Identification of a Metalloprotease-Chemokine Signaling System in the Ovarian Cancer Microenvironment: Implications for Antiangiogenic Therapy

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Abstract

Ovarian cancer is a lethal gynecologic malignancy that may benefit from new therapies that block key paracrine pathways involved in tumor-stromal interactions and tumor vascularity. It was recently shown that matrix metalloprotease-1 (MMP1) activation of the G protein–coupled receptor protease-activated receptor-1 (PAR1) is an important stimulator of angiogenesis and metastasis in peritoneal mouse models of ovarian cancer. In the present study, we tested the hypothesis that MMP1-PAR1 promotes angiogenesis through its paracrine control of angiogenic chemokine receptors. We found that MMP1-PAR1 activation induces the secretion of several angiogenic factors from ovarian carcinoma cells, most prominently interleukin (IL)-8, growth-regulated oncogene-α (GRO–α), and monocyte chemoattractant protein-1. The secreted IL-8 and GRO–α acts on endothelial CXCR1/2 receptors in a paracrine manner to cause robust endothelial cell proliferation, tube formation, and migration. A cell-penetrating pepducin, X1/2pal-i3, which targets the conserved third intracellular loop of both CXCR1 and CXCR2 receptors, significantly inhibited endothelial cell proliferation, tube formation, angiogenesis, and ovarian tumor growth in mice. Matrigel plugs mixed with MMP1-stimulated, OVCAR-4–conditioned media showed a dramatic 33-fold increase in blood vessel formation in mice. The X1/2pal-i3 pepducin completely inhibited MMP1-dependent angiogenesis compared with a negative control pepducin or vehicle. Conversely, a vascular endothelial growth factor–directed antibody, Avastin, suppressed angiogenesis in mice but, as expected, was unable to inhibit IL-8 and GRO–α-dependent endothelial tube formation in vitro. These studies identify a critical MMP1-PAR1-CXCR1/2 paracrine pathway that might be therapeutically targeted for ovarian cancer treatment. Cancer Res; 70(14); OF1–11. ©2010 AACR.

Introduction

Chemokines have traditionally been viewed as attractants for inflammatory leukocytes, however, accumulating evidence suggests that they play important roles in modulating the tumor-stromal microenvironment (1–3). Ovarian cancer is a lethal disease that recruits a network of chemokines, proteases, and their receptors to grow and metastasize with no anatomic barriers to prevent peritoneal dissemination (4). Inflammatory chemokines such as CXCL8 [interleukin (IL)-8], CXCL6 (IL-6), CXCL1 [growth-regulated oncogene-α (GRO–α)], and CCL2 (monocyte chemoattractant protein-1) are highly upregulated in epithelial ovarian cancers (5–7) and are postulated to play pivotal functions in tumor growth, inflammation, angiogenesis, and metastasis (2, 6, 8).

IL-8 and GRO–α, ligands for the CXCR1 and CXCR2 chemokine receptors, are produced by the ovulating follicle to attract leukocytes and assist in ovulation (9), and IL-8 and IL-8 antibodies have been detected in the serum of patients with ovarian cancer (10). Upregulated IL-8 expression has been shown to be a marker of poor prognosis in breast cancer (2, 11), and more recently, has been associated with poor clinical outcome and worse survival in patients with ovarian cancer (12). IL-8 is a strong stimulator of capillary tube formation via both CXCR1 and CXCR2 receptors (13) and coculturing of cancer cells with stromal fibroblasts causes an induction in IL-8, GRO–α, and GRO–β oncogenes (14). Moreover, GRO–α is expressed at higher levels in the tissue and serum of patients with ovarian cancer as compared with normal women (15).

Recent work has shown that thrombin activation of protease-activated receptor-1 (PAR1) causes an induction of GRO–α in breast carcinoma and melanoma (16). PARs are a unique class of G protein–coupled receptors that are activated by proteolytic cleavage of their extracellular domains (17). PAR1 has been identified as an oncogene and a potent invasogenic receptor for breast, ovarian, melanoma, and prostate...
carcinoma cells (18–24). We recently found that matrix metalloprotease-1 (MMP1) also functions as a protease agonist of PAR1 cleaving the receptor (22, 25) to generate PAR1-dependent migration, invasion, and angiogenesis in breast and ovarian tumors (22, 23, 26, 27). Furthermore, we found that fluids from patients with ovarian cancer harbor high levels of MMP1 which correlates with malignancy. Inhibition of PAR1 results in a pronounced reduction of stromal infiltration and angiogenesis in mouse models of breast and ovarian cancer. However, little is known regarding the mechanism of the MMP1-PAR1 signaling system in paracrine communication to the endothelial cells and angiogenesis.

