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Original Paper

Endothelin Expression and Responsiveness in Human Ovarian Carcinoma Cell Lines

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To elucidate the potential role of endothelins (ETs) as growth regulators in ovarian carcinoma cells in culture, expression of endothelins and their receptors were measured in two ovarian cancer cell lines (PEO4 and PEO14), together with the effect of the exogenous addition of endothelins on the growth of these cell lines *in vitro*. RT-PCR analysis of mRNA prepared from PEO4 and PEO14 indicated the presence of ET-1 and ET-3 mRNA. Immunoreactive ET-1-like peptide was found in media from cultures of both PEO4 (1.7 ± 0.4 fmol/10^6 cells/72 h) and PEO14 (20.2 ± 6.8 fmol/10^6 cells/72 h) cell lines. Radioligand binding studies using ^{125}I-ET-1 and membrane fractions were consistent with PEO4 cells having two receptor sites of either high affinity ($K_d = 0.065$ nM, $B_{max} = 0.047$ pmol/mg protein) or lower affinity sites ($K_d = 0.49$ nM, $B_{max} = 0.23$ pmol/mg protein). Studies using membrane fractions of PEO14 cells indicated that this cell line has only a single lower affinity binding site ($K_d = 0.56$ nM, $B_{max} = 0.31$ pmol/mg protein). However, RT-PCR analysis indictaed the presence of mRNA from both ET_A and ET_B receptors in PEO4 and PEO14 cell lines. Exogenous addition of ETs to PEO4 and PEO14 cells at concentrations of 10^{-10}–10^{-7}M resulted in specific dose-dependent increases in cell number for ET-1 (with maximum effects at 10^{-10} and 10^{-9}M for PEO4 and PEO14, respectively) and ET-2 (maximum effects at 10^{-8} and 10^{-9}M for PEO4 and PEO14, respectively) but not for ET-3. Experiments on the growth of PEO14 cells using BQ123 (ET_A-R) antagonist and "antisense" oligonucleotide against the ET_A-R, in the absence of exogenous ETs, suggested that immunoreactive ET-1-like material secreted by PEO14 cells can affect their growth in an autocrine manner. These results would be consistent with ET-1 acting as a possible autocrine growth regulator in human ovarian carcinoma cells. © **1997 Published by Elsevier Science Ltd. All rights reserved.**

Key words: endothelins, ovarian cancer

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INTRODUCTION

ENDOTHELINS (ETS) comprise a family of three 21-amino acid peptides: ET-1, -2 and -3 [1–4]. These interact with two populations of receptors (ET_A and ET_B) which have different affinities for the three peptides (ET_A shows decreasing receptor affinity for ET-1, ET-2 and ET-3, respectively, whereas ET_B demonstrates similar affinities for all three endothelins) [5]. ET-1 was originally isolated from the medium of porcine aortic endothelial cells and is a potent vasoconstrictor. However, local production of, and response to, ET-1 in a variety of tissues and cell lines (predominantly malignant) have led to the suggestion that the peptide may have a role in autocrine and paracrine growth regulation. In contrast, the roles of ET-2 and ET-3 are not as well defined. However, to our knowledge, there is only a single report on the effect of endothelins in human ovarian cancer [6]. The present study, therefore, presents further evidence for the production of, and response to, endothelins in two novel ovarian cancer cell lines.

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MATERIALS AND METHODS

Cell culture

The human ovarian PEO4 and PEO14 carcinoma cell lines were established and characterised as previously described [7]. These 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 pH indicator. The medium was supplemented with 10% fetal calf serum (FCS), L-glutamine (2 mM), penicillin (100 IU/ml) and streptomycin (100 μg/ml). To generate conditioned media for radioimmunoassay of endothelins, cells were collected in mid-log phase, plated in 25 cm^2 plastic tissue culture flasks and incubated under the conditions mentioned above until they reached 90% confluence. Media were then replaced with serum-free DMEM (10 ml), in which cells were incubated for a further 72 h. The resulting conditioned media were collected and cells were trypsinised and counted.

Extraction and radioimmunoassay of media

Conditioned media were applied to C-18 cartridge columns which had been previously activated with methanol and washed with distilled water. Bound material was then eluted with 3 ml of 60% acetonitrile/0.1% TFA (trifluoroacetic acid) and collected in glass tubes. The eluent was then dried overnight using a Univap Uniscience centrifuge and stored at 4°C until assayed. Radiolabelled ^{125}I-ET-1 was used to monitor the efficiency of the extraction method; recovery was found to be approximately 90%. Measurement of immunoreactive endothelin was performed using an ET-1 radioimmunoassay kit (Peninsula Laboratories Ltd, St Helens, U.K.). Assays were carried out on serially diluted conditioned media (250 μl); standard curves were plotted as detailed in the RIA kit. The cross-reactivities of ET-1 with ET-2 and ET-3, 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 and 18% for ET-2. The sensitivity of the assay was 1.5 pg/tube and mean intra-assay and inter-assay variations, as calculated using an undiluted sample of conditioned media, were found to be 5.6 and 6.8%, respectively.

