Pergamon

European Journal of Cancer, Vol. 35, No. 9, pp. 1381–1387, 1999

Printed in Great Britain
0959-8049/99/$ - see front matter

PII: S0959-8049(99)00131-8

Original Paper

Paracrine Regulation of Ovarian Cancer by Endothelin

S. Moraitis, W.R. Miller, J.F. Smyth and S.P. Langdon

Imperial Cancer Research Fund Medical Oncology Unit, Western General Hospital, Edinburgh EH4 2XU, U.K.

Previous studies have demonstrated that endothelin (ET) isoforms (ET-1, ET-2 and ET-3) can act in an autocrine manner in ovarian cancer while in breast cancer their role has been proposed to be that of a paracrine mitogen. To explore the possibility that endothelin isoforms might function not only as autocrine regulators but also as paracrine mitogens in ovarian cancers, we investigated their effects on the growth of ovarian fibroblasts derived from ovarian carcinomas, the interaction between ovarian carcinoma and fibroblast cells and the location of the isoform expression in primary ovarian tumours. ET-1, ET-2 and ET-3 stimulated the growth of three ovarian fibroblast cell lines at concentrations ranging from 10^{-12} M to 10^{-7} M. Inhibition of ^{125}I-ET binding by the ET_A receptor antagonist BQ123 and the ET_B receptor antagonist BQ788 suggested the presence of both types of ET receptors in fibroblast cells. In the absence of ET-1, neither BQ 123 nor BQ 788 inhibited growth. However, both antagonists inhibited ET-1 stimulated growth suggesting the involvement of both receptor types in ET-1 growth regulation. In contrast to carcinoma cells which secrete measurable levels of ET-1, fibroblast cell lines did not secrete detectable protein. Co-culture experiments (using porous membrane insert wells) of fibroblasts with carcinoma cells demonstrated that growth of both populations of cells was increased compared with either grown in isolation. In this system, growth of the fibroblast cell line was partially inhibited by both BQ123 and BQ788, whilst growth of the PE014 carcinoma cell line was inhibited by only BQ123. RT-PCR measurements detected the presence of the ET_A receptor subtype in 10/10 primary ovarian cancers but the presence of ET_B receptor in only 6/10 cancers. Using specific antibodies, ET-1 was found in 11/15, ET-2 in 5 of 7 and ET-3 in 5/7 primary ovarian cancers predominantly in the epithelial cells but with some stromal expression. These data indicate that the ET isoforms may stimulate growth of the fibroblast population within an ovarian cancer in addition to stimulating the epithelial cells and since the ETs are expressed in the majority of ovarian cancers, this paracrine effect may contribute to the overall growth of the tumour. © **1999 Elsevier Science Ltd. All rights reserved.**

Key words: endothelin, ovarian cancer, paracrine regulation
Eur J Cancer, Vol. 35, No. 9, pp. 1381–1387, 1999

INTRODUCTION

OVARIAN CANCER is the most fatal gynaecological malignancy. Current therapy is unsatisfactory, but cell biology studies continue to identify regulators of this disease that might provide useful targets for new therapies. Previous studies have demonstrated that endothelins are secreted by ovarian carcinoma cells and these peptides can influence growth in an autocrine manner [1, 2]. The endothelin family consists of three 21-amino acid peptides, ET-1, ET-2 and ET-3 [3, 4]. These peptides act through two distinct receptors, ET_A and ET_B [5, 6]. The ET_A receptor binds ET-1 and ET-2 with high affinity but has only low affinity for ET-3, whilst the ET_B receptor binds all three isoforms with equal affinity [5, 6]. ET-1 was originally identified as a potent vasoconstrictor produced by endothelial cells but, together with its related isopeptides, it has been shown to be present and act as a mitogen in many cancer systems [7, 8]. These peptides have been shown to have roles as autocrine and paracrine regulators in the normal ovary particularly influencing granulosa cell function, steroidogenesis and luteolysis [9–12]. Recent

Correspondence to S.P. Langdon, e-mail: s.langdon@icrf.icnet.uk
Received 17 Dec. 1998; revised 21 Apr. 1999; accepted 27 Apr. 1999.

studies have demonstrated that ET-1 can act as an autocrine regulator of growth in ovarian cancers [1, 2]. This contrasts with its role in breast cancer where a paracrine function has been assigned [13, 14].

In this study, the interaction between ovarian epithelial and fibroblast cells obtained from ovarian carcinomas was investigated to obtain evidence that the ETs may act in a paracrine manner in this disease type. Primary ovarian tumours were then examined to determine whether endothelin isoforms were present and in which cells they were expressed.

MATERIALS AND METHODS

Cell lines

The human ovarian carcinoma cell lines PE04 and PE014 were established and characterised as previously described [15]. All these lines were routinely cultured at 37°C in an atmosphere of 5% CO_2/95% air in Dulbecco's Modified Eagle Medium (DMEM) containing phenol red indicator. The medium was supplemented with 10% fetal calf serum (FCS), L-glutamine (2×10^{-3} M), penicillin (100 IU/ml) and streptomycin (100 μg/ml).

