Selective Blockade of Matrix Metalloprotease-14 with a Monoclonal Antibody Abrogates Invasion, Angiogenesis, and Tumor Growth in Ovarian Cancer

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Abstract
Most patients with ovarian cancer are diagnosed late in progression and often experience tumor recurrence and relapse due to drug resistance. Surface expression of matrix metalloprotease (MMP)-14 on ovarian cancer cells stimulates a tumor–stromal signaling pathway that promotes angiogenesis and tumor growth. In a cohort of 92 patients, we found that MMP-14 was increased in the serum of women with malignant ovarian tumors. Therefore, we investigated the preclinical efficacy of a MMP-14 monoclonal antibody that could inhibit the migratory and invasive properties of aggressive ovarian cancer cell lines. MMP-14 antibody disrupted ovarian tumor–stromal communication and was equivalent to Avastin in suppressing blood vessel growth in mice harboring Matrigel plugs. These effects on angiogenesis correlated with downregulation of several important angiogenic factors. Furthermore, mice with ovarian cancer tumors treated with anti-MMP-14 monotherapy showed a marked and sustained regression in tumor growth with decreased angiogenesis compared with immunoglobulin G (IgG)-treated controls. In a model of advanced peritoneal ovarian cancer, MMP-14–dependent invasion and metastasis was effectively inhibited by intraperitoneal administration of monoclonal MMP-14 antibody. Together, these studies provide a preclinical proof-of-concept for MMP-14 targeting as an adjuvant treatment strategy for advanced ovarian cancer. Cancer Res; 73(8); 2457–67. ©2013 AACR.

Introduction
Ovarian cancer remains the fifth leading cause of death in women and the most fatal gynecologic cancer, accounting for more than 15,000 deaths in the United States annually (1). The vast majority of patients with ovarian cancer are diagnosed in late stages and experience recurrences and relapses due to drug resistance despite initial response to surgical debulking and standard first-line chemotherapy (2). In addition, there are no established and effective treatments of platinum refractory ovarian cancer. Therefore, identification of novel targeted therapies that block critical signaling pathways are important for improving survival in ovarian cancer (3).

Angiogenic and inflammatory markers such as interleukin (IL)-8, IL-6, growth-related oncogene (GRO)-α, monocyte chemoattractant protein (MCP)-1, VEGF, and matrix metalloprotease (MMP)-1 are highly upregulated in epithelial ovarian cancers (4–7). These markers are postulated to play a pivotal role in tumor growth, inflammation, angiogenesis, and metastasis and are associated with poor survival (8, 9). Angiogenesis inhibitors, as exemplified by the VEGF-antibody Avastin, have recently emerged as potential treatments of recurrent ovarian and primary peritoneal cancer (10). But, anti-VEGF therapy has not improved overall survival (11) and one of the reasons is that decreased tumor size has been associated with increased invasiveness and propensity to metastasize (12).

MMPs are zinc-dependant endopeptidases that play a key role in cancer cell invasion, metastases, and tumor angiogenesis (13, 14). Abundant evidence has indited MMPs including MMP-1, -2, -9, and -14 as critical mediators of invasion, blood vessel penetration, and metastasis of many solid tumors including ovarian cancer (4, 13, 15–18). MMP-14, (MT1-MMP), is a transmembrane collagenase, which is not detected in normal ovarian surface epithelium but is widely expressed on ovarian carcinomas of all histologic types (18). High expression of epithelial MMP-14 by immunohistochemistry is associated with poorer prognosis and shorter disease survival in patients with ovarian cancer (19). MMP-14 is critical to the acquisition of the invasive (20) and metastatic phenotype of breast, prostate, melanoma, renal,
and ovarian carcinomas (21–23). MMP-14 promotes a collagen-invasive phenotype (24) in ovarian carcinoma, which triggers peritoneal dissemination of metastatic ovarian tumors (23). MMP-14 is known to activate gelatinase MMP-2 (25) that has been implicated in the mesothelial attachment and metastatic spread of ovarian cancer cells (20, 26). We have recently shown that ovarian cancer cell MMP-14 leads to activation of proMMP-1 (4) via MMP-9, which culminates in chemokine production, angiogenesis (4, 16, 27), and ovarian tumor growth (4, 27). Endothelial cell MMP-14 has been implicated in angiogenesis (28), however, the mechanism of cancer cell produced MMP-14 in stimulating angiogenesis in the ovarian tumor microenvironment is unknown.

