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Advances in Brief

Matrix Metalloproteinases (MMP9 and MMP2) Induce the Release of Vascular Endothelial Growth Factor (VEGF) by Ovarian Carcinoma Cells: Implications for Ascites Formation[1]

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Abstract

This study investigated the functional interplay between vascular endothelial growth factor (VEGF) and metalloproteinases (MMPs) in ovarian carcinomas. Levels of MMP9 (pro and activated form) and proMMP2 in ascites correlated with VEGF and with the ascitic volume in nude mice bearing human ovarian carcinoma xenografts (HOC22 and HOC8). The MMP inhibitor batimastat (BB-94) reduced VEGF release and ascitic fluid formation. Exogenous, activated MMP9, and, to a lesser extent, MMP2, increased VEGF release by SKOV3 and OVCAR3 ovarian carcinoma cells. The effect was dose and time dependent and inhibited by BB-94. MMP9-released VEGF was biologically active, because it induced endothelial cell motility, and its activity was prevented by the VEGF inhibitor SU5416. Our results indicate that MMPs, mainly MMP9, play a role in the release of biologically active VEGF and consequently in the formation of ascites.

Introduction

Ovarian cancer progression is associated with the accumulation of ascites in the peritoneal cavity. At least three different pathological events cause ascites: (*a*) reduced lymphatic drainage from the peritoneal cavity caused by the obstruction of lymphatic vessels by tumor cells; (*b*) hyperpermeability of microvessels lining the peritoneal cavity; and (*c*) angiogenesis (1). Various factors are involved. VEGF,[3] also known as vascular permeability factor that enhances vascular permeability and promotes new vessel growth, is thought to be one of the factors responsible for ascites formation and angiogenesis (2). As a result of alternative splicing of a single gene, VEGF exists as homodimers (M_r 42–34,000) of monomers containing 121, 145, 165, 189, and 206 amino acid residues ($VEGF_{121}$, $VEGF_{145}$, $VEGF_{165}$, $VEGF_{189}$, and $VEGF_{206}$) (3). Because the splicing involves exon 7 that codes for the heparin binding domain, the various isoforms of VEGF differ in their affinity for heparin and ECM components. $VEGF_{121}$ and $VEGF_{165}$ are diffusible proteins that are secreted into biological fluids, whereas $VEGF_{189}$ and $VEGF_{206}$ have high affinity for heparin and are bound to heparan sulfate proteoglycans in the ECM (4).

The expression of VEGF in ovarian carcinoma has been associated with tumor growth and invasion (5). High levels of VEGF have been found in serum or plasma and ascites of ovarian cancer patients (6). A correlation between ascites volume and VEGF levels has been reported in several experimental models (2). Inhibitors of VEGF activity reduced the formation of malignant ascites in human ovarian carcinoma xenograft models (7).

MMPs are zinc-dependent endopeptidases with a similar domain structure secreted in a latent (pro) form and activated by proteolytic removal of the NH_2-terminal propeptide. MMPs promote cancer progression by boosting cancer cell growth, migration and invasion, and metastasis and angiogenesis. During these processes, proteolytic enzymes act at different levels. They degrade basement membranes allowing cancer cell invasion and exposing cryptic sites within matrix molecules (8), increase the bioavailability of growth factors and cytokines (9), and regulate the function of bioactive molecules by proteolytic processing (10). The role of MMP2 and MMP9 has been particularly highlighted in the last few years using different *in vivo* and *in vitro* experimental models (11).

Ovarian cancer cells express MMP2 and MMP9 (12), and their increased expression is associated with their invasive and metastatic potential (13). We and others have reported that MMP2 and MMP9 are expressed in ascites and plasma of ovarian cancer patients (6). Furthermore, experimental studies have shown that animals bearing ovarian carcinoma xenografts in the peritoneal cavity and treated with MMP inhibitors formed fewer ascites and survived longer (14). A recent study showed that stromal MMP9 contributes to the malignant behavior of ovarian cancers by promoting neovessel sprouting and tumor growth (11). In the same study, VEGF was more expressed in ovarian tumors growing in nude mice with wild-type MMP9 than in mice that lacked an intact MMP9 gene (11). These findings suggest an interplay between VEGF and MMP9. However, the direct relationship between MMPs in general and specifically MMP9 activation and VEGF levels in the progression of ovarian carcinomas, particularly in ascites formation, still needs to be demonstrated.