The fibroblast cell lines PE012F, PE014F and PE027F were initiated from ascitic cells obtained from patients with primary ovarian cancer. PEO12F and PEO27F were obtained from patients with serous cystadenocarcinoma whilst PEO14F cells were derived from a patient with adenocarcinoma. Ascitic cells which had been washed in DMEM + 10% FCS were placed into 25 cm^2 flasks and allowed to adhere. These cell lines were maintained in 15% FCS. The fibroblast cells were selected on the basis that they attached to the plastic substrate more rapidly than epithelial cells; media containing unattached cells was poured off after 2–4 h, leaving a predominantly fibroblastic population.

The original ascites preparations contained leucocytes, carcinoma cells and fibroblasts. After the above culture selection, the final populations of fibroblasts were found to be >99% pure with less than 1% contamination by leucocytes or tumour cells as indicated by immunocytochemistry.

The initial growth experiments and ET measurement experiments used all three fibroblast cell lines, but since greater numbers of PE012F cells were available, this cell line was selected for the subsequent binding and co-culture experiments.

Immunocytochemistry

Once the cultures were established, after 1–2 passages, multispot slides were prepared and immunocytochemistry was used to compare the samples of the fibroblast cultures with the original ascites population. Cells were incubated with either 5B5 mouse antihuman fibroblast monoclonal antibody (used at 1:100, Dako, Ely, U.K.), 2B11 mouse antihuman leucocyte common antigen (used at 1:10, Dako), E29 mouse antihuman epithelial membrane antigen (used at 1:40, Dako) or tris buffered saline (TBS: as control) for 30 min at room temperature. After washing in TBS, multispots were treated with rabbit antimouse biotinylated antibody diluted 1:100 in TBS, followed by avidin–biotin peroxidase complex (ABC) made up in TBS; both incubations being for 30 min at room temperature and were followed by washing in TBS. After the final wash, multispots were treated with a solution of 3,3′-diaminobenzidine (1 mg/ml) (DAB) containing 5% hydrogen peroxide for 5 min. Multispots were then dehydrated, cleared and mounted under coverslips with DPX mounting medium.

In studies investigating the presence of ET isoforms in ovarian cancers, paraffin-fixed sections were used. These were obtained from the primary tumours of patients with ovarian cancer and were processed as follows. Paraffin-fixed sections of tumour samples were dewaxed in xylene for 10 min and washed in ethanol before being placed in 1% hydrogen peroxide for 15 min and washed in water. The sections were washed in TBS for 5 min and incubated with 20% FCS in TBS for 10 min. Excess FCS/TBS was drained off and sections were treated with antibodies targeted to ET-1, ET-2 or ET-3 (all from Peninsula Laboratoies Ltd, St Helens, U.K. and used at 1:200 to 1:400 dilutions) for 30 min at room temperature followed by TBS washing. Sections were then covered with ABC dissolved in TBS for 20 min, washed and treated with DAB containing 5% hydrogen peroxide as above. Sections were then counterstained with haematoxylin and mounted as above.

Extraction and radioimmunoassay of media

Conditioned media were collected from cells growing in 25 cm^2 plastic tissue culture flasks at 90% confluence. Serum free DMEM (10 ml) was added to cells for 72 h. The resulting conditioned media were collected and cells were trypsinised and counted.

Conditioned media were applied to C-18 cartridge columns which had previously been activated with methanol and washed with distilled water. Bound material was eluted with 3 ml of 60% acetonitrile/0.1% trifluoroacetic acid and collected in glass tubes. The eluents were dried down using a Univap Uniscience and stored at 4°C until assayed. The efficiency of the extraction method was measured using radiolabelled ET-1 (^{125}I-ET-1) and was calculated to be approximately 90%. Immunoreactive endothelin was measured using an ET-1 radioimmunoassay kit (Peninsula Laboratories Ltd, St Helens. U.K.). Standard curves were plotted as detailed in the RIA kit protocol and conditioned media were assayed as serially diluted samples (250 μl). The cross reactivities of ET-2 and ET-3 with ET-1 in the assay, as determined by running serial dilutions of authentic ET-2 and ET-3 peptide standards as samples through the radioimmunoassay, together with serial dilutions of the standard ET-1, were estimated to be 10% for ET-3, 18% for ET-2 and less than 1% for big ET-1. The sensitivity of the assay was 1.5 pg/tube and mean intra-assay and interassay variations, as calculated using an undiluted sample of conditioned media, were found to be 5.6 and 6.8%, respectively.

