ETS-1 Protein Regulates Vascular Endothelial Growth Factor-induced Matrix Metalloproteinase-9 and Matrix Metalloproteinase-13 Expression in Human Ovarian Carcinoma Cell Line SKOV-3

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Background: The mechanism of vascular endothelial growth factor (VEGF)-regulated expression of MMPs followed by cancer cell scattering/invasion is poorly understood.

Results: VEGF induces MMP-9, MMP-13, and ETS-1 through PI3K/AKT and p38 MAPK pathways in SKOV-3 cells.

Conclusion: VEGF induces ETS-1, which activates specific MMPs, leading to the invasion/scattering in SKOV-3 cells.

Significance: This study provides useful information that reveals the molecular mechanism of ovarian cancer metastasis.

Matrix metalloproteinase-mediated degradation of extracellular matrix is a crucial event for invasion and metastasis of malignant cells. The expressions of matrix metalloproteinases (MMPs) are regulated by different cytokines and growth factors. VEGF, a potent angiogenic cytokine, induces invasion of ovarian cancer cells through activation of MMPs. Here, we demonstrate that invasion and scattering in SKOV-3 cells were induced by VEGF through the activation of p38 MAPK and PI3K/AKT pathways. VEGF induced the expression of MMP-2, MMP-9, and MMP-13 and hence regulated the metastasis of SKOV-3 ovarian cancer cells, and the activities of these MMPs were reduced after inhibition of PI3K/AKT and p38 MAPK pathways. Interestingly, VEGF induced expression of ETS-1 factor, an important trans-regulator of different MMP genes. ETS-1 bound to both MMP-9 and MMP-13 promoters. Furthermore, VEGF acted through its receptor to perform the said functions. In addition, VEGF-induced MMP-9 and MMP-13 expression and in vitro cell invasion were significantly reduced after knockdown of ETS-1 gene. Again, VEGF-induced MMP-9 and MMP-13 promoter activities were down-regulated in ETS-1 siRNA-transfected cells. VEGF enriched ETS-1 in the nuclear fraction in a dose-dependent manner. VEGF-induced expression of ETS-1 and its nuclear localization were blocked by specific inhibitors of the PI3K and p38 MAPK pathways. Therefore, based on these observations, it is hypothesized that the activation of PI3K/AKT and p38 MAPK by VEGF results in ETS-1 gene expression, which activates MMP-9 and MMP-13, leading to the invasion and scattering of SKOV-3 cells. The study provides a mechanistic insight into the prometastatic functions of VEGF-induced expression of relevant MMPs.

Ovarian cancer is the second most common gynecological malignancy and the leading cause of death from gynecological cancer. Initial stages of ovarian cancer often remain clinically silent, and about 75% cases are diagnosed at an advanced stage with metastasis (1). Therefore, a detailed understanding of the mechanisms involved in invasion and metastasis of ovarian cancer might help develop new therapeutic strategy and thereby reduce mortality and morbidity caused by the disease. MMPs³ degrade various components of the extracellular matrix (ECM), including collagen, laminin, fibronectin, vitronectin, elastin, and proteoglycans. Thereby, MMPs play a critical role in invasion and migration of cancer cells and in metastasis and tumorigenesis. Among the five different types of MMPs, MMP-2 (gelatinase A) and MMP-9 (gelatinase B) are the major proteases that degrade ECM and basement membrane. Several experimental and clinical studies documented that ovarian tumor aggression significantly correlates with increased levels of MMP-2, MMP-9, and MMP-13 (2–8).

Different environmental influences from the surrounding stroma, ECM proteins, systemic hormones, and other factors regulate the activities of MMP (3, 9). VEGF, a potent angiogenic cytokine, is one of them. VEGF expression strongly correlates with that of MMPs (11). Different in vitro studies have demonstrated that VEGF increases expression as well as activities of different MMPs in ovarian carcinoma cell lines (12, 13). Studies have demonstrated that some ovarian carcinoma cells express both VEGF and VEGF receptors (14). The VEGF members show multiple interactions with receptor tyrosine kinases, namely VEGFR1 (Flt-1), VEGFR2 (Flk1), and VEGFR3 (Flt4, 15–19). However, VEGF-A, the major form of VEGF, binds to and signals through VEGFR2 and helps in the maintenance of...
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the vascular network (20) and hence is essential for cellular function. Recent studies have shown a distinct function of VEGF/VEGFR2 in mediating major growth and permeability in cancer cells (21). Inhibition of VEGF by its neutralizing antibodies was found to reduce in vitro ovarian cell proliferation as well as migration (22, 23). Again, studies have shown that VEGF and MMP regulate each other during tumor progression (24, 25); however, the precise mechanism underlying the VEGF-MMP cross-talk remains largely unknown.

Several protein kinases play an important role in different essential cellular processes, including cell migration and metastasis (26, 27). The mitogen-activated protein kinases are serine/threonine-specific protein kinases that respond to extracellular stimuli and regulate various cellular activities, such as gene expression, mitosis, differentiation, proliferation, and cell survival/apoptosis (28). Among different MAPK pathways, the p38 MAPK pathway has been reported to be associated with cell migration or metastasis (29) and apoptosis (30). PI3K signaling also plays a crucial role in cytoskeletal rearrangement and subsequent cell motility by different growth factors (31, 32). Several studies have indicated the important function of the PI3K/AKT signal cascade in tissue invasion/metastasis. The role of the ERK/MEK1 pathway in cancer cell invasion and metastasis is also well evidenced (33, 34). Although the involvement and importance of different signaling pathways in cancer cell invasion have been established, the precise molecular mechanism by which VEGF affects these pathways and thereby promotes invasive ovarian carcinomas remains elusive. Considering this background information, we investigated the signal transduction pathways that are activated during VEGF-regulated invasion of SKOV-3 epithelial ovarian carcinoma cells. The cyto- kine-induced secretory activities of MMPs in SKOV-3 cells have been reported earlier (35). It has been reported previously that VEGF activates MMP-2 in SKOV-3 cells (36). However, the detailed mechanism of the activation is still not clearly known. Moreover, information about the activation of other MMPs by VEGF in this cell line is lacking.