A causal relationship between MMP expression and cancer progression led to the evaluation of MMP inhibitors in several large clinical trials but met with limited efficacy and treatment failure (29, 30). These small-molecule MMP inhibitors elicited debilitating musculoskeletal toxicities that precluded adequate dosing and poor patient compliance (31, 32). Toxicity was assumed to be due to the broad-spectrum nature of these compounds, which led to off-target inhibition of protective MMPs such as MMP-8 and -12 leading to worse clinical outcomes (29). Moreover, most of the patients recruited to the trials were in late stages (stage III–IV) of cancer, which could potentially miss the positive therapeutic benefits of blocking MMP pathway at earlier stages in cancer (33).

In this study, we used a monoclonal antibody to inhibit MMP-14 on the ovarian carcinoma cells. MMP-14 inhibition led to suppression of both migratory and invasive propensity of ovarian carcinoma cells. MMP-14 antibody suppressed ovarian cancer cell-stimulated angiogenesis in vitro and in mice. Further, MMP-14 antibody treatment led to a marked regression in ovarian tumor growth and tumor blood vessel formation in a mouse xenograft model indicating that MMP-14 may be an attractive target for antiangiogenic therapy in ovarian cancer and perhaps other solid tumors. Furthermore, in a peritoneal model of aggressive ovarian cancer, combination intraperitoneal therapy of MMP-14 antibody and doxetaxel inhibited invasion through the mouse diaphragm and thoracic metastasis. We believe, blockade of MMP-14 as monotherapy or as combinatorial therapy along with conventional chemotherapy has direct clinical relevance for adjuvant and metronomic treatment regimens in ovarian cancer.

Materials and Methods

Reagents

MMP-14 monoclonal antibodies were obtained from Calbiochem (EMD Biosciences), R&D (MAB 9181, 918), and Millipore (MAB 3319). Isotypic immunoglobulin G (IgG) control was purchased from R&D System and fluorescein isothiocyanate (FITC)–conjugated rabbit anti-mouse from Invitrogen. MMP-7 (Ab-2), MMP-8 (Ab-1), and TIMP-1 (Ab-1) mouse monoclonal antibodies were obtained from Oncogene. Human MMP-14 ELISA was obtained from USCN (E92056Hu; Uscn Life Science, Inc.) and human Quantikine MMP-9 and VEGF ELISA from R&D Systems and used as per manufacturer’s protocol. RNA interference reagents (RNAi) against Luciferase, MMP-14, and MMP-9 have been described (4) and were obtained from Dharmaco.

Cell culture

Ovarian cancer cell lines OVCAR-4, SKOV-3, and OVCAR-3 were obtained from the National Cancer Institute and were grown in RPMI with 10% FBS. Cells were serum-starved overnight in 0.25% bovine serum albumin (BSA) for migration and invasion assays. Human umbilical vein endothelial cells (HUVEC) were bought from Cambrex (Lonza) and cultured in EBM2 medium with Bullet kit and 10% FBS. HUVEC cells were serum-starved for 1 hour before tube formation. Gelatinase activity was assayed from conditioned media by measuring the cleavage of fluorescein-conjugated DQ gelatin (Molecular Probes). Gelatinase assays contained 10 μg DQ gelatin in 50 mmol/L Tris–HCl, pH 7.6, 150 mmol/L NaCl, 5 mmol/L CaCl2, and 0.2 mmol/L Na3 and cleavage was monitored at 538 nm using a fluorescence microplate reader with excitation at 485 nm at 37°C. Gelatinase activity was expressed as percentage of the control group. Experiments were repeated 3 times in triplicates.

Detection of MMP-14 in patient samples

All patient blood samples were collected in full compliance with Tufts and Lahey Institutional Review Board guidelines. Blood was collected from women presenting at Tufts Medical Center (Boston, MA) or Lahey Clinic (Burlington, MA) with benign or malignant pelvic masses, before surgery. Blood was processed to collect serum that was stored at ~80°C till further use. Several serum samples were also obtained from Gynecology Oncology Group Tissue bank. Discarded patient fluids were collected from patients undergoing a peritoneal and pulmonary tap at Tufts Medical Center. Serum samples were diluted 10-fold and MMP-14 levels were quantified using ELISA (USCN E92056Hu). Groups were compared with one-way ANOVA followed by t test. *, P < 0.05. Patient fluids and serum samples were normalized using Bradford assay. Two microliter of normalized samples (2.2 mg/mL) were mixed with citrate buffer, EDTA, and 5× sample buffer in a 10 μL volume, heated for 40 minutes at 37°C, and immunoblotted with monoclonal MMP-14 antibody (Ab; MAB 918).