Growth assays

Measurements of growth of cell lines were based on changes in cell number. Cells were seeded in 24-well plates (at a concentration of 2.5×10^4 cells/well) in serum-free conditions for 24 h and allowed to grow under limited serum conditions (1% FCS) for a further 5-day period in the presence or the absence of increasing concentrations (10^{-13}–10^{-6} M) of ET-1, ET-2 or ET-3 (all ETs were obtained from Peninsula Laboratories Ltd). Media and peptides were changed 72 h after the initial addition. In the experiments in which ET receptor antagonists were used, cells were seeded at the same concentrations as before and cultured for 24 h, incubated with 0.5 ml of PBS and detached from the plates with trypsin/versene. Trypsinised cells were collected and cell counts were

determined using a ZF Coulter counter. BQ123 is an ET_A receptor antagonist (Peninsula Laboratories Ltd) and BQ788 is an ET_B receptor antagonist (Sigma, Poole, U.K.).

Preparation of cell membranes and binding inhibition studies

Cells were grown to 70% confluence under 15% FCS conditions in 125 cm^2 flasks and subsequently cultured in serum-free conditions until confluent. Cells were trypsinised and collected by centrifugation at 1000*g* for 1 min. The pellet was then re-suspended in buffer-1 (10^{-2} M Tris-HCl, 1.5×10^{-3} M EDTA, 10^{-3} M benzamidine, 0.002% soybean trypsin inhibitor, pH 7.4), sonicated and centrifuged at 1000*g* for 10 min at 4°C. The resulting supernatant was ultracentrifuged at 100 000*g* for 30 min at 4°C. The pellet was re-suspended in buffer-2 (5×10^{-2} M Tris-maleate, 10^{-2} M $MgSO_4$, 10^{-3} M benzamidine, 0.002% soybean trypsin inhibitor, pH 7.4) and stored at − 80°C until assayed.

For binding inhibition studies, cell membrane samples were incubated with ^{125}I-ET-1 (5×10^{-10} M) and increasing concentrations of ET-1, ET-3, BQ123 and BQ788. At the end of the incubation period, samples were placed on ice and ice-cold 0.5% (w/v) IgG solution (0.5 ml) followed by 12.5% (w/v) polyethylene glycol (PEG) (1 ml) were sequentially added. Mixed samples were centrifuged at 3000 rpm for 15 min in order to separate the bound and free ^{125}I-ET-1. The supernatant (free ^{125}I-ET-1) was aspirated and the remaining pellet was counted in a Packard Cobra gamma counter. Non-specific binding was determined in the presence of 1 μM ET-1.

Co-culture experiments

PEO12F fibroblasts were seeded in 24-well plates (5×10^4 cells/well) whilst PEO14 ovarian epithelial cancer cells were seeded (at 2.5×10^4 cells/well insert) in 24-well plate inserts (8 μm diameter pores, Falcon) placed in separate wells. All cells were initially cultured at 10% serum. After 24 h, the inserts containing the epithelial cells were then transferred into the wells containing the fibroblast cells and cultured together for 5 days in 1% serum in the presence or absence of BQ123 or BQ788 ET-receptor antagonists (10^{-7} M). At the end of the 5-day period cells were trypsinised and counted using a Coulter Counter.

RNA isolation and reverse transcriptase-polymerase chain reaction (RT-PCR)

Total RNA was prepared from cells and primary tumours using the Tri Reagent™ kit (Molecular Research Centre, Oxford, U.K.). A mixture of RNA (5 μg) with oligo(dT) primer (0.5 μg), 2 μl of Taq buffer (5×10^{-2} M KCl, 10^{-2} M Tris, 0.1% Triton X-100), 2 μl deoxy-NTPs (10^{-3} M each) and 4 μl $MgCl_2$ (5×10^{-3} M) was heated at 65°C for 5 min (for the RNA to anneal to oligo-dT primers) and left on ice for 2 min. RNAsin (0.5 μl of 1 U/μl) and reverse-transcriptase (0.5 μl of 0.5 U/μl) (RT) were then added and first strand cDNA synthesis was carried out at 42°C for 1 h followed by 1 min at 95°C to inactivate RT. For PCR amplification, a Techne PHC-3 programmable thermal cycler was used (Techne Cambridge Ltd, U.K.). The total volume of the reaction mixture was 20 μl consisting of 4 μl of the RT product, Taq buffer (2 μl of 5×10^{-2} M KCl, 10^{-2} M Tris, 0.1% Triton X-100), dNTP mix (0.6 μl of 3×10^{-4} M each), Taq (0.5 μl of 0.125 U/μl), distilled water (10.2 μl), 3′ and 5′ primers (0.6 μl of 2×10^{-5} M for each) and a drop of light mineral oil (RT-PCR kit supplied by Promega, Southampton, U.K.) and primers supplied by the ICRF (Clare Hall Laboratories, South Mimms, U.K.). Products were collected after 30 three-step amplification cycles (25 for β-actin), (93°C for 1 min, 61°C for 1 min and 72°C for 1 min), separated by agarose gel electrophoresis and visualised using ethidium bromide. The sequences of the specific primers used in the PCR reactions are shown in Table 1. Samples were scored as positive when a PCR product of the correct molecular size was amplifed following electrophoresis. Samples were sized using a 100 bp ladder (Gibco, U.K.). As an additional control, PCR of β-actin was performed to confirm the integrity of transcribed RNA.