Measurement of endothelin binding using cell membranes

Cells were grown to 70% confluence in 125 cm^2 flasks in 10% FCS and subsequently cultured in serum-free conditions until fully confluent. Cells were then trypsinised and collected by centrifugation at 1000*g* for 10 min. The pellet was then resuspended in buffer-1 (10 mM Tris-HCl, 1.5 mM EDTA, 1 mM benzamidine, 0.002% soybean trypsin inhibitor, pH 7.4), sonicated and recentrifuged at 1000*g* for 10 min at 4°C. The resulting supernatant was ultracentrifuged at 100 000*g* for 30 min at 4°C and the pellet resuspended in 0.5 ml of buffer-2 (50 mM Tris-maleate, 10 mM $MgSO_4$, 1 mM benzamidine, 0.002% soybean trypsin inhibitor, pH 7.4) before being stored at −80°C, until assayed. Duplicate aliquots (2 × 200 μl) of membrane preparations were incubated at 26°C for 90 min with increasing concentrations (50–9950 pM) of non-radioactive ET-1 added to a fixed concentration (50 pM) of ^{125}I-ET-1. For the binding inhibition studies, cell membrane samples were incubated with ^{125}I-ET-1 (50 pM) and increasing concentrations of ET-1, ET-3, BQ123 (an ET_A receptor antagonist—Peninsula Laboratories Ltd) and BQ788 (an ET_B receptor antagonist—Sigma, Poole, U.K.). 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. Samples were mixed and 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.

Growth assays

Measurements of growth were based on changes in cell number. Cells were grown in 24-well plates and responses to the exogenous additions of ETs were investigated under 1% FCS conditions in the absence of phenol red pH indicator. Cells were plated at a concentration of 2.5×10^4 cells/ml in 10% FCS and cultured for 24 h. Cells were then washed and incubated in 0.5 ml/well of 1% FCS for a further 24 h. A range of concentrations of ET-1 (10^{-13}–10^{-6} M) or ET-2 or ET-3 (10^{-10}–10^{-7} M) were then added to the cells (all ETs were obtained from Peninsula Laboratories Ltd). Media and peptides were changed after a further 72 h. In the experiments where ET receptor antagonists were used, cells were seeded at the same concentration as before and cultured for 24 h, then incubated with 0.5 ml of serum-free medium in the absence or presence of ET-1 and/or antagonist (100 nM) (BQ123, ET_A receptor antagonist or BQ788, ET_B receptor antagonist) for 72 h. Cells were then washed twice with 0.5 ml of PBS and detached from the plates using trypsin/versene. Trypsinised cells were collected and cell counts were determined using a ZF Coulter counter.

For the antisense experiments, 24-well plates were prepared as described previously for growth experiments. Additions of "sense", "antisense" and "random" oligonucleotides were made (at concentrations of 10 and 30 μM for each oligonucleotide) on days 3 and 5 and cells were counted on a ZF Coulter counter on day 7. The sequences of the oligonucleotides are shown in Table 1.

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

Total RNA was prepared from PEO4 and PEO14 cells 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 (50mM KCl, 10 mM Tris, 0.1% Triton X-100), 2 μl deoxy-NTPs (1 mM each) and 4 μl $MgCl_2$ (5 mM) 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 for the inactivation of RT. For the PCR amplification, a Techne PHC-3 programmable thermal cycler was used (Techne, Cambridge Ltd, Duxford, Cambridgeshire, 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 50 mM KCl, 10 mM Tris, 0.1% Triton X-100), $MgCl_2$ (1.5 μl of 1.8 mM), dNTPmix (0.6 μl of 0.3 mM each), Taq (0.5 μl of 0.125 U/μl), dH_2O water

Table 1. Sequences and general information on oligonucleotides used in anti-sense experiments

Oligonucleotide	Sequence (5′–3′)
ET_A-R sense	ATGGAAACCCTTTGC
ET_A-R antisense	GCAAAGGGTTTCCAT
ET_B-R sense	ATGCAGCCGCCTCCA
ET_B-R antisense	TGGAGGCGGCTGCAT
Random	GAACTCGAGGTTCAT

(10.2 μl), 3′ and 5′ primers (0.6 μl of 20 μM for each) and a drop of light mineral oil (RT-PCR kit supplied by Promega (Southampton, U.K.) and primers supplied by ICRF (Clare Hall, London). 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 of the three ETs and the two ET receptor types (ET_A and ET_B) are shown in Table 2. The primers used for the identification of ET_A, ET_B and β-actin were originally designed by Pekonen and associates [23]. The primers for ET-1, ET-2 and ET-3 were designed using the published ET-1, ET-2 and ET-3 DNA gene sequences by Bloch and associates [18–20]. β-Actin acted as a positive control for both cell lines. All the experiments were repeated on at least three occasions.

RESULTS

Production of immunoreactive ET in ovarian cancer cells

Conditioned media from both PEO4 and PEO14 cells were used to detect the production of ET-1. Concentrations of immunoreactive ET-1-like material secreted from PEO4 and PEO14 cell lines were 1.7 ± 0.4 (mean ± S.D.) and 20.2 ± 6.8 (mean ± S.D.) fmol/10^6 cells/72 h, respectively. Although PEO14 secreted much higher concentrations of ET-1-like material, the release of the ET-1-like material as a function of time was similar for the two cell lines, beginning to plateau at around 24 h (Figure 1). Conditioned media from both cell lines diluted in parallel with standard ET-1 in the radioimmunoassay (data not shown).