We investigated a functional link between gelatinase activation, VEGF release, and ascites formation in human ovarian carcinoma models. Two xenografts of human ovarian carcinoma (HOC22 and HOC8) produced ascites after transplantation in the peritoneal cavity of nude mice. The ascites volume was directly proportional to the activation status of MMP9 and the VEGF levels in ascites. We also found that activated MMP9, and to a lesser extent MMP2, increased the release of soluble VEGF by the human ovarian carcinoma cell lines SKOV3 and OVCAR3 *in vitro*. Finally, the release of VEGF was inhibited by the metalloproteinase inhibitor BB-94 both *in vivo* and *in vitro*.

Materials and Methods

Animals. Female NCr-nu/nu mice were obtained from the animal production colony of the National Cancer Institute (National Cancer Institute-BTB-

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[3] The abbreviations used are: VEGF, vascular endothelial growth factor; HUVEC, human umbilical vein endothelial cell; MMP, matrix metalloproteinase; RT-PCR, reverse transcription-PCR; ECM, extracellular matrix.

Developmental Therapeutics Program), Frederick Cancer Research and Development Center (Frederick, MD). Mice were used at 6–8 weeks of age. They were housed in filtered-air laminar-flow cabinets and manipulated using aseptic procedures. Procedures involving animals and their care were conducted in conformity with the institutional guidelines that are in compliance with national (D. L. No.116, G. U., Suppl. 40, Feb. 18, 1992; Circolare No.8, G. U., July, 1994) and international laws and policies (EEC Council Directive 86/609, OJ L 358. 1, Dec. 12, 1987; Guide for the Care and Use of Laboratory Animals, United States National Research Council, 1996).

Cell Lines and Xenografts. HOC22 and HOC8 xenografts, established and maintained i.p. in nude mice as described previously (15), grew in the peritoneal cavity, producing ascites. As these xenografts do not grow in tissue culture, the human ovarian carcinoma cell lines SKOV3 and OVCAR3 were used for *in vitro* studies. Both the cell lines were obtained from the Division of Cancer Treatment, Tumor Repository (National Cancer Institute). Cells were cultured in RPMI 1640 (Life Technologies, Inc., Europe, Paisley, Scotland) supplemented with 10% FCS and 5 mM glutamine. HUVECs were isolated from umbilical cord veins and grown as described previously (16).

Reagents and Drugs. Batimastat (BB-94) was provided by British Biotech Ltd. (Oxford, England). For *in vivo* experiments, BB-94 was formulated in 2.5% ethanol, 2.5% PEG400, and 1% methylcellulose and further dissolved in 5% glucose. For *in vitro* experiments, it was dissolved in DMSO (stock solution 5×10^{-3} M) and diluted with medium before the assay.

SU5416 was provided by SUGEN (San Francisco, CA). The compound was dissolved in DMSO (stock solution 4×10^{-2} M) and diluted with medium before use. Human recombinant MMP2 and MMP9 were purchased from R&D Systems (Minneapolis, MN).

Preparation of Conditioned Medium. Tumor cells were seeded in duplicate in 24-well plates and growth in culture medium for 48 h. Subconfluent cultures were then washed three times with saline and incubated for different times in serum-free medium. Conditioned medium was collected, centrifuged, and kept frozen at −80°C until analysis for VEGF content.

i.p. Tumor Growth. Nude mice were inoculated i.p. with 10×10^6 HOC22 and HOC8 cells (day 0). At different times after injection, mice were euthanized by carbon dioxide inhalation. Ascites was harvested and centrifuged, and the volume of fluid and number of cells in the pellet (representative of tumor burden) were recorded for each mouse. Ascitic fluid was stored at −80°C until analysis. Where indicated, BB-94 at the concentration of 60 mg/kg and the corresponding vehicle were administered i.p. every 7 days for three times starting 4 days after tumor cell inoculation. Three days after the last treatment, ascites were harvested and processed as described above.