Flow cytometry

Flow cytometry was conducted as described before (4) using a mouse monoclonal antibody specific to MMP-14 (R&D Systems MAB9181).

Migration and invasion

Chemotactic migration and invasion was conducted using an 8 μm Transwell apparatus (Corning), as described previously (16). Of note, 50,000 serum-starved ovarian cancer cells were treated with 10 μg/mL of antibody and placed in the top chamber of the Transwell in 200 μL of 0.25% BSA in RPMI. The cells were migrated toward stromal matrix (fibroblast conditioned media) or malignant ovarian ascites, as previously described (16) for a period of 5 hours. For chemoinvasion, wells were coated with 20 μg of reconstituted matrix (Matrigel; BD Biosciences; ref. 4) and cells were migrated
similarly for 20 hours. For collagen invasion assays, Transwell were coated with 30 µg of rat tail high concentration type I collagen (BD Biosciences) and cells were migrated for 40 hours. Migrated and invaded cells on the lower surface of the Transwell were fixed, stained, and counted and data were compared with the respective IgG controls. One-way ANOVA followed by Student t test was used to compare the effect of treatment with the control. *, P < 0.05; **, P < 0.005.

**Tube formation assay**

MatTek plates were chilled to 4°C and coated with 100 µL of Matrigel per well. Freshly passaged HUVEC cells (35,000; P2-5) in EBM2 media with 0.25% BSA were plated on Matrigel-coated MatTek plates and stimulated with conditioned media from OVCAR-4 or SKOV-3 cells treated with either MMP-14 antibody, IgG or RNAi. Endothelial tube formation was observed after 18 hours and photographed under phase contrast microscopy using an inverted Olympus microscope. Digital images were used to count total tubes per 5 × 40 fields and quantified for tube length and branching complexity using NIH ImageJ software. Measures were expressed in number of branch points and actual tubal length. *, P < 0.05; **, P < 0.005.

**RNA interference**

Cells were transfected with Luci, MMP-14, or MMP-9 RNAi using oligofectamine as previously described (4). Thirty hours after recovery, the cells were either used for RNA preparation or serum-starved for conditioned media.

**Matrigel plugs and ovarian cancer xenograft models**

All animal experiments were carried out in full compliance with Tufts Medical Center Institutional Animal Care and Use Committee. Female NCR Nu/Nu mice (5–7 weeks) were purchased from Taconic farms. Mice were injected with 300 µL Matrigel plugs of equal parts conditioned media and Matrigel in their flanks subcutaneously. Animals were subsequently treated 24 hours later with either MMP-14 antibody, IgG, or Avastin (2.5 mg/kg twice weekly) in a 100 µL volume. At the end of 7 days, mice were euthanized and plugs were excised and fixed in 10% formalin/PBS and stained with CD31 (Chemicon, MAB 1398Z) for confocal microscopy, as previously described (27). For ovarian xenograft experiments, mice were injected subcutaneously with 3.5 million OVCAR-4 cells in their flanks. Mice were randomized into 2 treatment groups and 24 hours later injected with 2.5 mg/kg of IgG or MMP-14 antibody, intraperitoneally (i.p.), twice weekly till day 26. Tumor sizes were measured twice weekly initially and every other day after day 26 for the duration of the experiment. Tumor volume was calculated using the formula: \( V = length \times diameter^2 / 2 \). Mice were euthanized on day 35 and tumors and tissues were collected. Tumors were stained for endothelial marker CD31 (760–4378; Ventana Medical Systems, automated as per manufacturer’s protocol) and evaluated by a pathologist in a blinded manner.

For the Peritoneal Ovarian Tumor Model, mice were injected into the peritoneal cavity with 1.5 million OVCAR-4 cells. Animals were treated 24 hours later with intraperitoneal injections of 2.5 mg/kg of either IgG or MMP-14 antibody twice a week with concomitant docetaxel (5 mg/kg at day 21). At the end of 35 days, tumors and organs were excised from euthanized mice, fixed in 10% formalin/PBS, and imbedded in paraffin. Tissue sections (5 µm) from omentum, diaphragm, and thoracic organs were prepared and stained with hematoxylin and eosin and examined in a blinded manner by 2 pathologists.

