Vascular Endothelial Growth Factor Is a Promising Therapeutic Target for the Treatment of Clear Cell Carcinoma of the Ovary

Seiji Mabuchi¹, Chiaki Kawase¹, Deborah A. Altomare⁴, Kenichirou Morishige¹, Masami Hayashi¹, Kenjiro Sawada¹, Kimihiko Ito², Yoshito Terai³, Yukihiro Nishio³, Andres J. Klein-Szanto⁵, Robert A. Burger⁴,⁶, Masahide Ohmichi³, Joseph R. Testa⁵, and Tadashi Kimura¹

Abstract

This study examines the role of vascular endothelial growth factor (VEGF) as a therapeutic target in clear cell carcinoma (CCC) of the ovary, which has been regarded as a chemoresistant histologic subtype. Immunohistochemical analysis using tissue microarrays of 98 primary ovarian cancers revealed that VEGF was strongly expressed both in early-stage and advanced-stage CCC of the ovary. In early-stage CCCs, patients who had tumors with high levels of VEGF had significantly shorter survival than those with low levels of VEGF. In vitro experiments revealed that VEGF expression was significantly higher in cisplatin-refractory human CCC cells (RMG1-CR and KOC7C-CR), compared with the respective parental cells (RMG1 and KOC7C) in the presence of cisplatin. In vivo treatment with bevacizumab markedly inhibited the growth of both parental CCC cell-derived (RMG1 and KOC7C) and cisplatin-refractory CCC cell-derived (RMG1-CR and KOC7C-CR) tumors as a result of inhibition of tumor angiogenesis. The results of the current study indicate that VEGF is frequently expressed and can be a promising therapeutic target in the management of CCC. Bevacizumab may be efficacious not only as a first-line treatment but also as a second-line treatment of recurrent disease in patients previously treated with cisplatin.

Introduction

Ovarian carcinoma is the fourth most common cause of cancer death among women in the United States, with more than 21,550 new cases diagnosed and 14,600 deaths estimated for 2009 (1). Cytoreductive surgery followed by platinum-based chemotherapy usually combined with paclitaxel is the standard initial treatment and has improved survival in patients with epithelial ovarian cancer (2). However, although this treatment regimen is initially effective in a high percentage of cases, most patients will ultimately experience a relapse due to the development of chemoresistance (3).

Clear cell carcinoma (CCC) of the ovary, which was first recognized by the World Health Organization as a distinct histologic subtype in 1973 (4), is known to show poorer sensitivity to platinum-based chemotherapy and to be associated with a worse prognosis than the more common serous adenocarcinoma (SAC). A recent retrospective review of six randomized phase III clinical trials has shown that patients with stage III CCC treated with carboplatin-paclitaxel in the setting of first-line chemotherapy had a significantly shorter survival compared with those with other histologic subtypes of epithelial ovarian cancer (5). Another important problem in the clinical management of CCC is the lack of effective chemotherapy for recurrent CCC after first-line treatment with platinum-based chemotherapy. A recent report showed that the response rate for various regimens in the setting of second-line chemotherapy for recurrent platinum-resistant CCC was only 1% (6). Therefore, to improve the survival of patients with CCC, the development of novel treatment strategies in the setting of both first-line treatment and salvage treatment for recurrent disease are needed.

One possible treatment strategy that may improve patient outcome is the use of angiogenesis-targeted agents. Among the variety of potential targets of angiogenesis, vascular endothelial growth factor (VEGF)
and its signaling pathway is reported to be a promising target in anticancer therapy (7–9). In a murine model of ovarian cancer, Zhang et al. (10) showed that VEGF overexpression was associated with tumor growth, angiogenesis, ascites formation, and tumor cell survival. Clinically, it has been previously reported that VEGF is overexpressed in most ovarian tumors (11–13). High levels of VEGF have also been found in the serum, plasma, and ascites of ovarian cancer patients and are associated with poor patient prognosis (14–16). However, because most tumors investigated in previous studies have been from ovarian SACs (11–13), the expression rate of VEGF and its prognostic significance in CCC of the ovary remain unknown. Moreover, although bevacizumab, a humanized monoclonal antibody against human VEGF, has shown significant single-agent activity in phase II trials involving patients with recurrent ovarian cancer, little information is available regarding its antitumor efficacy in patients with CCC. Thus, the therapeutic potential of bevacizumab or other VEGF inhibitors in patients with CCC is unknown (7, 8, 17).

