Expression of Endothelin 1 and Endothelin A Receptor in Ovarian Carcinoma: Evidence for an Autocrine Role in Tumor Growth

Anna Bagnato, Debora Salani, Valeriana Di Castro, Jinshyun R. Wu-Wong, Raffaele Tecce, Maria Rita Nicotra, Aldo Venuti, and Pier Giorgio Natali

ABSTRACT

In the present study, we have investigated the expression of endothelin 1 (ET-1) and the ET$_A$ receptor (ET$_A$R) and ET$_B$ receptor (ET$_B$R) in primary (n = 30) and metastatic (n = 8) ovarian carcinomas and their involvement in tumor growth. By reverse transcription-PCR and Northern blot analysis, we detected ET-1 mRNA in 90% of primary and 100% of metastatic ovarian carcinomas. ET-1 mRNA expression was significantly higher in tumors than in normal ovarian tissues (n = 12; P < 0.01). ET$_A$R mRNA was also detected in 84% of the carcinomas examined, whereas ET$_B$R mRNA was expressed in 50% of the tumors. The in vivo presence of mature ET-1 and ET$_A$R was confirmed by immunohistochemistry, demonstrating a higher expression in primary and metastatic cells. Ten primary cultures of ovarian tumors secreted ET-1 and were positive for ET-1 and ET$_A$R mRNA, whereas only 40% expressed ET$_B$R mRNA. Radioligand binding studies showed that ET-1-producing cells also expressed functional ET$_A$R, whereas no specific ET$_B$R could be demonstrated. ET-1 stimulated dose-dependent $[^3]$H]thymidine incorporation and enhanced the mitogenic effect of epidermal growth factor. The ET$_A$R-selective antagonist BQ 123 strongly inhibited ET-1-stimulated growth and substantially reduced the basal growth rate of unstimulated cells, whereas the ET$_B$R-selective antagonist BQ 788 had no effect. In conclusion, the present data demonstrate a novel mechanism in the growth control of ovarian carcinoma in vivo mediated by the ET-1 autocrine loop that selectively occurs via the ET$_A$R.

INTRODUCTION

An extensive characterization of growth factors and cognate receptors that regulate the growth of malignant ovarian epithelium is mandatory to the development of more effective approaches for the control of ovarian cancer, which represents the most fatal of gynecological malignancies (1). Proliferative advantage can be achieved by changes in growth factor production or receptor expression that circumvent the normal mechanisms of cell growth (2–4). In an attempt to characterize the growth factors involved in the malignant ovarian cancer phenotype, we identified ET-1 as an important autocrine growth factor in vitro. The ETs (ET-1, ET-2, and ET-3) are 21-amino acid peptides that were originally identified as potent vasoconstrictors produced in vascular endothelial cells (5). ETs bind at least two subtypes of G protein-coupled receptors: (a) a selective ET$_A$R that binds ET-1 and ET-2 with high affinity and ET-3 with low affinity; and (b) a nonselective ET$_B$R that binds all ET isopeptides with equal affinity (6). In addition to the well known activities of ETs in the cardiovascular system, ET-1 has been shown to represent a potent mitogen in vascular and nonvascular cells and to stimulate proto-oncogene expression. As a growth-regulatory peptide, ET-1 directly influences cell proliferation and can also act synergistically with growth factors that have been implicated in cancer progression (7, 8). Furthermore, the expression and release of ET-1 and its receptors in human cancer cell lines and human tumors suggest a potential role for ET-1 in tumoral growth promotion or maintenance through an autocrine or paracrine mechanism (9, 10). Recently, we have demonstrated that ET-1 is produced by human ovarian cancer cell lines and acts through ET$_A$R to stimulate proliferative responses in vitro (11). In these cells, ET-1 rapidly induces calcium signaling, activates mitogen-activated protein kinase and p125FAK through a G$_{13}$ protein, and enhances the expression of immediate early response genes (12). In the present report, we tested the hypothesis that ET-1 overexpression has a pathophysiological function in ovarian carcinoma, acting as an autocrine growth factor through specific ET receptors, which could be targeted for therapy. Therefore, we tested ET-1 production at the mRNA and protein levels in many primary and metastatic human ovarian tumors and quantified the specific $^1$H$^1$-labeled ET-1 binding sites in primary ovarian carcinoma cell cultures. Studies of the mechanism of autonomous growth inhibition are necessary to develop new treatment strategies that are clinically applicable. Therefore, in the present study, we evaluated the receptor subtype mediating the mitogenic effects of ET-1 on tumor cell proliferation and investigated whether specific antagonists inhibit the autocrine growth of ovarian cancer cells.