**Results**

**Serum MMP-14 is increased in patients with invasive ovarian cancer**

We have previously shown that MMP-14 initiates the activation of a MMP cascade in the ovarian tumor microenvironment that leads to angiogenesis and ovarian tumor growth (4). High expression of epithelial MMP-14 by immunohistochemistry was associated with poor outcome in patients with ovarian cancer (19). Therefore, we investigated whether MMP-14 could be detected as a tumor biomarker in the serum and malignant ascites of patients with ovarian carcinoma. These patients are known to produce copious amounts of peritoneal ascites and we explored whether we could detect MMP-14 or a cleaved part of MMP-14 in malignant ovarian ascites. We collected fluids from 14 patients with malignant and benign disease (malignant ovarian ascites, n = 5; malignant ovarian cyst, n = 1; malignant pleural effusions from patients with ovarian cancer, n = 4; benign ascites, n = 3; and benign ovarian cyst, n = 1) and found that the levels of MMP-14 were more than 3-fold increased in the malignant fluids of patients with ovarian cancer compared with benign fluids (Fig. 1A). Because MMP-14 was increased in the malignant ovarian ascites, we further explored whether it could be detected in the serum of patients with malignant ovarian cancer. In a cohort of 92 patients comprising healthy females (n = 34), those with benign ovarian tumors (n = 32) and malignant ovarian cancer (serous epithelial cancer, stages II–IV; n = 26), we found that serum MMP-14 was significantly increased in patients with malignant ovarian carcinoma (Fig. 1A). To our knowledge, this is the first report of increased MMP-14 in the serum and malignant ascites of patients with ovarian carcinoma and has important implications not only as a biomarker of the disease but also as a tumor marker for monitoring response to therapy. We also confirmed the presence of MMP-14 in these samples of malignant ovarian ascites, malignant pleural effusion, and the serum of patients with ovarian carcinoma using immunoblot analysis (Fig. 1B and C). Immunoblotting for MMP-14 showed several fragments of MMP-14 exodomain as previously described (34). Our data correlate with the increased expression of MMP-14 found by immunohistochemistry in metastatic ovarian lesions as compared with primary ovarian tumors (23) and suggests an important role for MMP-14 in ovarian cancer progression. We also tested aggressive OVCAR-4, SKOV-3, or OVCAR-3 ovarian cancer cells and found a robust expression of MMP-14 by flow cytometry (Fig. 1D). This finding reiterates the fact that MMP-14 can be detected as a tumor biomarker in malignant ovarian cancer and emphasizes the importance of this MMP in ovarian carcinogenesis.
Monoclonal MMP-14 antibody inhibits migration and invasion of ovarian cancer cells

MMP-14, when overexpressed, promotes cancer cell migration and invasion (24, 35) critical to the acquisition of the metastatic phenotype in ovarian cancer (23). MMP-14 silencing suppresses migration and invasion of tumor cells in vitro (36) and pharmacologic inhibition of MMPs, including MMP-14, using broad-spectrum MMP inhibitors such as prinimostat inhibits tumor progression (37). However, as broad-spectrum MMP inhibitors have not been successful in clinical trials (38), we investigated whether selective blockade of MMP-14 using a monoclonal antibody could inhibit the migratory ability of ovarian carcinoma cells. We treated OVCAR-4 and OVCAR-3 cells with monoclonal MMP-14 antibody or corresponding IgG and allowed them to migrate toward fibroblast conditioned media (Fig. 2A) or malignant ovarian ascites (Fig. 2B). Treatment with MMP-14 antibody significantly decreased migration by 46% to 69% of OVCAR-3 (P < 0.05) and OVCAR-4 (P < 0.005) cancer cells, respectively, compared with IgG controls (Fig. 2A).

We also used several other relevant monoclonal antibodies as controls. MMP-7 is a matrilysin important for inducing ovarian cancer invasion (39), MMP-8 is tumor collagenase such as MMP-14, which is known to play a protective role in cancers (40) but is not expressed in our ovarian cancer cells (Supplementary Fig. S1A) and TIMP-1 (tissue inhibitor of metalloproteinase-1) inhibits most MMPs except membrane-tethered MMPs such as MMP-14, -16, and -25 (41). Unlike MMP-14 antibody, antibodies against MMP-7, -8, and TIMP-1 did not suppress migration of either cell line (Fig. 2A), suggesting that the migratory ability of ovarian cancer cells was dependent on MMP-14. Anti-VEGF therapy is currently being used as first-line therapy in ovarian cancer (10) along with standard chemotherapeutic regimens but has not yet improved overall survival perhaps due to increased local invasion and metastasis (12). We therefore tested whether antibodies against VEGF (Avastin) would inhibit migration of ovarian
cancer cells. Anti-VEGF antibody had no effect on the migration of OVCAR-4 cells, whereas they had a nonsignificant decrease on the migration of OVCAR-3 cells (Fig. 2A).