^{125}I-ET-1 binding to PEO4 and PEO14 cell membranes

The specific binding of ^{125}I-ET-1 to membrane fractions from PEO4 and PEO14 cell lines was both time and temperature dependent, reaching maximum values after a 60 min incubation at 26°C (data not shown). In all experiments, non-specific binding was calculated in the presence of 1 μM unlabelled ET-1 and was less than 12% of the total binding. Scatchard plots for both cell lines demonstrating the binding of ET-1 are shown in Figure 2a,b. The assay for the PEO4 cell line produced a plot which suggested the presence of two distinct classes of receptor binding sites (Figure 2a). The higher affinity receptor site had a dissociation constant value of 0.065 nM (B_{max} = 0.047 pmol/mg protein), whereas the lower affinity receptor site had a dissociation constant value of 0.49 nM (B_{max} = 0.23 pmol/mg protein). For the PEO14 cell line, the Scatchard analysis indicated the presence of a single class of lower affinity receptor binding site with a dissociation constant of 0.56 nM (B_{max} = 0.31 pmol/mg protein) (Figure 2b). To help delineate further the types of receptors involved, ^{125}I-ET-1 binding to PEO4 and PEO14 cells was monitored in competition with ET-1, ET-3, BQ123 (ET_AR) and BQ788 (ET_BR) receptor antagonists (Figure 3a,b). Half maximal inhibitory concentrations for ET-1, ET-3, BQ123 and BQ788 were, respectively, 0.17 nM, 10 nm, 0.85 nM, >1000 nM for PEO14 and 0.04 nM, 3 nM, 0.2 nM, >1000 nM for PEO4 cells.

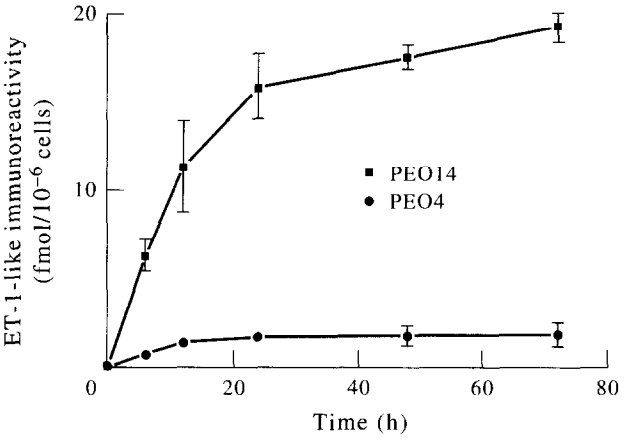

Figure 1. Release of immunoreactive ET-1-like material from PEO4 and PEO14 cells growing under serum-free conditions as a function of time. Conditioned media were collected at 6, 12, 24, 48 and 72 h. The results represent mean values of three distinct conditioned media collected at different times (S.D.).

Effect of the exogenous addition of ETs and ET receptor inhibitors on the growth of PEO4 and PEO14 cell lines

Concentrations of ET-1 of 10^{-11}–10^{-7} M stimulated proliferation of PEO4 and PEO14 cells in culture. In PEO4

Table 2. Oligonucleotide sequences and general information on primers used in PCR amplification

RNA	Oligonucleotide sequence	Nucleotide number	Product size (bp)	Reference
ET-1	5′ primer: ATGGATTATTTGCTCATGATTTT	268–291	582	[18]
	3′ primer: CTTGGGATCATGAAAAGATGATTT	826–849		
ET-2	5′ primer: AGGAAGAGATAGTGTCGTGAGC	596–617	197	[20]
	3′ primer: ACACAGAACTGCCTTGGACGT	773–792		
ET-3	5′ primer: TGTGAGTGTGGAGATGTTATCC	1662–1683	493	[19]
	3′ primer: TTCTCTCTGATACCATCTTGCC	2134–2154		
ET_A-R	5′ primer: CACTGGTTGGATGTGTAATC	38–57	368	[23]
	3′ primer: GGAGATCAATGACCACATAG	386–405		
ET_B-R	5′ primer: TCAACACGGTTGTGTCCTGC	308–327	530	[23]
	3′ primer: ACTGAATAGCCACCAATCTT	818–837		
β-actin	5′ primer: CCCAGGCACCAGGGCGTGAT	154–173	262	[23]
	3′ primer: TCAAACATGATCTGGGTCAT	396–415		

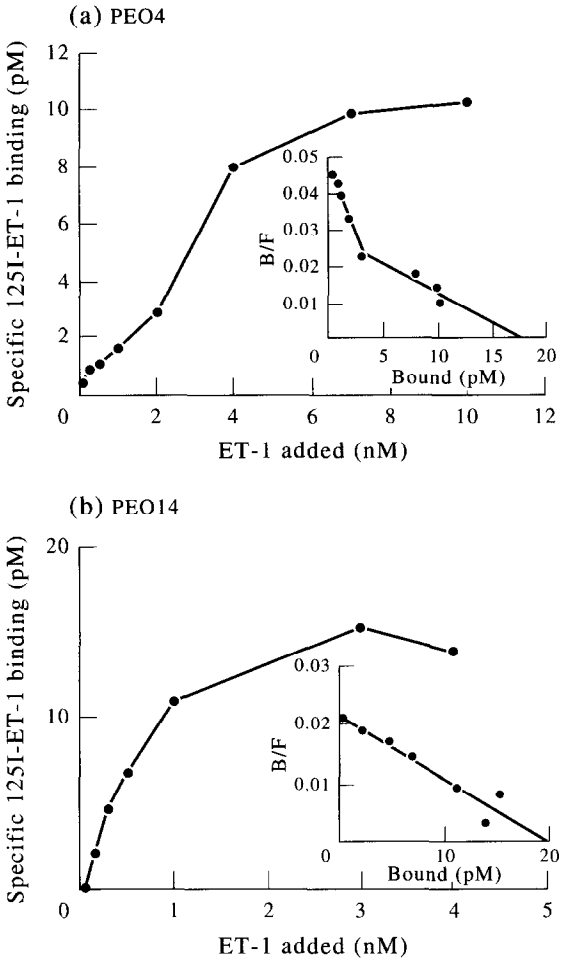

Figure 2. Saturation curve of binding of ^{125}I-ET-1 to PEO4 (a) and PEO14 cells (b). Increasing concentrations of labelled ET-1 were added to membranes prepared from cells in duplicate in the presence (non-specific) or absence (total binding) of unlabelled (1 μM) ET-1. Cells were incubated at 26°C for 60 min. The Scatchard plot was plotted with values obtained by the "LIGAND" program.