Analysis of VEGF in Ascites and Conditioned Medium. VEGF was measured by ELISA (Quantikine; R&D Systems), according to the manufacturer's instructions. This assay specifically recognizes human $VEGF_{121}$ and $VEGF_{165}$. The sensitivity of the assay was 9 pg/ml. Each sample was analyzed in duplicate. Western blotting was performed using a polyclonal antibody (Santa Cruz Biotechnology, Inc.) raised against a peptide mapping at the NH_2 terminus of VEGF of human origin and reacting with the 121, 165, and 189 amino acid splice variants of VEGF. Analysis of VEGF expression in tumor cells: 1 μg of total RNA, purified from ovarian tumor cells harvested from the peritoneal cavity of nude mice, was retro-transcribed in 20-μl reaction mixture using M-MuLV reverse transcriptase and random hexamer primers accordingly to manufacturer's instructions (RT-PCR core Kit; Applied Biosystems, Warrington, United Kingdom). Aliquots (2 μl) of RT-PCR products were subsequently used for PCR amplifications carried out in PTC-200 thermal cycler (MJ Research; Celbio, Milan, Italy). To amplify the different VEGF isoforms, primer sequences and amplification conditions were selected on data reported previously (17). As an internal reference, the housekeeping gene β-actin was amplified with the set of primers published previously (18).

Analysis of MMP Expression in Ascites. Zymography was done using SDS-polyacrylamide (10%) gels copolymerized with 1 mg/ml gelatin (Sigma Chemical Co.). The sample volumes were adjusted according to the protein concentration. Conditioned medium of WM983A melanoma cells and NIH3T3 fibroblasts activated or not with p-aminophenylmercuricacetate were used as reference standard, respectively, for human and murine MMP-9 and MMP-2. Western blotting was performed using monoclonal antibodies (Immunological Sciences, Rome, Italy) recognizing pro- and active forms of MMP9 and MMP2.

Cell Migration. Endothelial cell migration was evaluated using Boyden chambers and 8-μm pore size, gelatin-coated polycarbonate Nucleopore filters, as described (19). Conditioned medium of SKOV3 treated or not with MMP9 was used as the chemoattractant in the bottom compartment of the chamber. HUVEC 1×10^6/ml were added to the top compartment. SU5416 was incubated with the cells for 30 min before and throughout the assay. After a 4-h incubation at 37°C, filters were stained with Diff Quick (Merz-Dade, Dudingen, Switzerland), and the number of migrated cells in 10 high-power fields was counted.

Statistical Analysis. Statistical significance was determined by the two-tailed Mann-Whitney *U* test. The limit of statistical significance was $P \leq 0.05$.

Results

MMP9, MMP2, and VEGF in the Peritoneal Cavity of Nude Mice Transplanted with Human Ovarian Carcinomas Correlated with Tumor Burden and Ascites. The relationship between MMPs and VEGF expression during tumor progression was analyzed in the xenograft model (HOC22) of human ovarian carcinoma that produces ascites in the peritoneal cavity of nude mice. VEGF levels in ascites increased with time after tumor injection, showing a direct correlation with tumor burden and ascites volume (Fig. 1, *a* and *b*).

Cells harvested from the peritoneal cavity of nude mice at different times after tumor transplantation express the four VEGF isoforms of 121, 165, 189, and 206 amino acid residues (Fig. 1*c*). Accordingly, the different isoforms of VEGF were detectable in the ascitic fluid, and their expression increased with time of tumor progression (Fig. 1*d*). The amount of MMP2 and MMP9 released in ascites, measured in zymography, also correlated with tumor burden and ascites volume (Fig. 1*e*). Both human and murine proforms of MMP2 and MMP9 were detected in ascites of mice bearing HOC22. The activated form of MMP9 was detectable 24 and 34 days after tumor injection when VEGF levels were high, but the activated form of MMP2 was never detectable. MMP and VEGF levels and the ascites reached a plateau at the same time, 24 days after tumor transplant. Similar results were obtained with the other xenograft model HOC8 (data not shown).

