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# Endothelin-1 Induces Tumor Proteinase Activation and Invasiveness of Ovarian Carcinoma Cells[1]

**Laura Rosanò, Marco Varmi, Debora Salani, Valeriana Di Castro, Francesca Spinella, Pier Giorgio Natali, and Anna Bagnato[2]**

*Laboratories of Molecular Pathology and Ultrastructure [L. R., M. V., D. S., V. D. C., F. S., A. B.] and Immunology [P. G. N.], Regina Elena Cancer Institute, 00158 Rome, Italy*

## ABSTRACT

**Endothelin-1 (ET-1) is present at high concentrations in ovarian cancer ascites and is overexpressed in primary and metastatic ovarian carcinoma. In these cells, ET-1 acts as an autocrine mitogenic and angiogenic factor selectively through the $ET_A$ receptor ($ET_AR$). We investigated at mRNA and protein levels whether ET-1 could affect the expression and activation of metastasis-related proteinases and whether this process was associated with ovarian tumor cell invasion. ELISA, gelatin zymography, Western blot, and reverse transcription-PCR analyses demonstrated that in two ovarian carcinoma cell lines (HEY and OVCA 433), the expression of matrix metalloproteinase (MMP) -2, -9, -3, -7, and -13 was up-regulated and activated by ET-1. Moreover we observed that ET-1 was able to enhance the secretion and activation of membrane-type metalloproteinase-1, a critical mediator of invasiveness. The secretion of tissue inhibitor of metalloproteinase-1 and -2 was decreased by ET-1, which increased the net MMP/tissue inhibitor of metalloproteinase balance and the gelatinolytic capacity. In addition, ET-1 induced overexpression of urokinase-type plasminogen activator, its receptor, and plasminogen activator inhibitor type-1 and -2. Finally, we demonstrated that, in HEY and OVCA 433 cells, ET-1 dose-dependently increased migration and MMP-dependent invasion through Matrigel. BQ123, an antagonist of the $ET_AR$, inhibited the ET-1-induced tumor protease activity and subsequent increase in cell migration and invasion. These findings demonstrate that ET-1 promotes ovarian carcinoma cell invasion, acting through the $ET_AR$ by up-regulating secretion and activation of multiple tumor proteinases. Therefore, ET-1 may represent a key component of more aggressive ligand-induced invasiveness of ovarian carcinoma.**

## INTRODUCTION

Ovarian carcinoma frequently is diagnosed at advanced clinical stage when intra-abdominal spread has occurred, accompanied by the formation of ascites (1). ET-1[3] is a peptide produced primarily by vascular cells and in elevated amounts by different cancer cells (2). ET-1 acts through two distinct subtypes of G protein-coupled receptors, namely $ET_AR$ and $ET_BR$. The $ET_AR$ binds selectively ET-1, whereas the $ET_BR$ binds both ET-1 and ET-3. We have previously demonstrated that ET-1 is overexpressed in primary and metastatic ovarian carcinomas compared with normal ovarian tissues. In ovarian tumor cells, ET-1 acts as an autocrine growth factor selectively through the $ET_AR$, as demonstrated by the inhibitory proliferative effects induced by specific $ET_AR$ antagonists (3, 4). Binding of ET-1 to the $ET_AR$ results in activation of a pertussis toxin-insensitive G protein that stimulates phospholipase C activity and promotes $Ca^{2+}$/protein kinase catalytic subunit signaling.

Among downstream events after $ET_AR$ activation in ovarian carcinoma cell, ET-1 causes phosphorylation and activation of MAP kinase and stimulates the phosphorylation of $p125^{FAK}$ and paxillin, which are thought to transduce signals involved in tumor cell invasion (5, 6). We also observed that ET-1 stimulates neovascularization in ovarian carcinoma cells through direct angiogenic effects on endothelial cells and in part through the stimulation of vascular endothelial growth factor via $ET_AR$ binding (7, 8). Because high concentrations of ET-1 are present in ascitic fluids, this peptide could also enhance the secretion of ECM-degrading proteinases and thereby facilitate cell invasiveness and progression of ovarian carcinoma.

Invasion and metastasis in ovarian cancer require the action of tumor-associated proteases that promote the dissolution of the surrounding matrix and the basement membranes (9, 10). Both serine and matrix metalloproteinases have been implicated in the complex integrated events underlying tumor invasion. The MMP family contains 18 human members, which can be classified into subgroups of collagenases, gelatinases, stromelysins, MT-MMPs, and novel MMPs according to their substrate specificities and structures (11). MMPs associated with ovarian carcinomas include MMP-2 and -9 (gelatinase A and B), MMP-3 (stromelysin-1), MMP-7 (matrilysin), and MMP-13 (collagenase-3), which have been shown to correlate with high invasive and metastatic potential in ovarian carcinoma (12–18). MT1-MMP, described as proteinases anchored on plasma membranes, has been shown to be the major physiological activator of other MMPs, such as pro-MMP-2 and -13, and it has been speculated that MT1-MMP produced by ovarian carcinoma cells may initiate pro-MMP-2 activation, thereby facilitating motility, invasion, and metastasis (19, 20). All MMPs are produced in a latent form (pro-MMP) that requires activation for catalytic activity, a process that is usually accomplished by proteolytic removal of the propeptide domain. Once activated, all MMPs are specifically inhibited by a group of endogenous TIMPs that revert the unbalanced proteolytic activity of tumors, thus preventing local invasion and metastasis formation. TIMPs bind to the zinc-binding catalytic site of the MMPs in a 1:1 molar ratio. In addition, TIMP-2 and -1 can bind to the hemopexin domain of latent MMP-2 and -9, respectively. TIMP-1 inhibits the activity of most MMPs, with the exception of MT1-MMP and MMP-2. TIMP-2 also inhibits the activity of most MMPs, except MMP-9. The balance between the levels of activated MMPs and TIMPs is one control of MMP-dependent proteolysis (21).

uPA is a 54-kDa serine protease (22). Similar to many proteinases, tumor cells synthesize and secrete uPA in its pro form, which binds to specific receptors on the tumor cell surface. After binding, pro-uPA is activated by cathepsin B. In turn, receptor-bound, active uPA converts the proenzyme plasminogen to the active form of plasmin, which degrades components of the tumor stroma. Furthermore, uPA has been implicated in a cascade resulting in the activation of metalloproteinases. Its specific inhibitors PAI-1 and -2 regulate the activity of uPA. Binding of PAI-1 or -2 to uPA results in the subsequent internalization of the ternary complex, which regulates cell surface plasmin generation, thus constituting an effective proteolytic enzyme

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[2] To whom requests for reprints should be addressed, at Laboratory of Molecular Pathology and Ultrastructure, Regina Elena Cancer Institute, Via delle Messi d'Oro 156, 00158 Rome, Italy. Phone: 39-06-52662565; Fax: 39-06-52662505; E-mail: bagnato@ifo.it.