Table 1. *Oligonucleotide primers and probes used in the RT-PCR experiments*

RNA	Oligonucleotide sequence	Nucleotide number	Product size (BPs)	[Ref.]
ET-1	5′ primer: ATGGATTATTAGCTCATGATTTT	253–275	582	[16]
	3′-primer: CTTGGGATCATGAAAAGATGATTT	811–834		
	Oligo probe: GCACGTTGTTCCGTATGGACTTGG	486–509		
ET-2	5′ primer: AGGAAGAGATAGTGTCGTGAGC	596–617	197	[17]
	3′-primer: ACACAGAACTGCCTTGGACGA	773–792		
	Oligo probe: GACTCTCTGCCTGCTTCCTGGACC	680–704		
ET-3	5′ primer: TGTGAGTGTGGAGATGTTATCC	1662–1683	493	[18]
	3′ primer: TTCTCTCTGATACCATCTTGCC	2134–2154		
	Oligo probe: ATTTATTGTGAAACTGTTCTCCAC	1950–1974		
ET_AR	5′ primer: CACTGGTTGGATGTGTAATC	111–130	366	[19]
	3′ primer: GGAGATCAATGACCACATAG	457–476		
	Oligo probe: GCACAACTATTGCCCACAGCAGAC	268–291		
ET_BR	5′ primer: TCAACACGGTTGTGTCCTGC	538–557	530	[19]
	3′ primer: ACTGAATAGCCACCAATCTT	1048–1067		
	Oligo probe: TGTGTAAGCTGGTGCCTTTCATAC	748–770		
β-actin	5′ primer: CCCAGGCACCAGGGCGTGAT	154–173	262	[19]
	3′ primer: TCAAACATGATCTGGGTCAT	396–415		

Base pair positions are from sequences cited in the GenBank/EMBL databank. Accession numbers for these sequences are as follows: ET-1, Y00749; ET-2, M65199; ET-3, J05081; ET_AR, S63938; ET_BR, S44866; β-actin, X00351.

The identities of transcript sequences were confirmed by Southern blot analysis using specific [^{32}P] 5′ end labelled probes (24 mers) targeted to unique sequences within the transcripts (Table 1).

RESULTS

Effects of the ET peptides on the growth of ovarian cancer fibroblasts

We have previously shown that the epithelial cells within an ovarian carcinoma population are stimulated by ET-1 and ET-2 but not ET-3 [2]. To test whether the stromal fibroblast population is also sensitive to the ETs, we examined the effects of ET-1, ET-2 and ET-3 on the growth of fibroblast cell lines derived from ovarian carcinomas. Endothelins were added at concentrations ranging from 10^{-13} to 10^{-6} M to the PE012F, PE014F and PE027F fibroblast cell lines (Figure 1). All three endothelins produced statistically significant increases in cell number relative to control untreated cells after 5 days treatment at concentrations ranging from 10^{-12} M to 10^{-7} M. Maximum stimulation was typically a 60–70% increase in cell number relative to no treatment and was produced at 10^{-9} M–10^{-10} M for ET-1, 10^{-8}–10^{-9} M for ET-2 and at 10^{-9}–10^{-10} M for ET-3.

RT-PCR expression of mRNA for ET receptors and ^{125}I-ET-1 binding to ovarian fibroblast cell membranes

Expression of mRNA for both the ET_A and ET_B receptors was detected in the PE012F, PE014F and PE027F cell lines (data not shown). To delineate the type of receptors present in the ovarian fibroblasts, ^{125}I-ET-1 binding to PEO12F cells was monitored in competition with ET-1, ET-3 and also BQ123 (ET_A-R) and BQ788 (ET_B-R) receptor antagonists. The half maximal inhibitory concentrations for ET-1, ET-3, BQ123 and BQ788 were respectively, 3, 20, 140 and 500 nM (Figure 2).

Effect of endothelin receptor antagonists on the growth of ovarian cancer fibroblasts

To help identify which ET receptors were functional in mediating the growth effects of ETs in fibroblast cells, the ET_A-R antagonist BQ123 and the ET_B-R antagonist BQ788 were used. In the absence of ET-1, neither BQ123 (10^{-7} M) nor BQ788 (10^{-7} M) had any effect on the growth of PEO12F cells (Figure 3). When 10^{-9} M ET-1 was added to these cells it increased the cell number by 50% relative to control cell number. The addition of BQ123 (10^{-7} M) in the presence of 10^{-9} M ET-1 partially blocked the growth stimulatory effect of ET-1, resulting in an increase of only 20% of cell number compared with control. Similarly, the addition of BQ788 (10^{-7} M) to PEO12F cells in the presence of ET-1 also resulted in partial blockade of the stimulation seen with 10^{-9} M ET-1 in the absence of antagonists, showing an increase of only 16% compared to control cell number. Both antagonists combined produced a complete blockade of ET-1 stimulated growth.