The major limitation in conducting researches on ovarian CCC is the rarity of this histologic subtype. The precise incidence of CCC is unknown, but it is reported to be approximately 5% of all histologic subtypes among epithelial ovarian cancers in Western countries (18). However, in Japan, it is the second most frequent histologic subtype. More than 20% of ovarian cancers are classified as CCC (19).

Using clinical samples obtained in Japan, we have recently reported that mTOR is more frequently activated in ovarian CCCs than in SACs (87% versus 50%; ref. 20). It has also been reported that ovarian endometriosis, from which CCC is thought to arise, is characterized by hyperactivation of the AKT-mTOR pathway (21). Moreover, it has been recently reported that hypoxia-inducible factor 1α (HIF-1α) expression levels are significantly higher in CCC than in other histologic subtypes of ovarian cancer (22). Because AKT-mTOR signaling has been shown to stimulate the expression of HIF-1α and VEGF, leading to tumor angiogenesis essential for tumor growth, invasion, and metastasis (23), the VEGF pathway holds promise as a target in the therapy of CCC. In the current investigation, we examined the expression of VEGF in both early-stage and advanced-stage CCC, and determined its correlation with patient prognosis. Moreover, we investigated the therapeutic potential of bevacizumab in both cisplatin-sensitive and cisplatin-resistant CCC cells in vitro and in vivo.

Materials and Methods

Reagents/antibodies

Bevacizumab was obtained from Genentech, Inc. ECL Western blotting detection reagents were purchased from Perkin-Elmer. Anti-VEGF antibody (A-20) was obtained from Santa Cruz Biotechnology. Antibodies recognizing poly(ADP-ribose) polymerase (PARP) and β-actin were obtained from Cell Signaling Technology. Anti-CD31/platelet/endothelial cell adhesion molecule 1 (PECAM-1) antibody was obtained from Abcam. Cell Titer 96-well proliferation assay kit was obtained from Promega. Cisplatin was purchased from Sigma.

Drug preparation

Bevacizumab was diluted to the appropriate concentration in PBS before addition to cell culture. For animal studies, 5 mg/kg bevacizumab were diluted in 200 μL of PBS before administration.

Clinical samples

Tumor samples were obtained from patients undergoing primary cytoreductive surgery at the Osaka Medical College Hospital, Wakayama Rosai Hospital, Osaka Police Hospital, Kansai Rosai Hospital, and Sakai Municipal Hospital before any other therapeutic intervention between 1991 and 2006. All surgical specimens and clinical data were collected and archived according to protocols approved by the institutional review boards of these hospitals. Appropriate informed consent was obtained from each patient. Histologic diagnosis was based on World Health Organization criteria. The tumors included 52 CCCs and 46 SACs for reference as described previously (20). Based on the International Federation of Gynecology and Obstetrics criteria, 27 CCCs were stage I-II tumors and 25 were stage III-IV tumors. Among SACs, 22 were stage I-II tumors and 24 were stage III-IV tumors. The duration of overall survival was measured from the date of diagnosis to death or censored at the date of last follow-up.

Immunohistochemistry

Primary ovarian tumors obtained from patients were fixed in 10% neutral buffered formalin (10% formaldehyde, phosphate-buffered) overnight and then embedded in paraffin. From each case, a representative tissue block consisting of predominantly viable tumor tissue was selected from hematoxylin and eosin (H&E) slides. Ovarian cancer tissue microarrays consisting of two cores from each tumor sample were prepared by the Tumor Bank Facility at Fox Chase Cancer Center, as described previously (20, 24, 25). For each tumor, 5-μm-section slides stained with H&E were used to locate representative malignant areas. Two cores (0.6 mm) were punched from the morphologically representative area of the donor block and then placed in the recipient paraffin tissue microarray block. Fresh 4-μm sections were obtained from each tissue microarray block, mounted on slides, and processed for either H&E or immunohistochemical staining. For immunohistochemical studies, sections were incubated with the primary antibody, followed by the appropriate peroxidase-conjugated secondary antibody, as reported previously (20, 25). The primary antibody used was anti-VEGF antibody at 1:50 dilution. Surrounding nonneoplastic stroma served as an internal negative control for each slide. The slides were scored semiquantitatively by a pathologist who was blinded to
the clinical outcome. A score of 0 indicated no staining, +0.5 indicated weak focal staining (less than 10% of the cells were stained), +1 indicated focal staining (10–50% of the cells were stained), +2 indicated clearly positive staining (more than 50% of the cells were stained), and a score of +3 indicated intensely positive staining, as described in detail elsewhere (20). The slides were examined using a bright field microscope by two observers (A.J.K. and K.M.) who were blinded to the clinical data of the patients. Tumors with a staining of +2 or +3 were grouped as a strong-staining group, whereas tumors with a staining of +0.5 or +1 were grouped as a weak-staining group. When the two cores from the same tumor sample showed different positivity results, the lower score was considered valid.