[3] The abbreviations used are: ET-1, endothelin-1; $ET_AR$ and $ET_BR$, endothelin A and B receptor, respectively; ECM, extracellular matrix; MMP, matrix metalloproteinase; MT, membrane type; TIMP, tissue inhibitor metalloproteinase; uPA, urokinase plasminogen activator; PAI, plasminogen activator inhibitor; uPAR, urokinase plasminogen activator receptor; RT-PCR, reverse transcription-PCR; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; MMPI, MMP inhibitor; FAK, focal adhesion kinase.

system. Thus, the complex of uPA and PAI-1/-2 modulates the invasive and metastatic phenotype of cancer cells (23–26).

Under pathological conditions, regulation of MMP and uPA system expression involves several factors, including steroid hormones, cellular oncogenes, cytokines, and growth factors (11). In this study we demonstrate that ET-1 induces overexpression and activation of a panel of proteinases known to be associated with highly invasive and metastatic ovarian carcinoma. This effect is followed by a marked increase in ovarian tumor cell migration and invasion. The enhanced proteolytic capability of ovarian carcinoma cells induced by ET-1 suggests a biochemical mechanism by which disruption of local tissue architecture to allow tumor growth and spread of ovarian carcinoma may be mediated by ET-1 through $ET_AR$ binding.

## MATERIALS AND METHODS

**Cells.** The human ovarian carcinoma cell lines OVCA 433 and HEY were a gift from Dr. G. Scambia (Catholic University School of Medicine, Rome, Italy) and were cultured in DMEM containing 10% FCS and 1% penicillin-streptomycin. All culture reagents were from Life Technologies, Inc. (Paisley, Scotland). For analysis of proteinases, cells were plated in 100-mm Petri dishes.

**Preparation of Ovarian Cancer Cell-conditioned Medium.** Subconfluent cultures of HEY and OVCA 433 cells were starved for 24 h in DMEM not supplemented with FCS to reach quiescence. The medium was discarded, fresh serum-free DMEM was added alone or in addition of different concentrations of ET-1 (Peninsula Laboratories, Belmont, CA), and the ovarian carcinoma cells were incubated for an additional 24 h. The conditioned medium was then collected, centrifuged, and stored in aliquots at −20°C. The cells were counted, and the data were corrected for the cell number. The conditioned medium was then processed for ELISA, zymography, and Western blot.

**Measurement of MMPs and uPA/PAI-1 Protein by ELISA.** Gelatinase activities in conditioned medium were determined by a MMP Gelatinase Activity Assay Kit (Chemicon International, Inc., Temecula, CA), according to the manufacturer's instructions. The sensitivity of the assay is $<5$ ng/ml MMP in a range of 10–200 ng/ml.

MMP-2 in conditioned medium was measured by a BioTrak Human MMP-2 ELISA kit (Amersham, Arlington Heights, IL), according to the manufacturer's instructions. MMP-2 may be measured in the range 1.5–24 ng/ml, and the sensitivity of the assay is 0.37 ng/ml. The assay does not cross-react with MMP-1, -3, -7, -8, and -9 or MT1-MMP. MMP-9 in conditioned medium was measured by a MMP-9 ELISA kit (Oncogene Research Products, Cambridge, MA), according to the manufacturer's instructions. MMP-9 may be measured in the range 0.625–20 ng/ml, and the sensitivity of the assay is 0.1 ng/ml. uPA and PAI-1 were determined by commercially available ELISA kits (American Diagnostica, Greenwich, CT), according to the manufacturer's instructions. uPA may be measured in the range 0.1–1 ng/ml, and the sensitivity of the assay is 10 pg/ml. PAI-1 may be measured in the range 0.01–10 ng/ml, and the sensitivity of the assay is 50 pg/ml. The uPA activity was evaluated by an uPA Activity Assay Kit (Chemicon International), according to the manufacturer's instructions. The assay is sensitive over a range of 0.05–50 units of uPA activity.

**RT-PCR Analysis.** Total RNA was prepared using the TRIzol reagent (Life Technologies), according to the manufacturer's instructions. The RT-PCR was performed with a SUPERSCRIPT One-Step RT-PCR System (Life Technologies) according to the manufacturer's instructions. Briefly, 1 μg of RNA was reverse-transcribed. The primer sets used were as follows: for MMP-2, 5′-TTTGGACTGCCCCAGACAGG-3′ and 5′-GCTGCGGCCAGTATCAGTGC-3′; for MMP-9, 5′-TCCTGGTGCTGGCTTGCTGC-3′ and 5′-CAATGTCAGCTTCGGGGCCG-3′; for MT1-MMP, 5′-CCCTATGCCAACATCGGTGA-3′ and 5′-TCCATCCATGACTTGGTTTAT-3′; for uPA, 5′-GGCAGCAATGAACTTCATCAAGTTCC-3′ and 5′-TATTTCACAGTGCTGCCCTCCG-3′; for uPAR, 5′-ACAGGAGCTGCCCTCGCGAC-3′ and 5′-GAGGGGGATTTCAGGTTTAGG-3′; for PAI-1, 5′-CTTTGGTGAAGGGTCTGC-3′ and 5′-CTCCACCTCTGAAAAGTCC-3′; for PAI-2, 5′-ATGGAGGATCTTTGTGTG-3′ and 5′-GGGGAATTCTTTTGCAGAA GCA-3′. *GAPDH* was used as an internal control, and the primer sets used was 5′-TGAAGGTCGGTGTCAACGGA-3′ and 5′-GATGGCATGGACTGTGGTCAT-3′. Each RT-PCR included a cDNA synthesis and predenaturation cycle at 55°C for 30 min and at 94°C for 2 min; the cDNA was amplified for 30 cycles involving a denaturation step at 94°C for 1 min; a primer annealing step at 56°C for 30 s (uPA), 60°C for 1 min (uPAR), 55°C for 1 min (PAI-1/-2), or 62°C for 1 min (*GAPDH*); and an extension step at 72°C for 1 min. The PCR products were analyzed by electrophoresis on a 2% agarose gel containing ethidium bromide and visualized and photographed under UV light. In all experiments, two control reactions, one containing no mRNA and another containing mRNA but no reverse transcriptase or Taq, were included. All 5′ primers covered splice junctions, thus including the amplification of genomic DNA. Densitometric scanning was performed with a Mustek MFS-6000CX apparatus, and the data were analyzed with Phoretix 1D software and normalized to those of *GAPDH*. The semiquantitative analysis was performed essentially as described by Rieckmann *et al.* (27). The mRNA values are expressed as relative units calculated according to the following formula: density of the amplification product/density of the *GAPDH* amplification product × 100. To compare results from different experiments, optimal cycle conditions for linear amplification were determined by semiquantitative assay of the amplified products at 20, 25, 30, and 35 cycles. Thirty-cycle products, which were within the linear phase of the amplification curve, were chosen for the comparative analysis.