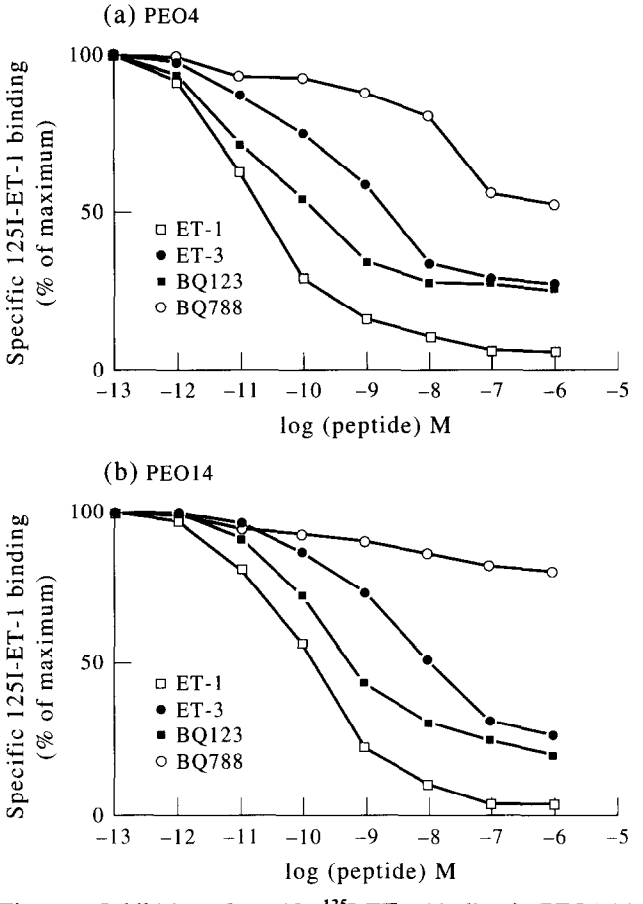

Figure 3. Inhibition of specific ^{125}I-ET-1 binding in PEO4 (a) and PEO14 (b) cell membrane preparations by ET-1, ET-3, BQ123 (ET_A-R antagonist) and BQ788 (ET_B-R antagonist). Cell membranes were incubated at 37°C for 60 min with 50 pM ^{125}I-ET-1 in the presence or absence of these peptides at the concentrations indicated. This is a representative experiment which has been repeated three times. Binding of ^{125}I-ET-1 is expressed as the percentage of the specific binding in the absence of unlabelled peptide.

cells, concentrations of ET-1 of 10^{-12}–10^{-10} M resulted in an increasing growth stimulatory effect, reaching a maximum at 10^{-10} M (53% increase in control cell number), while the stimulatory effect was decreased between concentrations of 10^{-9}–10^{-6} M (Figure 4a). A similar pattern of effects was seen in PEO14 cells with increasing effect shown between concentrations of 10^{-12}–10^{-9} M, a maximum growth stimulatory effect occurring after the addition of 10^{-9} M of ET-1 (52% increase in control cell numbers) and decreasing effects seen at 10^{-8} and 10^{-7} M concentrations (Figure 4b). Addition of exogenous ET-2 also resulted in an increase in cell number in both cell lines, although the effect on PEO14 cells was greater than in PEO4 cells. ET-2 addition resulted in an increasing effect at concentrations of 10^{-9}–10^{-7} in PEO4 cells reaching a maximum at 10^{-8} M addition (20% increase) (Figure 5a). In PEO14 cells, increasing effects were seen after the addition of 10^{-10} M (23% increase) and 10^{-9} M ET-2 (46%, maximum effect) and decreasing effects at 10^{-8} (43%, not statistically significant compared to 10^{-9} M) and 10^{-7} M ET-2 (40%, statistically significant compared to 10^{-9} M) (Figure 5b). Addition of ET-3 did not cause any notable effect in proliferation in either cell line (Figure 5a,b). Competition experiments (Figure 6a,b) in which ET_A (BQ123) and ET_B (BQ788) receptor antagonists have been included, showed that effects of ET-1 on the proliferation of PEO14 cells were not affected by the addition of the ET_B receptor antagonist, BQ788 (100 nM), but were almost completely blocked by the addition of ET_A receptor antagonist, BQ123 (100 nM), suggesting that the receptors involved are of the ET_A receptor type. In the case of PEO4 cells, addition of BQ123 ET_A receptor antagonist blocked the effect of ET-1 as in PEO14 cells, suggesting a similar mechanism of action through the same receptor type. However, in PEO4 cells, a minimal, but consistent, partial blockage of the ET-1 effect on the proliferation of these cells was observed on the addition of the BQ788, ET_B receptor antagonist, possibly suggesting an effect of ET-1 on proliferation through the ET_B receptor subtype. Slight, statistically significant, inhibition of growth (10%) was observed in PEO14 cells incubated in the presence of 100 nM BQ123 ET_A antagonist but absence of exogenous ET-1. No such inhibition was observed with the addition of BQ788 ET_B receptor antagonist in the same cell line or with either antagonists in PEO4 cells. Varying the concentrations of the antagonists in these experiments did not alter the effects significantly.