BB-94 Inhibits the Release of VEGF in Ascites and the Formation of Ascitic Fluid in Ovarian Carcinoma Xenografts. We had shown previously that the treatment of nude mice bearing HOC22 or HOC8 with BB-94 delayed tumor growth and increased mice survival (14). Fig. 2 shows that treatment of HOC22-bearing mice with BB-94 reduced the ascites (median 5 and 3 ml in vehicle-treated and BB-94-treated mice, respectively) but not the tumor burden (median 44×10^6 and 46×10^6 cells; Fig. 2, *a* and *b*). Despite the similar tumor burden, VEGF levels were significantly lower in the ascites of BB-94-treated mice (median 1936.8 pg/ml) than in mice treated with vehicle (median 14036.7 pg/ml; Fig. 2*c*). Therefore, the decrease in VEGF levels and ascitic volume was not caused by a reduction in tumor burden but might conceivably have been caused by inhibition of protease activity.

Activated MMP9 Increases VEGF Release in SKOV3 Conditioned Medium. To further investigate the role of MMPs in regulating VEGF release in ovarian carcinoma, we used the human ovarian carcinoma cell line SKOV3 that *in vitro* produces VEGF. To investigate whether MMP2 and MMP9 might directly influence the levels of VEGF released in the conditioned medium, SKOV3 cells were exposed to the recombinant activated form of MMP9 and MMP2. At a concentration of 6.6 ng/ml, activated MMP9 increased VEGF release by 40–80% after 6 h and by 150–300% after 24 h (Fig. 3*a*). MMP2 had less effect on VEGF release, increasing it by 20–40% after 24 h (Fig. 3*a*). The effect of MMP9 treatment on VEGF release was dose dependent. (Fig. 3*b*).

To study whether the effect of MMPs on VEGF secretion required their proteolytic activity, SKOV3 cells were treated with BB-94. Noncytotoxic concentrations of BB-94 (10^{-6} M) (20) added to the