**Gelatin Zymography.** The ovarian tumor cell supernatants were electrophoresed for analysis in 9% SDS-PAGE gels containing 1 mg/ml gelatin. The gels were washed for 30 min at 22°C in 2.5% Triton X-100 and then incubated in 50 mM Tris (pH 7.6), 1 mM $ZnCl_2$, and 5 mM $CaCl_2$ for 18 h at 37°C. After incubation the gels were stained with 0.2% Coomassie Blue. Enzyme-digested regions were identified as white bands on a blue background and quantified by computerized image analysis of the band. Molecular sizes were determined from the mobility, using gelatin zymography standards (Bio-Rad Laboratories, Richmond, CA).

**Western Blotting.** The presence of MMPs, TIMPs, uPA, uPAR, and PAIs in conditioned medium was analyzed by Western blotting. Twenty μl of concentrated medium diluted with an equal amount of Laemmli (Bio-Rad) sample dilution buffer were boiled and electrophoresed under reducing conditions on an 11% SDS-polyacrylamide gel. Anti-MMP-2 and anti-MMP-9 (NeoMarkers, Fremont, CA) were used at a 1:400 dilution. Anti-uPA, anti-uPAR, anti-PAI-1, anti-PAI-2 (Santa Cruz Biotechnology, Inc., Santa Cruz, CA), anti-TIMP-1, anti-TIMP-2, anti-MMP-3, anti-MMP-7, and anti-MMP-13 (Chemicon International) was used at a 1:1000 dilution. To test for the presence of MT1-MMP, whole-cell protein extracts of HEY cultures grown in the absence or presence of 100 nM ET-1 were scraped directly into Laemmli loading buffer; 30 μg of each extract were boiled, separated by 11% SDS-PAGE, and revealed by an anti-MT1-MMP antibody (Chemicon) at a 1:1000 dilution. To check the amount of proteins transferred to the nitrocellulose membranes, β-actin was used as control and detected by an anti-β-actin monoclonal antibody (Ab-1, clone JLA20; Oncogene) at a 1:10,000 dilution. Peroxidase-labeled antigoat, antimouse, and antirabbit antibody (Santa Cruz Biotechnology) was used according to the manufacturer's instructions. Blots were developed with an ECL kit (Amersham). The relative amounts of the transferred proteins were quantified by scanning the autoradiographic films with a gel densitometric scanner (Bio-Rad) and normalized to the related β-actin amounts.

**Chemotaxis and Chemoinvasion Assay.** Chemotaxis and chemoinvasion were assessed with a 48-well modified Boyden chamber (NeuroProbe, Pleasanton, CA) and 8 μm pore size polyvinyl pyrrolidone-free polycarbonate Nucleopore filters (Costar, New York, NY) as described previously (28). For chemotaxis, the filters were coated with gelatin by overnight immersion in a solution of 100 μg/ml gelatin in 0.1% acetic acid and then dried. For the chemoinvasion assay, the filters were coated with an even layer of 0.5 mg/ml Matrigel (Becton Dickinson, Milan, Italy). The lower compartment of the chamber was filled with chemoattractants (at different concentrations) or inhibitor (27 μl/well). Serum-starved HEY and OVCA 433 cells ($5 \times 10^5$ cells/ml) were harvested in trypsin-EDTA solution, collected by centrifugation, resuspended in DMEM, and placed in the upper compartment (55 μl/well). BQ123 (Peninsula Laboratories), an $ET_AR$ antagonist, was previously added to the cells and preincubated for 15 min at 37°C. After 4 h (chemotaxis) or 6 h (chemoinvasion) of incubation at 37°C, filters were stained with Diff-Quick (Merz-Dade, Dudingen, Switzerland), and the migrated cells in 10 high-power fields were counted. Each experimental point was analyzed in triplicate. In selected experiments, invasion was quantified in the presence of GM6001, also known as Ilomastat (Chemicon International), a chemical broad-spectrum inhibitor of MMP activity.

**Statistical Analysis.** All statistical analyses were performed using a two-tailed Student's *t* test and the Inplot software system (GraphPad Software Inc., San Diego, CA).

## RESULTS

**ET-1 Enhances Secretion and Activation of MMP-2 and -9 in Ovarian Carcinoma Cell Lines.** Using human MMP ELISA Kits, we measured the effect of exogenous ET-1 on the secretion of MMP-2 and -9 by two ovarian carcinoma cell lines, OVCA 433 and HEY, which express abundant high-affinity receptor for ET-1 ($k_d = 0.10$ nM and 45,500 receptors/cell in OVCA 433 cells and $k_d = 0.1$ nM and 36,500 receptors/cell in HEY cells). As shown in Fig. 1, ET-1 enhanced MMP-2 and -9 in a dose-dependent manner. The difference in MMP-2 and -9 secretion between stimulated and unstimulated cells was significant at all ET-1 concentrations tested. A selective $ET_AR$ antagonist, BQ123, was used to determine whether the $ET_AR$ was involved in the stimulation of MMP-2 and -9 secretion in ovarian carcinoma cells. ET-1-stimulated MMP-2 and -9 secretion was completely blocked by the addition of BQ123 (1 $\mu$M).

To identify the role of ET-1 in the activation status of MMP-2 and -9, conditioned media from HEY and OVCA 433 cells were analyzed by gelatin zymography. Ovarian carcinoma cell lines secreted high levels of pro-MMP-2 (gelatinase A) and pro-MMP-9 (gelatinase B). When 100 nM ET-1 was added, zymography showed that both HEY and OVCA 433 cells secreted high levels of gelatinolytic proteases corresponding to the active forms of MMP-2 and -9 (2.5- and 3.5-fold increase, respectively; Fig. 2*A*). These results were confirmed by Western blotting, demonstrating that ET-1 treatment led to the processing of endogenous pro-MMP-2 (72 kDa) and pro-MMP-9 (92 kDa) to molecular masses corresponding to their active forms (66 and 86 kDa, respectively; Fig. 2*B*). These results demonstrate that ET-1 induces a significant increase in the secretion of MMP-2 and -9 and has a direct role in the activation of both pro-gelatinases in ovarian cancer cells. Evaluation of MMP activity by a MMP gelatinase activity kit confirmed the net increase in MMP activity, showing a 2.5-fold increase in ET-1-treated cultures (Fig. 2*C*). The addition of BQ123 (1 $\mu$M) completely blocked the MMP activity, suggesting that ET-1 was able to enhance secretion and activation of MMP-2 and -9 in ovarian carcinoma cells through the $ET_AR$.