Here, we tested the hypothesis that MMP1-activated PAR1 is a critical regulator of cancer-stromal communication and angiogenesis in ovarian carcinomas through paracrine control of chemokine production and endothelial CXCR1/2 receptors. To block these angiogenic chemokine receptors, we used cell-penetrating pepducins based on the intracellular loops of CXCR1 and CXCR2 (28). Pepducins are lipidated peptides that act on the inside surface of the cell membrane and block signaling between their cognate receptor and its G protein effectors. The first (i1) and third (i3) loops of CXCR1 and CXCR2 receptors are identical and pepducins based on these loops are potent antagonists of both receptors. Pepducins have been extensively studied in the context of PAR and chemokine receptor signaling, and have been shown to possess drug-like properties through pharmacologic and efficacy studies in various animal models of angiogenesis, cancer, thrombosis, and inflammation (22, 23, 25–30).

We found that MMP1-PAR1 stimulation of ovarian carcinoma cells leads to the secretion of angiogenic chemokines, in particular, IL-8 and GRO-α. Medium from MMP1-stimulated ovarian carcinoma cells produced a florid angiogenesis both in vitro and in mice. The X1/2pal-i3 pepducin completely inhibited the effects of MMP1 in angiogenesis models, indicating that the MMP1-PAR1-CXCR1/2 paracrine system might be an attractive new target to block angiogenesis in ovarian cancer.

Materials and Methods

Pepducins

The CXCR1/2 pepducins X1/2pal-i3 (C15H31CONH-RTLF-KAHMGQKHRAM-RH2), X1/2LCA-i1 (lithocholic-CONH-YSRVGRSVT-DH2), and the PAR1-based negative control pepducin P1pal-19E (C15H31CONH-RCESSSAEARKKE-RELF-NH2) were synthesized by standard N1-(9-fluorenyl) methoxycarbonyl solid phase methods as described (29, 30). Palmitic acid and lithocholic acid were dissolved in 50% N-methyl pyrrolidone/50% methylene chloride and coupled overnight to the deprotected NH2-terminal amine of the peptide. After cleavage from the resin, palmitoylated peptides were purified to >95% purity by C18 or C4 reverse phase liquid chromatography and dissolved in DMSO as described (28).

Reagents

Quantikine ELISAs for human IL-8, GRO-α, and vascular endothelial growth factor (VEGF)-A-165, were obtained from R&D Systems and used as recommended by the manufacturer. Recombinant human IL-8 and GRO-α were commercially obtained from PeproTech. Pure pro-MMP1 was obtained from EMD Biosciences, FN-439 (MMP Inh-1) from Calbiochem and human α-thrombin was from Haematologic Technologies and 3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyltetrazolium bromide (MTT) was obtained from Sigma-Aldrich.

Angiogenesis array

The human angiogenesis antibody array (AAH-ANG-1) was bought from RayBiotech. The array membranes were incubated with either MMP1 or buffer-stimulated OVCAR-4 medium and used as recommended by the manufacturer. Results were calibrated using ImageJ by comparing signal intensities in the membranes after simultaneous exposure to X-ray film. Stock solutions of 800 nmol/L of pro-MMP1 were activated with 2 mmol/L of aminophenylmercuric acetate (APMA) in 50 mmol/L of Tris (pH 7.7), 5 mmol/L of CaCl2, 0.2 mol/L of NaCl, 50 mmol/L of ZnCl2 at 37°C for 30 minutes before being kept on ice. The APMA was then removed by overnight dialysis in 10 kDa molecular weight cutoff Mini Slide-A-Lyzers (Pierce) at 4°C as previously described (23).

Cell culture

OVCAR-4, OVCAR-3, and IGROV-1 cells were obtained from the National Cancer Institute (Frederick, MD) and were grown in RPMI with 10% fetal bovine serum. Cells were serum-starved for 7 hours in RPMI with 0.1% bovine serum albumin (BSA) then stimulated with MMP1 or vehicle and conditioned media (CM) was collected 18 hours later. OVCAR-4 PAR1 shRNAi cells were stable cell lines prepared by transfecting OVCAR-4 cells with PAR1 shRNAi from Sigma using OligofectAMINE and selecting clones with puromycin (0.5 μg/mL). Human umbilical vein endothelial cells (HUVEC) were bought from Cambrex (Lonza) and cultured in EB2 medium with Bullet kit and 10% fetal bovine serum. HUVEC cells were serum-starved in EB2 medium with 0.5% BSA.