ETS transcription factors are helix-turn-helix proteins with a highly conserved ETS domain that binds to the core consensus sequence GGA(A/T). Many MMP genes are reported to have an ETS binding sequence (EBS) in their promoter region. Here, we wanted to investigate whether ETS binds to specific MMPs. Again, signaling pathways, such as the mitogen-activated protein kinases ERK1/2, p38 MAPK, and JNK; the phosphatidylinositol 3-kinases; and Ca2+ -specific signals, activated by growth factors or cellular stresses converge on the ETS family of factors, controlling their activity and downstream signaling (37). The ETS family of transcription factors consists of over 200 target genes and is involved in several biological processes, such as angiogenesis, development, and apoptosis (38 – 40). Among the different types in the class, ETS-1, ETS-2, and PEA3 are reported to have prometastatic functions. Therefore, we aimed to study these factors in VEGF-mediated MMP gene regulation. ETS-1 has important role in angiogenesis (41), and its expression varies positively with metastatic potential of different types of cancers, viz. prostate (42), gall bladder (43), breast (44), lung (45), and esophageal cancers (46). A few reports describe the importance of ETS-2 (47) and PEA3 in invasive cancer (48). ETS is co-expressed with VEGF in different types of cancers and was found to be involved in the transcriptional regulation of VEGF receptors (49). However, regarding the involvement of PI3K and p38 MAPK of the ERK signaling pathways in VEGF-mediated MMP expression, a clear picture is unavailable.

Although several cytokines elicit MMP expression in ovarian carcinoma, VEGF is particularly important because it plays major role in ovarian cancer (50). However, the transcriptional regulation of MMP gene upon VEGF stimulation has not been studied yet. Our study provides evidence that MMP-2, MMP-9, and MMP-13 are overexpressed in VEGF-activated SKOV-3 cells. Using inhibitors of different signaling pathways, we concluded that PI3K and p38 MAPK might be involved in VEGF-induced ETS-1 and MMP gene regulation. These observations show how the activation of ETS-1 is caused by PI3K/p38 MAPK signaling. Finally, we report here that the ETS-1 transcription factor contributes to the VEGF-mediated regulation of expression and function of different MMPs in SKOV-3 cells.

MATERIALS AND METHODS

Cell Culture and Treatment—The human ascitic ovarian adenocarcinoma cell line SKOV-3 was obtained from the National Centre for Cell Science, Pune, India. The PA-1 human ovarian ascitic teratocarcinoma cells were purchased from the American Type culture Collection (ATCC), and the OAW-42 human ovarian serous papillary cystadenocarcinoma cells were purchased from Sigma-Aldrich. SKOV-3 cells were cultured at 37 °C and in a 5% CO2 atmosphere in McCoy’s 5A medium (Sigma) with 10% FBS, 100 units/ml penicillin, and 100 μg/ml streptomycin. The PA-1 and OAW-42 cells were grown in minimum Eagle’s medium and DMEM, respectively, and the rest of the culture conditions were the same as those for SKOV-3 cells. The human recombinant VEGF-A (hereafter referred to as VEGF) was purchased from R&D Systems, Minneapolis, MN and used at a concentration of 20 ng/ml unless otherwise specified. To study the effect of VEGF receptor tyrosine kinase inhibitor-II (Calbiochem), the cells were pretreated with a 20 nm concentration of this inhibitor for 30 min followed by measurement of VEGF-induced cell invasion, scattering, and gene expression.

Analysis of Matrigel Invasion and Cell Scattering—In vitro cell migration was studied using a Matrigel invasion chamber (BD Biosciences) following the manufacturer’s guidelines. In brief, 2.5 × 105 cells were seeded in the inserts of the Matrigel invasion chamber and treated with VEGF. The invasion chamber was incubated at 37 °C in a 5% CO2 atmosphere for 22 h, and a Matrigel invasion assay was performed.

The cell scattering assay was performed as described previously (51). Briefly, 10,000 cells/well were plated in 6-well plates. After 3 days of incubation, small, cohesive, and discrete colonies were formed. The cells were treated with different inhibitors (Calbiochem), viz. 25 μM LY294002 (LY), 50 μM PD98059 (PD), or 10 μM SB203580 (SB) for 30 min (32), and incubated with 20 ng/ml VEGF (R&D Systems) for 24 h. The dose of VEGF was selected as described before (24). The cells were washed with PBS, fixed with methanol, and stained with crystal violet (Sigma). Scattered colonies were judged by the typical change in
morphology characterized by cell-cell dissociation and the acquisition of a migratory, fibroblast-like phenotype. Scattering activity was measured by counting the total number of scattered colonies formed from 50 colonies under a light microscope.