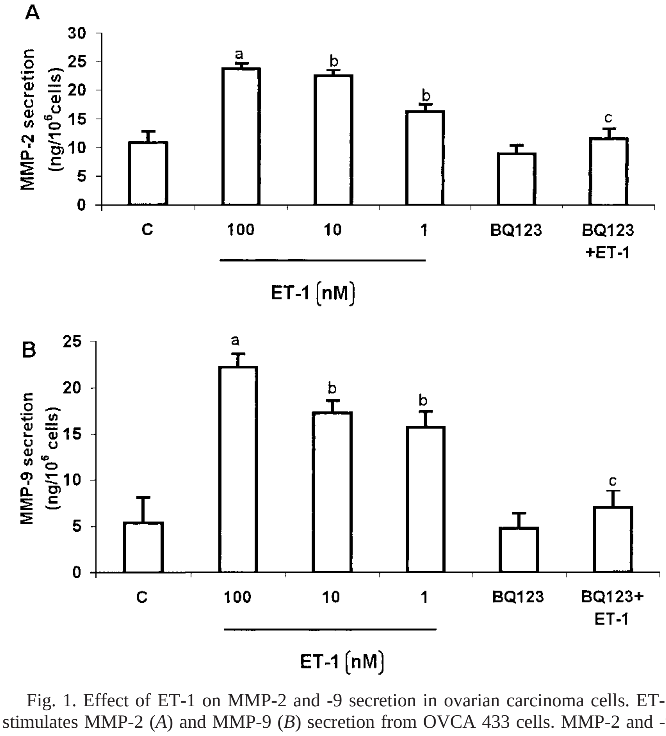

Fig. 1. Effect of ET-1 on MMP-2 and -9 secretion in ovarian carcinoma cells. ET-1 stimulates MMP-2 (*A*) and MMP-9 (*B*) secretion from OVCA 433 cells. MMP-2 and -9 were measured in conditioned media from cells treated with different concentrations of ET-1 for 24 h, using ELISA kits. $ET_AR$ antagonist BQ123 (1 $\mu$M) was preincubated 15 min prior to the addition of 100 nM ET-1. Data are presented as mean of results from three experiments each performed in duplicate. *Bars*, ± SD. *a*, $P < 0.0001$; *b*, $P < 0.005$ compared with control; *c*, $P < 0.0002$ compared with 100 nM ET-1.

To determine whether the ET-1-induced overexpression of MMP-2 and -9 was transcriptionally regulated, we established a sensitive RT-PCR assay to detect mRNA transcripts for MMP-2 (518 bp) and MMP-9 (467 bp) genes in the two ovarian carcinoma cell lines. The RT-PCR-amplified cDNA fragments for MMP-2 and -9 were detectable in all samples of untreated cells as a single band at the expected size (Fig. 2*D*). Primers for the amplification of the *GAPDH* gene were used as controls. Densitometric analysis of these bands and comparison with the intensity of the *GAPDH* bands indicated an up-regulation of MMP-2 and -9 mRNA after 8 h of stimulation with ET-1 (100 nM) in both cell lines. These results correlate closely with the protein expression levels observed by ELISA, zymography, and Western blotting, suggesting that MMP up-regulation by ET-1 was at the transcriptional level.

**ET-1 Enhances Secretion and Activation of MMP-3, -7, and -13.** To screen for the role of ET-1 on the secretion and activation of other metalloproteinases (MMP-3, -7, and -13) associated with ovarian carcinoma, conditioned media from HEY and OVCA 433 cells were analyzed by Western blotting. Unstimulated cells exhibited both latent and active forms of MMP-3 (59- and 57-kDa forms), MMP-7 (28- and 18-kDa forms), and MMP-13 (60- and 48-kDa forms; Fig. 3). The results demonstrated that 100 nM ET-1 was able to induce overexpression of both the latent and active forms of MMP-3 and -7 and the active form of MMP-13 in ovarian carcinoma cells with respect to the untreated cells. In addition, we observed that ET-1 treatment led to the complete processing of pro-MMP-13 to a molecular weight corresponding to the active form. These results suggest that ET-1 induces an increase in the secretion of MMP-3, -7, and -13 and is involved in their activation in ovarian carcinoma cells.

**ET-1 Enhances Secretion and Activation of MT1-MMP.** To examine the effect of ET-1 on MT1-MMP mRNA expression, we established a sensitive RT-PCR assay (Fig. 4*A*). ET-1 at a concentration of 100 nM induced up-regulation of MT1-MMP mRNA, as determined by densitometric analysis of these bands and comparison with the intensity of the *GAPDH* bands. To further verify the expression level of the MT1-MMP protein, we analyzed cell lysates from cells treated with ET-1 for 24 h. In cell lysates from ET-1-treated cells, we detected two bands, at 65 and 63 kDa, which corresponded to the latent and active MT1-MMP forms, indicating that ET-1 induced MT1-MMP activation (Fig. 4*B*). These results demonstrated that ET-1 was able to up-regulate, at the mRNA and protein level, the expression of MT1-MMP and to induce an activation of this critical mediator of invasive activity.

**Effect of ET-1 on the Secretion of TIMP-1 and -2.** Because the TIMPs inhibit active MMPs, conditioned media from HEY and OVCA 433 cells were examined by Western blotting. TIMP-1 and -2 were made constitutively by both ovarian carcinoma cells. The addition of ET-1 decreased the secretion of both TIMP-1 and -2 in the HEY and OVCA 433 cell-conditioned media (Fig. 5). Interestingly, the inhibition of TIMPs was concomitant with the activation of MMPs by ET-1. Thus, in ovarian carcinoma cells, ET-1 induced a strong increase in the net balance between gelatinases and TIMPs.