Cell culture

Human ovarian CCC cell lines RMG1 and KOC7C were kindly provided by Dr. H. Tamochi (Tottori University, Tottori, Japan). These cells were cultured in phenol red–free Dulbecco’s modified Eagle’s medium (DMEM Ham’s F-12, Gibco Ltd.) with 10% fetal bovine serum (FBS), as reported previously (26–28). Human umbilical vein endothelial cells (HUVEC) were isolated by trypsin digestion of umbilical veins from fresh umbilical cords and maintained in HuMedia-EG2 medium (Kurabo Industries) as described previously (29). Subcultures were obtained by trypsinization and were used for experiments at passages 3 to 5. To expose cells to hypoxia, cultures were placed into a multigas incubator (SANYO) that was infused with a mixture of 1% O₂, 5% CO₂, and 94% N₂, and incubated at 37°C.

Establishment of cisplatin-refractory cell lines

As a preclinical model of recurrent CCCs after the first-line platinum-based chemotherapy, cisplatin-refractory sublines from RMG1 and KOC7C were developed in our laboratory by continuous exposure to cisplatin, as described previously (20, 30). Briefly, cells of both lines were exposed to stepwise increases in cisplatin concentrations. Initial cisplatin exposure was at a concentration of 10 nmol/L. After cells had regained their exponential growth rate, the cisplatin concentration was doubled, and then the procedure was repeated until selection at 10 μmol/L was attained. The resulting cisplatin-refractory sublines, dubbed RMG1-CR and KOC7C-CR, were subcultured weekly and treated monthly with 10 μmol/L cisplatin to maintain a high level of chemoresistance.

Cell proliferation assay

An MTS assay was used to analyze the effect of VEGF or bevacizumab on cell viability as described (31). Cells were cultured overnight in 96-well plates (1 × 10⁴ cells per well). Cell viability was assessed after addition of bevacizumab or cisplatin at the indicated concentrations for 48 hours. The number of surviving cells was assessed by determination of the A₄₉₀ nm of the dissolved formazan product after addition of MTS for 1 hour as described by the manufacturer (Promega). Cell viability is expressed as follows: Aₑₓᵖ group/Aₑₓᵖ control × 100.

Western blot analysis

Cells were treated with either PBS or the indicated concentrations of cisplatin for 24 hours. 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 Na₃VO₄, 1 mmol/L β-glycerophosphate, 2.5 mmol/L sodium Pi, 1 mmol/L 4-(2-aminoethyl) benzenesulfonyl fluoride hydrochloride, 10 μg/mL aprotinin, 1 μg/mL leupeptin, and 1% Triton X-100] for 10 minutes at 4°C. Lysates were centrifuged at 12,000 × g at 4°C for 15 minutes, and protein concentrations of the supernatants were determined using the Bio-Rad protein assay reagent. Equal amounts of protein were separated by SDS-PAGE and transferred to nitrocellulose membranes. Blocking was done in 5% nonfat milk in 1× Tris-buffered saline. 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 an enhanced chemiluminescence Western blotting system (Perkin-Elmer).

Tube formation assay

Tube formation assay was done as described previously (29). Briefly, the surfaces of 96-well plates were coated with 30 μL of growth factor–reduced Matrigel matrix (BD Biosciences). Then, 1 × 10⁵ serum-starved HUVECs were plated. Various agents were added at the time of plating. After 8-hour incubation, tube formation was visualized under an inverted microscope (×40) and the images were analyzed.

