ABT-627, a potent endothelin receptor A antagonist, inhibits ovarian carcinoma growth in vitro

Debora SALANI*, Laura ROSANO*, Valeriana DI CASTRO*, Francesca SPINELLA*, Aldo VENUTI†, Robert J. PADLEY‡, Maria Rita NICOTRA§, Pier Giorgio NATALI|| and Anna BAGNATO*

*Laboratory of Molecular Pathology, Regina Elena Cancer Institute, Via delle Messi d’Oro 156, 00158 Rome, Italy, †Laboratory of Virology, Regina Elena Cancer Institute, Via delle Messi d’Oro 156, 00158 Rome, Italy, ‡Abbott Laboratories, 200 Abbott Park Road, Abbott Park, IL 60064-6181, U.S.A., §Biotechnology Institute, Consiglio Nazionale delle Ricerche, Rome, Italy, and ||Laboratory of Immunology, Regina Elena Cancer Institute, Via delle Messi d’Oro 156, 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 carcinomas. In these tumours the presence of ET-1 is associated with enhanced neovascularization and with vascular endothelial growth factor (VEGF) expression. In these tumour cells, ET-1 acts as an autocrine growth factor selectively through the receptor ETA, which is predominantly expressed in tumour cells. Furthermore, ET-1 produced by ovarian tumour cells stimulates VEGF production and VEGF-mediated angiogenic effects through ETA binding. These results demonstrate that activation of the ETA in ovarian carcinoma cells promotes cell proliferation, neovascularization and invasion, which are the principal hallmarks of malignant transformation. The present study was designed to investigate the effects of the ETA-selective antagonist ABT-627 on the ET-1-induced mitogenic effect in both primary cultures (PMOV1 and PMOV2) and cell lines (OVCA 433 and HEY) of ovarian carcinoma. All tumour cells express the components of the ET-1 system and secrete ET-1. ETA blockade by ABT-627 inhibits ET-1-induced mitogenic effects. The ET B antagonist BQ-788 is ineffective although all cell lines express both ETA and ETB mRNAs. In conclusion, our results demonstrate that ABT-627 is capable of inhibiting the proliferative activity of ET-1, suggesting that this potent ETA antagonist may provide a novel approach to the multidisciplinary treatment of ovarian carcinoma.

INTRODUCTION

Ovarian cancer has the highest mortality rate of all gynecological malignancies, which reflects the fact that most patients are diagnosed with advanced cancer. Extensive metastatic spreading and formation of ascites have been established within the peritoneal cavity at the time of initial diagnosis in most patients [1].

Endothelin-1 (ET-1) is a peptide produced primarily by vascular cells and in elevated amounts by a variety of cancer cells [2]. ET-1 acts through two distinct subtypes of G-protein-coupled receptors, namely ET A and ET B. ET A binds selectively ET-1 whereas ET B 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 [3].
these tumour cells, ET-1 acts as an autocrine growth factor selectively through ET\textsubscript{A} which is predominantly expressed on tumour cells [4]. We also observed that the presence of ET-1 correlates with tumour vascularity and vascular endothelial growth factor (VEGF) expression, and that ET-1 stimulated neovascularization in ovarian carcinoma cells through direct angiogenic effects on endothelial cells and, in part, through the stimulation of VEGF via ET\textsubscript{A} binding [5]. Our previous findings demonstrated that ET-1 is present at elevated concentrations in the ascitic fluids of patients with ovarian carcinoma, suggesting that ET-1 could enhance the secretion of extracellular matrix-degrading proteinases and thereby facilitate cell invasion and progression of ovarian carcinoma. We demonstrated that ET-1 consistently induces the overexpression and activation of two families of proteinases (matrix metalloproteinases and urokinase-type plasminogen activator system) at several levels: mRNA transcription, zymogen secretion and pro-enzyme activation. Furthermore, a specific ET\textsubscript{A} antagonist blocked the ET-1-induced activation of tumour proteases, indicating that ET-1 contributes to metastatic progression of ovarian carcinoma through ET\textsubscript{A} [6]. The autocrine production of ET-1 by ovarian cancer cells and the selective binding to ET\textsubscript{A}, mainly expressed on ovarian cancer cells, leads to an increased potential for tumour cell proliferation, neovascularization and cellular invasive activity. Because activation of ET\textsubscript{A} is involved in the proliferation, migration and invasion of ovarian carcinoma cells, we tested whether the potent ET\textsubscript{A} antagonist ABT-627 ([trans,trans]-2-(4-methoxyphenyl)-4-(1,3-benzodiazol-5-yl)-1-(dibutylaminocarbonylmethyl)-pyrrolidine-3-carboxylic acid) [7] may inhibit ovarian carcinoma cell growth in culture.