Western Blot Analysis—The SKOV-3 cells were treated with 20 ng/ml VEGF for 24 h in serum-free medium. The cells were then harvested, lysed in lysis buffer supplemented with protease inhibitors (1 μg/ml each of aprotnin, pepstatin, leupeptin, and trypsin inhibitor and 1 mM phenylmethylsulfonyl fluoride) and 1% Triton X-100 (all from Sigma-Aldrich) as described earlier (52). The homogenate was then centrifuged at 8000 × g for 10 min at 4 °C. The supernatant was collected (an aliquot was used for protein concentration estimation), resolved by SDS-PAGE, and transferred to PVDF membrane (Millipore Corp., Bedford, MA). The membranes were incubated overnight at first with 5% blocking solution (TBS containing 0.1% Tween 20 and 5% nonfat dried milk) and then with antibodies for MMP-2, p38 MAPK, phospho-p38 MAPK, AKT1, phospho-AKT1 (all from Santa Cruz Biotechnology, Santa Cruz, CA), MMP-9, MMP-13, ETS-1, ETS-2, PEA3, and GAPDH (all from Cell Signaling Technology Inc., Beverly, MA) at a dilution of 1:1000. The immunocomplex was detected with alkaline phosphatase-conjugated IgG as the secondary antibody (1:2000 dilution; Santa Cruz Biotechnology) using nitro blue tetrazolium/5-bromo-4-chloro-3-indolyl phosphate substrate.

RNA Isolation, cDNA Preparation, RT-PCR, and Quantitative Real Time PCR (Q-PCR)—Total RNAs were isolated from cultured cells using TRI Reagent (Sigma-Aldrich) following the standard protocol. The cDNA was synthesized using SuperScript III reverse transcriptase (Invitrogen) following the manufacturer’s instructions. The PCR was performed with initial denaturation at 94 °C for 5 min followed by 35 cycles of denaturation (at 94 °C for 30 s), annealing (at a specific temperature for each set of primers mentioned in Table 1 for 45 s), and extension at 72 °C for 30 s). Final extension was carried out for 10 min at 72 °C in the last cycle (PerkinElmer Life Sciences 9700 thermal cycler).

Q-PCR was performed on a 7500 real time PCR system (Applied Biosystems) using Power SYBR Green (Applied Biosystems). In brief, 1 μg of RNA was reverse transcribed as described above followed by Q-PCR. The conditions were as follows: initial denaturation step (95 °C for 5 min) and cycling step (denaturation at 94 °C for 15 s, annealing at a specific temperature for each set of primers for 30 s, and extension at 72 °C for 30 s repeated for 35 cycles) followed by melting curve analysis (45–90 °C). The internal control (GAPDH) was amplified in a separate tube. The CT value was calculated as described previously (53). The oligonucleotide primer sequences, gene accession numbers, amplicon sizes, and annealing temperatures of different genes that were used for PCR analyses are listed in Table 1. RT-PCR and Q-PCR were performed three times, and the mean ± S.D. value are shown.

MMP-9 Activity Assay by Gelatin Zymography—2 × 10^5 cells/well were seeded in a 24-well tissue culture plate (Nunc). After 24 h of incubation in regular culture medium, the cells were washed, refed with serum-free medium, and treated as described under “Analysis of Matrigel Invasion and Cell Scattering.” After 24 h of treatment, culture supernatants were collected, centrifuged at 4000 × g for 10 min to remove debris, stored at −20 °C, and then concentrated 10 times (54). The sample was then mixed with SDS sample buffer in the absence of any reducing agent, incubated at 37 °C for 30 min, and electrophoresed on 10% SDS-polyacrylamide gels co-polymerized with 0.2% gelatin at 4 °C. After electrophoresis, gels were washed twice in 2.5% Triton X-100 and incubated at 37 °C for 17 h in 0.15 M NaCl, 10 mM CaCl2, and 50 mM Tris–HCl buffer (pH 7.5). Gels were stained with 0.1% Coomassie Blue R-250 and destained in 5% isopropanol and 8% acetic acid in H2O. Activity of the gelatinolytic enzyme MMP-9 was detected as transparent bands on the blue background of the Coomassie Blue-stained gel.

MMP-2 and MMP-13 Activity Assay—MMP-2 and MMP-13 concentrations in the control and treated SKOV-3 cells were measured using the commercially available MMP-2 and MMP-13 ELISA kits (Calbiochem) following the manufacturer’s guidelines. For ELISA, 2 × 10^5 cells were cultured in a 6-well cell culture plate and then treated for 24 h with different inhibitors and VEGF as described above. The cell-free spent media were collected and subjected to ELISA. Samples were assayed in triplicate and calibrated against a standard curve.

Immunofluorescence Microscopy—Immunofluorescence microscopy was performed as described before (55). SKOV-3 cells were cultured on poly-l-lysine-coated coverslips placed on the wells of a 6-well plate and treated for 24 h as described for the invasion and scattering experiment. The medium was removed, and cells were fixed with PBS containing 4% paraformaldehyde. The cells were permeabilized with 0.1% Triton X-100 in PBS and incubated with anti-goat polyclonal ETS-1, ETS-2, and PEA3 antibodies (Santa Cruz Biotechnology; dilution, 1:100) for 3 h and then with corresponding secondary antibodies conjugated with FITC (donkey anti-goat; dilution, 1:50; Santa Cruz Biotechnology).
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Biotechnology) for 1 h. Rigorous washing was performed with PBS after the incubations. The cells were stained with 1 μg/ml DAPI/well. The stained cells were observed under a confocal microscope (Leica DMIRB and TCS SP2, Germany).