MATERIALS AND METHODS

Tissues and Cell Lines. Tumor specimens were obtained with informed consent from 30 patients (age range, 27–67 years) undergoing surgery for ovarian carcinomas at the Regina Elena Cancer Institute. Primary tumors included 10 adenocarcinomas and 9 serous, 5 endometrioid, 6 mucinous, and 8 omental metastases derived from 5 adenocarcinomas and 3 serous adenocarcinomas. Tissue samples were immediately snap-frozen in liquid nitrogen and stored at −80°C for RNA extraction and immunohistochemical studies. Fixed sections were either used immediately or kept frozen at −20°C with no loss of immunoreactivity. Six primary ovarian carcinoma cells (PMOV1–PMOV6) were derived from ascitic fluid that was freshly obtained from six additional untreated patients. Briefly, cells were harvested by centrifugation at 200 × g for 5 min at room temperature, resuspended in Dulbecco’s PBS, and then centrifuged through Ficoll-Histopaque 1077 (Sigma, St. Louis, MO). Interface cells were washed in culture medium, and 5 × 10$^6$ viable cells were seeded in 75-cm$^2$ culture flasks. Two primary (OPV1 and OPV2) and two metastatic (OVMI and OVM2) ovarian carcinoma cells were derived from solid tumor tissue. A single cell suspension was obtained by mechanical dispersion and filtered through a 30-μm polyester mesh in RPMI 1640 (Whitaker Bioproducts, Inc., Walkersville, MD) containing 1% penicillin-streptomycin and 10% FCS. All experiments were conducted between the first and second in vitro passage. The purity of primary cultures was assessed by immunophenotyping with a panel of monoclonal Abs recognizing ovarian tumor-associated antigens by the alkaline phosphatase-peroxidase-antiperoxidase method. Normal human ovarian tissues were obtained after hysterectomy for non-neoplastic diseases. The SKBr3 cell line was obtained from the American Type Culture Collection (Rockville, MD). The human ovarian
carcinoma cell line OVCA 433 and the melanoma cell line Mel 201 were generous gifts from Dr. Giovanni Scambia (Catholic University School of Medicine, Rome, Italy) and Dr. Michele Maio (CRO, Aviano, Italy), respectively. All cell lines were maintained in RPMI 1640 at 37°C in 5% CO2, 95% air. The OVCA 433 cell line was grown in DMEM.

**RNA Extraction and RT-PCR.** Total RNA was isolated from ovarian cancers, normal ovaries, SKBr3, and Mel 201 by the guanidinium thiocyanate-phenol chloroform extraction method. RT-PCR was performed with a GeneAmp RNA PCR kit (Perkin-Elmer Corp., Norwalk, CT) according to the manufacturer’s instructions. Briefly, 1 μg of RNA was reverse-transcribed using the antisense primer. The primer sets were as follows: (a) ET-1, 5'-TGCTCCTCTGTCCTTGATGAGTTAAGAG-3' and 5'-GGTGCACATAACCCGTCTGAGGGTTCT-3'; (b) ET-2, 5'-CACTGGTTGATGTGTAATC-3' and 5'-GAGATCAATGCAACATAGG-3'; and (c) ETβ, 5'-TCACACGCTGTGTTCTGCC-3' and 5'-ACTGAAATGGCACAACATTT-3' (13). GAPDH was used as an internal control. The semiquantitative analysis was done essentially as described by Rieckmann et al. (14). The amplified products were analyzed in a 3% agarose gel and visualized by ethidium bromide. Densitometric scanning was performed with a Mystek MPS-6000CX apparatus, and data were analyzed with Phoretix 1D software and normalized to those of GAPDH. The mRNA values were expressed as relative units calculated according to the following formula: density of the amplified products at 20, 25, 30, and 35 cycles. Thirteen-cycle conditions for linear amplification were determined by a semiquantitative assay of the amplified products at 20, 25, 30, and 35 cycles. The intra- and interassay variations were 8 and 13%, respectively.