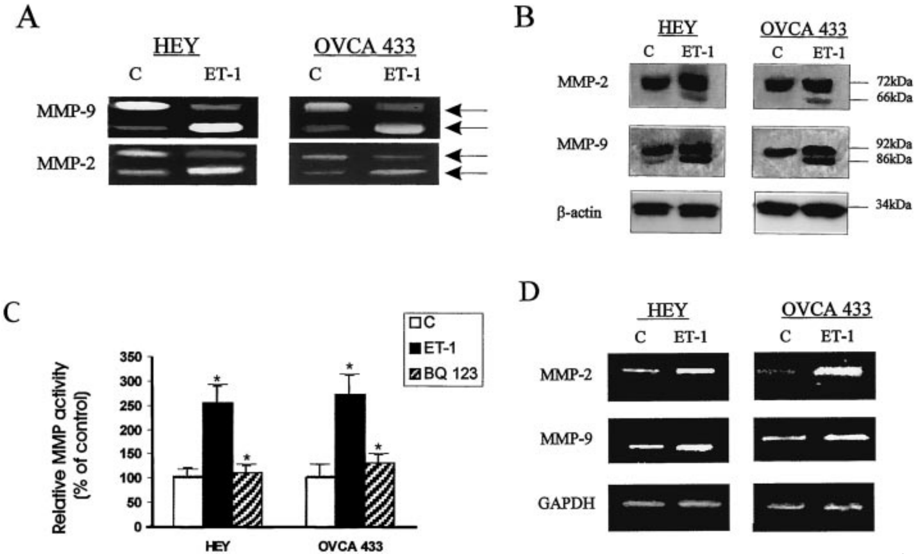

Fig. 2. Effect of ET-1 on MMP-2 and -9 secretion, activation, and mRNA expression in ovarian carcinoma cell lines. *A*, enzymatic activity of MMP-2 and -9 was studied in conditioned media from HEY and OVCA 433 cells by SDS-PAGE gelatin zymography, and gelatin lysis bands (pro-MMP and active) were measured by densitometric analysis. Both ovarian cancer lines, after starvation, were grown in serum-free medium for 24 h in the absence (*Lane C*) or presence of 100 nM ET-1 (*Lane ET-1*). *Arrows*, migration positions of pro- (*top*) and active (*bottom*) MMP-2 and MMP-9. *B*, conditioned media from HEY and OVCA 433 cells grown in the absence (*Lane C*) or the presence of 100 nM ET-1 (*Lane ET-1*) were analyzed for MMP-2 (latent form, 72 kDa; active form, 66 kDa) and for MMP-9 (latent form, 92 kDa; active form, 86 kDa) by Western blot analysis. The relative amounts of transferred MMPs were quantified and normalized to the corresponding β-actin protein amounts. *C*, MMP gelatinase activities were measured in conditioned media from both HEY and OVCA 433 cells treated with 100 nM ET-1 for 24 h, using a MMP Gelatinase Activity Assay Kit (■). BQ123 (1 μM) was preincubated 15 min prior to the addition of 100 nM ET-1 (▨). Data are presented as means of results from three experiments each performed in duplicate. *Bars*, ± SD. *, $P < 0.005$. *D*, expression of 518- and 467-bp mRNA transcripts for MMP-2 and -9, respectively, was detected by RT-PCR analysis. Primers for the amplification of *GAPDH* gene were used as controls. Data shown are PCR products of HEY and OVCA 433 cells, respectively, grown in serum-free medium for 8 h in the absence (*Lane C*) and in presence of 100 nM ET-1 (*Lane ET-1*). PCR products for MMPs and *GAPDH* were shown as visualized by ethidium bromide.

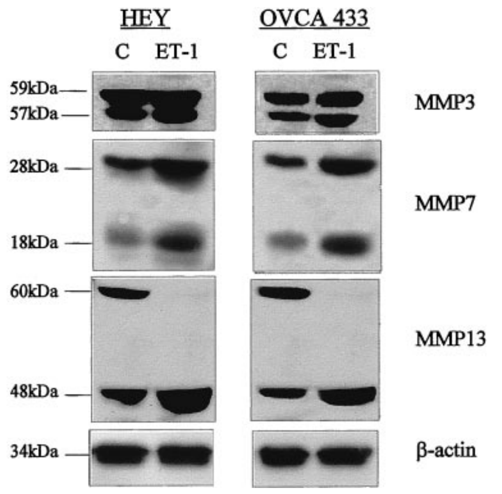

Fig. 3. Effect of ET-1 on the secretion and activation of MMP-3, -7, and -13 by ovarian cancer cells. Conditioned media from HEY and OVCA 433 cells alone (*Lane C*) or with addition of ET-1 100 nM (*Lane ET-1*) were tested for MMP-3 (the 59-kDa proform and the 57-kDa active form), MMP-7 (the 28-kDa proform and the 18-kDa active form), and MMP-13 (the 60-kDa proform and the 48-kDa active form) by Western blotting. The relative amounts of transferred MMPs were quantified and normalized to the corresponding β-actin protein amounts.

**ET-1 Enhances uPA, uPAR, and PAIs Levels in Ovarian Carcinoma Cell Lines.** uPA and its receptor uPAR have been proposed to play a role in ovarian carcinoma cell invasion (29). Conditioned media from HEY and OVCA 433 cell lines were analyzed by Western blotting. When treated with 100 nM ET-1 for 24 h, both HEY and OVCA 433 cells exhibited an increase in uPA and uPAR production (Fig. 6*A*). In addition to uPA/uPAR, high levels of PAIs have been correlated with the progression and metastasis of ovarian cancer. A strong increase above control levels of PAI-1 and PAI-2 was observed in both ovarian carcinoma cell lines treated with 100 nM ET-1 (Fig. 6*A*). The stimulation induced by ET-1 on uPA and PAI-1/-2 secretion by HEY cells was also measured by ELISA kits. This effect was dose dependent in the range between 0.1 and 100 nM with a maximum increase (~2.5-fold) observed at a concentration of 100 nM ET-1 (Fig. 6*B*). Pretreatment of cells with an ET$_A$R antagonist, BQ123, prevented the stimulation of uPA and PAI-1/-2 secretion induced by ET-1, confirming that the ET$_A$R subtype mediates stimulation of the uPA system.

Because ET-1 increased the levels of uPA, uPAR, PAI-1, and PAI-1/-2 proteins, we investigated whether ET-1 could affect the expression of their transcripts. The RT-PCR-amplified cDNA fragments for uPA (130 bp), uPAR (1046 bp), PAI-1 (409 bp), and PAI-2 (1280 bp) were detectable in all samples of untreated cells as a single band at the expected size in both cell lines with the exception of PAI-1 in HEY cells. We found that ET-1 at a concentration of 100 nM induced up-regulation in the levels of all PCR products for the uPA system, as determined by densitometric analysis (Fig. 6*C*). Taken together, these findings strongly suggest that ET-1 up-regulated uPA system mRNA levels and promoted the concomitant elevation of protein secretion.