In vitro VEGF protein quantitation by enzyme-linked immunosorbent assay

RMG1 or KOC7C cells (5 × 10⁴) were incubated in DMEM Ham’s F-12 medium containing 1% FBS for 24 hours. Then, the culture supernatants were collected and levels of VEGF (corrected for cell number) were determined using the Quantikine Human Vascular Endothelial Growth Factor Immunoassay (R&D Systems) according to the manufacturer’s protocol. The remaining monolayers were trypsinized, and the cells were counted to normalize VEGF protein values. VEGF values were derived from a standard curve of known concentrations of recombinant human VEGF. Each sample was analyzed in duplicate and averaged.

Subcutaneous xenograft model

All procedures involving animals and their care were approved by the Institutional Animal Care and Usage Committee of Osaka University, in accordance with institutional and NIH guidelines. Five- to 7-week-old nude mice (n = 48) were inoculated subcutaneously (s.c.) into the right flank with 5 × 10⁶ RMG1, RMG1-CR, KOC7C,
or KOC7C-CR cells in 200 μL of PBS. When tumors reached a size of about 50 mm³, mice were randomly assigned into two treatment groups, with 12 mice in each group. The first group was treated with PBS twice weekly. The second group was treated with bevacizumab (5 mg/kg) twice weekly. Bevacizumab was administered intraperitoneally as described previously (32). Caliper measurements of the longest perpendicular tumor diameters were done every week to estimate tumor volume using the following formula: \( V = L \times W \times D \times \pi / 6 \), where \( V \) is the volume, \( L \) is the length, \( W \) is the width, and \( D \) is the depth as described previously (20, 25, 32).

**Quantification of microvessel area**

Subcutaneous tumors harvested at autopsy were processed for immunostaining using anti-CD31/PECAM-1 antibody at a 1:50 dilution and appropriate peroxidase-conjugated secondary antibodies. The tissue sections were viewed at x100 magnification, and images were captured. Four fields per section were analyzed, excluding necrotic regions. The percentage of CD31-positive microvessel area (MVA) in each field was calculated as described previously (33). The mean value of MVA in each group was calculated from four tumor samples.

**Statistical analysis**

Cell proliferation was analyzed by the Wilcoxon exact test. Differences in VEGF concentrations and the effects of bevacizumab on tumor volume and MVA were analyzed by Student’s \( t \) test. Data are expressed as the mean ± SD. Immunoreactivity was analyzed using Fisher’s exact test. Survival rates were examined using Kaplan-Meier plots, and statistical differences between the survival rates of groups were assessed by the log-rank test. A \( P \) value of <0.05 was considered significant.

**Results**

**VEGF expression in CCCs and SACs**

Immunohistochemical analysis of ovarian cancer tissue microarrays for VEGF expression was done using 52 ovarian CCCs and 46 ovarian SACs as described above. Representative photomicrographs of the CCCs and SACs are shown in Fig. 1A. VEGF immunoreactivity was scored semiquantitatively (Fig. 1B). When analyzed according to surgical-pathologic stage (Table 1), immunoreactivity for VEGF was greater in advanced-stage CCCs than in early-stage CCCs. Among the 27 early-stage CCCs, 7 (26%) were scored as +0.5 or +1, 14 (52%) were scored as +2, and 6 (22%) were scored as +3. In contrast, among the 25 advanced-stage CCCs, 15 (60%) were scored as +2 and 10 (40%) were scored as +3 (Fig. 1C). Similar VEGF immunoreactivity was observed in SACs. Among the 22 early-stage SACs, 4 (18.2%) were scored as +0.5 or +1, 15 (68.2%) were scored as +2, and 3 (13.6%) were scored as +3. In contrast, among the 24 advanced-stage SACs, one (4.2%) was scored as +1, 19 (79.1%) were scored as +2, and 4 (16.7%) were scored as +3. When CCCs were compared with SACs, the frequency of strong VEGF immunoreactivity was slightly higher in CCCs than in SACs in both early-stage and advanced stage; however, the differences were not statistically significant. Collectively, these results indicate that VEGF can be a therapeutic target not only in patients with SAC as shown previously in clinical trials (7, 8, 17) but also in most patients with CCC.