Flow cytometry

A rabbit polyclonal antibody specific to PAR1 was purified by peptide affinity chromatography as previously described (20). FITC-conjugated goat anti-rabbit antibody was purchased from Zymed. Flow cytometry was performed on ovarian carcinoma cells as described (23). CXCR1 (IL8-RA) and CXCR2 (IL8-RB) chemokine receptor antibodies were bought from R&D Systems.

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 EB2 media with 0.5% BSA were plated on matrigel-coated MatTek plates and stimulated with either IL-8, GRO-α, or MMP1-activated OVCAR-4 CM. MMP1-activated CM was quenched with FN-439 before adding the CM to the HUVEC cells. Endothelial tube morphogenesis was carried out in the presence or absence of X1/2pal-i3, X1/2LCA-i1, P1pal-19E pepducins (300 nmol/L), Avastin (0.25 mg/mL), or vehicle (0.2% DMSO). Endothelial tube formation was estimated by ImageJ by comparing signal intensities in the membranes after simultaneous exposure to X-ray film.
observed after 18 hours and endothelial tubes were photographed under phase contrast microscopy using an inverted Olympus microscope. Quantification of the digital images was performed in a blinded manner by counting total tubes per five 40× fields and quantified for tube length and branch complexity using NIH ImageJ software. Tube formation was expressed as fold change or percentage over the controls.

**MTT assay**

HUVEC cells were plated in 96-well plates and subjected to various treatment conditions or vehicle (0.2% DMSO) for 72 hours. MTT reagent was added at a concentration of 0.5 mg/mL and allowed to incubate at 37°C for 5 hours. The resulting formazan crystals were dissolved with 100% DMSO and absorbance was measured on a SPECTRmax 340 microplate reader (Molecular Devices).

**Matrigel plug assays and ovarian cancer xenografts in mice**

All animal experiments were conducted 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 s.c. into their flanks with 300 μL matrigel plugs consisting of equal volumes of CM and matrigel. Animals were subsequently treated with either X1/2pal-i3, X1/2 LCA-i3, P1pal-19E pepducin (5 mg/kg/d × 7 d), Avastin (5 mg/kg), or vehicle (15% DMSO) in 100-μL volumes. At the end of 7 days, mice were euthanized and plugs were excised and fixed in 10% formalin/PBS. For ovarian cancer xenografts, OVCAR-4 cells were injected s.c. into each flank of female NCR nu/nu mice. The mice were treated 24 hours later with either X1/2pal-i3 or vehicle (15% DMSO) until day 30. Tumor volume was calculated using the formula, \( V = \text{length} \times \text{diameter}^2 / 2 \). On day 30, mice were euthanized and tumors were harvested for assessment of angiogenesis.

**Confocal microscopy of matrigel plugs and ovarian cancer xenografts**

Subcutaneously implanted matrigel plugs or tumors were removed, cleaned, and fixed overnight with 10% formalin. Plugs were blocked for 1 hour with 5% goat serum in TBS containing 0.3% Triton X-100 (TBST) and incubated overnight at 4°C with primary antibody CD31 (Millipore) diluted 1:1,000 in TBST. After several washes with TBST, plugs or tumors were then incubated with fluorescently tagged secondary antibody Cy3 anti-hamster (Jackson Immunolabs) diluted in TBST for 4 hours. Plugs and tumors were washed and postfixed with 4% paraformaldehyde for 10 minutes. Whole mounted matrigel plugs and tumors were imaged using a Leica TCS SP2 confocal microscope. Confocal images were constructed into three-dimensional projections of Z stacks. Quantification of images was performed using NIH ImageJ.

**Statistical analysis**

All in vivo and in vitro data are presented as mean ± SD or mean ± SE. Comparisons were made with Wilcoxon rank sum Student’s t test following ANOVA analyses. Statistical significance was defined as *, \( P < 0.05 \) and **, \( P < 0.005 \).

