Published in final edited form as:

Mol Cancer Ther. 2008 September ; 7(9): 2746–2757. doi:10.1158/1535-7163.MCT-08-0177.

Targeting a Metalloprotease-PAR1 Signaling System with Cell-Penetrating Peptiducins Inhibits Angiogenesis, Ascites, and Progression of Ovarian Cancer

Anika Agarwal^{1,2}, Lidija Covic^{1,2}, Leila M Sevigny^{1,5}, Nicole C Kaneider¹, Katherine Lazarides¹, Gissou Azabdaftari³, Sheida Sharifi^{3,4}, and Athan Kuliopulos^{1,2,5}

¹ Molecular Oncology Research Institute, Tufts Medical Center, Boston, MA

² Division of Hematology-Oncology, Department of Medicine, Tufts Medical Center, Boston, MA

³ Department of Pathology, Tufts-New England Medical Center, Boston, MA

⁴ Department of Pathology Mount Auburn Hospital Cambridge, MA

⁵ Departments of Biochemistry and Genetics, Tufts University Medical School Boston, MA

Abstract

Gene chip and proteomic analyses of tumors and stromal tissue has led to the identification of dozens of candidate tumor and host components potentially involved in tumor-stromal interactions, angiogenesis, and progression of invasive disease. In particular, matrix metalloproteases have emerged as important biomarkers and prognostic factors for invasive and metastatic cancers. From an initial screen of benign versus malignant patient fluids, we delineated a metalloprotease cascade comprising MMP-14, MMP-9 and MMP-1 that culminates in activation of PAR1—a G protein-coupled protease-activated receptor upregulated in diverse cancers. In xenograft models of advanced peritoneal ovarian cancer, PAR1-dependent angiogenesis, ascites formation and metastasis was effectively inhibited by intraperitoneal administration of cell-penetrating peptiducins based on the intracellular loops of PAR1. These data provide an *in vivo* proof-of-concept that targeting the metalloprotease-PAR1 signaling system may be a novel therapeutic approach in the treatment of ovarian cancer.

Keywords

Ovarian cancer; PAR1; MMP-1; MMP-9; MMP-14; angiogenesis; endothelium; peptiducins

Introduction

Ovarian cancer is a particularly deadly malignancy because the vast majority of patients are diagnosed well after the cancer has spread throughout the peritoneal cavity (1). Despite excellent initial response rates to chemotherapy, most patients experience relapses due to drug resistance. This has sparked an upsurge in interest in developing targeted therapies that block key signaling pathways involved in tumor-stromal interactions, tumor vascularity and invasion (2,3). Abundant evidence has pointed to matrix metalloproteases (MMPs) as playing critical

Requests for Reprints: A Kuliopulos, Box 7510, Molecular Oncology Research Institute, Tufts Medical Center, Boston, MA 02111. E-mail: athan.kuliopulos@tufts.edu.

Conflict of Interest Statement: Tufts Medical Center has out-licensed the peptiducins used in this paper.

roles in invasion, blood vessel penetration, and metastasis of many solid tumors including ovarian cancer (4,5). MMPs are zinc-dependent proteases secreted by both tumor and host cells that are required for tumor cell invasion through the basement membrane and stromal tissue. Secreted MMPs are produced in the pro-form as inactive zymogens which are activated on the cell surface by other proteases and membrane-tethered MMPs (4). However, the mechanism of activation of individual proMMPs remains largely unknown and may differ depending on the tumor type and stage. Human ovarian cancer cells and surrounding stroma have been shown to secrete proMMP-2 and proMMP-9 (6,7) and the increased expression of these gelatinases is associated with high invasive and metastatic potential (8,9) and short overall survival of ovarian cancer patients (10). Peritoneal inflammatory cells also supply metalloproteases such as macrophage-derived MMP-9 which increases tumorigenesis and angiogenesis in genetically-modified mice (11).

In addition to MMP-2 and MMP-9, one of the most promising of the new candidate biomarkers for metastatic and invasive cancer is the fibroblast collagenase, MMP-1. High MMP-1 expression in tumors is associated with poor prognosis in patients with colorectal, esophageal and breast cancer (12,13,14). MMP-1 was also identified as a predictive marker for breast cancer where it is expressed in reactive stromal tissue and ductal lavage fluids of patients with no prior history of breast cancer (15). A commonly occurring G/2G promoter polymorphism which causes enhanced *MMP1* transcription (16) was recently shown to be associated with shortened disease-free and overall 5-year survival in ovarian cancer patients (17), however it is not clear whether MMP-1 collagenase plays a direct role in the progression of ovarian cancer.

From a screen of fluids derived from patients with benign versus malignant ovarian diseases, a metalloprotease cascade was identified which culminates in activation of proMMP-1 to MMP-1. MMP-1 in turn directly activated protease-activated receptor-1 (18)—a cell surface receptor coupled to G proteins (19). PAR1 is not normally expressed in non-malignant epithelia including ovarian (20,21) but was found to be highly upregulated in invasive ovarian carcinomas *in vitro* and when passaged in mice. PAR1-dependent angiogenesis and ascites formation, invasion and metastasis, was effectively inhibited downstream of the MMP cascade by a cell-penetrating lipopeptide ‘pepducin’ based on the third intracellular loop of PAR1. These results provide *in vivo* evidence that PAR1 may be potential therapeutic target in ovarian cancer.

Materials and Methods

Pepducins

N-palmitoylated peptides, P1pal-7 ($C_{15}H_{31}CONH-KKSRALF-NH_2$), P1pal-12 ($C_{15}H_{31}CONH-RCLSSSAVANRS-NH_2$) and P1pal-19EE ($C_{15}H_{31}CONH-RCESSAEANRS KKERELF-NH_2$) were synthesized by standard fmoc synthetic methods with C-terminal amides. Palmitic acid was dissolved in 50% N-methyl pyrrolidone/50% methylene chloride and coupled overnight to the deprotected N-terminal amine of the peptide. After cleavage from the resin, palmitoylated peptides were purified to >95% purity by C_{18} or C_4 RP-HPLC as before (22).

RNAi

RNAi reagents have been previously described and characterized: *MMP-9* (5' CAUCACCUAUUGGAUCCAAdTdT3') (23), *MMP-14* (5' AUGCAGAAGUUUACGGCUUGUU3') (24), firefly *luciferase* (5' CGTACGCGGAATACTTCGA3') and *PAR1* (5'AAGGCUACUAUGCCUACUACU3') (18). The *PAR3* RNAi SMART pool L-005491 was synthesized by Dharmacon (Lafayette, Colorado). Total RNA was extracted for RT-PCR from OVCAR-4 cells with RNeasy mini kit

(Qiagen, Valencia, California). cDNA was prepared with 5µg of total RNA using MMLV reverse transcriptase. The desired products were amplified using Taq polymerase and the following primers: *hMMP-1* sense 5'CGACTCTAGAAACACAAGAGCAAGA3', antisense 5'AAGGTTAGCTTACTGTACACGCTT3'; *hMMP-2* sense 5'GTGCTGAAGGACACACTAAAGAAGA3', antisense 5'TTGCCATCCTTCTCAAAGTTGTAGG3'; *hMMP-9* sense 5'CACTGTCCACCCCTCAGAGC3', antisense 5'GCCACTTGTCGGCGATAAGG3', *hMMP14* sense 5'GCTTGCAAGTAACAGGCAAA3', antisense 5'AAATTCTCCGTGTCCATCCA3', as described (18,24), *hMMP-8* sense 5'-GACGCTTCCATTTCTGCTCTTACTCCAT-3', antisense 5'-TCCCCGTCACATTCAACCCAAAAA-3', *MMP-3* sense 5'-GATCTCTTCATTTTGGCCATCTCTTC-3', antisense 5'-CTTCCAGTATTTGTCTCTACAAAGAA-3'.