**MATERIALS AND METHODS**

**Cell cultures**

Two primary ovarian carcinoma cells (PMOV1 and PMOV2) were derived from ascitic fluid that was freshly obtained following informed consent from two untreated patients bearing ovarian carcinoma. Briefly, cells were harvested by centrifugation at 200 g for 5 min at room temperature, resuspended in Dulbecco’s PBS, and then centrifuged through F1 cell-Histopaque 1077 (Sigma, St Louis, MO, U.S.A.). Interface cells were washed in culture medium, and 5 × 10⁶ viable cells were seeded in 75 cm² culture flasks. 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 antibodies recognizing ovarian tumour-associated antigens by the alkaline phosphatase–peroxidase–antiperoxidase method. The human ovarian carcinoma cell lines OVCA 433 and HEY were generous gifts from Dr Giovanni Scambia (Catholic University School of Medicine, Rome, Italy). All cell lines were cultured in Dulbecco’s modified Eagle’s medium/10% foetal calf serum at 37 °C in 5% CO\textsubscript{2}/95% air.

**RNA extraction and reverse transcriptase (RT)-PCR**

Total RNA was isolated by the guanidinium thiocyanate/phenol/chloroform extraction method. RT-PCR was performed using a GeneAmp RNA PCR kit (PerkinElmer, Norwalk, CT, U.S.A.) according to the manufacturer’s instructions. Briefly, 1 μg of RNA was reverse-transcribed and amplified for 30 cycles. The primer sets used were: ET-1, 5’-TGCTCCTGCTGTCCTCTGATGGATAAGG-3’ and 5’-GGTCACATAACGCTCTCTGGGAGGGCTT-3’; ET\textsubscript{A}, 5’-CAC-TGGTTGGATGTGTAATC-3’ and 5’-GGGATCAATGCACACATAG-3’; ET\textsubscript{\textalpha}, 5’-TCAACACGGTTGTGTCCTGC-3’ and 5’-ACTGAAATGCCACACCATCCTT-3’; glyceraldehyde-3-phosphate dehydrogenase was used as an internal control.

**ELISA**

Cells were seeded at 1 × 10⁶ cells/dish in Dulbecco’s modified Eagle’s medium/10% foetal calf serum and were serum-starved for 24 h. After various times, cell supernatants were collected, centrifuged and frozen for subsequent use. The conditioned media were assayed in duplicate on microtitre plates by an ET-1 ELISA kit (R&D Systems, Minneapolis, MN, U.S.A.) according to the manufacturer’s instructions. The working range in the ELISA assay was 0–120 pg/ml.

**Binding assay**

Cells were cultured in 6-well plates until confluence was reached (5 × 10⁵ cells/well) and were then serum-starved for 24 h. After washing, cells were incubated at 25 °C for 60 min with \(^{125}\text{T}\)-labelled ET-1 (40 pM, 2200 Ci/mmol; Dupont New England Nuclear Research Products, Wilmington, DE, U.S.A.) and increasing concentrations of unlabelled ET-1 (Peninsula Laboratories, Belmont, CA, U.S.A.). At the end of the incubation period, cells were washed, solubilized with 1 M NaOH and analysed for bound radioactivity in a γ-spectrometer. ET-1 receptor affinity and the number of binding sites were determined using the LIGAND program.

