may provide insight into successful clinical strategies.

Fig. 3. Vascular architecture in OVCAR-3-inoculated nude mice treated with VEGF Trap and paclitaxel for 4 weeks, alone and in combination. A, control: extensive microvascular network comprised of numerous tortuous, irregular vessels; B, VEGF Trap: few, straight, short vessels; C, paclitaxel: irregular vessels, vascular leakage; D, VEGF Trap plus paclitaxel: scant, short, straight vessels.

Fig. 4. Apoptosis in OVCAR-3-inoculated nude mice treated with VEGF Trap and paclitaxel for 4 weeks, alone and in combination. A, control: solid sheets of tumor cells, no apoptosis/necrosis; B, VEGF Trap: areas of apoptosis (brown stain, Apoptag); C, paclitaxel: areas of apoptosis (brown stain, Apoptag); D, VEGF Trap plus paclitaxel: scant tumor cells, extensive necrosis and apoptosis (brown stain) with few remaining cells.
vasculature, the VEGF Trap allows better tumor perfusion, and thus paclitaxel is more successfully delivered to the tumor. A second possibility is that by decreasing vascular permeability, the VEGF Trap prevents a number of serum-derived growth factors that promote tumor cell survival from reaching the tumor parenchyma, thereby sensitizing the tumor to paclitaxel. A further explanation may be that paclitaxel, in addition to its antitumor effect, may have antiangiogenic activity (16), because the dividing, genetically stable endothelial cells of newly forming tumor vessels are believed to be sensitive to chemotherapeutic drugs (17), and blockade of VEGF, a factor that promotes endothelial cell survival, may enhance this sensitivity. Additional experiments will be required to determine if any or all of these mechanisms contribute to the success of combination therapy.

In this study, we used a dose of paclitaxel below which we had previously identified as the maximally tolerated dose, and we administered the drug for a longer period than that which would be considered a course of treatment. We believe that the dosing regimen we employed provides some of the benefits associated with “metronomic” drug dosing. Others have shown that low-dose continuous chemotherapy seems to sensitize tumors to antiangiogenic agents even in cases in which the tumors are resistant to the same cytotoxic drugs administered with intermittent high doses (18). It has been suggested that the low dose of chemotherapeutic drugs can provide a stable and safe way to overcome drug resistance in growing tumors, providing they are used in combination with a second antiangiogenic drug (19). Although we believe that the dosing regimen we employed may have afforded us some of the benefits of metronomic dosing, most of the mice in our survival study eventually succumbed to disease. Consequently, we believe that in future studies, treatment with low-dose paclitaxel should be continued for an extended period. We are currently exploring the possibility of eradicating ovarian cancer in our mouse model by coupling VEGF Trap treatment with metronomic paclitaxel. As there were some signs of tumor recurrence after a protracted period, tumor cells may have undergone mutations permitting their regrowth. This has been observed with other tumors and other chemotherapeutic agents in mouse xenografts (20–22).

Taken together, the combination of VEGF Trap and paclitaxel ultimately limits the blood supply and markedly increases tumor cell apoptosis and necrosis. This treatment dramatically reduces tumor burden, inhibits ascites and metastasis and greatly extends the life of the mice. Our data suggest that the combination of the VEGF Trap and paclitaxel may have novel clinical application in markedly reducing tumor growth and inhibiting ascites and metastasis over a prolonged period in patients with advanced ovarian cancer.

References
Vascular Endothelial Growth Factor Trap Combined with Paclitaxel Strikingly Inhibits Tumor and Ascites, Prolonging Survival in a Human Ovarian Cancer Model

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