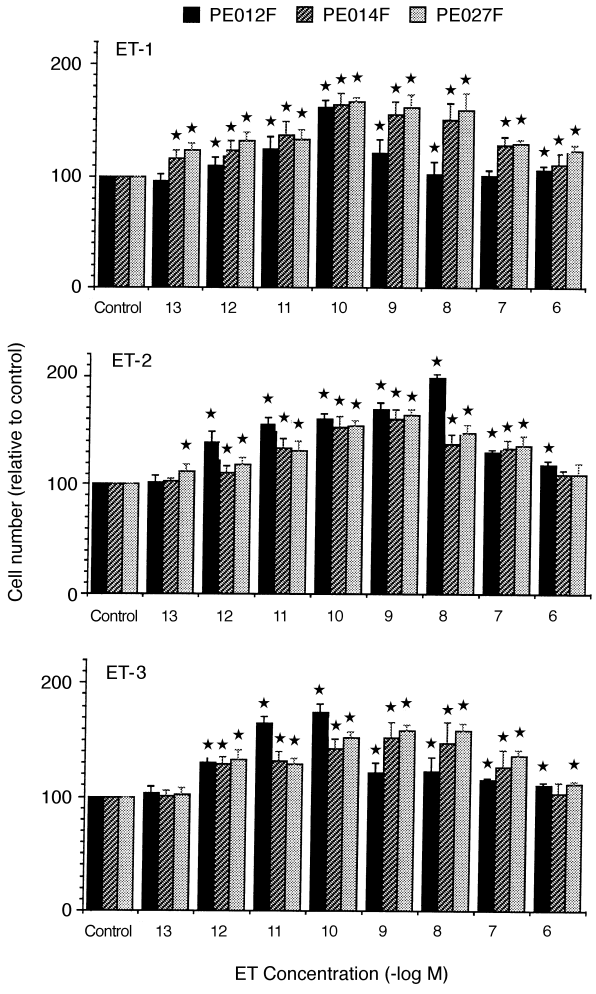

Figure 1. Effect of the addition of ET-1, ET-2 and ET-3 on the growth of three fibroblast cell lines derived from ovarian carcinomas. The PE012F, PE014F and PE027F cell lines were grown in 1% FCS for a 5-day period. The graph shows results from a representative experiment which was performed on at least three occasions. The standard error of the mean is indicated. *Represents values which are statistically different from the control ($P<0.05$, Student's *t*-test).

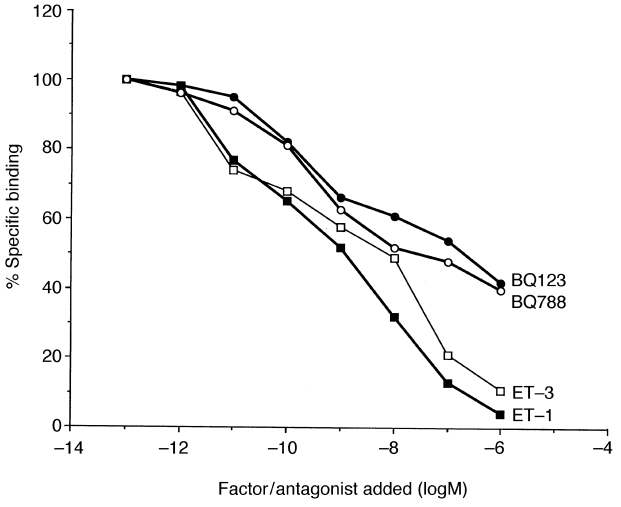

Figure 2. Inhibition of specific ^{125}I-ET-1 binding in PE012F fibroblast cells by ET-1, ET-3, BQ123 (ET_AR antagonist) and BQ788 (ET_BR antagonist). Cell membranes were incubated at 37°C for 60 min with 50 pM ^{125}I-ET-1 in the presence or absence of these peptides at the concentrations indicated. This is a representative experiment which was repeated twice. Binding of ^{125}I-ET-1 is expressed as the percentage of the specific binding in the absence of unlabelled peptide.

Table 2. Protein expression of endothelins-1, 2 and 3 in primary ovarian cancers

	ET-1		ET-2		ET-3	
Tumour	Epithelium	Stroma	Epithelium	Stroma	Epithelium	Stroma
HOV1	+	+	+	–	+	–
HOV2	++	–	–	–	+	–
HOV3	+	–	+	–	+	–
HOV4	+	+	–	–	–	–
HOV5	+	+	+	+	+	+
HOV6	++	++	+	–	–	–
HOV7	+	+	+	–	+	+
HOV8	+	–	ND	ND	ND	ND
HOV9	+	–	ND	ND	ND	ND
HOV10	–	–	ND	ND	ND	ND
HOV11	+	+	ND	ND	ND	ND
HOV12	+	–	ND	ND	ND	ND
HOV13	–	–	ND	ND	ND	ND
HOV14	–	–	ND	ND	ND	ND
HOV15	–	–	ND	ND	ND	ND

Intensity of staining is expressed as: strong positive (++), weak positive (+) or negative (–). ND, not done.