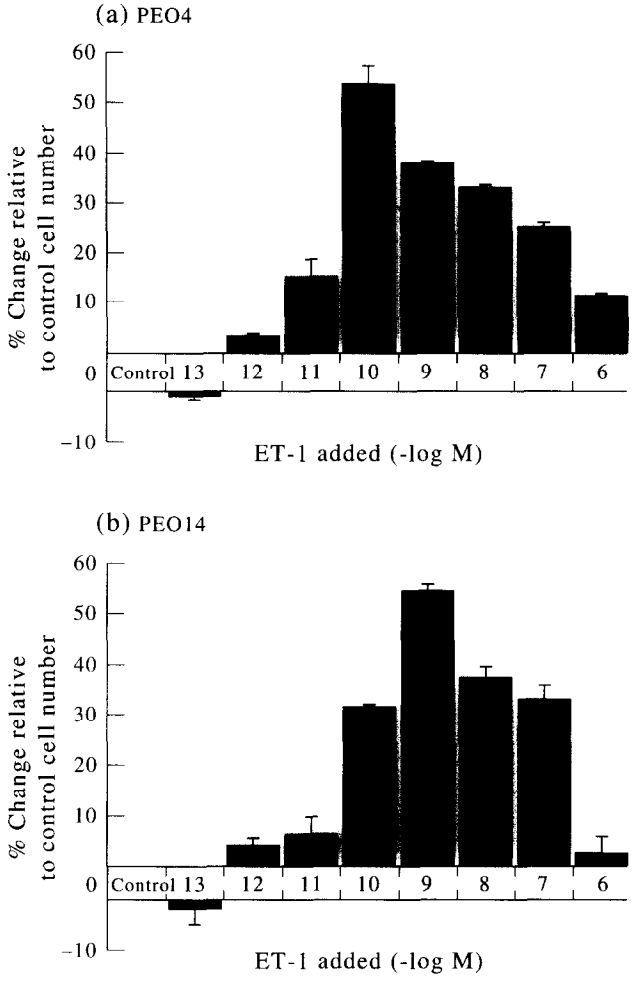

Figure 4. Effect of the exogenous addition of ET-1 on the growth of PEO4 cells (a) and PEO14 cells (b), growing in 1% FCS for a 5 day period. Results represent mean values of four experiments (bars: mean ± SEM).

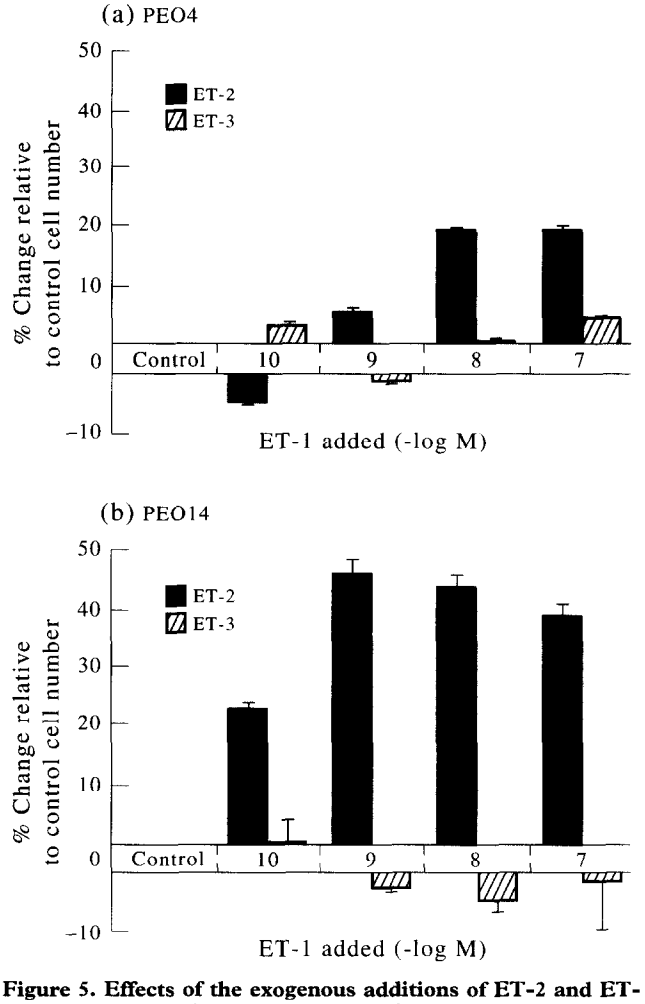

Figure 5. Effects of the exogenous additions of ET-2 and ET-3 on the growth of PEO4 (a) and PEO14 (b) cells growing in 1% FCS for a 5 day period. Results represent mean values of four experiments (bars: mean ± SEM).

The growth inhibition of PEO14 cells by BQ123 suggested autocrine regulation via the ET_A receptor. To investigate this further, "antisense" oligonucleotide sequences designed to block ET_A-R and ET_B-R RNA specifically (and therefore the production of ET receptors in both cell lines) were tested against both PEO4 and PEO14 cell lines. The specificity of the oligonucleotides was investigated by using "sense" and "random" sequences as controls which would theoretically not bind the RNA for ET receptors. Results for PEO14 cells shown in Figure 7 suggest that while "sense" and "random" sequences for both ET receptors and an "antisense" sequence for ET_B-R had no effect on the growth of PEO14 cells (at either 10 or 30 μM), the "antisense" oligonucleotide targeted to ET_A-R (10 and 30 μM concentrations) resulted in a 70% inhibition of growth ($P < 0.05$). In PEO4 cells (Figure 7a), "sense", "antisense" and "random" oligonucleotides for ET_A and ET_B receptors (added at either 10 or 30 μM) had no effect on the growth of the cells.