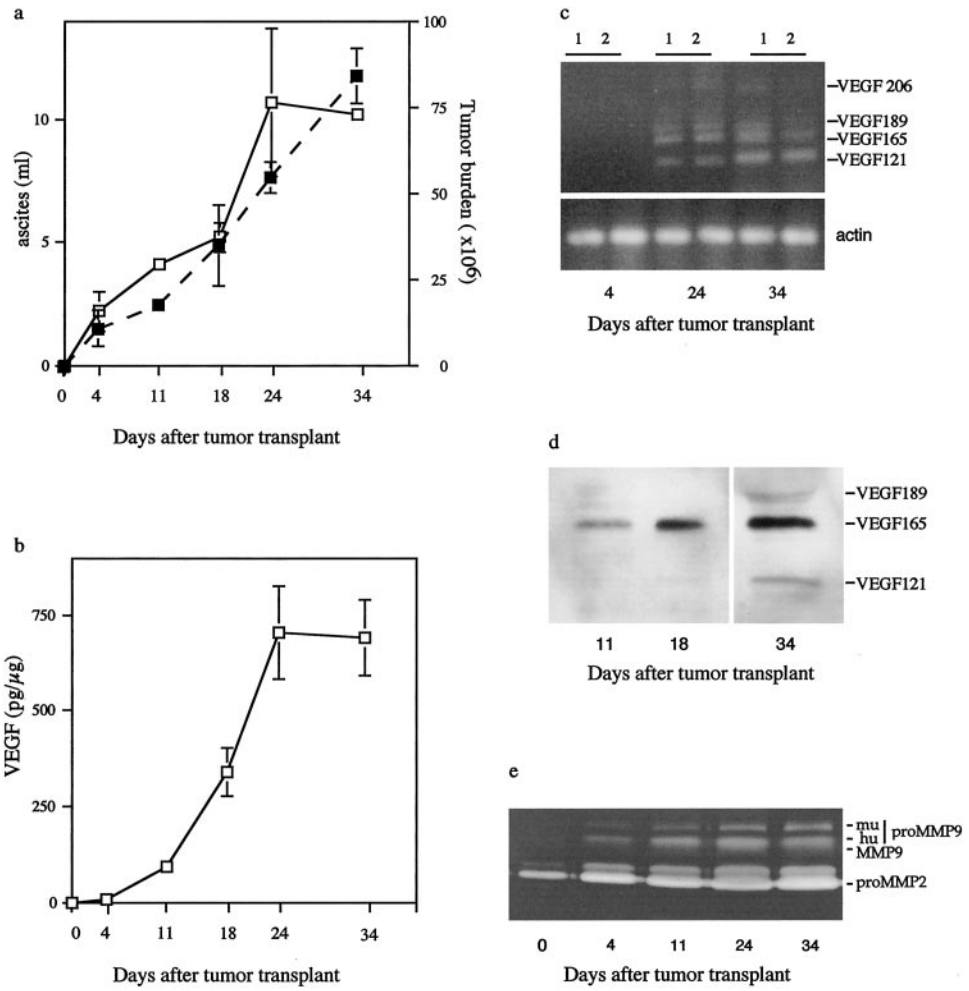

Fig. 1. VEGF, MMP9, and MMP2 released in ascites of ovarian carcinoma xenografts. Nude mice were transplanted i.p. with HOC22 xenograft. *a,* tumor burden, estimated as number of cells harvested from the peritoneal cavity (■), and volume of ascites (□), were evaluated at different times after tumor transplant. VEGF in ascitic fluids were evaluated at different times after tumor transplant by ELISA (*b*) and Western blot (*c*). Results in *a* and *b* are the mean ± SD of three mice per group. *d,* RT-PCR showing the expression of the different VEGF isoforms in cells harvested from the peritoneal cavity of nude mice at different times after tumor transplant (two mice/time). *e,* zymography showing a representative example of MMP expression and activation in ascites.

cells simultaneously with activated MMP9 prevented MMP9-induced release of VEGF, confirming that the proteolytic activity of MMP9 is indeed required. (Fig. 3*c*). BB-94 also blocked MMP2-stimulated release of VEGF by SKOV3 cells (data not shown).

Similar results were obtained with another ovarian carcinoma cell line, OVCAR-3, in which exogenous activated MMP9 and MMP2 increased the release of VEGF by 250 and 80% respectively. In this case too, the effect was dose and time dependent and inhibited by BB-94 (data not shown).

Conditioned Medium-induced Endothelial Cell Migration Is Increased by MMP9 Treatment. We next investigated whether the increased release of VEGF induced by MMP9 in ovarian carcinoma cells resulted in a true increment of the angiogenic potential of the tumor cells by testing the ability of SKOV3 conditioned medium to stimulate the migration of endothelial cells. Conditioned medium from SKOV3 treated with MMP9 induced greater endothelial cell migration than its untreated counterpart (Fig. 4*a*). SU5416, an inhibitor of VEGF-R2 (KDR/flk), was used to prove the specificity of the effect. SU5416 caused 70–90% inhibition of cell migration in response to MMP9-treated SKOV3 conditioned medium, proving that the effect was indeed mediated by VEGF (Fig. 4*b*). As expected, SU5416 selectively inhibited HUVEC motility induced by VEGF but not that induced by fibronectin. These findings indicate that VEGF released after MMP9 treatment was biologically active. Recombinant MMP9 used as chemoattractant in a range of concentration greater or equal to that contained in SKOV3 conditioned medium (between 10 and 1.25 ng/ml) did not stimulate HUVEC migration (data not shown), thus excluding a direct role of MMP in the induction of migration.

Discussion

There is evidence of a relationship between MMPs and VEGF in tumor progression. Here, we show that MMPs, mainly MMP9, induced the release of biologically active VEGF in the culture medium of ovarian tumor cells and in ascites of ovarian tumor-bearing mice. This was associated with ascites formation, providing direct evidence that MMPs, through the release of VEGF by ovarian tumor cells, contribute to the formation of ascites.