**Cell proliferation**

Cells were seeded in 96-well plates until confluence was reached (5 × 10⁵ cells/well) and were incubated in serum-starved medium for 24 h. Mitogenic stimuli were added and, after 18 h, 1 μCi of \(^{3}\text{H}\)thymidine (6.7 Ci/mmol; Dupont NEN) was added to each well for 6 h. ABT-627 was synthesized at Abbott Laboratories (North Chicago, IL, U.S.A.) and was...
RESULTS

To test whether ABT-627 was able to reverse the ET-1-induced proliferative effect, we performed thymidine incorporation assays on primary cultures of ovarian carcinoma and on cell lines of ovarian carcinoma that had been shown to produce elevated amounts of ET-1 and express abundant high-affinity ET\textsubscript{A}, as demonstrated by competitive displacement binding studies [3,4]. In this study we further characterized HEY, an ovarian carcinoma cell line, for the expression of the various components of the ET system and for ET-1 secretion. Using RT-PCR analysis, we detected amplified ET-1, ET\textsubscript{A} and ET\textsubscript{B} cDNA fragments of the predicted size, as shown in Figure 1(A). The levels of ET-1 mRNA are mirrored by the secretion of ET-1. HEY cells release ET-1 into the culture media as a function of time over a 48-h period, with a maximum of production at 48 h (43 ± 9.5 pg/10\textsuperscript{6} cells) (Figure 1B). The ET receptor competition studies demonstrated that \textsuperscript{125}I-labelled ET-1 was preincubated for 15 min prior to the addition of ET-1. After incubation, cells were washed with PBS and then fixed with ice-cold 10\% trichloroacetic acid for 15 min, and the cell-associated radioactivity was then determined.

Figure 1  Production of ET-1, ET\textsubscript{A} and ET\textsubscript{B} in HEY cells

(A) ET-1, ET\textsubscript{A} and ET\textsubscript{B} mRNAs detected by RT-PCR in HEY cells. PCR products in HEY cells of 462 bp (ET-1), 367 bp (ET\textsubscript{A}) and 535 bp (ET\textsubscript{B}) are shown (visualized by ethidium bromide staining).  
(B) Release of ET-1 from HEY cell lines as a function of time. Cells were incubated for 24 h in serum-free medium prior to the experiment. The conditioned media were collected at the indicated times and the ET-1 concentration was determined by ELISA. Each point represents the mean value for two individual conditioned media each performed in duplicate. Error bars represent the S.D.

Figure 2  Effect of ABT-627 on \textsuperscript{[3]H}thymidine incorporation

Quiescent cell lines [OVCA 433 (A) and HEY (B)] and primary cultures [PMOV1 (C) and PMOV2 (D)] of ovarian carcinoma were treated with ET-1 at the indicated concentrations for 24 h before measuring \textsuperscript{[3]H}thymidine incorporation. Cells were incubated for 15 min with 1 \mu M ABT-627 in the absence or in the presence of 100 nM ET-1. Bars represent the S.D. of data from three independent experiments, each of which was performed six times. a, \(P < 0.0001\) compared with control; b, \(P < 0.001\) compared with control; c, \(P < 0.0001\) compared with 100 nM ET-1.
obtained a significant ($P < 0.0001$) inhibition of cell proliferation (Figure 2), demonstrating that the potent ET$_A$ receptor antagonist ABT-627 is capable of reversing mitogenic responses induced by ET-1.

**DISCUSSION**

In the two primary cultures and in the two cell lines of ovarian carcinoma examined, activation of ET$_A$ by ET-1 resulted in the highest proliferative activity of tumour cells. Interestingly, we found that addition of a potent and highly selective ET$_A$ antagonist, ABT-627, blocked the ET-1-induced proliferative responses. It has been reported previously that ABT-627 is capable of inducing receptor internalization, which possibly further enhances the ability of an antagonist to block ET-1-induced effects [8].

We demonstrated previously that ET-1-induced stimulation of mitogen-activated protein kinase and mitogenic responses in ovarian cancer cells are mediated in part by the transactivation of the epidermal growth factor receptor through ET$_A$ binding [9,10], suggesting that ET$_A$ antagonists can inhibit cell proliferation and possibly other ET-1- and/or epidermal growth factor-associated processes that contribute to maximal growth advantage in ovarian carcinoma cells.

In conclusion, we demonstrated that ABT-627 is able to reverse ET-1-induced proliferative responses. The autocrine production of ET-1 by ovarian cancer cells and the selective activation of the ET$_A$ receptor, mainly expressed on ovarian cancer cells, lead to an increased cell proliferation, neovascularization and invasive activity. Thus, the therapeutic use of ABT-627 may provide an additional approach to the treatment of ovarian carcinoma through ET$_A$ blockade, which may result in the reduction of tumour growth and invasion.

**REFERENCES**