**Northern RNA Blot Analysis.** RNA (20 μg) from ovarian carcinomas, metastases, OVCA 433, and SKBr3 was electrophoresed on 1% agarose-0.7% formaldehyde gels, transferred to nylon membranes by capillary blotting, and hybridized with a 462-bp specific primer for ET-1. Subsequently, rehybridization was performed with the human GAPDH probe. The filters were stripped and rehybridized with the human GAPDH probe.

**Immunohistochemistry.** Immunoperoxidase labeling of ET-1 or ET A R was carried out on acetone-fixed 4-μm frozen tissue sections stored at −20°C. Two Abs for ET-1 were used: (a) a rabbit polyclonal Ab (Peninsula Laboratories, Belmont, CA); and (b) a mouse monoclonal Ab (clone TR.ET.48.5; Affinity Bioreagents, Golden, CO; cross-reactivity for ET-3, 7 and 2%, respectively). The primary Ab for ET-R was rabbit polyclonal Ab raised against a decapeptide (DNPERYSTNL) of the extracellular NH₂-terminal domain of ET A R (Abbott Laboratories). Sections were preincubated in goat or horse serum, followed by incubation with polyclonal or monoclonal ET-1 Abs (1:200 and 1:200) or polyclonal ET-R Ab (1:200) overnight at 4°C. Different Ab dilutions ranging from 1:20 to 1:200 were assayed to optimize the signal:background ratio. After the primary Ab, biotinylated anti-IgG (Vector Laboratories, Burlingame, CA) was applied, followed by detection using the avidin-biotin complex method. The enzymatic reaction was detected using 3-aminobenzidine and 3-ethylcarbazole as the chromogen. Nuclear counterstaining was done with Mayer’s hematoxylin. To assure specificity, the primary Ab was preabsorbed with synthetic ET-1 (10−8 M), omitted, or substituted with nonspecific IgG (negative control in all cases). The immunoreactivity of large ovarian vessels was used as a positive ET-1 stain.

**Extraction of ET-1.** The conditioned media were acidified with 0.1% trifluoroacetic acid, and the supernatant was applied to Sep-Pak C18 cartridges (Water Associates, Milford, MA). After an elution with 2 ml of 60% acetonitrile/0.1% trifluoroacetic acid, the lyophilized material was analyzed for ET-1 content by RIA. The recovery of synthetic ET-1 through the extraction procedure was 78%.

**RIA.** The lyophilized ET-1 samples were reconstituted in an assay buffer consisting of 0.1 M phosphate buffer (pH 7.4) containing 0.05 mM NaCl, 0.1% BSA, 0.1% Triton X-100, and 0.01% sodium azide. Samples were equilibrated for 24 h at 4°C with specific ET-1 Ab (Peninsula Laboratories), followed by the addition of 20,000 cpm of 125I-labeled ET-1 (2,200 Ci/mmol; DuPont New England Nuclear Research Products, Wilmington, DE). After incubation for an additional 48 h, free and Ab-bound tracers were separated by the addition of sheep antiserum to rabbit immunoglobulin and an additional 2-h incubation at room temperature. The cross-reactivity of the antisera for ET-related peptides (versus ET-1 = 100%) was: ET-2, 7%; ET-3, 7%; and big ET-1, 17%. The sensitivity of ET-1 RIA was 1.9 pg/tube, and the 50% intercept was 36 pg/tube. The intra- and interassay variations were 8 and 13%, respectively.