A functional interaction between VEGF and MMPs has been observed in other tumor models. Specifically, MMP9 increased the bioavailability of VEGF in the K14-HPV16 skin cancer model (21) and RIP1-Tag2 insulinoma model (9), although it is not known exactly how. In ovarian cancer, the MMP-VEGF relationship is indicated by several observations. Huang *et al.* (11), using *in vivo* models of ovarian carcinoma, found an important role for macrophage-derived MMP9 in angiogenesis and ovarian tumor growth; ovarian tumors from mice with a homozygous null mutation in the MMP9 gene had lower microvessel density, and tumor cells expressed lower levels of VEGF than tumors from mice wild type for the MMP9 gene. However, they did not describe direct functional connection between MMP9 activation, VEGF release, and ascites formation.

Our finding that activated MMP9 is associated with high VEGF bioavailability in cultured and transplanted ovarian carcinoma

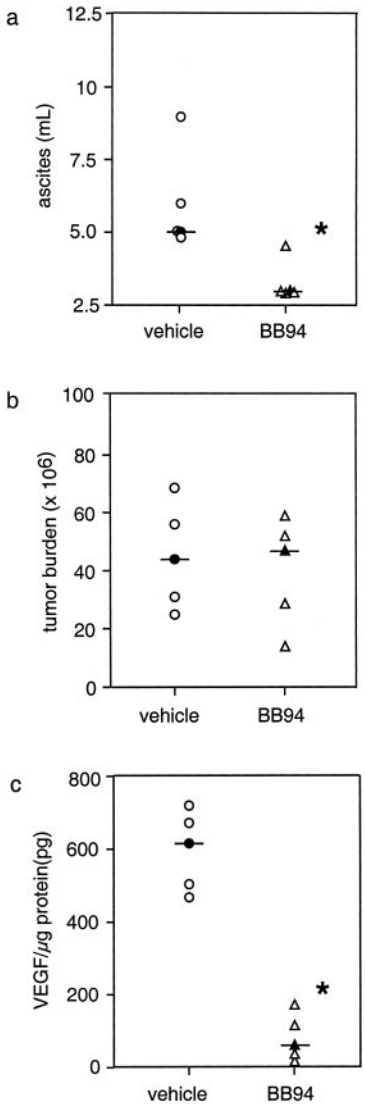

Fig. 2. Effect of BB-94 on VEGF release, ascites formation, and tumor burden in ovarian carcinoma xenografts. HOC22 was transplanted i.p. in nude mice, and treatment with BB-94 started 4 days later. Five mice per group were treated with BB-94 (60 mg/kg i.p.) and vehicle every 7 days for three times (days +4, +11, +18). On day +22, the animals were killed to record volume of ascitic fluid (*a*), tumor burden as number of cells in the ascites (*b*), and VEGF levels in ascites (*c*). Results are representative of two independent experiments. $*P < 0.05$ compared with vehicle (Mann-Whitney *U* test).

strongly supports a direct role of MMP in ascites formation. Activated MMP9 accumulated in the peritoneal cavity of mice 24 and 34 days after i.p. injection of HOC22 or HOC8 ovarian tumor cells, when large amounts of VEGF were present in ascites. Zymographic analysis enabled us to distinguish between human MMP9 secreted by the tumor cells xenografted in nude mice and murine MMP9 derived from host cells. In line with recent data showing that stromal MMP9 promotes angiogenesis and growth of ovarian cancer xenografted in nude mice (11), we detected high levels of host-derived, murine MMP9 in ascites, increasing with the ascites formation. However, as human MMP9 was also secreted in the ascitic fluid, it is possible that MMP9 from both origins influence VEGF release and ascites formation. The relative roles of host- and tumor-derived MMP9 need to be defined.

Only MMP2 proform was present in ascites, its activated form never being detectable, suggesting a secondary role for this MMP in VEGF release *in vivo*. This was confirmed *in vitro* where exogenous MMP2 was less effective in inducing VEGF release than MMP9. A fundamental role of MMP9 in VEGF release has been reported previously in the angiogenic switch of the RIP1-Tag2 insulinoma model (9).

SKOV3 cells constitutively release VEGF, MMP9, and MMP2 in culture medium in a time-dependent fashion (data not shown). These findings are in favor of a role for endogenous MMPs in the spontaneous release of VEGF.