Electrophoretic Mobility Shift Assay—EMSA was performed to examine ETS-1 binding activity to human MMP-9 and MMP-13 promoter elements in SKOV-3 cells according to the published method (56). In brief, cells were harvested, washed with PBS, resuspended in sucrose buffer, and mixed well by pipetting. Then nuclei were spun down by centrifugation at 500 × g for 5 min. The wash buffer was aspirated, and the cells were resuspended in 30 μl of low salt buffer and mixed well. Then 30 μl of high salt buffer was added, and the sample was incubated at 4 °C for 45 min with shaking. The supernatant was collected, and the protein content was quantified and stored at −80 °C until use. Complementary oligonucleotides of human MMP-9 promoter ( promoter and 3′-ACTCGTTCCCTCCCTGTC-3′ where EBS is underlined) and those of human MMP-13 promoter ( and 3′-TTAAGGT-GTCCATGTATCAT-3′ where EBS is underlined) were used. Mutated EBSs ( and 3′-TTAAGGT-GTCCATGTATCAT-3′ and 3′-TTAAGGT-GTCCATGTATCAT-3′ where EBSs were mutated) were used as a negative control.

The sense and antisense oligos were end-labeled with [γ-32P]ATP (Bhaba Atomic Research Centre, Mumbai, India) using T4 polynucleotide kinase (New England Biolabs, Beverly, MA). The labeled oligonucleotides were purified using the QIAquick Nucleotide Removal kit (Qiagen, Valencia, CA) according to the manufacturer’s guidelines. For labeling, 10 μg of nuclear extract and 32P-labeled (3000 Ci/mmole) double strand oligomers (50 fmol) were incubated in binding buffer (20% glycerol, 20 mM HEPES, 100 mM KCl, 20 mM Tris-HCl, 1 mM EDTA, and 1 mM DTT) with or without unlabeled competitors for 30 min at room temperature. To check antibody-mediated supershift experiments, 10 μg of the ETS-1 antibody (Santa Cruz Biotechnology) was added to the lysate, and the mixture was incubated on ice for 1 h before addition of the probe. To verify specific ETS-1 activity, competitive EMSA was performed with a 100-fold excess of unlabeled oligonucleotide in the binding reaction, and the reaction proceeded for 20 min before addition of 32P-end-labeled oligonucleotide. After a 30-min prerun of the gel, samples were loaded onto 5% native polyacrylamide gels in 0.5× Tris borate-EDTA buffer and run at 120 V for 3 h at 4 °C. The gel was then dried and exposed to a phosphorimaging analyzer (Typhoon Trio, GE Healthcare). To study the effect of VEGF on ETS-1 and MMP-9/MMP-13 promoter binding, SKOV-3 cells were treated with 0, 20, and 50 ng/ml VEGF for 4 h, and nuclear extracts were prepared followed by EMSA.

siRNA Transfection followed by Q-PCR and Invasion Study—Subconfluent SKOV-3 cells were transfected in a 10-cm dish with either 20 nm ETS-1 siRNA (catalog number 4392422; siRNA ID s4849) or nonspecific control siRNA (catalog number AM4611) purchased from Ambion, Austin, TX. The delivery of siRNA to SKOV-3 cells was achieved following Ambion’s general transfection protocol. After 24 h of transfection, the cells were stimulated with or without VEGF (20 ng/ml) and then harvested followed by Q-PCR to detect the expression of MMP-2, MMP-9, MMP-13, and GAPDH genes. The cells were treated similarly, and a Matrigel invasion assay was performed.

Luciferase Assay—Cells were plated in a 24-well plate on the day before transfection at a density of ～0.5 × 105/well in 500 μl of complete medium. The MMP-9 and MMP-13 promoters cloned in pGL3 vector were a kind gift from Dr. Ernst Lengyel, University of Chicago (57, 58) and Dr. Hee-Jeong Im Sampen, University of Illinois at Chicago, respectively. On the day of transfection, 200 ng of plasmid DNA was diluted with 100 μl of serum-free medium, mixed with 100 μl of serum-free medium containing 1 μl of Lipofectamine 2000 (Invitrogen), and incubated at room temperature for 30 min. To understand the effect of ETS-1, cells were co-transfected with 5 pmol of ETS-1 siRNA (Ambion) as described before (59). Then the DNA-lipid complex was added to the wells and incubated at 37 °C in a CO2 incubator for 24 h. The cells were then washed with fresh serum-free medium, and VEGF was added at a concentration of 20 ng/ml in the respective wells. The cells were then harvested, and a luciferase assay was performed using the Dual-Luciferase Assay System (Promega, Madison, WI). The relative luciferase activity was calculated by normalization against the co-transfected internal control vector pRL-null.

The results shown are the mean ± S.E. of three independent transfection experiments.

Statistical Analysis—All data were expressed as mean ± S.D., and statistical analyses were performed by Student’s t test. A p value <0.05 was considered to be significant. The experiments were repeated at least three times unless otherwise stated. To make the variance independent of the mean, statistical analysis of quantitative RT-PCR data is presented after logarithmic transformation.

RESULTS

VEGF Induces Invasion and Scattering of SKOV-3 Cells—Matrigel invasion and scattering assays were performed to demonstrate the invasion and scattering of SKOV-3 cells upon VEGF treatment. VEGF (20 ng/ml for 24 h) increased the cellular invasion significantly compared with that in untreated cells (Fig. 1a). The scattering response is a prerequisite for cell invasion through basement membrane, and it was observed that cell scattering was significantly induced upon VEGF treatment (Fig. 1b). Next we checked whether the VEGF receptor is expressed in SKOV-3 cells and whether VEGF acts through its receptor to induce invasion and scattering. The copy number of VEGFR2 in these cells was found to be high (data not shown). The cells were then induced by VEGF alone or in combination with VEGF receptor inhibitor (RI). The results suggest that the VEGF-induced cellular invasiveness (Fig. 1a) and scattering (Fig. 1b) were significantly reduced when the cells were pre-treated with the said inhibitor.