Cell Culture

OVCAR-4, SKOV-3, OVCAR-3, IGROV-1, OVCAR-5, and OVCAR-8 cells were obtained from the NCI (Frederick, Maryland). NIH-3T3 murine fibroblasts were from ATCC (Manassas, Virginia). Cells were grown in RPMI with 10% FBS and transfected with OligofectAMINE using 20µM RNAi per 100 mm plate. Migration assays were conducted 48 h following transfection. Human recombinant IL-8 was expressed in *E. coli* using the pET31 system (25) and purified and reconstituted as previously described (26).

Flow Cytometry

Rabbit polyclonal PAR1 and PAR3 antibodies were purified by peptide affinity chromatography as described previously (27). Fluorescein isothiocyanate (FITC)-conjugated goat anti-rabbit antibody was purchased from Zymed (San Francisco, CA). Flow cytometry was performed on ovarian carcinoma cells as before (27,28).

Cell Migration and MMP Assays

Ovarian and hepatic ascites, ovarian cystic fluid, and pleural effusions were collected from 16 patients with all patient identifiers removed as approved by the Tufts Medical Center Institutional Review Board (Supplemental Table I). Conditioned media was prepared from fibroblasts or OVCAR-4 cells after 2 d of cell culture as described (18). Chemotactic migration and chemo-invasion (29) assays were conducted using a 8 µm pore size transwell apparatus (Corning, Costar) as previously described (27). Chemotactic index measurements (ratio between the distance of migration toward chemoattractants over that toward medium alone) were performed with a 48 blindwell microchemotaxis chamber (Neuroprobe) equipped with 8 µm pore nitrocellulose filters as previously described (26). Pure proMMP-1, proMMP-2, proMMP-3, proMMP-7, and proMMP-9 were obtained from EMD BioSciences (San Diego, California). MMP-200 was from Enzyme Systems Products (Livermore, California) and FN-439 (MMP Inh-1), MMP 2 Inh, MMP 9/13 Inh-1, MMP-3 Inh-III were from Calbiochem (La Jolla, California). Human α-thrombin was from Haematologic Technologies (Essex Junction, VT). Stock solutions of 400 nM proMMP-1 were activated with 2 mM APMA in 50 mM Tris, pH 7.7, 5 mM CaCl₂, 0.2 M NaCl, 50 µM ZnCl₂ at 37 °C for 30 min and then transferred to an ice bath prior to use. Prior to migration assays, the APMA was removed by overnight dialysis in 10 kDa MWCO Mini Slide-A-Lyzers (Pierce, Rockford, IL) at 4 °C. Quantikine human proMMP-1, total MMP-2, total MMP-3, total MMP-8, total MMP-9 and total MMP-13 ELISAs were purchased from R&D systems (Minneapolis, MN) and was used according to the manufacturer's protocol. Collagenase activity was assayed from lysed cells and in conditioned media by measuring the cleavage of fluorescein conjugated DQ collagen (Molecular Probes). Collagenase assays contained 10 µg DQ collagen in 50 mM Tris-HCL,

pH 7.6, 150 mM NaCl, 5 mM CaCl₂ and 0.2 mM NaN₃ and cleavage was monitored at 538 nm using a fluorescence microplate reader with excitation at 485 nm at 25°C. One unit of collagenase activity was defined as the cleavage of 1 µg collagen per min at 25°C.

Endothelial Monolayer Permeability Assays

Immortalized HUVECs (EA.hy 926) were plated on 3 µm polycarbonate transwell membranes (Corning Inc, Corning, NY) in EBM2 supplemented with 10% FBS. After reaching confluence, the cell coated membranes were placed into a dual chamber system and were either treated with MMP-1 or conditioned media from peritoneal fibroblasts (HPF) in the presence or absence of 5 µM FN-439, 5 µM RWJ-56110, or 3 µM P1pal-7. After a 2 h incubation period, the media in the upper chamber was removed and DMEM containing 30 mg/mL Evans blue was added to the upper well and PBS was added to the lower compartment of the chamber. After 15 min incubation, Evans blue was quantified in the lower wells by OD650.

Peritoneal Ovarian Tumor Models

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 into the peritoneal cavity with 1.5 million OVCAR-4 or SKOV-3 cells. Animals were subsequently treated every other day with either intraperitoneal injections of P1pal-7 pepducin or FN-439 in 100 µL 10% DMSO vehicle with or without concomitant docetaxel. At the end of 6 weeks, tumors and organs were excised from euthanized mice, fixed in 10% formalin/PBS and imbedded in paraffin. Tissue sections (5 µm) from omentum, diaphragm, abdominal and thoracic organs were prepared and stained with hematoxylineosin or immunostained using a rabbit polyclonal anti-von Willebrand Factor (DakoCytomation, N1505) for micro-vessel quantification and staged in a blinded manner by two independent pathologists.

Results and Discussion

Matrix Metalloproteases in Benign Versus Malignant Ovarian Fluids

Patients with ovarian cancer often produce copious amounts of peritoneal ascites or cystic fluid which may contain metalloproteases that aid in the dissemination of the cancer. Fluid was collected from the peritoneal cavity (ascites), ovarian cysts, or pleura from sixteen patients with malignant or benign diseases. Protein levels of the collagenases (proMMP-1, total MMP-8, proMMP-13), gelatinases (total MMP-2, total MMP-9) and stromelysin (total MMP-3) were then measured in the patient fluids. Of the six MMPs tested, proMMP-1 levels had the strongest positive correlation with malignancy (Fig. 1). Likewise, there was a significant correlation between proMMP-1 levels in the patient samples and ability to induce migration of highly invasive OVCAR-4 carcinoma cells ($R=0.85$, $P=0.016$). Malignant ovarian cyst fluid and ovarian ascites had proMMP-1 levels that were 8–13 fold higher than those in benign ovarian cyst fluid. Conditioned media from peritoneal fibroblasts isolated from ovarian cancer patients had up to 200-fold higher proMMP-1 levels than media from the OVCAR-4 cancer cells themselves (Fig. 1). Conversely, protein levels of the other collagenases, MMP-8 and MMP-13, did not correlate with either malignancy nor ability to induce migration. Levels of the MMP-2 gelatinase were 90 to 550-fold higher than proMMP-1 in the patient fluids but did not correlate as strongly with malignancy nor migration. MMP-9 gelatinase was abundant in nearly all tested benign and malignant patient fluids (71 ± 20 ng/mL) and was produced at very high levels by OVCAR-4 cells. MMP-3 levels did not correlate with malignancy nor migration.