RT-PCR for mRNA of endothelins and their receptors

The presence of mRNA for ET-1, ET-2, ET-3 and ET_A, ET_B receptors in PEO4 and PEO14 cell lines was investigated using RT-PCR technology. Figure 8 shows the PCR products separated on an agarose gel. ET-1 and ET-3 specific primers amplified mRNA bands of the predicted sizes (582 and 493 bp, respectively) and primers for ET_A and ET_B receptors also amplified products at 368 and 530 bp, respectively (bands correspond to predicted sizes) (Table 1). No bands were detected for ET-2 in either cell line, but the same ET-2 primers amplified a specific band for ET-2 (197 bp) in other systems (data not shown). β-Actin was used as a positive control in both cell lines and the use of specific primers for its mRNA produced bands of the predicted size (262 bp). Negative controls containing no RNA but including specific primers produced no bands for any of the mRNA sequences excluding the possibility of the presence of the bands due to contamination during the several steps of the RT-PCR. Samples of extracted mRNA not taken through the RT step but amplified for 30 cycles in a PCR reaction also produced no specific and significant bands, excluding the possibility of DNA contamination in the RNA preparations. The specificities of the bands for ET-1, ET-3, ET_AR and ET_BR were confirmed by Southern blotting (data not shown).

DISCUSSION

The results show that PEO4 and PEO14 ovarian carcinoma cell lines respond, in terms of growth, to exogenous addition of ET-1 and ET-2, but not ET-3. Both cell lines

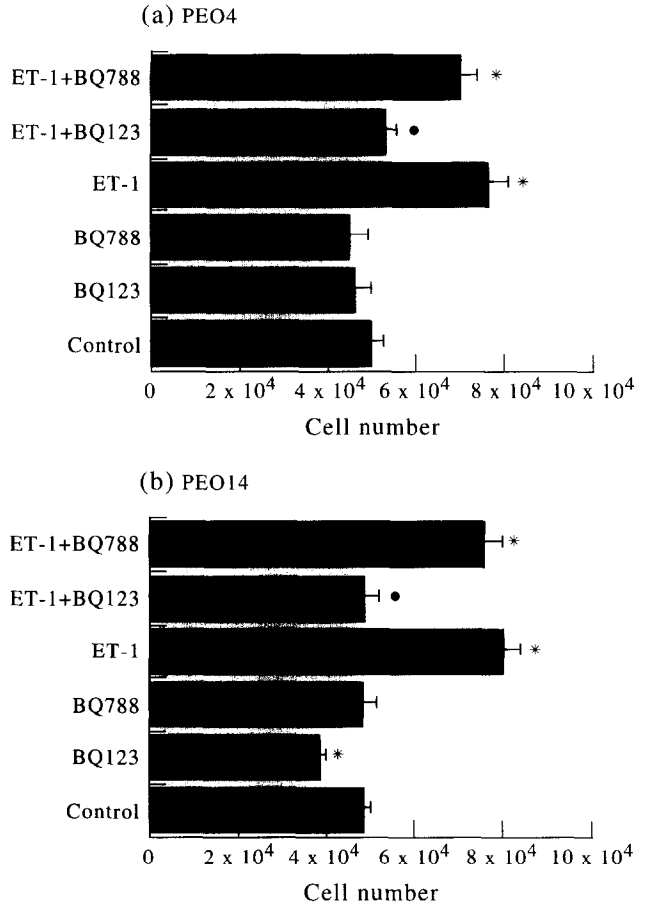

Figure 6. Effects of ET-1 and/or BQ123 and BQ788 receptor antagonists on growth of PEO4 (a) and PEO14 (b) ovarian carcinoma cells. Cells were incubated in 1% serum conditions with the indicated concentrations of ET-1 alone (1 nM), ET-1 with BQ123 ET_A antagonist (100 nM), ET-1 with BQ788 ET_B antagonist (100 nM) or either of the antagonists alone (100 nM) for 72 h. Cells were harvested and cell numbers were counted in a ZF Coulter counter. The results represent mean values of three experiments. (bars: mean ± SEM) and asterisks (*) represent statistical significance ($P < 0.05$) compared to controls, while points (•) represent statistical significance compared to ET-1.