VEGF Induces Cell Invasion and Scattering through p38 MAPK and PI3K/AKT Pathways—To further investigate the involvement of various signaling pathways, such as PI3K/AKT, ERK, and p38 MAPK, in cell invasion and motility, the
SKOV-3 cells were pretreated with different concentrations of inhibitors specific for these pathways as described under “Materials and Methods.” Inhibitor treatment was for 30 min followed by VEGF treatment for 24 h, and then the invasion and scattering experiments were performed. The results showed that inhibition of PI3K/AKT and p38 MAPK pathways by LY294002 and SB203580, respectively, reduced VEGF-induced cell invasion (Fig. 2a) in vitro, and particu-
larly the p38 MAPK inhibition was more effective here (Fig. 2b). However, inhibition of MEK1 (by PD98059) could not reduce VEGF-induced cell invasion (Fig. 2b). Similarly, VEGF-induced cell scattering was also clearly reduced by treatment with SB203580 and LY294002 but not with PD98059 (Fig. 2, c and d).
We further investigated the time-dependent activation of the AKT and p38 MAPK pathways by VEGF. To achieve this, we detected the phosphorylated form of AKT and p38 MAPK by Western immunoblot using phosphospecific antibodies in the cellular proteins after treatment with VEGF for 15, 30, 60, 120, and 180 min. Along with their phosphorylated forms, the expression of total AKT1 and p38 MAPK was also determined. As shown in Fig. 3, a and b. The phosphorylated forms of AKT1 and p38 MAPK were maximal upon VEGF stimulation for 60 and 120 min, respectively, and after that, the activation was reduced gradually.

**VEGF Induces MMP-2, MMP-9, and MMP-13 Genes in SKOV-3 Cells in Correlation with Invasion and Scattering**—As MMP expression correlates with the invasive behavior in advanced ovarian cancers, we examined the expression profile of specific and relevant MMPs after VEGF treatment. Accordingly, we checked the expression status of MMP-2, MMP-9, and MMP-13 upon VEGF stimulation, and the data suggest that the expression levels of these genes were significantly increased upon VEGF treatment (Fig. 4a) in SKOV-3 cells compared with untreated cells. Here the cells were treated with 20 ng/ml VEGF for 24 h. The Western blot data showed moderate and significantly high induction of pro- and active MMP-2 levels, respectively, upon VEGF treatment compared with control (Fig. 4b, panel i). Similarly, the pro-MMP-9 fraction was also induced by VEGF treatment but not to the extent of active MMP-9 (Fig. 4b, panel ii). However, the MMP-13 level was increased significantly upon VEGF treatment (Fig. 4b, panel iii). In this experiment, the GAPDH expression level was used as an internal control (Fig. 4b, panel iv).

**VEGF-induced MMP-2/MMP-9/MMP-13 Activities Are Regulated by PI3K/AKT and p38 MAPK Signaling Pathways**—To determine the signaling pathways involved in VEGF-induced MMP-2/MMP-9/MMP-13 activities, SKOV-3 cells were pretreated with a specific concentration of inhibitors for 30 min prior to VEGF treatment for 24 h. The result of gelatin zymography (Fig. 5a) showed that VEGF-induced MMP-9 activity was decreased upon treatment with PI3K pathway inhibitor (LY) and p38 MAPK inhibitor (PD). The ELISA data (Fig. 5b and c) showed a significant decrease of VEGF-induced MMP-2 and MMP-13 activities in the presence of either of the inhibitors of
the PI3K and p38 MAPK pathways. However, blockade of the ERK pathway by SB203580 had no effect on the activities of these MMPs.

VEGF Induces ETS-1 Expression—The promoters of different MMP genes consist of the EBS. We have already shown that VEGF regulates expression of MMP genes associated with invasion and scattering. To know the exact mechanism, we investigated whether the expression of major tumor-promoting ETS factors, viz. ETS-1, ETS-2, and PEA3, is regulated by VEGF in SKOV-3 cells. To do so, the cells were treated with VEGF (20 ng/ml for 24 h), and total RNAs or proteins were isolated for Q-PCR and Western immunoblot assay. The Q-PCR data (Fig. 6a) show that the ETS-1 gene expression was significantly increased after VEGF treatment. Here PEA3 gene expression was also increased; however, the expression of ETS-2 remained unchanged. The Western immunoblot data (Fig. 6b) also show the significant increase of the ETS-1 protein in VEGF-treated cells (Fig. 6b, panel i). Here two isoforms of ETS-1 protein were observed that showed differential expression in both control and VEGF-treated cells. The PEA3 expression was also significantly increased (Fig. 6b, panel iii), but ETS-2 expression remained unchanged (Fig. 6b, panel ii).

Induced Expression of MMP-9, MMP-13, and ETS-1 Genes by VEGF Is Mediated by Its Receptor in SKOV-3 Cells—Next we checked whether VEGF acts through its receptor to induce the expression of relevant genes involved in cellular invasion and scattering. Hence, the cells were induced by VEGF in the presence or absence of RI. The VEGF-induced expression of MMP-9, MMP-13, and ETS-1 genes was inhibited in the presence of RI (Fig. 7). This suggests that VEGF acts through its receptor (VEGFR2 or Flk1) to induce ETS-1, MMP-9, and MMP-13 genes, which may lead to the induced invasiveness and scattering in SKOV-3 cells.