**Binding of 125I-labeled ET-1.** Ovarian carcinoma cells were cultured in 6-well plates until confluent (5×104 cells/well) and then serum-starved for 24 h. After washing with HBSS supplemented with 0.2% BSA and bacitracin (100 mg/ml; Sigma), cells were incubated at 25°C for 60 min with increasing concentrations of the radioactive tracer in the presence or absence of an excess (10−4 M) of unlabeled ET-1 (Peninsula Laboratories). In binding inhibition studies, cells were incubated with tracer and ET-related peptides including ET-1, ET-2, ET-3, and Sgc (Peninsula Laboratories) as well as an unrelated peptide, bombesin (Sigma). At the end of the incubation period, cells were washed three times with cold PBS containing 0.2% BSA, solubilized with 1 M sodium hydroxide, and analyzed by bound radioactivity in a γ-spectrometer. ET-1 receptor affinity and the number of binding sites were determined by Scatchard analysis using the LIGAND program. Data points represent the mean of three experiments, each of which was performed in triplicate.

**Thymidine Incorporation Assay.** Cells were seeded in 96-well plates at approximately 80% confluence (2×104 cells/well) and incubated in serum-free medium for 24 h to induce quiescence. Mitogenic molecules were then added, and after 18 h, when the cells were confluent, 1 μCi of [methyl-3H]thymidine (6.7 Ci/mmol; DuPont New England Nuclear Research Products) was added to each well. Six h later, culture media were removed, and cells were washed three times with PBS, treated with 10% trichloroacetic acid for 15 min, washed twice with 100% ethanol, and solubilized in 0.4 N sodium hydroxide. The cell-associated radioactivity was then determined by liquid scintillation counting. Responses to all agents were assessed in sextuplicate, and results were expressed as the means of three separate experiments.

**Cell Proliferation Assay.** Tumor cells were seeded at 5×104 cells/well in 12-well plates, cultured for 24 h, and then incubated in 2 ml of 1% FCS in the

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RESULTS

Detection of ET-1, ET_{A}R, and ET_{B}R mRNAs by RT-PCR and Northern Blot Analysis. Using RT-PCR and Northern blot analysis, we detected ET-1 mRNA in primary (n = 30) and metastatic (n = 8) ovarian carcinomas as well as in normal ovarian tissues (n = 12). An autoradiograph of Northern blot analysis using the probe for ET-1 mRNA is shown in Fig. 1. In all primary tumors and metastases tested, we detected a 2.3-kb band, demonstrating the presence of ET-1 mRNA in ovarian carcinomas. RT-PCR-amplified cDNA fragments for ET-1, ET_{A}R, and ET_{B}R that were detectable in normal ovarian tissue and in ovarian carcinomas and metastases of different histotypes are shown in Fig. 2. The amplified 462-bp ET-1, 367-bp ET_{A}R, and 529-bp ET_{B}R PCR cDNA fragments were of the predicted molecular size. All tissues showed a positive signal for GAPDH mRNA (data not shown), demonstrating that negative results were not due to a degradation of mRNA. The specificity of RT-PCR amplification products for ET-1, ET_{A}R, and ET_{B}R was confirmed by Southern blot (data not shown), revealing the same pattern and intensity of the bands and showing an absence of cross-reactivity. The amplified signals for ET-1 and both receptor subtypes were detected in all normal tissues (12 of 12 tissues), although the ET-1 and ET_{B}R signals were weaker than the signal generated from the ovarian carcinomas. In our series of 30 ovarian carcinomas, 90% (27 of 30 carcinomas) were positive for ET-1 mRNA, and 84% (25 of 30 carcinomas) were positive for ET_{A}R mRNA, whereas only 50% (15 of 30 carcinomas) were positive for ET_{B}R mRNA. Because a notable difference in the expression of ET-1 was evident between normal and ovarian carcinoma tissues, we determined the level of ET-1 expression in 30 ovarian carcinomas and 12 normal tissues by semiquantitative RT-PCR on cDNA derived from total RNA. To quantitate the levels of ET-1 expression in both neoplastic and normal tissues, amplification efficiency had to be comparable. All primer pairs showed a nearly identical amplification efficiency, as indicated by the parallel regression lines in the linear amplification range of the curves (data not shown). All measurements were normalized on GAPDH expression to take differences in template integrity into account. The semiquantitative analysis of the amplified products showed a significantly increased expression of ET-1 mRNA in human ovarian carcinomas compared to that in normal ovaries (P < 0.01; Fig. 3).