**Results and Discussion**

**MMP1 induces chemokine production from ovarian cancer cells in a PAR1-dependent manner**

MMP1 activation of PAR1 has recently been implicated in tumor angiogenesis of breast and ovarian cancers (22, 23, 27), but the mechanism of action of PAR1-dependent tumor-endothelial cell communication is not well understood. Therefore, we first characterized the profile of angiogenic factors that resulted from the stimulation of PAR1 in ovarian cancer cells (Fig. 1A). We exposed a high PAR1-expressing ovarian carcinoma cell line, OVCAR-4, to MMP1 and found that several angiogenic factors were secreted into the CM. As shown in Fig. 1A, the CXCR1/2 chemokines IL-8 and GRO (α/β/γ), and the CCR2 chemokine monocyte chemo-attractant protein-1 were the most highly upregulated angiogenic/inflammatory factors with a 4- to 5.5-fold increase above baseline (\( P < 0.005 \)). Thrombin, a PAR1 agonist, is known to upregulate VEGF-A in chick allantoic membrane and human vascular smooth muscle cells (31, 32), however, we noted no significant change in VEGF-A levels (the two major isoforms, 165 and 121) following MMP1 stimulation and a slight increase in VEGF-D (Fig. 1A). Other chemotactic and angiogenic factors such as angiogenin were increased by 1.5- to 3-fold following MMP1 stimulation of OVCAR-4 cells (Fig. 1A). We focused on the CXCR1/2 chemokine receptors and their two major agonists, IL-8 and Gro-α, because we recently developed the first dual antagonist pepducins targeted against both receptors (28).

To confirm the findings of the cytokine array, we tested whether MMP1 stimulated IL-8 and GRO-α secretion in several ovarian cancer cell lines expressing varying levels of PAR1. PAR1 surface expression was quantified on the OVCAR-4 (high), IGROV-1 (medium), and OVCAR-3 (low) ovarian cancer cells by fluorescence-activated cell sorting using a PAR1-specific antibody (Fig. 1B). In addition, we performed stable knockdown of PAR1 in the high PAR1-expressing OVCAR-4 using shRNAi (Fig. 1B; Supplementary Fig. S1). ELISA analysis validated that MMP1 treatment caused increased secretion of IL-8 from PAR1-expressing OVCAR-4 and IGROV-1 cells (\( P < 0.005 \)) but had no effect on the low PAR1-expressing cell line, OVCAR-3, or following gene silencing of PAR1 in OVCAR-4 (Fig. 1C). A similar pattern in GRO-α secretion was confirmed by a GRO-α ELISA for OVCAR-4 and IGROV-1 cells, whereas the low PAR1 expressing OVCAR-3 and OVCAR-4/PAR1-shRNA cells did not show an increase in GRO-α when stimulated with MMP1 (Fig. 1D).

We also blocked the effect of PAR1 in the ovarian cancer cells with a PAR1 small molecule antagonist RWJ-56110 (33) and a PAR1 antagonist pepducin, P1pal-7 (23, 27, 29, 30). Pepducins are lipidated peptides based on the intracellular loops of the G protein–coupled receptor that have been previously validated in animal models of thrombosis, sepsis, and cancer (23, 27, 29, 30, 34–36). We observed that IL-8 induction by MMP1 and thrombin was inhibited by RWJ-56110 or P1pal-7.
MMP1-PAR1 stimulation of ovarian carcinoma cells triggers endothelial tube formation

We next investigated the angiogenic potential of IL-8 and GRO-α induction by the MMP1-PAR1 signaling system in endothelial cells. Medium from OVCAR-4 cells stimulated with either MMP1 or vehicle was added to freshly cultured HUVEC seeded on matrigel and incubated overnight. As MMP1 could stimulate endothelial cells directly (37, 38), we quenched any residual MMP1 activity with the MMP1 inhibitor FN-439 before adding the CM to the HUVECs. Consistent with our hypothesis, MMP1 CM containing elevated levels of IL-8 and GRO-α and basal levels of VEGF enhanced tube