PAR1, a Receptor for MMP-1, Is Correlated with Invasive Propensity of Ovarian Cancers

A recent study found that the G protein-coupled receptor, PAR1, is cleaved by MMP-1 which promotes breast cancer migration and invasion (18). To investigate whether PAR1 might play a role in metalloprotease-dependent chemotaxis of ovarian carcinoma cells, we characterized six ovarian cancer cell lines from the NCI-60 panel for PAR1 surface expression and correlated expression with cell migration toward fibroblast conditioned media (CM). There was a strong positive correlation ($R=0.96$, $P=0.0006$) between PAR1 expression and migration of OVCAR-5, OVCAR-3, SKOV-3, IGROV-1, OVCAR-8 and OVCAR-4 ovarian cancer cell lines (Fig. 2A). In contrast, there was a poor correlation ($R=0.33$, $P=0.47$) between the closely homologous PAR3 receptor (Fig. 2A) and migration of ovarian cancer cells to the fibroblast CM. OVCAR-4 cells had the highest levels of PAR1 surface expression and gave a robust calcium signal to the PAR1 ligand TFLLRN (data not shown). We also tested whether there was a correlation between the levels of a particular MMP in the patient fluids and chemo-invasion (29) of the ovarian carcinoma cells through matrigel. Consistent with the migration data, the only significant positive correlation between chemo-invasion and MMP level was observed for proMMP-1 and the high PAR1-expressing OVCAR-4 and IGROV-1 cells (Supplementary Table II).

Metastatic spread of cancer cells in mice often results in altered expression of malignancy-associated genes, therefore we tested whether passage of OVCAR-4 cells in the peritoneal cavity of nude mice would increase expression of PAR1. OVCAR-4ip1 cells, harvested at 40 d from nude mice, had a 2.3-fold further increase in migration and a corresponding 2.5-fold further increase in surface expression of PAR1 (Fig. 2A). In contrast, surface expression of PAR3 dropped by 50% in the ip-passaged OVCAR-4 cells relative to the parent OVCAR-4 cells. Gene silencing of *PAR1* in the OVCAR-4ip1 and parental OVCAR-4 cells with RNAi caused a 45–77% decrease in median surface expression of PAR1 relative to luciferase control RNAi-treated cells (Supplementary Fig. 1A) with an attendant 60–75% loss in cell migration (Fig. 2B). PAR3 RNAi treatment (Supplementary Fig. 1B) had no effect on the migration of either the OVCAR4 or OVCAR-4ip1 cells (Fig. 2). To validate the MMP1-PAR1 signaling axis, OVCAR-4 cells were treated with either PAR1 or luciferase RNAi for 48 h, then tested for ability to migrate toward MMP-1. The 2.3-fold increased migration toward 0.3 nM MMP-1 was completely blocked with *PAR1* RNAi treatment of the OVCAR-4 cells (Fig. 2C). To provide evidence that the decreased migration was a PAR1-specific event we found that chemotactic migration of the OVCAR-4 cells toward IL-8 was unaffected by *PAR1* RNAi treatment relative to *luciferase* RNAi (Supplementary Fig. 1C).

PAR1 Pepducins Inhibit Migration of Ovarian Cancer Cells Toward Malignant Ascites Fluid

To block PAR1-dependent migration of ovarian carcinoma cells toward ascites fluid from patients with ovarian cancer or fibroblast media, we used cell-penetrating pepducin antagonists based on the intracellular loops of PAR1 (22,30). Pepducins are cell-penetrating lipo-peptide conjugates based on the sequences of the intracellular loops of PAR1 and other GPCRs (22, 26,30,31). After delivering its cargo across the plasma membrane, the lipid moiety anchors the compound to the lipid bilayer thereby concentrating the pepducin to the target receptor-G protein interface (32). The P1pal-12 pepducin is a N-palmitoylated peptide based on the amino-terminal portion of the third intracellular loop (i3) of PAR1 that interdicts signaling between PAR1 and internally located G proteins (22). In addition, we tested a carboxy-terminal i3 loop pepducin, P1pal-7, that is a full antagonist of PAR1, and a negative control PAR1 pepducin, P1pal-19E (22). P1pal-12 and P1pal-7 blocked 90–94% of OVCAR-4 migration toward human ovarian ascites and fibroblast CM, whereas P1pal-19E was without effect (Fig. 2D). Likewise, a small molecule ligand-based antagonist of PAR1, RWJ-56110 (33), strongly inhibited migration of the OVCAR-4 cells. We also tested the ability of the PAR1-based pepducins to inhibit migration of other ovarian carcinoma cell lines to either malignant human ascites fluid

or fibroblast media. IGROV-1 cells were inhibited by up to 80% with the PAR1 peptidicins or RWJ-56110 whereas SKOV-3 was inhibited by 33–70% (Fig. 2D). The less pronounced inhibition of migration of the SKOV-3 cells by the PAR1 antagonists was correlated with the lower PAR1 expression levels in those cells as compared to the high PAR1-expressing OVCAR-4 cells. Addition of the PAR1 antagonists had no effect on the migration of the very low PAR1-expressing OVCAR-3 cells (Supplementary Fig. 2).

MMP-1 Activity From Malignant Ascites Fluid Stimulates the Migration of Ovarian Carcinoma Cells

PAR1 has been shown to be activated by several serine proteases from blood such as thrombin, plasmin, activated protein C, or by fibroblast-secreted matrix metalloprotease MMP-1 (18, 28,34). To provide evidence that the MMP-1 activity derived from the human ascites was responsible for the PAR1-dependent migration of the OVCAR-4 cells, a panel of protease inhibitors that block serine proteases (PMSF, broad spectrum serine protease inhibitor; hirudin, specific thrombin inhibitor) or Zn^{2+} -dependent matrix metalloproteases (MMP-200, broad spectrum MMP inhibitor; FN-439, inhibitor of MMP-1) were assessed for their ability to block migration. As shown in Figure 2E, neither hirudin nor PMSF inhibited migration, whereas MMP-200 and FN-439 prevented 65% of migration of OVCAR-4 cells toward ascites fluid from patients with ovarian cancer or CM from fibroblasts. The 35–40% residual motility of the OVCAR-4 cells may be due to other chemotactic factors or proteases that are not blocked by the protease inhibitors employed. To confirm that proteolytic activation of PAR1 is capable of stimulating migration of OVCAR-4 cells, we added exogenous activated MMP-1 or thrombin to serum-free media in the lower chamber of the chemotaxis apparatus. We found that low concentrations of MMP-1 or thrombin induced cell motility of OVCAR-4 with a peak at 0.3 nM for both proteases (Fig. 2F–G). MMP-1 and thrombin induction of OVCAR-4 cell mobility was abolished with the PAR1 inhibitor, RWJ-56110. MMP1-induced migration was unaffected by PMSF whereas thrombin-induced migration was completely blocked (Fig. 2F–G). Together, these data indicate that the observed PAR1-dependent chemotaxis of the ovarian carcinoma cells toward MMP-1 is independent of serine proteases.

Upstream Roles of MMP-9 and MMP-14 in Activating MMP1-Dependent Migration of Ovarian Carcinoma Cells

A panel of proMMPs were individually screened for ability to stimulate chemotactic migration of OVCAR-4 ovarian carcinoma cells. We found that proMMP-1 zymogen added to serum-free media in the bottom well was also able to induce nearly a 100% increase in chemotactic migration of OVCAR-4 cells (Fig. 3A). Unlike proMMP-1, addition of proMMP-2, proMMP-3, proMMP-7, or proMMP-9 zymogens to RPMI in the bottom well was not able to confer chemotaxis to the OVCAR-4 cells. A panel of metalloprotease inhibitors was used to determine whether active MMPs produced by the OVCAR4 cells themselves were essential for the chemotaxis towards proMMP-1. FN-439 completely blocked the ability of proMMP-1 to induce migration of the OVCAR-4 cells (Fig. 3B), indicating that active MMP-1 was required. Inhibitors against MMP-2 and MMP-3 were without effect, however, the MMP9/13 inhibitor completely blocked proMMP1-induced migration. RT-PCR showed that the OVCAR-4 cells did not express *MMP-1*, *MMP-2*, *MMP-3* or *MMP-8* but did express mRNA for *MMP-9* (Fig. 3C), consistent with the ELISA results in Figure 1. Together, these data suggested that the MMP-9 produced by the OVCAR-4 cells might be required for activating exogenous proMMP-1 to MMP-1.