We next examined the impact of VEGF expression on survival in patients with CCC. Although the correlation between VEGF expression and survival has been intensively investigated and reported in patients with SAC, to the best of our knowledge, it has never been examined in patients with CCC. Interestingly, as shown in Fig. 1C, immunoreactivity for VEGF significantly correlated with patient prognosis in patients with early-stage CCC. The overall survival in the group with weak expression of VEGF (mean 60 months) was significantly higher than that in the group with high expression of VEGF (mean 40 months). In patients with advanced-stage CCC, all tumor samples showed strong staining for VEGF; thus, we did not observe a correlation between VEGF immunoreactivity and patient prognosis in this subgroup. However, the overall survival in patients with advanced-stage CCC was significantly shorter than that observed in the early-stage, weak-expression group or in the early-stage, strong-expression group.

**VEGF production in CCC cells and antiangiogenic activity of bevacizumab in vitro**

Given the frequent VEGF expression found in human CCC tumor specimens (Fig. 1), we evaluated the in vitro expression of VEGF in two human CCC cell lines. For this purpose, we assessed the release of VEGF into the culture medium by enzyme-linked immunosorbent assay (ELISA). As shown in Fig. 2A, VEGF was secreted in the culture medium of both CCC cell lines tested, which is consistent with immunohistochemical results observed in tumor samples. The concentration of VEGF significantly increased in response to the exposure to hypoxia in all cell lines.

We next examined the proangiogenic activity of VEGF released by CCC cells. As shown in Fig. 2B (I) and (II), treatment with either recombinant VEGF or cultured medium containing VEGF secreted by CCC cells significantly stimulated the proliferation of HUVECs. Moreover, treatment with either recombinant VEGF or culture medium containing VEGF enhanced the tube formation activity of HUVECs (Fig. 2C). Collectively, these results suggest that VEGF produced by CCC cells have significant angiogenic activity and that VEGF inhibition may be a promising therapeutic strategy in the management of CCC. Therefore, we examined the antiangiogenic activity of bevacizumab in vitro. As shown in Fig. 2B and C, treatment with bevacizumab almost completely inhibited the growth-stimulating activity and tube formation activity of culture medium containing VEGF, consistent with the significant antiangiogenic activities of bevacizumab.
Effect of bevacizumab on the growth of ovarian CCC in vivo

To examine the in vivo growth-inhibitory effect of bevacizumab, we used a xenograft model in which athymic mice were inoculated s.c. with RMG1 or KOC7C cells. When tumors reached ~50 mm³, the mice were randomized into two treatment groups receiving PBS or bevacizumab. Drug treatment was well tolerated, with no apparent toxicity throughout the study. Tumor volume was measured weekly after the start of treatment (Fig. 3). Histologically, these subcutaneous tumors were CCCs (data not shown). At 4 weeks after the start of
treatment, the mean RMG1-derived tumor burden in mice treated with bevacizumab was 232.3 mm³ compared with 456.3 mm³ in PBS-treated mice, and mean KOC7C-derived tumor burden in animals treated with bevacizumab was 198.8 mm³ compared with 532.9 mm³ in PBS-treated mice. These results indicate that bevacizumab has significant antitumor effects as a single agent in this CCC model.

To investigate the mechanism by which bevacizumab inhibited the tumor growth in vivo, the endothelial marker CD31 in RMG1-derived subcutaneous tumors was assessed by immunohistochemistry (Fig. 3C). As shown, large CD31-immunopositive vessels were observed in tumors from PBS-treated mice, whereas fewer and smaller vessels were observed in tumors from bevacizumab-treated mice. There was a significant decrease of MVA in bevacizumab-treated tumors compared with control tumors (Fig. 3D). To exclude the possibility that bevacizumab directly inhibits the growth of CCC cells, we further examined the effect of bevacizumab on the proliferation of CCC cells in vitro. Treatment with either VEGF, bevacizumab, or the combination for 72 hours had no effect on the proliferation of CCC cells (data not shown). These results are consistent with the tumor-suppressive effect of bevacizumab being mediated primarily through inhibition of neovascularization.