ETS-1 Regulates VEGF-induced MMP-9 and MMP-13 Expression—To confirm the binding of ETS-1 to the promoters of MMP-9 and MMP-13 genes in SKOV-3 cells, EMSA was performed by incubating nuclear extracts with double-stranded oligonucleotides of those gene promoters that include EBS. The protein and these oligonucleotides formed a complex, and its mobility was shifted (supplemental Fig. 1a). Furthermore, addition of anti-ETS-1 antibody decreased the mobility of the protein-oligonucleotide complex. Addition of a 100 times molar excess of unlabeled specific competitor abolished the binding of ETS-1 to labeled MMP-9 promoter, whereas a 100 times molar excess of mutant competitor oligonucleotide also reduced the DNA binding activity (supplemental Fig. 1a). Similar results were obtained with oligonucleotide primers designed on the basis of MMP-13 promoter (supplemental Fig. 1b). Therefore, it was concluded that ETS-1 is able to interact with MMP-9 and MMP-13 promoters in SKOV-3 cells.

To confirm the above observation, we examined whether ETS-1 knockdown prevents VEGF-induced MMP-9 and
MMP-13 expression. The ETS-1 siRNA was found to be most effective at a 20 nM concentration (data not shown). Addition of 20 ng/ml VEGF for 24 h significantly increased MMP-9 (Fig. 8a) and MMP-13 (Fig. 8b) expression compared with nonspecific siRNA-treated control cells, whereas addition of ETS-1 siRNA along with VEGF treatment reduced these expressions by 93 and 25%, respectively, as shown by Q-PCR using gene-specific primers. ETS-1 siRNA alone also reduced the expression of these genes (Fig. 8, a and b). The cell migration was closely associated with the potential of tumor invasiveness and metastasis. We further observed that ETS-1 siRNA potently inhibited VEGF-induced invasion of SKOV-3 cells (Fig. 8, c and d) compared with that of nonspecific siRNA-treated control cells. Expectedly, the ETS-1 siRNA-treated cells showed significantly less invasion than that of control.

To confirm the requirement of endogenous ETS-1 protein for VEGF-induced MMP-9 and MMP-13 promoter activity, the ETS-1 gene was knocked down, and the reporter clones containing MMP promoters were transfected in SKOV-3 cells. Both MMP-9 and MMP-13 promoters were cloned into pGLuc reporter vector. We observed that the luciferase reporter activity was significantly increased compared with the pGL3-Basic vector having no promoter (data not shown). After exogenous addition of 20 ng/ml VEGF to the cells transfected with different promoter constructs in pGLuc, both MMP-9 (Fig. 8e) and MMP-13 (Fig. 8f) promoter activity was increased significantly; promoter activity was again reduced in ETS-1 siRNA-transfected cells. This means that ETS-1 recruitment to the putative MMP-9 and MMP-13 promoters enhances the transcription of these genes, and VEGF further augments the transcriptional activity. This is an indirect evidence for ETS-1 interaction with the MMP-9 and MMP-13 promoters in SKOV-3 cells.

**PI3K and p38 MAPK Pathways Are Involved in VEGF-induced ETS-1 Expression and Thereby Its Translocation**—To study the involvement of the PI3K and p38 MAPK pathways in VEGF-induced expression of ETS-1 factor, the cells were treated with VEGF alone (20 ng/ml for 4 h) or with different inhibitors individually (LY, SB, and PD; doses as described under “Materials and Methods”) for 4 h, or the cells were pretreated with inhibitors individually for 30 min followed by VEGF treatment (20 ng/ml for 4 h). The cellular proteins were isolated and immunblotted with ETS-1-specific antibody, and the specific bands are shown in Fig. 9a. The data suggest that the VEGF-induced expression of ETS-1 was significantly reduced in the presence of LY and SB. The GAPDH expression level was used as an internal control.

Our next approach was to study the effect of VEGF, if any, in nuclear translocation of ETS-1 by immunofluorescence microscopy. The cellular images show that ETS-1 localization to the nucleus upon VEGF treatment was increased, and this was inhibited by LY294002 (PI3K inhibitor) and SB203580 (p38 MAPK inhibitor), suggesting the involvement of these pathways in this event. The nuclear localization of ETS-1 was not observed upon VEGF treatment if the cells were pretreated with the inhibitors SB203580 and LY294002 (Fig. 9b). Similarly,
when only inhibitors were added, ETS-1 localization in the nucleus was not observed (Fig. 9b).

To confirm the nuclear localization of ETS-1 upon VEGF treatment, its expression profile was checked in the nuclear and cytoplasmic fractions after treatment with different doses of VEGF (0, 20, and 40 ng/ml, respectively) for 4 h. The data suggest that the ETS-1 content increases significantly in the nuclear fraction, whereas it is more or less unchanged in the cytoplasmic fraction upon VEGF treatment (Fig. 9c).

**DISCUSSION**

Most fatalities from ovarian cancer occur due to metastasis characterized by widespread intraperitoneal dissemination and malignant ascites formation. VEGF plays a major role in the progression of ovarian cancer by influencing tumor growth through promotion of tumor angiogenesis and ascites formation. MMPs, the extracellular proteinases, are the principal mediators of the alterations observed in the microenvironment during cancer progression, and they target a variety of ECM proteins and thereby regulate invasion as well as migration of cancer cells (60, 61). Migration, invasion, metastasis, and angiogenesis, the four important stages of cancer cells, are dependent on the surrounding microenvironment. Here MMPs play a critical role as they degrade various cell adhesion molecules, thereby modulating cell-cell and cell-ECM interactions (62). For growth and migration of cancer cells, formation of new blood vessels is necessary, and hence the elimination of physical barriers by ECM degradation and generation of proangiogenic factors are necessary. The angiogenic balance is accurately regulated by MMPs as they down-regulate blood vessel formation through the generation of degradation fragments that inhibit angiogenesis (63).