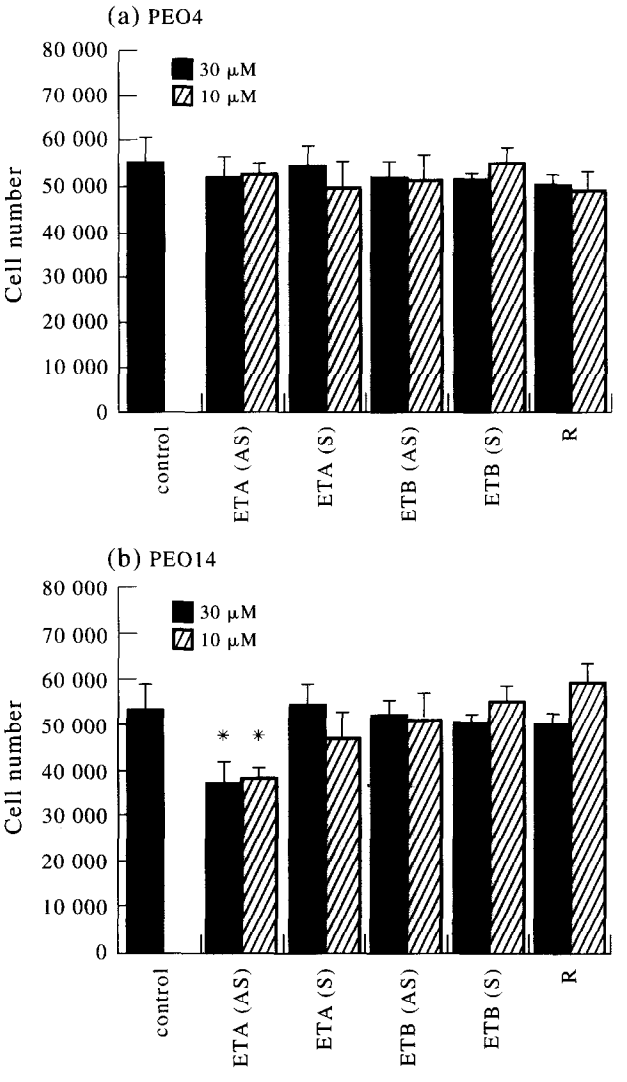

Figure 7. Effects of the use of "antisense" (AS), "sense" (S) and "random" (R) oligonucleotides for ET_A-R and ET_B-R at concentrations of 10 and 30 μM on the growth of PEO4 (a) and PEO14 (b) cells after a 7 day incubation period. Day zero counts for PEO14 and PEO4 controls were 29 836 ± 794 and 31 254 ± 879 cells, respectively. Results shown are mean ± SEM values from three experiments performed on separate occasions and the asterisk (*) represents statistical significance ($P < 0.05$) compared to control values at the end of the 7 day incubation period.

also bind endothelin with high affinity, consistent with the presence of endothelin receptors. Additionally, both PEO4 and PEO14 cell lines express mRNA for ET-1 and ET-3 and secrete ET-1-like material as determined by radioimmunoassay of conditioned media. It is, therefore, feasible that the growth of these cell lines may be mediated, at least in part, by an autocrine mechanism involving endothelin. Previous studies have also demonstrated the production of immunoreactive ET-1 in the pancreatic and colon carcinomas [1, 8], human breast cancer tissues [9], breast epithelial cells [10], HeLa and Hep-2 cells [2] and three ovarian carcinoma cell lines OVCA-433, A2780 and SK-OV-3 [6].

In our study, conditioned media from both PEO4 and PEO14 ovarian carcinoma cell lines contained substantial amounts of immunoreactive ET-1-like material which diluted in parallel with standard ET-1 as compared in a radioimmunoassay. PEO14 cells secreted 10 times the amount of peptide (20.2 fmol/10^6 cells/72 h) secreted by PEO4 cells (1.7 fmol/10^6 cells/72 h). These levels are comparable to the reported levels of ET-1 produced by other cell lines [1, 2, 6, 8–10] and are sufficient to produce mitogenic effects. The secretion of immunoreactive ET-1-like material in the conditioned media of both PEO4 and PEO14 cell lines reached a plateau after 24 h. Similar observations have been reported for other cell lines [5] and may be caused by the action of the degradation enzymes which have been shown to be produced in parallel with the secretion of ETs and were detectable as early as 6 h after the beginning of the incubation of the cells in the medium [5]. To identify the nature of the isoforms of endothelin expressed, the expression of mRNA for specific endothelins was examined by use of RT-PCR technology. Single bands for ET-1 and ET-3 were detected on an agarose gel using RNA from both cell lines.

The exogenous addition of ETs to PEO4 and PEO14 cells showed that ETs produced significant stimulatory

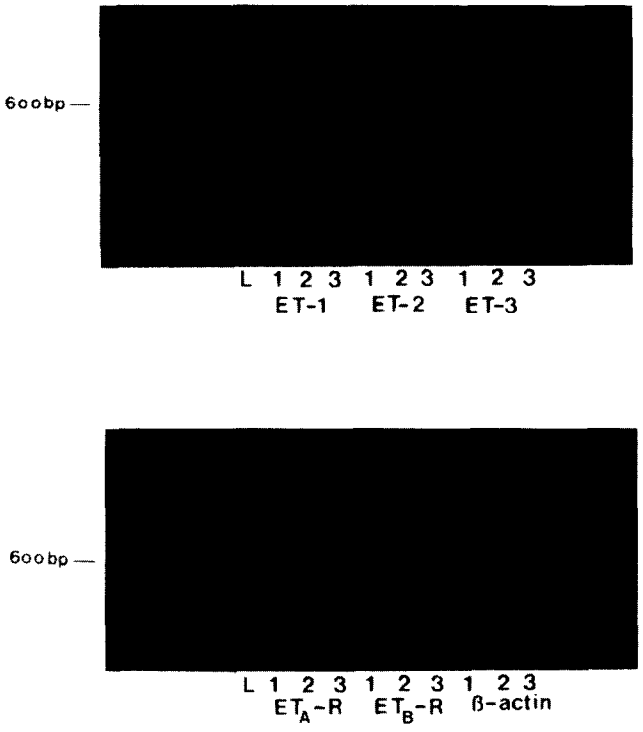

Figure 8. RT-PCR products of ET-1, ET-2, ET-3, ET_A receptor (ET_A-R), ET_B receptor (ET_B-R) and β-actin in PEO4 and PEO14 cell lines. Lane L: 100 bp DNA marker, lane 1: PEO4, lane 2: PEO14, lane 3: control samples without mRNA. The predicted sizes were: ET-1: 582 bp, ET-2: 197 bp, ET-3: 493 bp, ET_A-R: 368 bp, ET_B-R: 530 bp, β-actin: 262 bp.