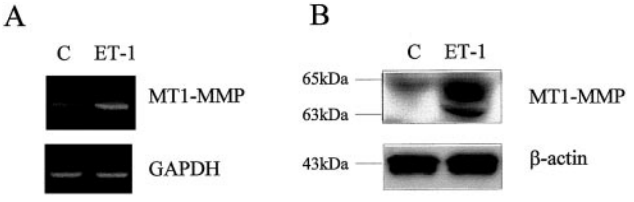

Fig. 4. Effect of ET-1 on the secretion and activation of MT1-MMP by ovarian cancer cells. *A*, effect of ET-1 on MT1-MMP mRNA expression in HEY cells. Data shown are PCR products for MT1-MMP (532 bp) and *GAPDH* (230 bp) of HEY cells grown for 8 h in the absence (*Lane C*) and presence of 100 nM ET-1 (*Lane ET-1*). *B*, cell extracts of HEY cells alone (*Lane C*) or treated with 100 nM ET-1 for 24 h (*Lane ET-1*) were tested for MT1-MMP (the 65-kDa latent form and the 63-kDa active form) by Western blotting. The relative amounts of transferred MT1-MMP proteins were quantified and normalized to the corresponding β-actin protein amounts.

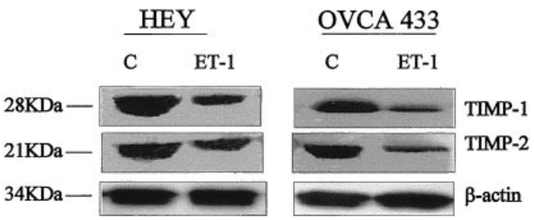

Fig. 5. Effect of ET-1 on the secretion of TIMPs by ovarian cancer cells. Conditioned media from HEY and OVCA 433 cells alone (*Lane C*) or with 100 nM ET-1 added (*Lane ET-1*) were tested for TIMP-1 (28 kDa) and TIMP-2 (21 kDa) by Western blotting. The relative amounts of transferred TIMPs were quantified and normalized to the corresponding β-actin protein amounts.

**Effect of ET-1 on Ovarian Carcinoma Cell Migration and Invasion.** The ability of ET-1 to consistently induce secretion and activation of tumor proteases led us to explore the effect of ET-1 on ovarian carcinoma motility and invasive capacity. Using the Boyden chamber assay to assess chemotactic mobility of HEY and OVCA 433 cells, we observed a significant dose-dependent increase in migration in the presence of 0.1–100 nM ET-1 (Fig. 7*A*). At a higher concentration of ET-1 (100 nM), we observed a 60% increase in the number of migrated cells. Matrigel-coated invasion chambers were used to examine the effect of ET-1 on *in vitro* ovarian cancer cell invasion. We observed that ET-1 markedly stimulated invasion of HEY and OVCA 433 cells in a dose-dependent fashion (Fig. 7*B*). At a higher concentration of ET-1 (100 nM), we observed maximal stimulation, corresponding to a 55% increase in the number of cells migrating through the Matrigel. Phenanthroline, used as a reference inhibitor of invasiveness, completely blocked the invasive capacity of OVCA 433 and HEY cells (data not shown). A selective $ET_AR$ antagonist, BQ123, was used to determine whether the $ET_AR$ was involved in the stimulation of ovarian cancer cell migration and invasion. ET-1-stimulated migration and invasion of both HEY and OVCA 433 cells was completely blocked by the addition of 1 $\mu$M BQ123 (Fig. 7, *A* and *B*). Ovarian cancer cell invasion induced by 10 mg/ml epidermal growth factor was not inhibited by the addition of 1 $\mu$M BQ123, indicating that the inhibitory effect induced by the $ET_AR$ antagonist was specific and was not the result of cytotoxicity (data not shown). To determine whether modulation of tumor protease activities had an impact on ET-1-induced ovarian cancer cell invasion, a potent chemical broad-spectrum MMPI, such as Ilomastat, was used. ET-1-stimulated invasion was reduced to control levels in the presence of 20 $\mu$M MMPI, providing evidence for the role of ET-1-induced MMP activity in ovarian cancer cell invasion (Fig. 7*C*). Taken together, these results demonstrate that ET-1 is able to induce tumor cell migration and MMP-dependent invasion through the $ET_AR$.

## DISCUSSION

Tumor progression to the metastatic phenotype is a complex process involving changes in cell adhesion and migration as well as degradation of the ECM (30). Overproduction of proteinases of the uPA and MMP families has previously been demonstrated in ovarian cancer cells (31); however, the regulation of each proteinase family remains unclear. Receptor-mediated induction of proteinases represents one possible mechanism linking the receptors to tumor progression and metastasis. Agonists binding to tyrosine kinase receptors, such as epidermal growth factor, basic fibroblast growth factor, transforming growth factor α and β, or to G protein-coupled receptors, such as lysophospha-