To provide direct evidence that MMP-9 from OVCAR-4 cells was required for MMP1-dependent migration, we suppressed *MMP-9* expression using a previously-described RNAi (23) (Supplementary Fig. 1D). As shown in Figure 3D, proMMP1-dependent migration, but not MMP1-migration, was nearly completely suppressed by *MMP-9* RNAi treatment of the

OVCAR4 cells as compared to *luciferase* RNAi controls. To demonstrate that MMP-9 was directly activating proMMP-1, we measured *in situ* production of collagenase activity from OVCAR-4 media. Addition of proMMP-1 to OVCAR4 cells significantly enhanced collagenase activity by 13 ± 2 U/mL above baseline (Fig. 3E). Conversely, knock-down of *MMP-9* expression from OVCAR-4 cells with RNAi inhibited 70% of the proMMP1-dependent collagenase activity.

Tumor-derived membrane-tethered MMP-14 (35,36) is known to convert proMMP-2 to MMP-2 (37,38,39) and may also be involved in the activation of the related proMMP-9 (40). We found that *MMP-14* mRNA was expressed in the OVCAR-4 cells (Fig. 3C). Therefore, we examined whether MMP-14 was also required for the promigratory effects of proMMP-1. As shown in Fig. 3D, treatment of OVCAR-4 cells with a RNAi against *MMP-14* (24) completely suppressed the promigratory effects of proMMP-1 and could be complemented by exogenous MMP-1 but not by proMMP-9 plus proMMP-1. Similarly, *MMP-14* gene suppression (Supplementary Fig. 1E) inhibited 80% of proMMP1-derived collagenase activity from OVCAR-4 cells (Fig. 3E). Taken together, these data support a proposed cascade whereby OVCAR4-produced MMP-14 and MMP-9 convert proMMP-1 to MMP-1 which in turn triggers migration.

A previous study indicated that peritoneal macrophages (11) can secrete proMMP-9, and we found that MMP-9 protein was abundant in malignant peritoneal fluids. Therefore, it is possible that the pro and/or active forms of MMP-9 supplied by the peritoneal fluids would be sufficient to activate chemotaxis of the ovarian carcinoma cells without a requirement of MMP-9 or MMP-14 from the carcinoma cells. To test this possibility, the chemotactic migration assays were conducted using ascites from patients with malignant ovarian cancer. Chemotactic migration of the OVCAR-4 cells to the malignant ascites fluid was inhibited by 75% by the MMP-1 inhibitor, FN-439 (Fig. 3F). *MMP-9* or *MMP-14* RNAi treatment of the OVCAR-4 cells ablated chemotaxis of the cells toward the malignant ascites fluid. In control experiments, neither *MMP-9* nor *MMP-14* RNAi treatments had an effect on chemotactic migration of OVCAR-4 cells toward IL-8 as compared to *luciferase* RNAi controls (Supplementary Fig. 1C). Together, these data indicate that MMP-9 or other MMPs from the human ascites fluid cannot complement the loss of OVCAR4-produced MMP-9 or MMP-14.

Effects of Peritoneal Fibroblast-Derived MMP-1 on PAR1-Dependent Endothelial Barrier Function

Peritoneal carcinomatosis is associated with the production of large volumes of ascitic fluid which has a severe negative impact on quality of life of patients with advanced ovarian cancer and may aid in peritoneal dissemination and carcinomatosis. It has been postulated that the ascitic fluid arising from the peritoneal tumors is a direct result of a leaky tumor vasculature (41,42). Endothelial PAR1 is an important modulator of endothelial barrier function (43,44, 45) and could potentially be involved in ascites formation. Moreover, tumor-derived MMP-1 was recently shown to activate PAR1-dependent von Willebrand factor release, calcium flux and IL-8 secretion from endothelial cells (46). We tested whether MMP-1 derived from patient peritoneal fibroblasts could stimulate PAR1-dependent increases in the barrier permeability of endothelial monolayers. First, we showed that addition of exogenous MMP-1 (0.5 nM) caused a 50% increase in endothelial barrier permeability which was inhibited by either the MMP-1 antagonist, FN-439, or the PAR1 antagonists, P1pal-7 or RWJ-56110 (Fig. 4A). We then incubated peritoneal fibroblast-derived CM overnight in the presence of OVCAR-4 cells. The OVCAR4-treated peritoneal fibroblast CM elicited a 2.2-fold increase in endothelial barrier permeability which could be nearly completely inhibited by FN-439, P1pal-7 or RWJ-56110 (Fig. 4B). Taken together, these *in vitro* data suggest that MMP-1 derived from peritoneal

fibroblasts can strongly stimulate PAR1-dependent increases in endothelial barrier permeability.

To test whether PAR1 was involved in ascites formation *in vivo*, we assessed the effects of 40 d treatment with the PAR1 pepducin, P1pal-7 (10 mg/kg i.p. every other day), on nude mice with OVCAR-4 peritoneal carcinomatosis. As shown in Fig. 4C-D, vehicle-treated nude mice with OVCAR-4 peritoneal tumors developed pronounced ascitic fluid accumulation (2.5 ± 0.7 mL) at the 40 d time point. Monotherapy with the P1pal-7 pepducin significantly reduced mean ascites fluid volume by 60% ($P = 0.0017$) as compared to vehicle, consistent with the predicted effects of blocking endothelial PAR1 on vascular leakage. Similar results were also seen in nude mice injected i.p. with SKOV-3 cells (11, 47). All vehicle-treated mice developed ascites, however, mice treated with P1pal-7 had a trend towards reduced ascites (65%; $P = 0.137$) at the 40 d time point (Supplementary Fig. 3).

The P1pal-7 Pepducin Blocks Angiogenesis of Peritoneal Ovarian Cancers

We examined the effect of PAR1 inhibition by P1pal-7 on microvessel density in i.p. ovarian tumors. OVCAR-4 tumors excised from peritoneal organs 6 weeks post-inoculation, revealed extensive replacement of normal tissue and had a pronounced serous cyst glandular morphology typical of ovarian adenocarcinoma. We found that P1pal-7 monotherapy caused a highly significant 84–96% ($P < 0.005$) reduction in blood vessel density in both the center and edge of the OVCAR-4 tumors as compared to vehicle-treated mice after a treatment period of 6 weeks (Fig. 5A). Likewise, P1pal-7 in combination with docetaxel caused a significant 73–92% reduction in blood vessel density in both the center and edge of the OVCAR-4 tumors as compared to docetaxel alone (Fig. 5B). MMP-1 inhibition with FN-439 was found to have similar effects as P1pal-7 on blocking tumor vascularity in the ovarian tumors (Fig. 5B).