Expression of ET-1 and ET_{A}R in Primary and Metastatic Ovarian Carcinomas. In 30 ovarian carcinomas, the majority of which were positive for the presence of ET-1 and ET_{A}R mRNA, we also studied the protein expression of ET-1 and ET_{A}R. The presence of mature ET-1 was found in 84% of ovarian carcinomas examined, including primary and metastatic lesions of different ovarian cancer histotypes (Fig. 4). ET-1 was localized in the cytoplasm of tumor and metastatic cells and in cells lining the tumor-feeding vessels, whereas ET-1 immunoreactivity in normal ovarian tissue was weak and confined to vascular elements (data not shown). Specific immunostaining identifying ET_{A}R was seen over the majority (89%) of cell cytoplasm in all areas of the tumors and metastases independent of the histological type and in some blood vessels adjacent to neoplastic cells (Fig. 5, A–D). These conclusions are supported by negative immunoreactivity after immunoabsorption, omission of the primary Ab, or substitution of a nonspecific IgG. Endothelial cells were a consistent positive internal control. This study found for the first time that specific ET-1 and ET_{A}R immunoreactivity was present in human ovarian carcinoma in vivo, and immunostaining for ET-1 and its receptor in same tissue cells raises the possibility of autocrine stimulation by coexpression of ET-1 and ET_{A}R.

Analysis of mRNA Expression by RT-PCR and Analysis of ET-1 Production in Primary Cultures of Ovarian Carcinoma Cells. Table 1 summarizes the expression of ET-1, ET_{A}R, and ET_{B}R mRNAs as detected by RT-PCR in 10 primary cultures of ovarian carcinoma cells isolated from ascites samples (PMOV1–PMOV6) and solid primary (OVP1 and OVP2) and metastatic (OVM1 and OVM2) tumors. All ovarian carcinoma-derived cell lines were positive for ET-1 and ET_{A}R mRNA, whereas only 40% were positive for ET_{B}R mRNA. The levels of ET-1 mRNA in different primary ovarian cancer cell lines are mirrored in their ET-1 secretion. All of these cell lines release ET-1 in the culture media as a function of time over a 48-h period.
period, with detectable production as early as 6 h after the replacement of the culture medium and maximum production at 24 h, with no further increases at 48 h (data not shown). The time-dependent release of immunoreactive ET-1 in the conditioned media from these cells is at concentrations ranging from 100–800 fmol/10^6 cells, which is higher than the biologically effective range for this peptide (15, 16).

125I-labeled ET-1 Binding in Primary and Metastatic Cultured Ovarian Carcinoma Cells. The ET receptor saturation studies demonstrated that 125I-labeled ET-1 was saturable and of high affinity in all four of the cultures of purified ovarian cancer cells examined. The approximate K_d and B_max ranged from 0.8 –1.2 nM and 18,504 –22,000 sites/cell, respectively, with no differences in receptor number between the primary and metastatic ovarian cancer cells (Table 1). Competition for 125I-labeled ET-1 binding to primary ovarian cancer cells (OVP1) by unlabeled ET-1, ET-2, ET-3, and S6c, a selective ETA R agonist, is shown in Fig. 6. Whereas the IC_{so} of ET-1 and ET-2 were 150 and 250 pm, respectively, the IC_{so} of ET-3 was 125 nm, and the IC_{so} of S6c was >1000 nm. The specificity of binding was indicated by the inability of the unlabeled peptide bombesin to inhibit the binding of 125I-labeled ET-1. Although ETA R mRNA can be detected in OVP1 cells, these data, which show differences in the displacement capacity of ET-1 and ET-2 compared with ET-3 and S6c, suggest that the predominant functional receptor present in these cells was the ETA subtype.