In the peritoneal cavity, malignant ovarian ascites serves as a rich chemoattractant for the migratory ovarian carcinoma cells. To examine the role of MMP-14 in migration toward ascites, we treated OVCAR-4 or OVCAR-3 cells with MMP-14 RNAi (36) and migrated them toward malignant ovarian ascites. MMP-14 silencing of OVCAR-4 and OVCAR-3 cells led to a 60% to 80% decrease in MMP-14 levels at the mRNA level (Supplementary Fig. S1B) and 80% to 95% decrease by flow cytometry (Supplementary Fig. S1C and S1D, respectively), which was confirmed by immunoblotting (Supplementary Fig. S1E and S1F). MMP-14 RNAi-treated OVCAR-4 and OVCAR-3 cells showed decreased migration toward ascites compared with the corresponding luciferase-treated cells (Fig. 2B), consistent with the MMP-14 antibody data, suggesting that MMP-14 is important for the movement of ovarian cancer cells in the peritoneal cavity.

We further explored the effect of MMP-14 antibody on ovarian cancer cell invasion. MMP-14 antibody-treated cells showed a more than 50% reduction in the invasion of OVCAR-4 and a 40% reduction in the invasion of OVCAR-3 cells through reconstituted basement membrane (Matrigel) compared with corresponding IgG controls. Antibodies against VEGF (Avastin), MMP-7, and TIMP-1 had no significant effect on Matrigel invasion (Fig. 2C). We also tested invasion through reconstituted type I collagen matrices, which is highly dependent on MMP-14 activity (42). We migrated MMP-14 antibody-treated OVCAR-4 and OVCAR-3 cells through a type I collagen matrix and found that MMP-14 antibody significantly inhibited more than 60% of the invasive propensity of OVCAR-4 (P < 0.005) and OVCAR-3 cells compared with respective IgG-treated controls (P < 0.05; Fig. 2D). These data suggest that selectively blocking MMP-14 may be physiologically relevant for inhibiting the migratory and invasive potential of ovarian carcinoma cells.

**Blockade of cancer cell MMP-14 inhibits endothelial angiogenesis**

Endothelial cell MMP-14 is known to be important for blood vessel formation (43) because MMP-14 knockout mice display skeletal defects due to impaired vascularization of the cartilage and endothelial cells from these mice show decreased fibroblast growth factor (FGF2)-induced capillary tube formation (44). We postulated that the cancer cell MMP-14 was regulating the release of angiogenic factors in the ovarian tumor microenvironment and stimulating blood vessel formation. To test the role of cancer cell MMP-14 in cancer cell–stimulated angiogenesis, we treated OVCAR-4 or SKOV-3 cells overnight with either MMP-14–blocking antibody or IgG (10 µg/mL), collected serum-starved conditioned media, and stimulated HUVECs on Matrigel-coated plates. HUVECs stimulated with conditioned media from MMP-14 antibody-treated OVCAR-4 or SKOV-3 cells showed 60% to 83% reduction in branching.
complexity and 50% or more reduction in tubal length compared with those stimulated with IgG-treated conditioned media (Fig. 3A and B). Furthermore, to ensure that these effects were a function of blockade of MMP-14 on the cancer cell, we also collected serum-starved conditioned media from MMP-14 and Luci RNAi-treated OVCAR-4 cells (Supplementary Fig. S1C and S1E) and stimulated tube formation. MMP-14 gene silencing of the ovarian cancer cells completely blocked carcinoma cell–stimulated angiogenesis (Fig. 3C) confirming that endothelial tube formation was at least partly regulated through cancer cell MMP-14. We conducted a mitochondrial activity assay (MTT) to rule out that MMP-14 antibody treatment affected the viability of treated cells. MMP-14 antibody- and IgG-treated endothelial and cancer cells did not show any differences in viability after 72 hours of treatment (Fig. 3D). This suggests that the cancer cell MMP-14 was regulating the production or release of angiogenic factors in the ovarian tumor microenvironment that promoted endothelial tube formation and branching complexity.

Furthermore, we characterized the MMP-14 antibodies used in this study (Calbiochem and R&D Systems) and found that they blocked the conversion of proMMP-2 to active MMP-2 (Supplementary Fig. S2B). Furthermore, MMP-14 antibody (R&D Systems) was specific in recognizing MMP-14 protein and did not cross-react with other MMPs such as MMP-3, -7, and -8 (Supplementary Fig. S2C).