Monotherapy with the PAR1 pepducin, P1pal-7, also caused a striking decrease (90%) in mean blood vessel density in i.p. SKOV-3 tumors as compared to vehicle-treated mice (Fig. 5C). Addition of P1pal-7 to docetaxel caused a further significant decrease in blood vessel density as compared to docetaxel alone. Thus, blockade of PAR1 signaling may provide a benefit in inhibiting angiogenesis either when used alone or in combination with docetaxel in mouse models of peritoneal carcinomatosis.

None of the nude mice that received P1pal-7 every other day for 6 weeks in the presence or absence of docetaxel, exhibited any obvious toxicity. To assess whether P1pal-7 was immunogenic or could affect the host immune response, we injected P1pal-7 (10 mg/kg i.p. 6 days per week) for 6 weeks into DBA/1J mice which are susceptible to developing autoantibodies and exhibit heightened inflammatory responses. Sera collected at the 6 week time point were not immunoreactive to P1pal-7 by dot-blot as compared to vehicle-treated mice (Supplementary Fig. 4A). Moreover, serum KC (Gro- α) levels—a marker of systemic inflammation in mice (26)—showed no differences between the P1pal-7 and control groups (Supplementary Fig. 4B). Pepducin-treated mice had normal joint mobility and no evidence of arthritis or swelling over the 6 week period (Supplementary Fig. 4C).

Combination Intraperitoneal Therapy of Docetaxel Plus a PAR1-Based Pepducin Inhibits Invasion and Metastasis of Ovarian Cancer in Nude Mice

Patients whose cancer has spread throughout the peritoneal cavity have a survival rate of only 28%, thus inhibition of peritoneal dissemination and metastatic progression is critical for the successful treatment of ovarian cancer. Docetaxel, or the related paclitaxel administered i.p. and used in combination with other therapies, is emerging as the standard-of-care chemotherapeutic agent for the treatment of advanced and recurrent ovarian cancer (48). Despite the significant inhibitory effects on ascites and angiogenesis, targeting PAR1 alone

with P1pal-7 had little effect on the metastatic spread of the OVCAR-4 cells through the peritoneal cavity of the mice (data not shown). It was possible that the residual blood vessels and ascites fluid observed in the mice treated with P1pal-7 alone was sufficient to provide the necessary factors for metastatic progression.

Therefore, we assessed whether inhibition of PAR1 versus MMP-1 in combination with docetaxel was able to slow progression of advanced ovarian cancer in mice. To quantify these effects, we developed a histological staging system that used the diaphragm as a marker of the progressive invasion and metastasis from the peritoneal cavity/omentum to the thoracic organs (Fig. 6A). Four intraperitoneal treatment groups were evaluated. In the first group, mice received vehicle alone every other day for the 40 d time period. A second group received docetaxel (10 mg/kg) i.p. once weekly starting on day 20 until the end of the 40 d period. The third treatment group received the identical docetaxel regimen plus P1pal-7 (3.2 mg/kg i.p. every other day for 40 d starting 24 h after tumor inoculation to allow implantation). The fourth treatment group received the identical docetaxel regimen plus FN-439 (5 mg/kg i.p. every other day starting 24 h after tumor inoculation). At the 40 d time point, all of the vehicle-treated mice had stage 4 disease with 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*. By comparison, mice treated with docetaxel alone had a marked decrease in metastatic progression but the cancer had largely spread to the peritoneal surface of the diaphragm (Stage 1b, Fig. 6A). Addition of the P1pal-7 pepducin to the docetaxel regimen caused a significant further decrease in invasion ($P = 0.01$) relative to docetaxel alone and slowed invasion into the diaphragm or metastases to the thoracic cavity (Fig. 6B). Likewise, addition of FN-439 to docetaxel conferred significant ($P = 0.003$) protection against invasion into the diaphragm with no evidence of thoracic metastases.

Targeting the Metalloprotease-PAR1 Axis in Ovarian Cancer

The interaction of ovarian carcinoma cells with peritoneal stromal components such as fibroblasts and endothelial cells provides a rich host environment that facilitates tumor growth, invasion and angiogenesis. Matrix metalloproteases in particular are well-adapted to serve as signaling conduits in the tumor-stromal microenvironment given their key roles in the complex processes of tissue invasion, blood vessel homeostasis, and metastasis. MMP-1, MMP-9 and MMP-14 have all been implicated in the progression of ovarian cancer. Our finding that these three stromal and tumor-derived metalloproteases act together in a metalloprotease cascade to activate PAR1 serves to reinforce the notion that carcinoma cells must closely coordinate their malignancy-associated activities with the surrounding stromal cells. The use of membrane-tethered MMP-14, which localizes to the cellular protrusions or invadopodia, might further augment the concentration gradients of the secreted MMP-1 and MMP-9 near the PAR1 receptor and also create an appropriate invasive microenvironment for outward tumor expansion and angiogenesis (49). In addition to MMP-1, PAR1 has several potential protease agonists including thrombin, activated protein C and plasmin. Thus, downstream blockade of PAR1 with a pepducin might be more advantageous as opposed to direct MMP-1 inhibition which does not impact other putative PAR1 (serine protease) agonists.

Matrix metalloprotease inhibitors are an attractive class of drug candidates for blocking tumor progression, but their approval as cancer therapeutics has been hampered due to toxicity in humans (50). Likewise, intravenously-administered chemotherapy often causes severe side effects arising from bone marrow and gastrointestinal toxicity. One strategy to limit systemic side effects and improve tumor-targeting in ovarian cancer patients is to deliver the anti-cancer agent directly to the peritoneal cavity. A recent clinical trial involving 415 patients with stage III ovarian carcinoma randomized patients to standard intravenous versus high-dose intraperitoneal chemotherapy (48). High-dose intraperitoneal cisplatin and paclitaxel

significantly increased the overall median survival from 50 to 66 months as compared to the intravenous treatment group. Therefore, it anticipated that future treatment regimens for ovarian cancer will further utilize intraperitoneal therapy. The present study showed that 6 week intraperitoneal administration of a PAR1 pepducin reduced ascites production and angiogenesis, and when given in combination with docetaxel inhibited metastatic progression of peritoneal ovarian cancer. An intraperitoneal treatment regimen that targets PAR1 may provide an orthogonal approach by inhibiting both tumor invasion and angiogenesis downstream of matrix metalloproteases in the peritoneal microenvironment.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

We are highly grateful to David Simon for conducting statistical analyses, Yoko Yoda for conducting PCR and FACS, John Yang, Katie Wakely and Lawrence Brinkerhoff for providing patient samples, Nga Nguyen for conducting PCR, Claudia Derian and Patricia Andrade-Gordon of Johnson & Johnson Pharmaceuticals for providing RWJ-56110 and Namita Jain for histopathology analysis.

Grant Support: NIH grants CA122992, HL64701, HL57905 (A. Kuliopulos), CA104406 (L. Covic), and by the Susan G. Komen Breast Cancer Foundation (A. Kuliopulos).