(Fig. 1C; Supplementary Fig. S2A), confirming that the protease-induced production of IL-8 was PAR1-dependent. The PAR1 inhibitors RWJ-56110 and P1pal-7 were also able to inhibit the GRO-α increases induced by MMP1 and thrombin (Figs. 1D and 2A; Supplementary Fig. S2B). By comparison, VEGF-A-165 levels (Fig. 1E) remained essentially unchanged (0.9- to 1.15-fold) following MMP1 stimulation in the ovarian cancer cell lines tested. As was observed with MMP1, thrombin stimulation of PAR1 caused the secretion of IL-8 and GRO-α, and gave only a slight induction in VEGF-A-165 in OVCAR-4 and OVCAR-3 cells (Supplementary Fig. S2C).
formation and branching complexity of the endothelial cells by 4-fold (Fig. 2A-B). Furthermore, the MMP1 paracrine effect on tube formation could be significantly attenuated by pretreating OVCAR-4 cells with the MMP1 inhibitor FN-439, or the PAR1 antagonists RWJ-56110 or P1pal-7 (Fig. 2A and B). Taken together, these findings suggest that the MMP1-induced paracrine communication between ovarian carcinoma cells and endothelial cells was mediated through PAR1.

A cell-penetrating CXCR1/2 pepducin blocks IL-8 and GRO-α-stimulated endothelial cell proliferation

The chemokine receptors CXCR1 and CXCR2 are expressed on the endothelium and are important for endothelial survival, proliferation, and angiogenesis (13, 39). We confirmed the presence of CXCR1/2 chemokine receptors on our endothelial cells using flow cytometry (Fig. 3A). IL-8 and GRO-α were highly potent inducers of endothelial cell proliferation, with EC\textsubscript{50} values of 200 and 600 pmol/L, respectively (Fig. 3B). The effects of IL-8 and GRO-α on endothelial cell proliferation were completely blocked by the X1/2pal-i3 pepducin (Fig. 3B), a palmitoylated pepducin based on the third intracellular loop of the CXCR1/2 chemokine receptors (28).

We then tested whether medium from the MMP1-stimulated OVCAR-4 cells could cause endothelial cell proliferation. As expected, the MMP1-OVCAR-4 media induced mitogenesis and proliferation by 3.5- to 4.5-fold (Fig. 3C). The mitogenic effect of MMP1 was not observed in the low PAR1-expressing OVCAR-3 cells (Fig. 3C). The MMP1-induced proliferation was inhibited by the addition of X1/2pal-i3 to the HUVECs (Fig. 3D), again demonstrating that the observed paracrine effect between the ovarian cancer cells and the endothelial cells was dependent on the CXCR1/2 receptors. Exogenous IL-8 and GRO-α also stimulated the migration of endothelial cells in a wound-healing migration assay which was blocked by X1/2pal-i3 (Supplementary Fig. S3).

The X1/2pal-i3 pepducin blocks IL-8 and GRO-α-stimulated endothelial cell tube formation

As MMP1 caused a 4-fold increase in both IL-8 and GRO-α secretion from carcinoma cells, we tested the ability of these chemokines to induce endothelial cell tube formation on

**Figure 2.** CXCR1/2 chemokines produced by MMP1 stimulation of OVCAR-4 cells lead to tube formation of endothelial cells. OVCAR-4 cells (0.2 million) were plated in each well of a six-well plate, serum starved, and then treated overnight with either PBS buffer or 1 nmol/L of MMP1 in the presence or absence of 0.2% DMSO vehicle, P1pal-7 (5 μmol/L), or RWJ-56110 (5 μmol/L). CM was collected and the residual MMP1 activity was quenched with FN-439 (5 μmol/L). CM was then added to HUVECs (30,000) on matrigel-coated 12-well MatTek plates and incubated overnight at 37°C. A, tube formation of HUVECs was visualized at 40× magnification. B, tube formation in A was quantified using ImageJ software in a blinded manner. As indicated, FN-439 (5 μmol/L) was also added to the OVCAR4 cells prior to MMP1 treatment. Columns, means are representative of two or more experiments performed in triplicate; bars, SD; *, P < 0.05 and **, P < 0.005.
matrigel-coated wells. Exogenous IL-8 and GRO-α caused a 23-fold increase in branch point complexity and a 13-fold increase in tube length over unstimulated controls (Fig. 4A). This effect was significantly inhibited by 300 nmol/L of X1/2pal-i3 but not by Avastin (Fig. 4A), suggesting that the effect was mediated through the CXCR1/2 receptors. In the reciprocal experiment, exogenous VEGF stimulated HUVEC tube formation, which was significantly inhibited by Avastin but not by X1/2pal-i3 (Supplementary Fig. S4). To validate the inhibitory effects of the CXCR1/2 pepducins on endothelial tube formation, we used neutralizing antibodies to the IL-8 receptors CXCR1 and CXCR2. The CXCR1 and CXCR2 neutralizing antibodies significantly (P < 0.05) suppressed IL-8-induced branch point complexity by 68% and tube length by 58% as compared with control IgG (Supplementary Fig. S5), quantitatively similar to the inhibition observed with the X1/2pal-i3 pepducin.