Production of immunoreactive ET

For the detection of immunoreactive ET (ir-ET), conditioned media were collected from cultures of PEO4 and PEO14 ovarian cancer cell lines and PEO12F, PEO14F and PEO27F ovarian fibroblasts. Concentrations of ir-ET secreted from PEO4 and PEO14 cell lines were 1.7 ± 0.4 (mean ± standard deviation, S.D.) and 20.2 ± 6.8 (mean ± S.D.) fmol/10^6 cells/72 h, respectively. In contrast, none of the ovarian fibroblast cell lines secreted measurable levels of the peptide.

Co-culture of ovarian carcinoma epithelial and fibroblast cell lines

To examine a possible paracrine role for ET between epithelial and fibroblast cells, the PE014 epithelial and PE012F fibroblastic cell lines were co-cultured. PE014 cells were grown in insert wells which were then placed into wells containing PE012F cells. After a 5-day incubation, cell counts of

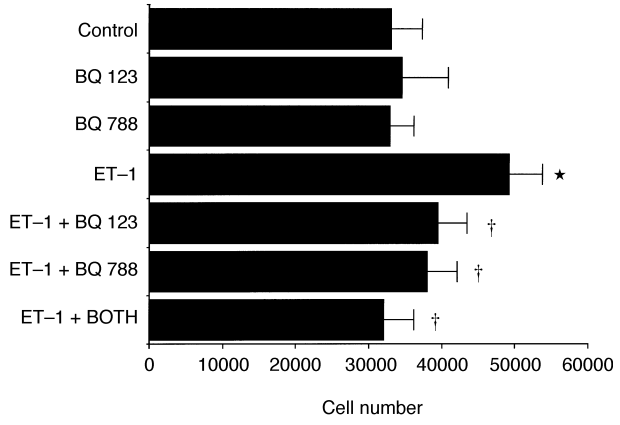

Figure 3. Effects of ET-1 and/or BQ123 and BQ788 receptor antagonists on the growth of PE012F fibroblast cells. Cells were incubated in 1% serum conditions with ET-1 alone (10^{-9} M), ET-1 with BQ123 ET_AR antagonist (10^{-7} M), ET-1 with BQ788 ET_BR antagonist (10^{-7} M), ET-1 with both antagonists or either antagonist alone (10^{-7} M) for 72 h. Cells were harvested and counted on a Coulter Counter. The results represent mean values of three experiments (bars: standard error of the mean. ⋆ and † represent statistically significant differences ($P<0.05$) compared with control and ET-1 groups respectively.

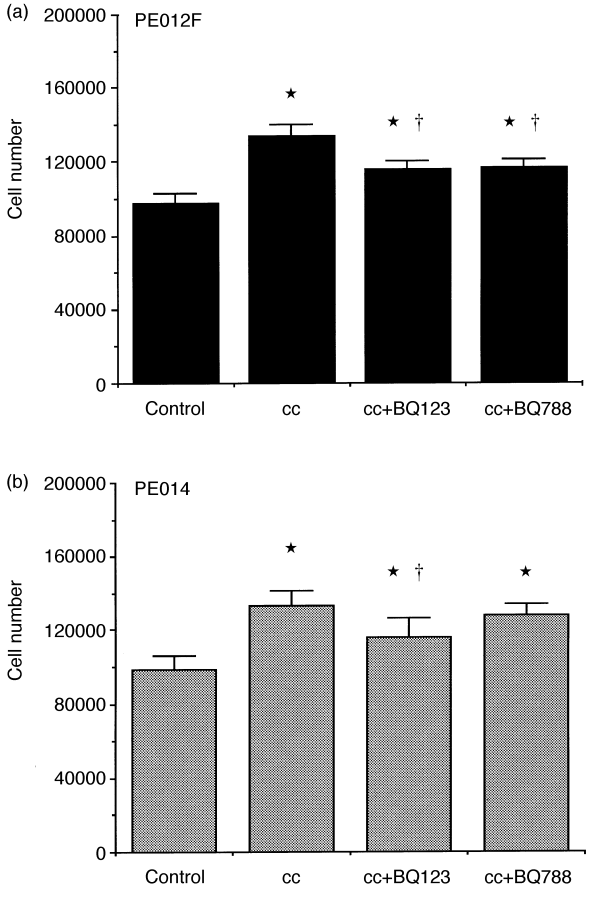

Figure 4. Growth of (a) PE012F fibroblast and (b) PE014 epithelial carcinoma cells in isolation (control) or in co-culture (CC), in the absence (cc) or presence of BQ123 (ET_AR antagonist; 10^{-7} M) or BQ788 (ET_BR antagonist; (10^{-7} M) for 5 days. Values shown are standard error of the mean of a representative of three experiments performed on separate occasions. ⋆ and † represent values that are significantly ($P<0.05$; Student's *t*-test) different from control and co-cultured cells, respectively.

both PE012F and PE014 cell populations were higher (35 and 37% respectively) when grown in the presence of the other cell line than when grown alone (Figure 4). To test whether ET contributed to this increase, BQ123 and BQ788 were added to the co-cultures. Both antagonists inhibited growth of the PE012F cells in co-culture (by 14 and 15% respectively) whilst only BQ123 inhibited growth of the PE014 cell line (by 13%).