References

1. Ozols RF, Bookman MA, Connolly DC, et al. Focus on epithelial ovarian cancer. *Cancer Cell* 2004;5:19–24. [PubMed: 14749123]
2. Sawiris GP, Sherman-Baust CA, Becker KG, Cheadle C, Teichberg D, Morin PJ. Development of a highly specialized cDNA array for the study and diagnosis of epithelial ovarian cancer. *Cancer Res* 2002;62:2923–2928. [PubMed: 12019173]
3. Conrads TP, Fusaro VA, Ross S, et al. High-resolution serum proteomic features for ovarian cancer detection. *Endocr Relat Cancer* 2004;11:163–178. [PubMed: 15163296]
4. Egeblad M, Werb Z. New Functions for the Matrix Metalloproteinases in Cancer Progression. *Nature Rev Cancer* 2002;2:161–174. [PubMed: 11990853]
5. Pei D. Matrix metalloproteinases target protease-activated receptors on the tumor cell surface. *Cancer Cell* 2005;7:207–8. [PubMed: 15766657]
6. Naylor MS, Stamp GW, Davies BD, Balkwill FR. Expression and activity of MMPS and their regulators in ovarian cancer. *Int J Cancer* 1994;58:50–56. [PubMed: 8014015]
7. Fishman D, Bafetti LM, Banionis S, Kearns AS, Chilukuri K, Stack MS. Production of extracellular matrix-degrading proteinases by primary cultures of human epithelial ovarian carcinoma cells. *Cancer* 1997;80:1457–1463. [PubMed: 9338470]
8. Huang LW, Garrett AP, Bell DA, Welch WR, Berkowitz RS, Mok SC. Differential Expression of Matrix Metalloproteinase-9 and Tissue Inhibitor of Metalloproteinase-1 Protein and mRNA in Epithelial Ovarian Tumors. *Gyn Oncol* 2000;77:369–376.
9. Graves LE, Ariztia EV, Navari JR, Matzel HJ, Stack MS, Fishman DA. Proinvasive properties of ovarian cancer ascites-derived membrane vesicles. *Cancer Res* 2004;64:7045–9. [PubMed: 15466198]
10. Lengyel E, Schmalfeldt B, Konika E, et al. Expression of Latent Matrix Metalloproteinase 9 (MMP-9) Predicts Survival in Advanced Ovarian Cancer. *Gyn Oncol* 2001;82:291–298.
11. Huang S, Arsdall MV, Tedjarati S, et al. Contributions of Stromal Metalloproteinase-9 to Angiogenesis and Growth of Human Ovarian Carcinoma in Mice. *J Natl Cancer Inst* 2002;94:1134–1142. [PubMed: 12165638]
12. Murray GI, Duncan ME, O'Neil P, Melvin WT, Fothergill JE. Matrix metalloproteinase-1 is associated with poor prognosis in colorectal cancer. *Nat Med* 1996;2:461–462. [PubMed: 8597958]

13. Murray GI, Duncan ME, O'Neil P, McKay JA, Melvin WT, Fothergill JE. Matrix metalloproteinase-1 is associated with poor prognosis in oesophageal cancer. *J Pathol* 1998;185:256–261. [PubMed: 9771478]
14. van't Veer LJ, Dai H, Vijver MJvd, et al. Gene expression profiling predicts clinical outcome of breast cancer. *Nature* 2002;415:530–536. [PubMed: 11823860]
15. Poola I, DeWitty RL, Marshalleck JJ, Bhatnagar R, Abraham J, Lefall LD. Identification of MMP-1 as a putative breast cancer predictive marker by global gene expression analysis. *Nat Med* 2005;11:481–483. [PubMed: 15864312]
16. Rutter JL, Mitchell TI, Buttice G, et al. A single nucleotide polymorphism in the matrix metalloproteinase-1 promoter creates an Ets binding site and augments transcription. *Cancer Res* 1998;58:5321–5. [PubMed: 9850057]
17. Six L, Grimm C, Leodolter S, et al. A polymorphism in the matrix metalloproteinase-1 gene promoter is associated with the prognosis of patients with ovarian cancer. *Gynecol Oncol* 2006;100:506–10. [PubMed: 16214204]
18. Boire A, Covic L, Agarwal A, Jacques S, Sharifi S, Kuliopulos A. PAR1 is a Matrix Metalloprotease-1 Receptor that Promotes Invasion and Tumorigenesis of Breast Cancer Cells. *Cell* 2005;120:303–313. [PubMed: 15707890]
19. Arora P, Ricks TK, Trejo J. Protease-activated receptor signalling, endocytic sorting and dysregulation in cancer. *J Cell Sci* 2007;120:921–928. [PubMed: 17344429]
20. Even-Ram S, Uziely B, Cohen P, et al. Thrombin Receptor Overexpression in Malignant and Physiological Invasion Processes. *Nature Med* 1998;4:909–914. [PubMed: 9701242]
21. Grisaru-Granovsky S, Salah Z, Maoz M, Pruss D, Beller U, Bar-Shavit R. Differential expression of protease activated receptor 1 (Par1) and pY397FAK in benign and malignant human ovarian tissue samples. *Int J Cancer* 2005;113:372–378. [PubMed: 15455382]
22. Covic L, Gresser AL, Talavera J, Swift S, Kuliopulos A. Activation and inhibition of G protein-coupled receptors by cell-penetrating membrane-tethered peptides. *Proc Natl Acad Sci (USA)* 2002a; 99:643–648. [PubMed: 11805322]
23. Sanceau J, Truchet S, Bauvois B. Matrix Metalloproteinase-9 Silencing by RNA Interference Triggers the Migratory-adhesive Switch in Ewing's Sarcoma Cells. *J Biol Chem* 2003;278:36537–36546. [PubMed: 12847101]
24. Ueda J, Kajita M, Suenga N, Fujii K, Seiki M. Sequence-specific silencing of MT1-MMP expression suppresses tumor cell migration and invasion: importance of MT1-MMP as a therapeutic target for invasive tumors. *Oncogene* 2003;22:8716–8722. [PubMed: 14647466]
25. Kuliopulos A, Walsh CT. Production, Purification, and Cleavage of Tandem Repeats of Recombinant Peptides. *J Am Chem Soc* 1994;116:4599–4607.
26. Kaneider NC, Agarwal A, Leger AJ, Kuliopulos A. Reversing Systemic Inflammatory Response Syndrome with Chemokine Receptor Pepducins. *Nature Med* 2005;11:661–665. [PubMed: 15880119]
27. Kamath L, Meydani A, Foss F, Kuliopulos A. Signaling from Protease-activated Receptor-1 Inhibits Migration and Invasion of Breast Cancer Cells. *Cancer Res* 2001;61:5933–5940. [PubMed: 11479236]
28. Kuliopulos A, Covic L, Seeley SK, Sheridan PJ, Helin J, Costello CE. Plasmin Desensitization of the PAR1 Thrombin Receptor: Kinetics, Sites of Truncation, and Implications for Thrombolytic Therapy. *Biochemistry* 1999;38:4572–4585. [PubMed: 10194379]
29. Albini A, Benelli R. The chemoinvasion assay: a method to assess tumor and endothelial cell invasion and its modulation. *Nat Protocols* 2007;2:504–511.
30. Covic L, Misra M, Badar J, Singh C, Kuliopulos A. Pepducin-based intervention of thrombin receptor signaling and systemic platelet activation. *Nature Med* 2002b;8:1161–1165. [PubMed: 12357249]
31. Leger A, Jacques SL, Badar J, et al. Blocking the Protease-Activated Receptor 1–4 Heterodimer in Platelet-Mediated Thrombosis. *Circulation* 2006;113:1244–1254. [PubMed: 16505172]
32. Covic, L.; Tchernychev, B.; Jacques, S.; Kuliopulos, A. Pharmacology and in vivo efficacy of pepducins in hemostasis and arterial thrombosis. Langel, U., editor. Taylor & Francis; New York: 2006. p. 245-257.