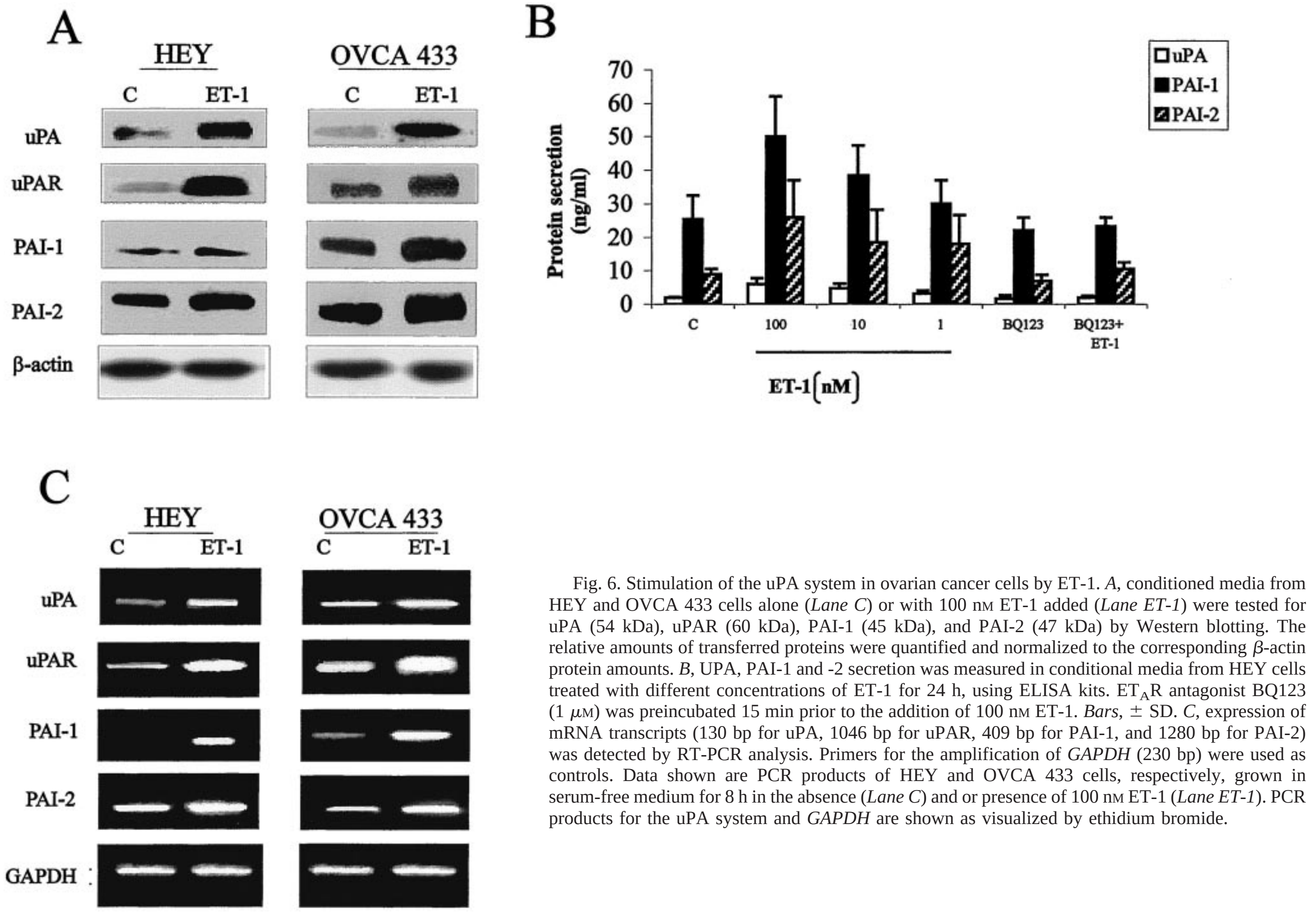

Fig. 6. Stimulation of the uPA system in ovarian cancer cells by ET-1. *A*, conditioned media from HEY and OVCA 433 cells alone (*Lane C*) or with 100 nM ET-1 added (*Lane ET-1*) were tested for uPA (54 kDa), uPAR (60 kDa), PAI-1 (45 kDa), and PAI-2 (47 kDa) by Western blotting. The relative amounts of transferred proteins were quantified and normalized to the corresponding β-actin protein amounts. *B*, UPA, PAI-1 and -2 secretion was measured in conditional media from HEY cells treated with different concentrations of ET-1 for 24 h, using ELISA kits. $ET_AR$ antagonist BQ123 (1 $\mu$M) was preincubated 15 min prior to the addition of 100 nM ET-1. *Bars*, ± SD. *C*, expression of mRNA transcripts (130 bp for uPA, 1046 bp for uPAR, 409 bp for PAI-1, and 1280 bp for PAI-2) was detected by RT-PCR analysis. Primers for the amplification of *GAPDH* (230 bp) were used as controls. Data shown are PCR products of HEY and OVCA 433 cells, respectively, grown in serum-free medium for 8 h in the absence (*Lane C*) and or presence of 100 nM ET-1 (*Lane ET-1*). PCR products for the uPA system and *GAPDH* are shown as visualized by ethidium bromide.

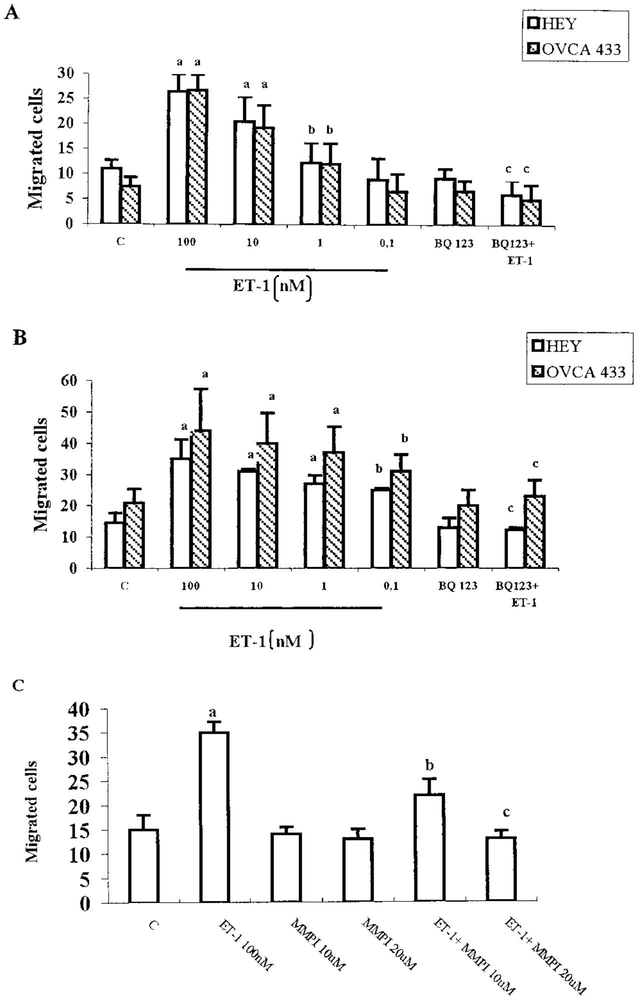

Fig. 7. Effect of ET-1 on ovarian carcinoma cell migration and invasion. *Columns* indicated by a *C* are controls. *A*, HEY and OVCA 433 cells ($5 \times 10^5$ cells/ml) pretreated for 15 min with 1 $\mu$M BQ123, an $ET_AR$ antagonist, were placed in the upper compartment of a 48-well Boyden chamber. Different doses of ET-1 were added in the lower wells. Cells migrating through the filter were counted after 4 h. Data are expressed as the number of migrated cells in 10 high-power fields and are means of results from three experiments, each performed in triplicate. *Bars*, ± SD. *a*, $P \leq 0.001$ compared with control; *b*, $P \leq 0.01$ compared with 100 nM ET-1; *c*, $P \leq 0.001$ compared with 100 nM ET-1. *B*, HEY and OVCA 433 cells ($5 \times 10^5$ cells/ml) pretreated for 15 min with 1 $\mu$M BQ123, an $ET_AR$ antagonist, were seeded on Matrigel layer in a Boyden chamber assay. Different doses of ET-1 were added in the lower wells. Cells migrating through the filter were counted after 6 h. *a*, $P \leq 0.001$ compared with control; *b*, $P \leq 0.02$ compared with 100 nM ET-1; *c*, $P \leq 0.001$ compared with 100 nM ET-1. *C*, HEY cells were seeded on a Matrigel layer in a Boyden chamber assay in the absence or presence of ET-1 (100 nM) or MMPI (10 or 20 $\mu$M), as indicated. Cells migrating through the filter were counted after 6 h. *a*, $P \leq 0.001$ compared with control; *b*, $P \leq 0.01$ compared with 100 nM ET-1; *c*, $P \leq 0.001$ compared with 100 nM ET-1.

tidic acid, act as positive regulators on tumor invasion, stimulating tumor-associated proteinases and tumor cell motility (32–35).