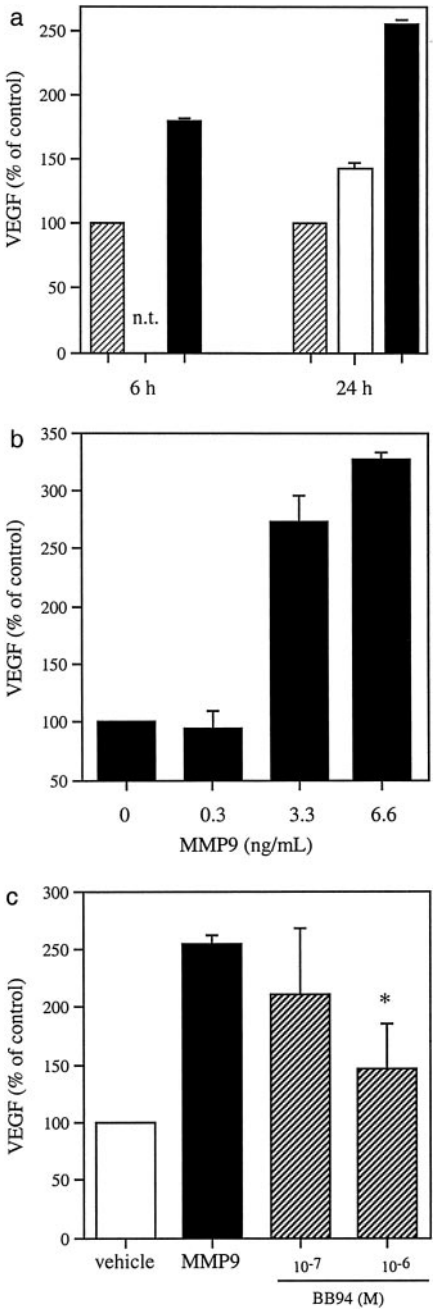

Fig. 3. MMP9 and MMP2 induce VEGF release from SKOV3 ovarian carcinoma cells in culture. *a,* VEGF levels in conditioned medium of SKOV3 cells were determined by ELISA in untreated cells (*striped columns*) or after 6 and 24 h of treatment with 6.6 ng/ml MMP9 (*black columns*) or MMP2 (*white columns*). VEGF in conditioned medium from untreated cells was 110 and 350 ng/ml at 6 and 24 h, respectively. *b,* effect of increasing doses of MMP9 on VEGF release in the conditioned medium of SKOV3 cells (24-h treatment). *c,* effect of BB-94 on MMP9-stimulated VEGF release from SKOV3 cells. BB-94 was added to the cells at the concentrations indicated together with exogenous activated MMP9 for 24 h. VEGF released in conditioned medium of cells treated with vehicle (*white column*), gelatinase alone (*black column*), or BB-94+gelatinase (*striped columns*). Results are the percentage of control (vehicle-treated cells) from one experiment, representative of at least two. $*P < 0.05$ compared with MMP9-treated cells.