Proliferative Effects of ET-1 on Primary Cultures of Ovarian Carcinoma Cells. In all primary cultures of ovarian carcinomas (n = 10), we found that increasing concentrations (10^{-9} to 10^{-7}) of ET-1 induced a marked dose-dependent increase in [3H]thymidine incorporation in serum-free medium. In this study, ET-1 (100 nm) was equipotent with EGF (10 ng/ml) in stimulating mitogenesis. When quiescent primary ovarian cancer cells were treated with 100 nm ET-1 in the presence of 10 ng/ml EGF, additive thymidine responses were observed, as shown for PMOV2 cells in Fig. 7. To examine whether the mitogenic action of ET-1 was mediated by ETA R, we coincubated the cells with ET-1 and the ETA R antagonist BQ 123. The stimulatory action of 100 nm ET-1 on [3H]thymidine incorporation by primary ovarian carcinoma cell cultures was completely blocked in the presence of 1 μM BQ 123. In the same experiment, 100 nm of selective ETA agonists ET-3 and S6c had no mitogenic effect (Fig. 7B). Taken together, these data confirm that mitogenic signaling by ET-1 is mediated mainly by the ETA R subtype.

Inhibitory Effects of ETA R Antagonist on ET-1-mediated Cell Growth. In addition to defining the receptor-mediated action of ET-1 on thymidine incorporation by the ovarian tumor cells, we also determined the extent to which such growth responses were manifested as an increase in cell proliferation. To determine whether endogenous ET-1 produced by the primary tumor cells could act as an autocrine growth factor, primary ovarian carcinoma cell cultures (PMOV2 and OVP1 cells) were incubated for up to 4 days in the absence or presence of ETA R and ETA R antagonists BQ 123 and BQ 788, respectively. In both ovarian cell lines, the spontaneous growth was significantly inhibited in the presence of ETA R antagonist BQ 123. The addition of ETA R antagonist caused only a slight decrease in the basal growth rate of the cells, even in OVP1 cells, which expressed mRNA

Fig. 4. Expression of ET-1 protein in sections of different histological types of ovarian carcinomas. Immunohistochemical localization is shown using a monoclonal Ab for ET-1 as detected by immunoperoxidase. ET-1 is expressed in serous (grade II; A), mucous (grade III; B), and endometrial (grade II; C) ovarian primary adenocarcinomas and in metastatic serous adenocarcinoma (grade III; D). All sections are counterstained with Mayer’s hematoxylin. Magnification, ×25.
for both ET receptors, demonstrating that ET-1 acts as an autocrine regulator of ovarian carcinoma cell proliferation only through ET\textsubscript{A}R (Fig. 8). These results are consistent with those obtained in the competitive displacement binding studies, confirming that the predominant functional receptor present in these cells was the ET\textsubscript{A}R subtype.

**DISCUSSION**

An abnormal expression of different growth factors and/or their receptors has been demonstrated to be involved in the pathogenesis of ovarian carcinoma (3, 4, 17, 18). Elevated expression of ET-1 has been reported in many tumors (9, 19, 20), and ET-1 expression in ovarian carcinoma cell lines has been previously demonstrated in vitro (11, 12, 21). The results of this study indicate for the first time that the expression of ET-1 mRNA is significantly increased in the majority of ovarian carcinomas compared with normal ovarian tissue. Using an ET-1-specific assay, we have confirmed that ET-1 produced by primary cultures of ovarian carcinoma accumulates in culture media to biologically active levels (15, 16). Elevated ET-1 expression was also documented immunohistochemically in primary and metastatic tumors. RT-PCR revealed that all ovarian carcinomas and primary ovarian cancer cell lines that express ET-1 coexpress mRNA encoding ET\textsubscript{A}R, whereas the expression of ET\textsubscript{B}R mRNA in malignant tissues was reduced. Competitive displacement experiments demonstrated

<table>
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<tr>
<th>Primary cells</th>
<th>Stage</th>
<th>Tumor type</th>
<th>PCR products</th>
<th>ET-1 (pmol/10\textsuperscript{6} cells)</th>
<th>RIA\textsuperscript{b}</th>
<th>Binding constants</th>
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<tr>
<td>PMOV1</td>
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<td>PMOV6</td>
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<td>ND</td>
<td>B\textsubscript{max}</td>
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</table>

\textsuperscript{a} \#1, presence of product; \#2, absence of product; ND, result not determined.