Cancer cell MMP-14 regulates angiogenesis in the ovarian tumor microenvironment

Cancer cell MMP-14 is known to convert proMMP-2 to MMP-2 (25). We have previously shown that MMP-14 on the ovarian cancer cells regulates activation of proMMP-1 to MMP-1, which upregulates chemokine production and ovarian cancer angiogenesis (4). Therefore, we speculated that cancer cell MMP-14 disrupts tumor–stromal communication and angiogenesis.

Figure 3. Blockade of MMP-14 disrupts tumor–stromal communication and angiogenesis. A and B, ovarian cancer cell–stimulated angiogenesis is inhibited by MMP-14 antibody. OVCAR-4 and SKOV-3 cells were treated overnight with either MMP-14 antibody or IgG (10 μg/mL) in serum-starved conditioned media. Ovarian cancer cell conditioned media was collected 18 hours later and used to stimulate HUVECs on Matrigel-coated MatTEK plates. C, OVCAR-4 ovarian cancer cells were treated with RNAi against MMP-14 and luciferase, and serum-starved conditioned media was collected to stimulate HUVEC cells. Results from A–C were quantified using NIH ImageJ. Tubal length and branch points were compared between the treatment groups using a t test. **, P < 0.01.

D, HUVECs, OVCAR-4, and SKOV-3 ovarian cancer cells were treated with either 10 μg/mL of IgG or MMP-14 antibody and allowed to grow for 72 hours. Mitochondrial activity for all the cells was assessed after 72 hours and expressed as a percentage of IgG controls.
cell MMP-14 was regulating angiogenesis by modulating the activity of other MMPs or angiogenic factors (45) in the ovarian tumor microenvironment. Having seen the unequivocal effects of MMP-14 antibody and MMP-14 RNAi on in vitro cancer cell–stimulated angiogenesis, we explored the angiogenic factors being regulated through MMP-14. It is well established that MMP-9 and VEGF are important for tumor angiogenesis (46), but it is not well known that MMP-14 regulates MMP-9 (47). We hypothesized that MMP-14 was suppressing blood vessel formation by downregulating production or release of MMP-9 or VEGF, or both of these factors. We tested MMP-14 antibody-treated OVCAR-4 cell conditioned media and confirmed the decrease in MMP-9 levels by ELISA (Fig. 4A). Because active MMP-9 is required for the release of VEGF from the cancer cells and for promoting angiogenesis (46), we further tested whether MMP-14 inhibition decreased MMP-9 activity by measuring in situ gelatinase (largely MMP-2 and -9) activity from RNAi-treated OVCAR-4 cell conditioned media.

MMP-14 gene suppression significantly reduced gelatin activity (Fig. 4B), part of which can be attributed to MMP-9 activity as OVCAR-4 cells lack MMP-2 (Supplementary Fig S2A and S2B).

We further tested whether MMP-14 blockade also had an effect on VEGF levels. MMP-14 knockdown also significantly decreased VEGF A (165) levels in serum-starved OVCAR-4 conditioned media (Fig. 4C) establishing the importance of MMP-14 in ovarian tumor angiogenesis. To ensure that these effects were a function of decreased MMP-9, we also collected serum-starved conditioned media from OVCAR-4 cells that had been treated with MMP-9 and Luci RNAi (Fig. 4D and Supplementary Fig. S3A) and stimulated tube formation. MMP-9 gene silencing of the ovarian cancer cell showed more than 75% decrease in tubal length and branching complexity (Fig. 4D). This suggested that at least a part of the MMP-14–regulated ovarian cancer angiogenesis was mediated through suppression of MMP-9 (Fig. 4A and B and Supplementary Fig. S3A).
S3B) especially as MMP-14 antibody added directly on the HUVECs did not significantly suppress tube formation (Supplementary Fig. S3C).

Having seen the importance of cancer cell MMP-14 in ovarian cancer angiogenesis and the inhibitory effects of MMP-14 antibody on in vitro tube formation, we tested whether this would be true for in vivo angiogenesis. We subcutaneously injected Matrigel plugs into the flanks of female nude mice. The plugs consisted of equal volumes of OVCAR-4 conditioned media and Matrigel along with HUVECs. The mice were then treated on day 2 with either MMP-14 antibody or corresponding IgG antibody or Avastin (VEGF) twice a week at 2.5 mg/kg i.p. At the end of 7 days, Matrigel plugs were excised, fixed overnight, and stained with CD31, a marker for endothelial cells (27). Both MMP-14 and VEGF antibody-treated mice showed a marked decrease in vascularization of Matrigel plugs as compared with the IgG-treated controls (Fig. 4E). The Matrigel plug experiment not only established the efficacy of the MMP-14 antibody in vivo, but also the adequate dose required for inhibition of blood vessels suggesting that blocking MMP-14 may be a therapeutic strategy to inhibit ovarian cancer angiogenesis.