33. Andrade-Gordon P, Maryanoff BE, Derian CK, et al. Design, synthesis, and biological characterization of a peptide-mimetic antagonist for a tethered-ligand receptor. *Proc Natl Acad Sci (USA)* 1999;96:12257–12262. [PubMed: 10535908]
34. Nguyen N, Kuliopulos A, Graham RA, Covic L. Tumor-Derived Cyr61(CCN1) Promotes Stromal Matrix Metalloprotease-1 Production and Protease-Activated Receptor 1-Dependent Migration of Breast Cancer Cells. *Cancer Res* 2006;66:2658–65. [PubMed: 16510585]
35. Davidson B, Goldberg I, Gotlieb WH, Ben-Baruch G, Nesland JM, Reich R. The prognostic value of metalloproteinase and angiogenic factors in ovarian cancer. *Mol Cell Endocrinol* 2002;187:39–45. [PubMed: 11988310]
36. Drew AF, Blick TJ, Lafleur MA, et al. Correlation of tumor- and stromal-derived MT1-MMP expression with progression of human ovarian tumors in SCID mice. *Gynecol Onc* 2004;95:437–448.
37. Cao J, Rehemtulla A, Bahou W, Zucker S. Membrane Type Matrix Metalloproteinase 1 Activates Pro-gelatinase A without Furin Cleavage of the N-terminal Domain. *J Biol Chem* 1996;271:30174–30180. [PubMed: 8939968]
38. Sternlicht MD, Werb Z. How Matrix Metalloproteinases Regulate Cell Behavior. *Annu Rev Cell Dev Biol* 2001;17:463–516. [PubMed: 11687497]
39. Wang P, Nie J, Pei D. The hemopexin domain of membrane-type matrix metalloproteinase-1 (MT1-MMP) Is not required for its activation of proMMP2 on cell surface but is essential for MT1-MMP-mediated invasion in three-dimensional type I collagen. *J Biol Chem* 2004;279:51148–55. [PubMed: 15381707]
40. Murphy G, Knauper V, Cowell S, et al. Evaluation of some newer matrix metalloproteinases. *Ann NY Acad Sci* 1999;878:25–39. [PubMed: 10415718]
41. Nagy JA, Masse EM, Herzberg KT, et al. Pathogenesis of Ascites Tumor Growth: Vascular Permeability Factor, Vascular Hyperpermeability, and Ascites Fluid Accumulation. *Cancer Res* 1995;55:360–368. [PubMed: 7812969]
42. Mesiano S, Ferrara N, Jaffe RB. Role of Vascular Endothelial Growth Factor in Ovarian Cancer. *Am J Pathol* 1998;153:1249–1256. [PubMed: 9777956]
43. Garcia JG, Davis HW, Patterson CE. Regulation of endothelial cell gap formation and barrier dysfunction: role of myosin light chain phosphorylation. *J Cell Physiol* 1995;163:510–22. [PubMed: 7775594]
44. Klarenbach SW, Chipiuk A, Nelson RC, Hollenberg MD, Murray AG. Differential actions of PAR2 and PAR1 in stimulating human endothelial cell exocytosis and permeability: the role of Rho-GTPases. *Circ Res* 2003;92:272–8. [PubMed: 12595338]
45. Kaneider NC, Leger AJ, Agarwal A, et al. Role reversal for the receptor PAR1 in sepsis-induced vascular damage. *Nature Imm* 2007;8:1303–1312.
46. Goerge T, Barg A, Schnaeker E-M, et al. Tumor-Derived Matrix Metalloprotease-1 Targets Endothelial Proteinase-Activated Receptor 1 Promoting Endothelial Cell Activation. *Cancer Res* 2006;66:7766–74. [PubMed: 16885380]
47. Suganuma T, Ino K, Shibata K, et al. Functional expression of the angiotensin II type 1 receptor in human ovarian carcinoma cells and its blockade therapy resulting in suppression of tumor invasion, angiogenesis, and peritoneal dissemination. *Clin Cancer Res* 2005;11:2686–94. [PubMed: 15814650]
48. Armstrong DK, Bundy B, Wenzel L, et al. Intraperitoneal cisplatin and paclitaxel in ovarian cancer. *N Engl J Med* 2006;354:34–43. [PubMed: 16394300]
49. Hotary K, Li X-Y, Allen E, Stevens SL, Weiss SJ. A cancer cell metalloprotease triad regulates the basement membrane transmigration program. *Genes Dev* 2006;20:2673–2686. [PubMed: 16983145]
50. Coussens LM, Fingleton B, Matrisian LM. Matrix Metalloproteinase Inhibitors and Cancer: Trials and Tribulations. *Science* 2002;295:2387–2392. [PubMed: 11923519]

Figure 1.

ProMMP-1 in patient fluids correlates with ovarian carcinoma cell migration. Various fluids were collected from 16 patients with benign or malignant disease. ProMMP-1, total MMP-2 (pro and active), total MMP-3 (pro and active), total MMP-8 (pro and active), total MMP-9 (pro and active), proMMP-13 levels were measured by ELISA from malignant ascites, n=4; malignant pleural effusion, n=4; benign ascites, n=4; benign ovarian cyst, n=3; malignant ovarian cyst, n=1. Primary peritoneal fibroblasts were cultured from ascitic taps obtained from patients with ovarian cancer. Conditioned media (CM) from 80% confluent peritoneal fibroblasts or OVCAR-4 ovarian carcinoma cells was collected after 2 d in RPMI. Data represent mean $\pm$ se. **Inserts**-The linear correlation of MMP levels (x-axis) versus migration

of OVCAR-4 cells (y-axis) toward the various fluids is shown for each MMP. OVCAR-4 cells (50,000) were allowed to migrate for 18 h toward each of the patient fluids or the CM from peritoneal fibroblasts or OVCAR-4 cells using a 8- μ m pore transwell apparatus. Statistical analyses were conducted by linear regression using the SAS System and the CORR procedure.

Figure 2. MMP1-PAR1-dependent migration of ovarian cancer cells toward malignant ascites fluid. **A**, surface PAR1 and PAR3 expression of the NCI-60 ovarian cancer cell lines ($n=6$) was measured by flow cytometry as before (27) and correlated with migration toward fibroblast-produced conditioned media (CM). Ovarian cancer cells (50,000 cells) were seeded onto a 8 μ m pore membrane in the upper well of a transwell apparatus and allowed to migrate for 5 h toward CM from NIH-3T3 fibroblasts. PAR1: $R=0.96$, $P=0.0006$; PAR3: $R=0.33$, $P=0.47$. **B**, silencing of PAR1 expression in OVCAR-4 and OVCAR-4ip1 cells with *PAR1* RNAi inhibits migration toward fibroblast CM. OVCAR-4 or OVCAR-4ip1 cells were transfected with *luciferase* (Luc), *PAR1* or *PAR3* RNAi for 48 h. Surface expression of PAR1 and PAR3

dropped by 45–77% as determined by flow cytometry, when cells were treated with their respective RNAi molecules (Supplementary Fig. 1A–B).

C, gene silencing of *PAR1* with RNAi blocks chemotaxis of OVCAR-4 cells toward MMP-1. OVCAR-4 cells were transfected with *luciferase* or *PAR1* RNAi for 48 h and then allowed to migrate toward 0.3 nM MMP-1 (APMA-activated) in RPMI/0.1% BSA for 18 h in a 48-well microchemotaxis chamber. Chemotaxis index is the ratio of directed over random migration through a 8- μ m pore filter.