VEGF expression in cisplatin-refractory cell lines

To evaluate the preclinical antitumor efficacy of bevacizumab on recurrent CCCs after the first-line treatment with platinum-based chemotherapy, we established cisplatin-refractory CCC cell lines as described above. To examine whether these sublines had acquired resistance to cisplatin, we first evaluated the sensitivity of these cell lines to cisplatin by the MTS assay. As shown in Fig. 4A, clear differential sensitivity to cisplatin was observed between parental cells and respective cisplatin-refractory sublines. Moreover, treatment with cisplatin induced cleavage of PARP in parental cells, but not in cisplatin-refractory sublines (Fig. 4B). We next examined the production of VEGF in both cisplatin-refractory sublines and parental cells by ELISA. As shown in Fig. 4C, in parental CCC cells, VEGF production was significantly inhibited by treatment with cisplatin. In cisplatin-refractory CCC cells, significantly higher concentrations of VEGF were observed than in their respective parental cells. Moreover, VEGF production was not inhibited by treatment with cisplatin.

Effect of bevacizumab on the cisplatin-refractory CCC in vivo

Given the strong VEGF expression found in cisplatin-refractory CCC cells, we next examined the in vivo effect of bevacizumab on cisplatin-refractory CCC. Athymic mice were inoculated s.c. with RMG1-CR or KOC7C-CR cells, and were randomized into two treatment groups receiving PBS or bevacizumab. Graphs depicting diminished tumor volumes in bevacizumab-treated mice relative to PBS-treated mice are shown in Fig. 5. Mean RMG1-CR-derived tumor burden in mice treated with bevacizumab was 173.1 mm³ compared with 496.8 mm³ in PBS-treated mice, and mean KOC7C-CR-derived tumor burden in animals treated with bevacizumab was 171.1 mm³ compared with 566.3 mm³ in PBS-treated mice. The antitumor effect of bevacizumab was similar both in cisplatin-refractory cell–derived

---

**Table 1. VEGF immunoreactivity by histology and clinical stage**

<table>
<thead>
<tr>
<th></th>
<th>No. of patients</th>
<th>Immunoreactivity</th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Zero, n (%)</td>
<td>Weak (+0.5 or 1), n (%)</td>
<td>Moderate (+2), n (%)</td>
<td>Strong (+3), n (%)</td>
<td></td>
</tr>
<tr>
<td><strong>CCCs</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Stage I-II</td>
<td>27</td>
<td>0</td>
<td>7 (26.9)</td>
<td>14 (51.9)</td>
<td>6 (22.2)</td>
<td></td>
</tr>
<tr>
<td>Stage III-IV</td>
<td>25</td>
<td>0</td>
<td>0</td>
<td>15 (60)</td>
<td>10 (40)</td>
<td></td>
</tr>
<tr>
<td><strong>SACs</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Stage I-II</td>
<td>22</td>
<td>0</td>
<td>4 (18.2)</td>
<td>15 (68.2)</td>
<td>3 (13.6)</td>
<td></td>
</tr>
<tr>
<td>Stage III-IV</td>
<td>24</td>
<td>0</td>
<td>1 (4.2)</td>
<td>19 (79.1)</td>
<td>4 (16.7)</td>
<td></td>
</tr>
</tbody>
</table>
CCC and in parental cell–derived CCC (Figs. 3 and 5). Collectively, these in vivo data indicate that inhibition of VEGF may be a reasonable treatment strategy for cisplatin-refractory CCCs.

We finally compared the antitumor effect of cisplatin with that of bevacizumab in vivo. As shown in Fig. 6, although treatment with cisplatin significantly decreased RMG1-derived tumor burden, the antitumor effect of cisplatin was minimal on RMG1-CR–derived tumors, which is consistent with the cisplatin-resistant nature of RMG1-CR cells shown in Fig. 4A. Importantly, the effect of bevacizumab on both RMG1-derived– and RMG1-CR–derived tumors was more profound than that of cisplatin, which may indicate that bevacizumab is clinically more efficacious for CCCs than cisplatin.

Discussion

CCC of the ovary was originally referred to as “mesonephroma” by Schiller in 1939 because it resembled renal carcinoma and was thought to be of mesonephric origin (34). Subsequent findings of its association with endometriosis have suggested that CCC is of Mullerian origin (35), and this tumor was recognized as a distinct histologic subtype of epithelial ovarian tumors by the World Health Organization in 1973 (3). A growing body of evidence has suggested that CCC is associated with a diminished sensitivity to platinum-based chemotherapy and a worse prognosis than other epithelial ovarian cancers. To improve survival, the development of new treatment strategies that target CCC more effectively is necessary.