Expression of ET isoforms in primary ovarian cancers

The expression and location of the ET isoforms was examined in a small series of primary ovarian carcinomas. ET-1 was detected in 11 of 15 (73%) tumours and subsequently both ET-2 and ET-3 were detected in 5/7 tumours. Staining patterns were similar for all three isoforms with expression predominantly found in the epithelial cells though stromal staining was also observed. Staining was generally cytoplasmic and diffuse.

RT-PCR followed by Southern blotting was also undertaken in a separate series of 10 tumours and indicated the expression of ET-1 and ET-3 in all 10 tumours, with ET-2 present in 6/10 tumours. Expression of the ET_A receptor was present in all 10 whilst that of the ET_B receptor was present in 6/10 tumours (data not shown).

DISCUSSION

Previous studies have suggested that the ETs can exert an autocrine role in ovarian cancer [1, 2]. This view was based on the observations that ovarian carcinoma cell can both secrete and respond to ETs in culture which contrasts with systems such as breast cancer wherein breast carcinoma cells secrete ETs but are receptor-deficient and do not respond to this factor [13, 14]. Within these systems, however, neighbouring fibroblasts which do express ET receptors are responsive allowing the possibility of paracrine regulation. The present study sought to see if such a mechanism might also be in place within ovarian carcinomas and the data obtained in this study do support such a view. Whilst fibroblasts do not appear to secrete measurable levels of peptide (at least in culture), there are several indications that they possess both receptors and are responsive to the effects of ET. Firstly all three isoforms of ET stimulate the growth of fibroblast cell lines in culture with maximal stimulation of approximately 60–70% at concentrations between 10^{-10} M and 10^{-8} M. Higher concentrations produced smaller stimulations and this may be explained by the downregulation of endothelin receptors after the addition of very high concentrations of peptide. The mitogenic effects in the fibroblast lines contrast with effects produced in two ovarian carcinoma epithelial cell lines (PE014 and PE04) wherein both ET-1 and ET-2 stimulated growth to a lesser degree (approximately 50% and 20–40% respectively), while ET-3 produced no effect [2]. The observation that ET-3 has a mitogenic effect equivalent to that of ET-1 and ET-2 in ovarian fibroblast lines suggests that these effects are probably being mediated at least in part through the ET_B receptor. The ET-1 stimulated growth of PE012F was partially inhibited by both receptor subtype antagonists, BQ123 and BQ788, and together these had a more potent effect. These data suggest the involvement of both forms of receptor. Expression of both subtypes was confirmed at the level of mRNA by use of RT-PCR. Insufficient numbers of fibroblast cells were available for Scatchard analysis but competition studies using ^{125}I-ET-1 provided further insight as to the nature of the receptors present. Both ET_A and ET_B receptor antagonists had comparable competitive activity (140 and 500 nM) against the PE012F line suggesting the binding of ET-1 to both receptor types whilst ET-3 had a $<$10-fold difference relative to ET-1 in potency. These data suggest the presence of both receptor types and contrast with those previously obtained for the PE014 and PE04 carcinoma cell lines wherein BQ123 was $>$1000-fold more effective than BQ788 in ^{125}I-ET-1 competition assays and there was an approximate 100-fold difference between ET-1 and ET-3 consistent with the presence of only ET_A receptors [2].

Having demonstrated that fibroblast cells were responsive to ETs, we next sought evidence that the ETs might have a mitogenic role on this cell type when in the presence of neighbouring epithelial cells. Co-cultures of fibroblast with epithelial cells produced enhanced growth in both populations consistent with release of diffusible mitogenic growth factors. Evidence that the ETs might be components of this system was provided by the partial blockade of this growth stimulation by both ET receptor-targeted antagonists for the fibroblast cells but only the ET_AR antagonist for the epithelial cells. Again, this observation is consistent with fibroblasts expressing both receptor types but the PE014 epithelial cells expressing only the ET_AR receptor [2]. Such data are consistent with the carcinoma cells secreting ETs which act in a paracrine manner on the fibroblast population and as autocrine regulators in the carcinoma cells.