\textsuperscript{b} ET-1 concentrations in conditioned media collected after a 48-h culture are given as the mean ± SD of three determinations.

Fig. 5. Expression of ET\textsubscript{A}R protein in sections of different histological types of ovarian carcinomas. Immunohistochemical localization is shown using an antipeptide Ab directed against ET\textsubscript{A}R in serous (grade II; A), mucous (grade III; B), and endometrioid (grade III; C) ovarian primary adenocarcinomas and in metastatic serous adenocarcinoma (grade III; D). All sections are counterstained with Mayer’s hematoxylin. Magnification: A, B, and D, \times 25; C, \times 40.
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Fig. 6. Concentration-dependent inhibition of 125I-labeled ET-1 binding by ET-1 and related peptides. Primary ovarian carcinoma cells (OVP1) were incubated for 60 min at 25°C in the presence of 40 pM 125I-labeled ET-1 and increasing concentrations of ET-1 ( ), ET-2 ( ), ET-3 ( ), S6c ( ), and bombesin ( ). Binding is expressed as a percentage of the bound radioligand in the absence of unlabeled peptide; each point is the mean of data from three experiments. Inset, Scatchard plot analysis of 125I-labeled ET-1 saturation binding data to OVP1.

Fig. 7. Stimulatory actions of ET-1 and EGF on [3H]thymidine incorporation. A, quiescent ovarian carcinoma cells in primary culture (2 x 10⁴ PMOV2 cells) were treated with ET-1 or EGF at the indicated concentrations for 24 h before measuring [3H]thymidine incorporation as described in "Materials and Methods." B, effects of ET-1 receptor agonists and antagonist on ET-1-stimulated incorporation of [3H]thymidine. Quiescent PMOV2 cells were treated with ETₐR agonists ET-3 and S6c or with an ETₐR antagonist in the absence or presence of 100 nM ET-1. Bars, mean ± SD of data from five independent experiments, each of which was performed in sextuplicate. *, P < 0.05; **, P < 0.01.

that the ETₐR subtype is the dominant functional receptor present in ovarian carcinoma cells producing ET-1, and that no specific ETₐ binding sites could be detected. We further characterized the expression of the ET receptor in ovarian carcinomas by immunohistochemistry. The intense in vivo immunostaining signals for ETₐR, for which ET-1 is the most important physiological ligand, were localized in tumors and metastases and on some blood vessels adjacent to nests of tumor cells. These results raise the possibility that ET-1 may act as a growth factor by autocrine or paracrine mechanisms on receptor-expressing tumor cells. In all primary cultures of ovarian carcinoma cells, ET-1 promotes tumor cell proliferation. Furthermore, this study suggests that ET-1 is one of the essential autocrine growth factors involved in the growth control of ovarian cancer, as demonstrated by the decrease of cell proliferation in the presence of the selective ETₐR antagonist. Early evidence for an autocrine action of ET-1 on tumor cell growth was described in two epithelial carcinoma cell lines derived from the human cervix (HeLa) and larynx (Hep-2; Ref. 10). This finding was confirmed in other human tumors including meningiomas (22), astrocytomas (23), and prostate adenocarcinoma (24–26). Interestingly, in all of these instances, the normal counterparts express mainly the ETₐR subtype, whereas in primary and metastatic tumor cells, ETₐR is likely to become the predominant form. The specific functional consequences of the reduced ETₐR expression in human cancer cells are unknown. It has been proposed that ETₐR exerts a variety of compensatory activities, including ET-1 clearance, inhibition of ET-1 secretion, and activation of signaling transduction pathways that counterregulate ET-1. On the contrary, the major function of ETₐR seems to be linked to mitogenic signal transduction (27, 28), rather than the clearance of ET-1 (29). Moreover, the ETₐR-mediated mitogenic and additive proliferative effect of ET-1 may be enhanced by methylation inactivation of the ETₐR gene and the loss of its unique negative regulatory functions (26). In the present study, RT-PCR and binding studies revealed that ovarian carcinoma-derived cells functionally express ETₐR but not the ETₐR, supporting the hypothesis that the expression and function of ETₐR or ETₐR, in malignant tissues, as in benign and normal tissues, may depend on the prevailing pathophysiological conditions. In this respect, ETₐR expression might represent a mechanism relevant to tumor proliferation.