We further tested whether X1/2pal-i3 was able to block endothelial cell tube formation induced by medium from MMP1-stimulated ovarian carcinoma cells. We used two different CXCR1/2 pepducins—X1/2pal-i3 and X1/2LCA-i1 (a lithocholic-tagged i1 loop of CXCR1/2), and a negative control pepducin P1pal-19E based on the i3 loop of PAR1 (29), which has a similar structure to X1/2pal-i3. Both X1/2pal-i3 and X1/2LCA-i1 significantly inhibited endothelial cell tube formation and branch complexity induced by the medium from MMP1-stimulated ovarian carcinoma cells, whereas P1pal-19E had no effect (Fig. 4B). These data provide further
evidence that the CXCR1/2 pepducins were specifically blocking the CXCR1/2 receptors to inhibit tube formation.

A CXCR1/2 pepducin suppresses angiogenesis in mice

Next, we tested whether the X1/2pal-i3 pepducin would inhibit blood vessel formation in mouse models of angiogenesis. As stimulation of ovarian carcinoma cells produced a marked increase in the secretion of both IL-8 and GRO-α, we first spiked unstimulated ovarian carcinoma cell media with IL-8 plus GRO-α versus buffer alone and added the mixtures to matrigel plugs, which were injected into the flanks of nude mice. Mice receiving the IL-8/GRO-α matrigel plugs were divided into three treatment groups. The mice were injected s.c. with either vehicle, X1/2pal-i3 or Avastin. On day 7, the matrigel plugs were harvested and stained for blood vessel formation. Unstimulated CM (buffer-treated) from OVCAR-4 cells containing basal levels of VEGF, IL-8, and GRO-α were unable to form blood vessels. The addition of IL-8/GRO-α to the unstimulated ovarian carcinoma cell media caused a dramatic increase ($P = 0.0016$) in angiogenesis within the plugs as compared with media with buffer alone, which was completely blocked by treatment with X1/2pal-i3 ($P = 0.0075$; Fig. 5A). Treatment with Avastin also significantly inhibited angiogenesis, suggesting that the basal levels of VEGF (~200 pg/mL) in the OVCAR-4 CM were contributing to the IL-8/GROα-driven angiogenesis in vivo. These basal levels of VEGF, however, were not sufficient to stimulate angiogenesis in the mice as the unstimulated CM from the OVCAR-4 cells did not support angiogenesis without supplementation with IL-8 and GRO-α.

Mice were then injected with matrigel mixed with media from MMP1-stimulated OVCAR-4 cells. These mice were divided into the following treatment groups: X1/2pal-i3 (5 mg/kg/d × 7 days), Avastin (5 mg/kg), or negative control i3 loop pepducin P1pal-19E (5 mg/kg/d × 7 days). Mice injected with MMP1-stimulated OVCAR-4 CM showed a significant 33-fold increase in blood vessel formation as assessed by confocal microscopy (Fig. 5B). As observed above, Avastin...
inhibited the MMP1-induced angiogenesis. Treatment of mice with the CXCR1/2 antagonist pepducin X1/2pal-i3 gave a striking reduction ($P = 0.002$) in blood vessel formation (Fig. 5B; Supplementary Fig. S6). However, the negative control i3 loop pepducin, P1pal-19E, had no effect on the mouse model providing further evidence that the observed angiogenesis was mediated by the CXCR1/2 receptors.

**X1/2pal-i3 blocks angiogenesis and inhibits tumor growth in ovarian cancer xenografts**

Lastly, we examined the effect of CXCR1/2 receptor blockade on ovarian cancer tumor growth and related these effects on tumor angiogenesis. OVCAR-4 ovarian carcinoma cells were s.c. injected into the flanks of female NCR nu/nu mice. The mice were then randomly divided into two treatment groups: X1/2pal-i3 or vehicle, and tumor volume was measured. After 30 days, the mice were euthanized and tumors assessed for angiogenesis. As shown in Fig. 6A and B, mice treated with the CXCR1/2 pepducin showed a significant decrease in tumor volume on day 23 ($P = 0.01$) and at the 30-day end point ($P = 0.02$). The X1/2pal-i3–treated tumors also showed a highly significant ($P < 0.0001$) 5-fold decrease in angiogenesis (Fig. 6C), confirming an important role for the CXCR1/2 receptors in ovarian tumor growth and angiogenesis.