MMP-14 antibody inhibits ovarian tumor growth and angiogenesis

Small-molecule MMP inhibitors have not been successful in clinical trials due to their broad-spectrum effects. As shown earlier, monoclonal MMP-14 antibody had striking effects on in vivo ovarian cancer angiogenesis and did not bind nonspecifically to other MMPs such as MMP-8 (Supplementary Fig. S2C); therefore, we further tested whether these effects could be translated into decreased tumor growth. We injected 3.5 million OVCAR-4 ovarian carcinoma cells subcutaneously into the flanks of nude mice. Mice were randomized 24 hours later into 2 treatment groups and treated with either MMP-14 antibody or corresponding IgG (2.5 mg/kg, twice per week, i.p.). MMP-14 antibody–treated mice showed a significant reduction in tumor growth (P < 0.05) from day 26 onward (Fig. 5A). As antibodies usually have long lasting effects, we stopped treatment in both groups at day 27 and let the tumors grow for another week. After halting treatment, the IgG-treated mice had exponential tumor growth, whereas the MMP-14 antibody-treated mice continued to show a significant reduction (P < 0.01) in tumor growth compared with the IgG-treated mice. Furthermore, within the MMP-14 antibody treatment group there was no significant increase in tumor size between the day 28 and 35 time point showing the long lasting effect of the MMP-14 antibody treatment on tumor growth. Mice were euthanized and tumors collected for CD31 immunohistochemistry on day 35. MMP-14-treated mice showed a marked reduction in blood vessels (P < 0.005) both at the tumor edge and the tumor center compared with the IgG-treated controls (Fig. 5B and C). We correlated decreased angiogenesis with reduced tumor growth in MMP-14 antibody-treated tumors compared with their IgG-treated counterparts. Furthermore, we examined the livers of mice in both treatment groups and found no obvious signs of toxicity (data not shown). These findings suggest that MMP-14 is an important regulator of ovarian cancer angiogenesis and tumor growth and could potentially be a novel target for antiangiogenic therapy in ovarian cancer.

Intraperitoneal combination of MMP-14 antibody plus docetaxol inhibits invasion and metastasis of ovarian cancer in nude mice

Patients whose cancer has spread throughout the peritoneal cavity have a poor survival rate, thus inhibition of peritoneal dissemination and metastatic progression is critical for the successful treatment of ovarian cancer. Therefore, we assessed whether inhibition of MMP-14 using a monoclonal antibody in combination with docetaxol was able to slow progression of peritoneal ovarian cancer in mice. We have previously developed a histologic staging system that used the diaphragm as a marker of the progressive invasion and metastasis from the peritoneal cavity/omentum to the thoracic organs (4). We injected 1.5 million aggressive OVCAR-4 ovarian cancer cells into the peritoneal cavity of mice. Starting on day 2, mice received either IgG or MMP-14 antibody (2.5 mg/kg i.p., twice a week) and 2 doses of docetaxel (5 mg/kg) i.p. on day 21 and 28 until the end of the experimental period. At the 35-day time point, all of the IgG-treated mice had complete penetration through the diaphragm and metastasis to the lungs and mediastinum, confirming that the OVCAR-4 cells were extremely aggressive and metastatic in vivo (Fig. 5D and Supplementary Fig. S4A). In comparison, mice treated with MMP-14 antibody had a marked decrease in metastatic progression (P = 0.01) and the cancer remained confined to the peritoneal surface of the diaphragm in 75% of the mice (Fig. 5D and Supplementary Fig S4A). Although we did not see a significant reduction in ascites volume between the two treatment groups, the ascites in the MMP-14–treated mice was straw colored as opposed to the bloody ascites in the IgG–treated mice (Supplementary Fig. S4B). MMP-14 antibody conferred significant (P = 0.01) protection against invasion into the diaphragm and thoracic metastases suggesting that MMP-14–targeted therapy maybe clinically relevant for inhibiting progression of advanced ovarian cancer.

Discussion

MMP-14: a new antiangiogenic molecular therapeutic target in human ovarian cancer

Ovarian cancers critically depend on blood vessels for their expansive growth and belong to the group of highly angiogenic tumors that respond to even monotherapy with antiangiogenic drugs (48). Bevacizumab, a monoclonal antibody against VEGF, has been increasingly used upfront for therapy in ovarian cancer (10). Despite early benefits seen with these anti-VEGF pathway targeting drugs, the clinical benefits in terms of overall survival have been modest and associated with enhanced local invasion and metastatic progression (12). Therefore, it is possible that therapies that target multiple, distinct angiogenic pathways are superior to those targeting singular angiogenic pathways. We believe MMP-14 could be such a target in ovarian cancer.