In a recent gene expression profiling study, it has been reported that renal CCC and ovarian CCC have remarkably similar expression patterns, and thus could not be distinguished statistically (36). Thus, theoretically, a relevant target molecule in the treatment of renal CCC may also hold promise as a therapeutic target in patients with CCC of the ovary (37).

Recently, bevacizumab, a monoclonal antibody to human VEGF, has shown significant antitumor activity in randomized clinical trials and has become the standard of care for patients with metastatic renal cancer (38), 90% of which is clear cell histology (renal CCC). Although significant clinical activity of bevacizumab has been shown in three phase II studies in patients with ovarian cancer (7, 8, 17), most of the patients enrolled...
in these trials had SAC. Only seven patients with CCC were enrolled in these trials; thus, the role of VEGF as a therapeutic target in CCC of the ovary is largely unknown.

Hypoxia commonly develops within tumors. HIF-1α plays a key role in helping hypoxic tumor cells to compensate for hypoxia at the molecular level by increasing the activity or the expression of variety of

![Figure 4. VEGF expression and its role as a therapeutic target in cisplatin-refractory CCC cells. A and B, establishment of cisplatin-refractory variant cell lines. Cisplatin-refractory sublines were established as described in Materials and Methods. A, parental (KOC7C and RMG1) and cisplatin-refractory variant (KOC7C-CR and RMG1-CR) cells were treated with the indicated concentrations of cisplatin in the presence of 5% FBS for 72 h. Cell viability was assessed by MTS assay. Points, mean; bars, SD (*, P < 0.05, **, P < 0.01, significantly different from control.). B, effect of cisplatin on cleavage of PARP in parental and cisplatin-refractory variant cell lines. KOC7C, KOC7C-CR, RMG1, and RMG1-CR treated with 10 μmol/L cisplatin or bevacizumab for 24 h. Cells were harvested, and then lysates were subjected to Western blotting using anti-PARP or anti-β-actin antibody. C, production of VEGF in cisplatin-refractory sublines and parental chemosensitive cells. Levels of secreted VEGF protein in the conditioned medium under normoxic or hypoxic condition were measured by ELISA assay. Columns, mean; bars, SD. *, P < 0.05, significantly different from control.]

www.aacrjournals.org

Mol Cancer Ther; 9(8) August 2010

Published OnlineFirst July 27, 2010; DOI: 10.1158/1535-7163.MCT-10-0169
proteins connected with angiogenesis. Recent work has shown that HIF-1α expression levels are significantly higher in CCC than in other histologic subtypes of ovarian cancer, including serous, mucinous, and endometrioid carcinoma (22). Because HIF-1α stimulates the expression of VEGF and promotes angiogenesis to meet the metabolic requirements for sustained tumor growth (39), there is a particularly strong clinical rationale for blocking VEGF in the treatment for patients with CCC.

In the present study, we show that VEGF was expressed in all stage III-IV CCCs and stage I-II CCCs. Strong VEGF immunoreactivity was observed more frequently in advanced-stage CCCs than in early-stage CCCs, suggesting that advanced-stage CCCs are more dependent on VEGF for tumor progression than early-stage CCCs. Importantly, as previously shown in patients with SAC (12, 13), immunoreactivity for VEGF was inversely correlated with patient prognosis in early-stage CCC. Patients whose tumor showed strong immunoreactivity had significantly shorter survival than those with weak immunoreactivity for VEGF (mean, 60 months versus 40 months, respectively).