D, effect of PAR1-based pepducins on migration of OVCAR-4, IGROV-1, and SKOV-3 ovarian carcinoma cells towards malignant ascites from ovarian cancer patients or fibroblast CM. Migration toward ascites or fibroblast CM was conducted in the presence of PAR1-antagonists based on either the extracellular ligand (3 μ M RWJ-56110) or cell-penetrating pepducins (3 μ M) derived from the PAR1 third intracellular loop (P1pal-12, P1pal-7 or control P1pal-19EE) in 0.2% DMSO vehicle. The source of the malignant ascites was from 2 different patients with ovarian adenocarcinoma.

E, migration (18 h) of OVCAR-4 cells toward fibroblast or malignant ovarian ascites in the presence of PMSF (100 μ M), hirudin (0.015 U), MMP-200 (200 nM), FN-439 (5 μ M) or vehicle.

F–G, low concentrations of MMP-1 or thrombin activate PAR1-dependent migration of OVCAR-4 cells. OVCAR-4 cells were allowed to migrate toward activated MMP-1 or thrombin (Thr) in RPMI/0.1% BSA for 18 h in the presence or absence of the PAR1 antagonist, 1 μ M RWJ-56110 or 100 μ M PMSF.

All data is expressed as mean $\pm$ 1 s.e. (n = 2–4) with assays done in duplicate, * P< 0.05, # P= 0.05–0.06 by the Student *t*-test.

Figure 3.

MMP-14 and MMP-9 activate proMMP1-dependent migration of ovarian cancer cells.

A, OVCAR-4 cells were placed in the top well of a 48-well microchemotaxis chamber and allowed to migrate for 18 h toward 20 nM of each proMMP in RPMI/0.1% BSA in the bottom well.

B, OVCAR-4 cells were allowed to migrate toward 20 nM proMMP-1 in RPMI/0.1% BSA in the presence of the indicated MMP inhibitors used at ≥ 3 -fold IC_{50} : 5 μ M FN-439, 5 μ M MMP-2 Inh, 5 μ M MMP-2/3 Inh, 5 nM MMP-9/13 Inh.

C, total RNA was extracted from OVCAR-4 cells, cDNA was prepared and amplified with PCR primers to MMP-1, MMP-2, MMP-3, MMP-8, MMP-9 or MMP-14, and separated on a 1.5% agarose gel.

D, OVCAR-4 ovarian cells were treated for 48 h with RNAi directed against *MMP-9*, *MMP-14* or *luciferase* (luci) and allowed to migrate for 18 h toward 20 nM proMMP-1, 0.3 nM active MMP-1 or a combination of 20 nM proMMP-1 and 20 nM proMMP-9 in RPMI/0.1% BSA.

E, OVCAR-4 cells were treated with RNAi against *luciferase*, *MMP-9* or *MMP-14* for 48 h, cells were washed, and then 20 nM proMMP-1 was added in RPMI/0.1% BSA on top of the cells. Conditioned media from the treated cells was then collected after 6 h and assayed for collagenase activity.

F, OVCAR-4 cell treated for 48 h with the indicated RNAi were allowed to migrate for 18 h toward malignant ovarian ascites in the absence or presence of 5 μ M FN-439.

All data is expressed as mean $\pm$ 1 s.e. (n = 2–4), * P < 0.05, ** P < 0.01 by the Student *t*-test.

Figure 4.

Effects of inhibiting MMP1-PAR1 on endothelial barrier function and ascites production in nude mice with peritoneal ovarian cancer.

A–B, immortalized HUVECs (EA.hy926) were grown to confluence on 3 μ m polycarbonate transwell membranes. Endothelium was then stimulated for 2 h with either (A) 0.5 nM MMP-1 or (B) peritoneal fibroblast CM harvested after overnight incubation with OVCAR-4 cells in the presence or absence of the MMP-1 antagonist FN-439 (5 μ M), the PAR1 antagonist RWJ-56110 (5 μ M), or P1pal-7 (3 μ M). Evans blue (30 mg/mL in DMEM) was then added to the upper well of the dual chamber system and leakage (15 min) into the bottom well relative to unstimulated control (vascular permeability) was measured by OD₆₅₀. * p <0.05, ** p <0.001, (n=3–5 in duplicate).

C, female NCR nu/nu mice were injected i.p. with either RPMI (left) or 1.5 million OVCAR-4 cells (right) in RPMI. After 40 d, mice injected with cancer cells showed abdominal tumor masses and pronounced ascitic fluid as indicated with arrows.

D, female NCR nu/nu mice were injected with 1.5 million OVCAR-4 cells and treated with either i.p. vehicle alone (20% DMSO A/D) or the PAR1 pepducin antagonist P1pal-7 (10 mg/kg, A/D) for 40 d and ascites fluid collected and volume determined.

Figure 5.

Effects of inhibiting MMP1-PAR1 on angiogenesis in mouse models of peritoneal ovarian cancer.

A–B, the PAR1 pepducin, P1pal-7, used either as monotherapy or in combination with docetaxel inhibits angiogenesis of peritoneal OVCAR-4 cancer in mice. Female NCR Nu/Nu mice were injected i.p. with 1.5 million OVCAR-4 cells and divided into treatment groups of vehicle (10% DMSO A/D, starting on day 2), P1pal-7 (3.2 mg/kg i.p. alternate day (A/D), starting on day 2), docetaxel alone (10 mg/kg) once per week starting at day 20, docetaxel (10 mg/kg) once per week starting at day 20 plus P1pal-7 (3.2 mg/kg i.p. alternate day (A/D), starting on day 2), docetaxel (10 mg/kg) once per week starting at day 20 plus FN-439 (5 mg/kg A/D, starting on day 2) (n=8–10). Intense staining of Weibel-Palade bodies within individual endothelial cells using a rabbit polyclonal anti-von Willebrand Factor was scored as a blood vessel in a blinded manner from paraffin embedded tissue sections from mice with peritoneal OVCAR-4 cancer. Representative sections and blood vessels (arrow heads) from tumor centers or tumor edge are shown. Vascular density was determined by counting 5–9 fields (160× magnification) from at least 5 tumors in each group.

C, P1pal-7 inhibits angiogenesis of peritoneal SKOV-3 cancer in mice. Female NCR nu/nu mice were injected with 1.5 million SKOV-3 cells and divided into the same 4 treatment regimens as above (n=5). After 40 days of treatment, the mice were sacrificed, peritoneal tissues collected for histopathology and assessment of vessel density as in (a). Mean blood vessel density per field is shown at the bottom.*P < 0.05, **P < 0.005.

Figure 6. Effect of a PAR1 pepducin in combination with docetaxel on invasion and metastasis in an *in vivo* peritoneal ovarian cancer model. **A**, histological staging system for peritoneal ovarian cancer in nude mice. **B**, the PAR1 pepducin, P1pal-7, or FN-439 MMP-1 inhibitor, used in combination with docetaxel inhibits invasion and metastasis of peritoneal ovarian cancer in mice. Six week treatment with vehicle, docetaxel alone, or docetaxel plus P1pal-7 or FN-439 was conducted as in Fig. 5A. Mice were killed and diaphragm, peritoneal, and thoracic organs were harvested, soaked in 10% formalin then sectioned and stained with hematoxylin & eosin for histopathological analyses. Staging was conducted by two independent pathologists who were blinded to the various treatment groups.