In the present study, we observed a significant increase in invasion (Fig. 1a) in SKOV-3 ovarian adenocarcinoma cells caused by VEGF. Cell scattering is prerequisite for invasion through basement membrane. Hence, we investigated whether VEGF promotes cell motility or scattering as well, and indeed it was increased (Fig. 1b). Moreover, VEGF increased the expression of MMP-2, MMP-9, and MMP-13 significantly in agreement with similar studies performed in other cell types or tis-
sues (64). The next obvious query was whether VEGF acts through its receptor to achieve these functions in SKOV-3 cells. Therefore, we checked whether VEGF receptor is expressed in SKOV-3 cells and found an abundant expression of VEGFR2 with high copy number (data not shown), which supports the earlier published report (14). VEGF-A signals through VEGFR2 (Flk1); therefore, we inhibited this particular receptor to check the downstream effect. Our data confirm that the VEGF-induced cellular activities as mentioned were significantly inhibited when a VEGF receptor blocker was used, suggesting that VEGF acts through its receptor to perform those activities (Figs. 1 and 7). However, the molecular mechanism by which VEGF regulates the expression of different MMPs associated with invasion and scattering of cancer cells is not clearly understood. Therefore, the present study focused on the investigation of the regulation of MMP gene expression, especially MMP-9 and MMP-13, by VEGF in SKOV-3 cells.

AKT/PKB, a major effector of the PI3K signaling pathway, plays an important role in VEGF-mediated cell migration essential for angiogenesis in endothelial cells (65). Moreover, AKT regulates the cell motility and ECM degradation in different cell types (66), including ovarian cancer cells (67). The present findings demonstrate that phosphorylation of both PI3K and p38 MAPK is induced by VEGF, and the subsequent pathways contribute to the VEGF-induced invasion and scattering in SKOV-3 cells (Figs. 2 and 3).

We have shown that VEGF activation induces the expression of MMP-2, MMP-9, and MMP-13 (Figs. 4 and 5) as well as activities of these proteins in PI3K- and p38 MAPK-dependent pathways. But the involvement of ETS-1 was not studied in this regard. In the previous studies, ETS-1 was shown to be co-expressed with and induce MMP-9 expression in different types of carcinomas (68) and induce MMP-13 expression in noncancerous (69) as well as cancer cells (70). As VEGF induces PEA3

**FIGURE 9. Induction of ETS-1 by VEGF through activation of PI3K/AKT and p38 MAPK pathways.** The cells were treated with VEGF (V) alone or in combination with LY, PD, or SB. In another set, LY, PD, and SB were added independently as described under "Materials and Methods." The cells were either subjected to Western immunoblot (a and c) or immunofluorescence microscopy (b). In Western immunodetection of ETS-1, its two isoforms were differentially expressed in the presence of the inhibitors as described. The intensities of each band were measured as ImageJ software and are shown as bars. Here, GAPDH expression is shown as a loading control (a). For immunofluorescence microscopy, the cells were treated with anti-ETS-1 antibody followed by Alexa Fluor 488-conjugated secondary antibody that shows green fluorescence where ETS-1 localizes (b, middle panel). In the upper panel, the cells were treated with the DNA-specific dye DAPI to localize the nucleus. In the lowest panel, some of the treated cells are presented at higher magnification to show the localization of ETS-1. The treatment condition of the cells is noted above the top panel. Here the symbols α, β, γ, δ, ε, and ζ are used in some cells in the middle and lower panels to indicate the magnified view of particular cells, for e.g., the α in the middle panel is magnified and shown as α in the lower panel and so on. The role of VEGF in the induction and accumulation of ETS-1 in the nucleus and cytoplasm is shown (c). The cells were treated with VEGF (0, 20, and 40 ng/ml) for 4 h, and the nuclear and cytoplasmic proteins were isolated followed by SDS-PAGE and Western immunodetection with ETS-1 antibody. The relative expression of ETS-1 versus GAPDH expression has been represented in the lower panels as bars. For a and c, the data are represented as the mean of three independent experiments ± S.D. (error bars) (*, p < 0.05; **, p < 0.01; ***, p < 0.001 versus untreated control cells (C)).
expression insignificantly compared with that of ETS-1, we did not proceed further with the PEA3 study (Fig. 6). Instead, a DNA-protein interaction study (EMSA) was performed to determine whether ETS-1 interacts with the upstream EBS present in the MMP-9 and MMP-13 promoters. Representative gel shift data (supplemental Fig. 1, a and b) demonstrated that ETS-1 interacts with the EBS in the MMP-9 and MMP-13 promoters. Therefore, VEGF-induced MMP-9 and MMP-13 gene expression is regulated by ETS-1, which was further confirmed by an siRNA-mediated ETS-1 knockdown experiment (Fig. 8, a and b). The VEGF-induced invasion was decreased after ETS-1 siRNA treatment (Fig. 8, c and d), and the expression of relevant MMPs correlates with the extent of cell invasion in SKOV-3 cells. The result of a luciferase reporter assay reveals that VEGF-induced MMP-9 and MMP-13 promoter activity was inhibited by ETS-1 siRNA treatment (Fig. 8, e and f). Therefore, ETS-1 and its associated proteins that form a complex of transcription initiation factors might be a potential therapeutic target for the treatment of ovarian cancer. Unlike the MMP-9 and MMP-13 promoters, the MMP-2 promoter does not have this element, and therefore, VEGF-induced MMP-2 expression in SKOV-3 cells may be mediated by an ETS-independent mechanism.