We evaluated the efficacy of bevacizumab in vivo, using s.c. xenograft models (Fig. 3). As predicted, intraperitoneal treatment with bevacizumab was well tolerated with no apparent toxicity. In mice inoculated s.c. with RMG1 or KOC7C cells, treatment with bevacizumab significantly inhibited tumor growth. These findings indicate that bevacizumab could have significant antitumor effects as a single agent for CCC in the setting of first-line therapy.
An additional important finding in our study is the antitumor activity of bevacizumab in cisplatin-refractory CCC. The lack of effective chemotherapy for recurrent CCCs after first-line platinum-based chemotherapy is a major clinical problem in the management of these patients. Therefore, identification of new treatment strategies for recurrent CCC of the ovary is urgently needed. In the current study, we found that cisplatin-refractory CCC cell lines exhibit higher VEGF expression compared with the corresponding parental cell lines (Fig. 4C). Similar findings have been reported by others. For example, VEGF production in cisplatin-resistant Lewis lung carcinoma cells has been reported to be 1.5-fold higher than that in cisplatin-sensitive parental cells (40). Moreover, 5-fluorouracil–resistant colon adenocarcinoma subclones were found to have increased expression of VEGF and enhanced proangiogenic activity compared with corresponding primary adenocarcinoma cells (41). These results suggest that cisplatin-refractory tumors might be good candidates for treatment with bevacizumab.

Our cisplatin-refractory CCC cell–derived tumors showed significant sensitivity to bevacizumab in vivo (Fig. 5). However, although cisplatin-refractory CCC cells express higher concentrations of VEGF than parental cells, the antitumor effect of bevacizumab on cisplatin-refractory cell–derived tumors was similar to that observed in parental cell–derived tumors. This finding is consistent with recent reports suggesting that VEGF expression may not be a reliable biomarker for predicting sensitivity to bevacizumab (42). Importantly, when we directly compared bevacizumab with cisplatin, the in vivo antineoplastic effect of bevacizumab on CCC-derived tumor growth was more profound than that for cisplatin (Fig. 6). The dose of cisplatin used in our experiment (3 mg/kg) is roughly equivalent to the standard clinical dose (50–75 mg/m^2) used in patients (43). Moreover, the RMG1-CR and KOC7C-CR cells used in this study mimic the clinical situation involving the development of resistance to cisplatin. Thus, our results suggest that bevacizumab might be clinically efficacious not only as a first-line treatment but also as a second-line treatment of recurrent disease in patients previously treated with cisplatin.

In conclusion, our collective findings indicate that bevacizumab is a promising agent for the treatment of CCC of the ovary both as a first-line treatment and as a salvage treatment for recurrence after platinum-based chemotherapy. Although bevacizumab is currently being evaluated by the Gynecologic Oncology Group (GOG) in protocol GOG 218 (9) to evaluate the benefit of bevacizumab in combination with first-line chemotherapy as well as when administered as maintenance therapy, to date, no clinical studies have been initiated to examine the antitumor effect of bevacizumab specifically in patients with CCC. We believe that our data provide significant rationale for future clinical trials with bevacizumab in patients with CCC of the ovary.

**Disclosure of Potential Conflicts of Interest**

No potential conflicts of interest were disclosed.

**Acknowledgments**

We thank Drs. Min Huang and Yulan Gong in the Tumor Bank Facility for preparing tissue microarrays, and Drs. M. Tsujimoto (Department of Pathology, Osaka Police Hospital), Y. Tsutsuma (Department of Pathology, Wakayama Rosai Hospital), Y. Hoshiba (Department of Pathology, Kansai Rosai Hospital), and H. Miwa (Department of Pathology, Sakai Municipal Hospital) for providing tumor specimens and clinical information.

**Grant Support**

This publication was supported in part by NIH grants P30 CA069274, CA74249, and P50 CA83638, and by an appropriation from the Commonwealth of Pennsylvania. Grant-in-aid for Young Scientists no. 21791554 from the Ministry of Education, Culture, Sports, Science and Technology of Japan. D.A. Altomare is supported in part by the Liz Tilberis Scholar Program, sponsored by the Ovarian Cancer Research Fund, Inc. 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.

Received 02/22/2010; revised 05/27/2010; accepted 06/04/2010; published OnlineFirst 07/27/2010.

**References**

11. Wong C, Wellman TL, Lounsbery KM. VEGF and HIF-1α expression

www.aacrjournals.org Mol Cancer Ther; 9(8) August 2010 2421

Published OnlineFirst July 27, 2010; DOI: 10.1158/1535-7163.MCT-10-0169

Downloaded from mct.aacrjournals.org on April 13, 2017. © 2010 American Association for Cancer Research.
are increased in advanced stages of epithelial ovarian cancer. Gynecol Oncol 2003;91:513–7.