Our previous studies demonstrated that the potent mitogen ET-1 is overexpressed in primary and metastatic ovarian carcinoma and is markedly elevated in ascites of patients with ovarian cancer, suggesting that this peptide could participate in the progression of ovarian carcinoma (4, 8). This study demonstrates that ET-1 consistently induces the activity of multiple metastasis-related proteinases at several levels: mRNA transcription, zymogen secretion, and proenzyme activation. Because members of the MMP family of proteinases are secreted as inactive zymogens, requiring cleavage of the $NH_2$-terminal propeptide to gain full activity, zymogen activation is postulated to be an important factor for invasion (36, 37). We demonstrated that ET-1 induces activation of MMP-2, -9, -3, -7, and -13, leading to the degradation of all known ECM components and thus enabling malignant ovarian carcinoma cells to break down basement membrane. In addition to soluble MMPs, ET-1 induces activation of MT1-MMP. The up-regulation of this protein was concomitant with the overproduction and activation of MMP-2 and -13. All of these results indicate that in ovarian cancer cells, ET-1 could participate in the coordinated secretion and activation of different MMPs and that the combination of this active enzymes could result in rapid degradation of ECM (38, 39). Overexpression of TIMPs by cancer cells has been shown to reduce their invasive and metastatic capacity by down-regulating the activity of MMPs (40, 41). The present results demonstrating the concomitant production of TIMP-1 and -2 associated with the up-regulation of MMPs induced by ET-1 in both ovarian carcinoma cell lines strongly support the pivotal role of ET-1 in regulation of the net MMP activity.

Recent evidence suggests that the uPA system is involved in tumor cell migration and invasion by different mechanisms, including interactions between uPA, uPAR, PAI-1/-2, ECM proteins, integrins, endocytosis receptors, and growth factors (23, 42). These interactions seem to allow temporal and spatial reorganization of the uPA system during cell migration and selective degradation of ECM during invasion. As in other cancers, the presence of the uPA correlates with the aggressiveness of ovarian cancer (43), and a positive correlation was observed between higher uPA levels and increased lymphonodal metastases, increased ascites, and higher grade tumors (44). Our results indicate the ability of ET-1 to enhance cancer cells by concomitant stimulation of the production and secretion of uPA and uPAR. Fibrinolysis by plasmin is controlled by several inhibitors, such as PAIs. Nevertheless, the results of studies of the significance of PAI in various cancers have been conflicting. Recent studies indicate that PAI-1 acts as a cell detachment factor, explaining in part the correlation between its increased levels and poor prognosis and the reduced tumor invasion and vascularization observed in PAI-1-deficient mice (45). The present results demonstrate that ET-1 stimulates PAI-1 and -2 synthesis and secretion in ovarian carcinoma cells, leading to increased degradation of ECM, which might lead to tumor growth and metastasis.

MMPs and the uPA system represent two families of proteinases that are known to function at multiple stages of tumor progression, affecting tumor growth, neovascularization, intravasion/extravasion, and metastasis. Recent studies focusing on the different roles of proteinases demonstrated the cooperation between MMPs and uPA. It is likely that some of these proteinases serve redundant functions, so that activation of multiple pathways might be required to elevate proteolytic activity (46–48). Some MMPs, such as MMP-3, are activated intracellularly by serine proteinases. Thereafter, in concert, plasmin and MMP-3 activate other MMPs (MMP-13, -9, and -7). Latent MMP-2 and -13 are activated by MT1-MMP. Therefore, two membrane-associated proteolytic systems, MT-MMPs and the uPA/plasmin system, can initiate the activation of MMPs that in turn can interact with each other and with plasmin in a complex cascade. Our results indicate that the proprotein ET-1-dependent activation of metalloproteinases may involve an uPA/plasminogen-independent proteolytic processing system. On the other hand, our studies have shown that ET-1 increased the uPA system in ovarian carcinoma cell lines, suggesting a parallel co-regulation of the enzymatic activities of MMPs. The interactions among different enzymatic systems, their receptors, activators, and inhibitors, and the action of ET-1 on their synthesis, secretion, and activation represent one possible mechanism linking ET-1 to ovarian carcinoma progression and metastasis.

In the two ovarian carcinoma cell lines examined, co-induction of the uPA system and MMPs by ET-1 produced the highest invasive potential of tumor cells through the Matrigel, suggesting that ET-1 plays a relevant role in ovarian tumor invasion. Interestingly, we found that addition of a specific $ET_AR$ antagonist, BQ123, blocked the ET-1-induced activation of MMPs and the uPA system. Furthermore, BQ123 blocked ET-1-

induced migration and invasion in both ovarian carcinoma cell lines, indicating that ET-1 contributes to the metastatic progression of ovarian carcinoma cells via activation of tumor proteases and the subsequent increase in cell migration and invasion through $ET_AR$ binding. We previously reported that in ovarian carcinoma cells, ET-1 stimulated FAK and paxillin phosphorylation through $ET_AR$ binding (5, 6). These effects on FAK phosphorylation directly correlate with ovarian cancer cell migration and invasion induced by ET-1 and suggest that $ET_AR$ antagonists can inhibit cell migration and possibly other FAK-associated processes that contribute to invasion and metastasis in ovarian carcinoma cells.

In conclusion, among the several factors that contribute to ovarian tumor invasiveness, we identified ET-1 as a tumor protease activity regulator. The autocrine production of ET-1 by ovarian cancer cells and the selective binding to $ET_AR$, mainly expressed on ovarian cancer cells, lead to an increased potential for tumor cell proliferation and cellular invasive activity. Thus, the therapeutic use of specific $ET_AR$ antagonists may provide an additional approach to the treatment of ovarian carcinoma through $ET_AR$ blockade, which may reduce tumor growth and invasion.

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