effects on the growth of both ovarian carcinoma cell lines. The effect of exogenous ET-1 addition was concentration dependent with the maximum effects (around 50% increase in control values) shown at 10^{-10}–10^{-9} M in these cell lines (Figure 4). The minimum ET-1 concentration necessary to produce a significant increase in cell number in both PEO4 and PEO14 was 10^{-12} M. This concentration is higher than the circulating ET-1 concentration in humans (10^{-13} M) [5] but 100 times less than the concentration (10^{-10} M) reported for producing mitogenesis in fibroblasts and mesangial cells [5]. ET-2 also stimulated an increase in cell numbers in a concentration-dependent manner, but quantitative differences were evident between the cell lines, PEO14 cells appearing more sensitive. Thus, 10^{-8} M was necessary to stimulate the maximum effect (20% increase in cell growth) in PEO4 cells compared to 10^{-9} M needed to stimulate the maximum effect (46% increase) in PEO14 cells (Figure 4). In Figure 3, the effects of ET-1 on the growth of PEO4 and PEO14 cell lines show a biphasic nature with a smaller effect being seen at higher exogenous concentrations (10^{-7} M) than lower exogenous concentrations (10^{-9} M) of the peptide added to the cells. Such "bell-shaped" effects on growth have been observed with other peptides and could be explained by the downregulation of endothelin receptors by the addition of high concentrations of ET-1. Indeed, such effects have also been described in endothelial and smooth muscle cells which secrete ET-1-like material and also express endothelin receptors on their membranes [11]. A previous report on ovarian cancer cell lines suggested increasing stimulatory effects of ET-1 addition at increasing concentrations of the peptide and maximum effects at 10^{-7} M. Exogenous additions of ET-3 did not result in any significant mitogenic response in either cell line and at any concentration (10^{-13}–10^{-6} M) tested (Figure 5). The nature of the receptor type(s) involved in the proliferative responses was investigated using specific antagonists (BQ123 and BQ788 for ET_A and ET_B receptors, respectively). The results with BQ123 receptor antagonist suggest that ET-1 exerted its mitogenic effects through ET_A receptors in both cell lines. In the case of PEO4 cells, a small percentage (20%) of the mitogenic effect of ET-1 was blocked by the addition of BQ788 ET_B receptor antagonist. This may be caused by a small number of ET_B receptors on the membrane of these cells. The small growth inhibition in PEO14 cells seen with the addition of BQ123 (ET_A receptor antagonist), in the absence of exogenously added ET-1, could represent blockade of the binding of ET-1 produced and secreted by the same PEO14 cells on the ET_A receptors on their membrane. The antisense experiments showed that antisense for ET_A receptor had a 60% inhibitory effect on the growth of PEO14 but not PEO4 cells. This suggested that ET-1 secreted by PEO14 cells acts through an autocrine mechanism and activates their growth through binding to the ET_A receptors expressed in the same cells. All these observations suggest the expression of at least one of the two types of ET receptors in both cell lines and most probably the ET_A receptor which shows slightly higher affinity for ET-1 than ET-2 and no affinity for ET-3.

Radiolabelled ET-1 (^{125}I-ET-1) was also used to identify ET receptors in membrane preparations from PEO4 and PEO14 cells. Results from PEO14 cells suggested the presence of a single type of receptor binding site (K_d = 0.56 nM, B_{max} = 0.31 pmol/mg protein), while studies on PEO4 cells suggested the presence of two distinct receptor binding sites, a lower affinity binding site, K_d = 0.49 nM, B_{max} = 0.23 pmol/mg protein, similar to the one seen in PEO14cells, and a higher affinity binding site, K_d = 0.065 nM, B_{max} = 0.047 pmol/mg protein. The nature of the receptors expressed on the surface of both cell lines was investigated in competition experiments where ET-1, ET-3, BQ123 and BQ788 were competing with ^{125}I-ET-1 for binding to membrane preparations from both cell lines. Increasing concentrations of ET-3 also inhibited ^{125}I-ET-1 binding but its effects were observed at higher concentrations compared to the same effects seen with ET-1. Results from these experiments support the presence of ET_A receptors in both cell lines. A slight (statistically significant) inhibition of ^{125}I-ET-1 binding to PEO4 cells in the presence of BQ788 might suggest (and support the suggestion made previously) the presence of a limited amount of ET_B receptors on the surface of these cells. Since PEO4 cells secrete only 1/10 the amount of ET-1 of PEO14 cells, this might explain a reduced dependence in PEO4 cells.

Combining the results from growth and binding experiments, we would suggest that the lower affinity binding site in PEO4 cells and the single similar affinity binding site on PEO14 cells may represent the ET_A receptor type present in both cell lines. The higher affinity binding site on PEO4 cells could, therefore, represent either the ET_B receptor type or a higher affinity ET_A receptor. RT-PCR analysis of mRNA from both cell lines suggests the presence of mRNA for both ET_A and ET_B receptors. However, the fact that the affinity of this higher receptor binding site is similar to reported values for ET_A type receptors [6], from antagonist

experiments on both binding and growth, and the fact that no mitogenic effects are observed with ET-3 exogenous additions on PEO4 cell lines, it is more reasonable to suggest that this represents a higher affinity ET_A receptor binding site. Similar ET_A receptors with two different affinity binding sites have also been reported in S3T3 fibroblasts [12]. A number of individual studies have identified specific ET receptors in S3T3 fibroblasts, $K_d = 1 \pm 0.2$ nM [13–15], porcine granulosa cells, $K_d = 0.59$ nM [3], HeLa and Hep-2 cells $K_d = 10$ nM and 300 nM, respectively [2]. In human ovarian carcinoma cell lines, it has been suggested that single affinity binding sites of the ET_A type are present in OVCA 433, $K_d = 0.02 \pm 0.004$ nM, A2780, 0.03 ± 0.005 nM, and in SK-OV-3, $K_d = 0.15 \pm 0.02$ nM [6].

In summary, both PEO4 and PEO14 ovarian carcinoma cells produce ET-1-like material, and also contain specific ET_A receptors on their membranes. Furthermore, as the growth of these cell lines is stimulated by ET-1 and ET-2, it is possible that tumour cell growth may be mediated through an autocrine mechanism involving ET-1. These data would suggest that ETs may play a role in the growth regulation of ovarian cancer.

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