The relevance of these observations in ovarian cancer was then tested by investigation of the expression and location of the endothelins and their receptors in a series of primary tumours. Expression of all three ET protein isoforms was found in approximately 70% of tumours by the use of immunohistochemistry. This was supported by RT-PCR data identifying the mRNA for ET-1 and ET-3 in 100% of tumours and 60% for ET-2. The higher mRNA expression for ET-1 and ET-3 may be due to increased sensitivity of this methodology relative to immunohistochemistry. Clearly, ET isoforms are present in the majority of ovarian tumours and immunohistochemistry indicated more marked expression within the epithelial cells. This is consistent with ET originating from the epithelial cells (as detected in the culture experiments) and being transported to areas of the stroma wherein it could stimulate fibroblast growth.

Together these data provide further support for the view that endothelins may be important mitogens in ovarian cancer and indicate that tumour growth may be stimulated not only through autocrine effects on the carcinoma cells but also by paracrine regulation utilising the supporting stromal fibroblasts.

1. Bagnato A, Tecce R, Moretti C, Di Castro V, Spergel D, Catt KJ. Autocrine actions of endothelin-1 as a growth factor in human ovarian carcinoma cells. *Clin Cancer Res* 1995, **1**, 1059–1066.
2. Moraitis S, Langdon SP, Miller WR. Endothelin expression and responsiveness in human ovarian carcinoma cell lines. *Eur J Cancer* 1997, **33**, 661–668.
3. Yanagisawa M, Kurihara H, Kimura S, *et al.* A novel potent vasoconstrictor peptide produced by vascular endothelial cells. *Nature* 1988, **332**, 411–415.
4. Inoue A, Yanagisawa M, Kimura S, *et al.* The human endothelin family: three structurally and pharmacologically distinct isopeptides predicted by three separate genes. *Proc Natl Acad Sci USA* 1989, **86**, 2863–2867.

5. Sokolovsky M. Endothelins and sarafotoxins: physiological regulation, receptor subtypes and transmembrane signaling. *Trends Biochem Sci* 1991, **16**, 261–264.
6. Sakurai T, Yanagisawa M, Masaki T. Molecular characterization of endothelin receptors. *Trends Pharmacol Sci* 1992, **13**, 103–107.
7. Kusuhara M, Yamaguchi K, Nagasaki K, *et al*. Production of endothelin in human cancer cell lines. *Cancer Res* 1990, **50**, 3257–3261.
8. Shichiri M, Hirata Y, Nakajima T, *et al*. Endothelin-1 is an autocrine/paracrine growth factor for human cancer cell lines. *J Clin Invest* 1991, **87**, 1867–1871.
9. Mancina R, Barni T, Calogero AE, *et al*. Identification, characterization, and biological activity of endothelin receptors in human ovary. *J Clin Endocrinol Metab* 1997, **82**, 4122–4129.
10. Girsh E, Wang W, Mamluk R, *et al*. Regulation of endothelin-1 expression in the bovine corpus luteum: elevation by prostaglandin F2 alpha. *Endocrinol* 1996, **137**, 5191–5196.
11. Kamada S, Blackmore PF, Kubota T, *et al*. The role of endothelin-1 in regulating human granulosa cell proliferation and steroidogenesis *in vitro*. *J Clin Endocrinol Metab* 1995, **80**, 3708–3714.
12. Kamada S, Kubota T, Hirata Y, *et al*. Endothelin-1 is an autocrine/paracrine regulator of porcine granulosa cells. *J Endocrinol Invest* 1993, **16**, 425–431.
13. Baley PA, Resink TJ, Eppenberger U, Hahn AWA. Endothelin messenger RNA and receptors are differentially expressed in cultured human breast and stromal cells. *J Clin Invest* 1990, **85**, 1320–1323.
14. Schrey MP, Patel KV, Tezapsidis N. Bombesin and glucorticoids stimulate human breast cancer cells to produce endothelin, a paracrine mitogen for breast stromal cells. *Cancer Res* 1992, **52**, 1786–1790.
15. Langdon SP, Lawrie SS, Hay FG, *et al*. Characterization and properties of nine human ovarian adenocarcinoma cell lines. *Cancer Res* 1988, **48**, 6166–6172.
16. Itoh Y, Yanagisawa M, Ohkubo S, *et al*. Cloning and sequence analysis of cDNA encoding the precursor of a human endothelium-derived vasoconstrictor protein peptide, endothelin: identity of human and porcine endothelin. *FEBS Lett* 1988, **231**, 440–444.
17. Bloch KD, Hong CC, Eddy RL, Shows TB, Quertermous T. cDNA cloning and chromosomal assignment of the endothelin-2 gene: vasoactive intestinal contractor peptide is rat endothelin-2. *Genomics* 1991, **10**, 236–242.
18. Bloch KD, Eddy RL, Shows TB, Quertermous T. cDNA cloning and chromosomal assignment of the gene encoding endothelin-3. *J Biol Chem* 1989, **264**, 18156–19161.
19. Pekonen F, Nyman T, Ammala M, Rutanen EM. Decreased expression of messenger RNAs encoding endothelin receptors and neutral endopeptidase 24.11 in endometrial cancer. *Br J Cancer* 1995, **71**, 59–63.