**Targeting MMP1-chemokine communication in ovarian cancer angiogenesis**

Angiogenesis inhibitors, as exemplified by the VEGF antibody Avastin, have recently emerged as potential treatments for recurrent ovarian and primary peritoneal cancer (40, 41). Nonetheless, anti-VEGF therapy has not universally been able
to translate response rates into cure rates in patients (42), necessitating the need to identify new pathways involved in tumor-stromal interactions. However, there is limited information regarding the involvement of other tumor-produced angiogenic factors and their cognate receptors in ovarian cancer angiogenesis. In this study, we found that the metalloprotease MMP1, a poor prognostic factor for ovarian cancer (43, 44), mediates tumor-stromal communication by stimulating the release of angiogenic chemokines IL-8 and GRO-α via the PAR1 receptor on the ovarian carcinoma cells. The secreted GRO-α and IL-8 chemokines then act in a paracrine manner to promote endothelial cell proliferation, migration, tube formation, and angiogenesis. A cell-penetrating X1/2pal-i3 pepducin directed against both the CXCR1 and CXCR2 receptors was able to block the MMP1-induced effects of IL-8 and GRO-α on the endothelial cells and inhibited tumor progression and angiogenesis in ovarian cancer xenografts. These findings are consistent with our previous observations that upstream blockade of MMP1-PAR1 also significantly inhibited angiogenesis in mouse models of ovarian peritoneal carcinomatosis (23).

Proangiogenic factors such as IL-8 and Gro-α are upregulated by mitogen-activated protein kinase activation of the NF-κB and activator protein-1 transcription factors in many cell types including inflammatory cells, endothelial, and carcinoma cells (45). Although little is known regarding PAR1-dependent mitogen-activated protein kinase signaling pathways in ovarian carcinoma cells, both thrombin and MMP1 have been shown to upregulate IL-8 gene expression in melanoma cells, presumably through PAR1 (21, 46). We found that MMP1 activation of PAR1 did not significantly stimulate VEGF-A production above basal levels from the ovarian carcinoma cells. Despite this, Avastin had a partial inhibitory effect on the IL-8/GRO-α-induced angiogenesis in mice. This inhibition might be due to the synergistic effects of VEGF-A on IL-8 in endothelial cells. Indeed, recent studies have shown that IL-8 could induce the physical interaction of CXCR1/2 with VEGFR2 and cause subsequent activation of RhoA (47). RhoA is critical for endothelial angiogenesis (48), suggesting that there may be a critical relationship between VEGF and IL-8 in promoting angiogenesis. Other reports showed that shRNAi knockdown of GRO-α impaired tumor growth and angiogenesis in melanoma (16), and silencing IL-8 expression with lipocalated RNAi decreased ovarian tumor growth (12).

Figure 6. A and B, X1/2pal-i3 inhibits tumor progression and angiogenesis of ovarian cancer xenografts. OVCAR-4 cells (3 × 10^6) were injected s.c. into each flank of female NCR nu/nu mice. Mice (n = 8) were treated 24 h later with either X1/2pal-i3 (5 mg/kg/d × 6/7 d) or vehicle (15% DMSO) until day 30, and tumor volume was measured with calipers. C, on day 30, mice were euthanized and tumors were harvested, fixed in formalin, and stained with CD31 and subjected to confocal microscopy to assess angiogenesis. Columns, mean; bars, SE.
as adjuvants in metronomic chemotherapy (50). However, the rich peritoneal milieu that promotes growth and angiogenesis for the spread of ovarian cancer provides multiple communication pathways that might need to be interrupted to prevent metastasis. As exemplified by the inhibitory effects of the CXCR1/2 pepducins, this study presents a potential orthogonal strategy to target ovarian cancer angiogenesis and progression.

Disclosure of Potential Conflicts of Interest

Tufts Medical Center has out-licensed the pepducins used in this study. L. Covic and A. Kuliopulos are Scientific Founders and consultants for Anchor (Ascent) Therapeutics. The other authors disclosed no potential conflicts of interest.

References

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