Human ovary expresses both subtypes of ET receptors (30), which are localized mainly in the blood vessels, indicating that the ET system is involved in the regulation of ovarian blood flow and not in steroidogenesis (31). Because ET-1 is a potent mitogen for vascular smooth muscle cells as well as for endothelial cells and is produced by neoplastic cells, it has been suggested that this peptide could stimulate angiogenesis. This has been shown to be the case in well-vascularized brain tumors such as gliomas or astrocytomas, in which the presence of ET-1 correlates with tumor vascularity and malignancy (32). Recent studies demonstrated that ET-1, predominantly through ETₐR, stimulated the synthesis of vascular endothelial growth factor in vascular smooth muscle cells and induced endothelial cell proliferation and invasion, indicating a novel mechanism by which ET-1 could modulate angiogenesis (33). These data suggest that the tumor-promoting effect of ET-1 could also be mediated through a paracrine pathway involving direct angiogenic effects on vascular smooth muscle and endothelial cells and/or through the stimulation of vascular endothelial growth factor, which is involved in ovarian tumor angiogenesis (18). The immunoreactivity of ETₐR detected in this study,
tumors renews interest in the possibility of restoring growth control to ovarian carcinoma. This potential point of therapeutic intervention for certain solid tumors raises the possibility that ET-1 might exert angiogenic effects against ovarian carcinoma targeting the receptor-ligand interaction. Early clinical trials using specific antagonists for ETA R have provided an additional approach to the control of cancer cell growth and invasion not only directly via tumor cell receptors, but also indirectly via its effects on vascular elements. The identification of ET-1 as an autocrine growth factor in ovarian carcinoma cell lines. PMOV2 and OVP1 cells were seeded at 5 x 10^4 cells/well in 12-well plates and cultured in 1% FCS medium in the absence (○) or presence of 100 nM of data from two independent experiments, each of which was performed in triplicate.

Fig. 8. Effects of BQ 123 and BQ 788 on the spontaneous growth rate of primary ovarian carcinoma cells. PMOV2 and OVP1 cells were seeded at 5 x 10^4 cells/well in 12-well plates and cultured in 1% FCS medium in the absence (○) or presence of 100 nM BQ 123 (●) and 100 nM BQ 788 (▲), and cell numbers were measured at the indicated times in a hemocytometer. Bars, mean ± SD of data from two independent experiments, which was prominent mostly in blood vessels adjacent to transformed epithelium, raises the possibility that ET-1 might exert angiogenic effects through this receptor, providing an ideal microenvironment for tumor growth.

In conclusion, this study demonstrates that the increased production of ET-1 by ovarian tumors along with the coexpression of ETaR can result in autocrine stimulation of ovarian carcinoma cell growth, providing a selective proliferative advantage for tumor growth in vivo. Overexpression of ET-1 by tumor cells could thus facilitate their growth and invasion not only directly via tumor cell receptors, but also indirectly via its effects on vascular elements. The identification of ET-1 and ETaR in primary and metastatic ovarian tumors as potential specific targets offers a rationale for new therapeutic strategies against ovarian carcinoma targeting the receptor-ligand interaction. Early clinical trials using specific antagonists for ETaR have provided an additional approach to the control of cancer cell growth (34). This potential point of therapeutic intervention for certain solid tumors renews interest in the possibility of restoring growth control to cancer cells by preventing the autonomous growth of autocrine-stimulated cells.

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Anna Bagnato, Debora Salani, Valeriana Di Castro, et al.


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