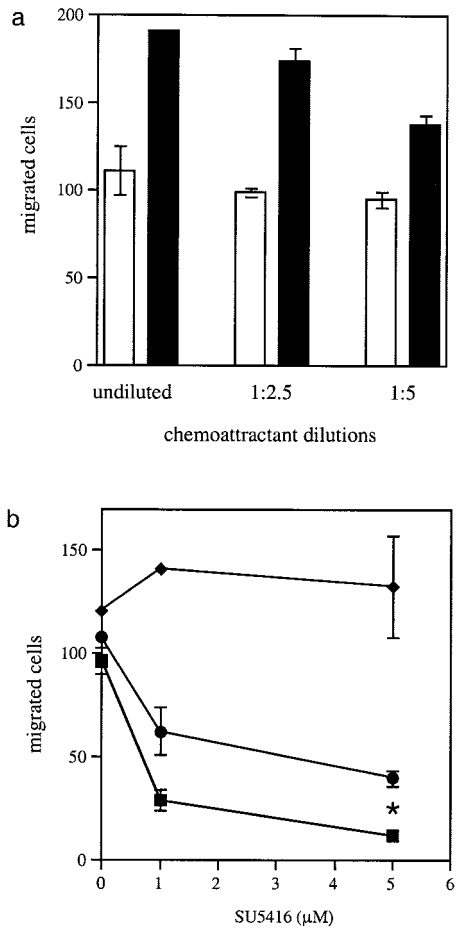

Fig. 4. Effect of MMP9-induced VEGF on the chemotactic potential of SKOV3 conditioned medium. *a,* conditioned medium from untreated (*white columns*) or activated MMP9-treated (*black columns*) SKOV3 cells was diluted as indicated and used as the attractant for HUVEC in a chemotaxis assay. Results are the number of cells migrated through the filter in 10 high-power fields. *b,* HUVECs were pretreated with different concentrations of SU5416 for 30 min before the chemotaxis assay. Conditioned medium of MMP9-treated SKOV3 cells (■), VEGF (5 ng/ml, ●), and fibronectin (20 μg/ml, ♦) were used as the attractants. Results are the number of migrated cells (means and SD of at least three values from one experiment representative of two). $*P < 0.05$ compared with control.

Zymography revealed a second band in the M_r 72,000 range whose expression increased with time after tumor transplantation. In Western blot analysis, this band was specifically recognized by an anti-MMP2 but not by an anti-MMP9 antibody (data not shown). Moreover: (*a*) it is a calcium-dependent protease, because the depletion of $CaCl_2$ in zymography completely inhibits the digestion band; and (*b*) it is probably of murine origin, because it is not expressed in ascites from ovarian carcinoma patients and in conditioned medium of human cancer cells *in vitro* but only in cells of murine origin (data not shown). Additional studies are necessary to characterize this molecule and its possible role in the release of growth factors.

VEGF is one of the most potent angiogenic factors. We found that conditioned medium from MMP9-treated tumor cells induced endothelial cell migration. Their migration was blocked by SU5416, a VEGF tyrosine kinase receptor inhibitor. This confirms the activity of VEGF released by MMP9. SU5416 is considered a potent selective inhibitor of the tyrosine kinase activity of VEGF receptor-2, but it inhibits the phosphorylation of other tyrosine kinase receptors too (22). Therefore, it is possible that besides VEGF, activated MMP9 causes the release of other angiogenic/growth factors whose activity is also inhibited by SU5416.

Different MMPs modify the bioavailability of angiogenic factors sequestered in the ECM (23) and process a variety of bioactive molecules, which are regulators of vascular growth or function, including fibroblast growth factor receptor type 1 (24), tumor necrosis factor (25), and heparin-binding epidermal growth factor (26). The role of gelatinases in VEGF release was confirmed by our finding that treatment of mice bearing ascites with the MMP inhibitor BB-94 lowered VEGF levels, with a reduction in ascitic volume. At the dose and schedule used in our studies, BB-94 did not affect tumor burden. We conclude that the decrease in VEGF release and, as a consequence, in ascites volume was exclusively caused by inhibition of gelatinolytic activity by BB-94. Accordingly, *in vitro,* BB-94 reduced the amount of VEGF released in SKOV3 conditioned medium at a concentration that did not affect tumor cell proliferation and survival, confirming that MMPs are indeed required. BB94 is a broad spectrum inhibitor of proteases, so we cannot exclude that other MMPs, beside MMP9, are involved in inducing VEGF release.

There are several possible mechanisms of action of MMPs: (*a*) directly stimulating VEGF release from tumor cells; (*b*) activating other factors involved in VEGF release; and (*c*) mobilizing VEGF from the extracellular compartments. The fact that also the larger isoforms of VEGF are detectable in ascites suggests an effect on the bioavailability of VEGF bound to the ECM. Additional studies are necessary to clarify this phenomenon.

In conclusion, our results provide direct evidence that MMPs, mainly MMP9, contribute to the formation of ascites through the release of VEGF. Targeting MMP9 in tumor cells or host-derived cells could offer a way to control tumor progression and ascites in human ovarian carcinoma.

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