MMP-14 has been associated with poor overall survival in breast cancer (22), and in this study, we found MMP-14...
to be increased in the serum of patients with malignant ovarian cancer compared with normal women and those with benign ovarian masses confirming its importance for ovarian carcinogenesis. MMP-14 is a 66-kDa membrane-tethered MMP and unlike other MMPs it is not known to be secreted into the tumor milieu. However, we postulated that other MMPs and serine proteases present in the ovarian tumor microenvironment might cleave the extra-cellular domain of MMP-14 (49) and allow for its detection in patient with ovarian cancer fluids and serum. We confirmed the increased presence of cleaved MMP-14 in malignant ovarian ascites, pleural effusions from patients with ovarian cancer, and serum of these patients using ELISA and immunoblot analysis using a MMP-14 monoclonal antibody. To our knowledge, this is the first study to report the detection of MMP-14 in the serum and malignant fluids of patients with ovarian cancer. Detection of MMP-14 in the serum is important for monitoring relapse and recurrence of the disease, remission and potentially, response to anti-MMP-14-targeted therapy.

Recently, there have been several reports of proinvasive consequences of antiangiogenic tumor therapy (50) that led the authors to suggest that a combination of different treatment regimens, which include anti-invasive therapy, may be more effective in blocking tumor progression. MMP-14, which is expressed on the invadopodia of cancer cells (20), could be such a therapeutic target. However targeting MMPs has been challenging and like other systemic therapy broad-spectrum MMP inhibitors failed to produce enduring efficacy in terms of either tumor shrinkage or long-term survival benefit (30, 32). In this study, we used commercially available monoclonal antibody against MMP-14 to significantly impair the migratory and invasive ability of MMP-14 expressing ovarian cancer cells. This is especially relevant for the spread of ovarian cancer cells within the rich milieu of the peritoneal cavity (23). These results show that antimigratory and anti-invasive property of
the MMP-14 antibody could be a significant advantage over the VEGF antibody for long-term antiangiogenic therapy.

Using a monoclonal antibody or RNAi against ovarian cancer cell MMP-14, we disrupted tumor-stromal communication that normally stimulated in vitro and in vivo angiogenesis. *In vivo*, MMP-14 antibody was equivalent to Avastin in blocking blood vessel growth within Matrigel plugs in mice. We confirmed that these effects were not due to inhibition of cellular proliferation but due to production of angiogenic factors. Cancer cell MMP-14 not only regulated MMP-9 and VEGF production and but also MMP-9 activity, which is important for VEGF release (46). Although OVCAR-4 cells do not express MMP-1, we have previously shown that MMP-14 on the ovarian cancer cell, along with MMP-9 activates stromal proMMP-1 to MMP-1 (4), which is known to be important for tumor angiogenesis (9, 16) directly and through the upregulation of several chemokines (27).

Furthermore, mice bearing OVCAR-4 ovarian cancer tumors when treated with MMP-14 antibody as monotherapy showed a marked regression in tumor growth. Even a week after the treatment had been stopped, there was no significant growth in the MMP-14 antibody-treated tumors, whereas the IgG-treated cohort continued to grow exponentially. The MMP-14 antibody-treated tumors showed a marked reduction in CD31-stained blood vessels compared with the IgG controls providing proof-of-concept for targeting MMP-14 for antiangiogenic therapy in ovarian cancer. In addition, in an aggressive, orthotopic mouse model of ovarian cancer, MMP-14 antibody along with docetaxel, inhibited invasion through the diaphragm and thoracic metastasis. We believe that MMP-14 blockade as monotherapy or as combinatorial therapy with standard-of-care drugs may have direct clinical relevance for patients with advanced ovarian cancer and implications for adjuvant therapeutic regimens not only for ovarian cancer but also for other solid tumors.

**Disclosure of Potential Conflicts of Interest**

No potential conflicts of interest were disclosed.

**Authors’ Contributions**

Conception and design: R. Aljumaily, A. Kulipolupus, C. Zarwan, A. Agarwal

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MMP-14 Antibody Inhibits Ovarian Tumor Progression


Selective Blockade of Matrix Metalloprotease-14 with a Monoclonal Antibody Abrogates Invasion, Angiogenesis, and Tumor Growth in Ovarian Cancer

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