Our investigation confirms that the VEGF-induced expression of MMP-9, MMP-13, and ETS-1 in SKOV-3 cells is carried out through VEGF receptor as the VEGF activities were significantly inhibited in SKOV-3 cells pretreated with an inhibitor of VEGFR2 (Fig. 7). We selected these three genes because they are involved in the VEGF-induced migration and scattering in the cell. To check whether the effects of VEGF in SKOV-3 cells are also present in other ovarian carcinoma cells, we selected two more cell lines, PA-1 and OAW-42, to perform some of the key experiments. VEGF-induced cellular invasion, scattering, and the expression levels of MMP-9, MMP-13, and ETS-1 genes were studied in these two cell lines. The results showed a similar pattern of cellular invasiveness and scattering and a similar MMP-9, MMP-13, and ETS-1 gene expression profile in PA-1 cells upon VEGF treatment (supplemental Fig. 2, a–c) as was observed in SKOV-3 cells. But in OAW-42 cells, the VEGF-induced invasion and scattering were not observed (supplemental Fig. 2b), and the mRNA levels of MMP-9, MMP-13, and ETS-1 were found to be negligible (data not shown). Earlier data also suggest that OAW-42 cells express a very low amount of MMP-9 and MMP-13 genes (71). It was also found that, like SKOV-3 cells, PA-1 cells are VEGFR2-positive, but the OAW-42 cells do not express a detectable quantity of this receptor (data not shown). Therefore, it is expected that the VEGF cannot induce the invasion and scattering in OAW-42 cells possibly due to the low abundance of its receptor. Hence, it may be concluded that the effect of VEGF to induce cellular invasion and scattering depends on the cell types.

In the present study, our results indicate that PI3K and p38 MAPK signaling is critical for VEGF-induced cell motility/invasion. Again, it was observed that blocking AKT by LY294002 significantly reduced VEGF-mediated ETS-1 expression (Fig. 9a) as well as its nuclear localization (Fig. 9, b and c); the same effect was observed when SB203580, the p38 MAPK inhibitor, was used (Fig. 9c). Thus, it appears that VEGF-induced ETS-1

![Hypothetical model showing VEGF-regulated MMP-9 and MMP-13 gene expression through ETS-1 following activation of PI3K/AKT and p38 MAPK pathways.](image-url)
expression as well as nuclear localization is regulated by both the PI3K and p38 MAPK pathways. The induction of ETS-1 protein by ERK1 inhibitor alone (Fig. 9a) might happen by certain alternative pathways activated by PD.

Earlier data suggest that a specific transcription factor regulates MMP-9 expression in a p38 MAPK-dependent pathway (72). When activated, these pathways regulate MMP-2, MMP-9, and MMP-13 gene expression as well as their activities (Figs. 4 and 5). Therefore, the complexity interaction mechanism should be understood properly to know the p38 MAPK-activated MMP expression. Again, the role of MMP-2, the major gelatinase involved in cancer cell migration, is not clearly understood. It was reported that the C-terminal domain, also conserved in the more distantly related ELK and ERG gene products (ETS transcription factors), is essential for both nuclear targeting and DNA binding activity of ETS-1 in vitro (74). Studies have revealed that the nuclear localization of ETS-1 is strongly regulated by epidermal growth factor and requires nuclear calcium (75). The data obtained from our experiments have been schematically represented in Fig. 10, and it is hypothesized that VEGF induces ETS-1 through the activation of the PI3K/AKT and p38 MAPK pathways, and after that ETS-1 is enriched in the nucleus followed by the activation of relevant MMP genes. However, the mechanism behind VEGF-induced nuclear localization of ETS-1 is yet to be understood and is an important area for future study.

In conclusion, our data provide a mechanistic insight into VEGF-regulated MMP gene expression followed by invasion and scattering in an ovarian carcinoma cell line. The major observations of this report are as follows: 1) VEGF induced SKOV-3 cell invasion and migration through the activation of the PI3K and p38 MAPK pathways. 2) VEGF increases MMP-2, MMP-9, and MMP-13 expression in SKOV-3 cells. 3) VEGF induces ETS-1 expression, which in turn binds to both MMP-9 and MMP-13 promoters. 4) VEGF acts through its receptor to perform all these activities. 5) Knockdown of ETS-1 reduces VEGF-induced MMP-9 and MMP-13 promoter activity and expression as well as cell invasion. 6) VEGF enriches the ETS-1 in the nuclear fraction in a dose-dependent manner. 7) The expression as well as nuclear localization of ETS-1 induced by VEGF treatment requires the coordinated activation of PI3K and p38 MAPK pathways. Considering the enormous role of MMPs in several steps of cancer progression, research is being done to develop safe and effective agents targeting MMPs. In most cases, these agents that target MMPs showed poor results in clinical trials, and hence thorough research is necessary prior to clinical trials to find strategies to target MMPs for cancer therapy (10, 62). Thus, these results provide valuable insights into the mechanisms of VEGF-induced signaling networks in ovarian cancer cells and may contribute to the development of new therapies and to early detection of the disease.

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