

Stimulation of colorectal cancer cell line growth by ET-1 and its inhibition by ET_A antagonists

H Ali, M Loizidou, M Dashwood, F Savage, C Sheard, I Taylor

Abstract

Background—The vasoactive peptide endothelin 1 (ET-1) acts via two receptors, endothelin receptors A (ET_A) and B (ET_B). ET-1 is overexpressed by human cancers in vivo and in vitro and may be mitogenic for cancer cells.

Method—To elucidate if ET-1 is a growth regulator the following were investigated in human colorectal cancer cell lines (LIM1215 and HT29): ET-1 production by ELISA; ET receptor expression using radioligand autoradiographic techniques; and responsiveness to ET-1, and to ET_A and ET_B antagonism by growth measurements.

Results—ET-1 was produced by LIM1215 and HT29 cells (21.3 and 41.7 fmol/ml/10⁶ cells (24 hours); 22.6 and 71.7 fmol/ml/10⁶ cells (48 hours), respectively). ET_A and ET_B receptors were expressed by both cell lines. Addition of ET-1 resulted in a dose dependent increase in cell numbers which was significant at 10⁻⁸–10⁻⁹ M for LIM1215, with the greatest increase at 10⁻⁸ M (32.7% and 28.4% increase above controls at 48 hours and 72 hours; p<0.05) and at 10⁻⁸–10⁻⁹ M for HT29, with the greatest increase at 10⁻⁹ M (13.4% and 15.7% increase above controls at 48 hours and 72 hours; p<0.05). ET_A antagonists BQ123 and BQ610, but not the ET_B antagonist BQ788, inhibited ET-1 induced proliferation of both LIM1215 and HT29 (p<0.05).

Conclusion—ET-1 can stimulate the proliferation of colorectal cancer cell lines via the ET_A, but not the ET_B, receptor.

(Gut 2000;47:685–688)

Keywords: endothelin-1; endothelin receptor; colorectal cancer; colorectal cancer cell lines

Department of Surgery, Royal Free and University College Medical School, London, UK

H Ali
M Loizidou
F Savage
C Sheard
I Taylor

Departments of Molecular Pathology and Clinical Biochemistry, and Surgery, Royal Free and University College Medical School, London, UK
M Dashwood

Correspondence to: Professor I Taylor, Department of Surgery, Royal Free and University College Medical School, Charles Bell House, 67–73 Riding House Street, W1P 7LD, London UK. m.loizidou@ucl.ac.uk

Accepted for publication 9 May 2000

colonic, pancreatic, ovarian, breast, stomach, and prostate.^{13–16} Shichiri and colleagues have proposed that ET-1 acts as an autocrine growth factor.¹⁷ Specifically in ovarian cancer cells, ET-1 has been shown to give an autocrine mitogenic stimulus via the ET_A receptor.¹⁸

In colorectal cancer, raised levels of ET-1 have been detected in the plasma of patients with and without metastasis to the liver. Furthermore, ET-1 has been demonstrated immunohistochemically in tumour epithelial cells, stroma, and the blood vessels of both primary colorectal cancer and liver metastases.^{19–21} Autoradiographic studies have detected the presence of binding sites (putative receptors) for ET-1 in colorectal cancer specimens and normal tissue, including the nerve supply of the colon.^{22–24} Although colorectal cancer cells have been shown to secrete ET-1 in vitro, it is not known if this peptide acts as a mitogen for these cells.

The aims of our study were to investigate: firstly, the production of ET-1 and expression of ET_A and ET_B receptors by two colorectal cancer cell lines; secondly, the ability of ET-1 to stimulate the proliferation of these cell lines; and finally, the potential of ET receptor antagonism as an inhibitor of growth.

Materials and methods

The human colorectal cancer cell lines HT29 (ECACC, Salisbury, Wiltshire, UK) and LIM1215 (kindly donated by Dr M O'Hare, Ludwig Institute for Cancer Research, London, UK) were used. The cells were routinely cultured at 37°C with 5% CO₂ in 95% air in Dulbecco's modified Eagle medium (DMEM), supplemented with 10% fetal calf serum (FCS), L-glutamine 2 mM, penicillin 100 IU/ml and streptomycin 100 µg/ml (all from Imperial Laboratories, Basingstoke, Hants, UK).

ET-1 PRODUCTION

Cells were plated to obtain 85–90% confluence by 72 hours. They were seeded and left to grow in 10% FCS containing medium for the first 24 hours. This was replaced by serum free medium and supernatants were collected after 24 and 48 hours and stored at -70°C until assayed. At each time point, the number of cells were counted on a haemocytometer in order to relate production of ET-1 to cell numbers. ET-1 in the media was measured using an ET-1 kit for an ELISA (Nycomed Amersham, Little Chalfont, Buckinghamshire, UK) and the

The endothelins are a family of 21 amino acid peptides. There are three members in the endothelin family: endothelin 1, 2, and 3 (ET-1, ET-2, and ET-3). These peptides act via the G protein coupled to receptor subtypes A (ET_A) and B (ET_B); ET-1 and ET-2 bind to ET_A with a higher affinity than ET-3 and all isopeptides display similar affinities for ET_B.^{1–3} ET-1 was first isolated from porcine aortic endothelial cells and has potent vasoactive activity.⁴ However, ET-1 is now recognised as having multiple pathophysiological activities, including mitogenesis.^{5–7} Increased ET-1 levels have been detected in plasma and tissue samples from patients with solid malignant tumours, including liver, lung, prostate, and breast cancers.^{8–12} ET-1 is also produced in vitro by several cancer cell lines, including

Abbreviations used in this paper: ET, endothelin; ET_A, endothelin receptor A; ET_B, endothelin receptor B; DMEM, Dulbecco's modified Eagle medium; FCS, fetal calf serum; PBS, phosphate buffered saline.

absorbance read at 550 nm on a plate reader (MRX Denley, Billingham, West Sussex, UK). Baseline readings of ET-1 were provided by assaying serum free DMEM. The assay has a sensitivity of 1–32 fmol per well for a 96 well plate and specificity of 98% for ET-1; cross reactivity with ET-2 is <2%, and with ET-3 <0.01%.

ENDOTHELIN RECEPTOR EXPRESSION

Cultured cells were trypsinised and cytopspins were prepared using 10^6 cells per spot. The slides were incubated with 150 pM ^{125}I ET-1 (specific activity 2000 Ci/mM) and non-specific binding was determined by coincubation of alternate slides with 500 nM unlabelled ET-1 at room temperature. After incubation, cells were dried and autoradiographs generated by apposing to Hyperfilm ^3H (Nycomed Amersham) for up to eight days. Film was processed according to the manufacturer's instructions and cells were stained with haematoxylin and eosin for histology. Cellular binding was achieved by post fixing incubated cells with paraformaldehyde vapour (80°C for two hours), coating them with molten nuclear emission (Ilford K2, Mobberly, Cheshire, UK), and exposing them for eight days, after which emulsion was processed and cells stained with Hand E.²⁵ Endothelin receptor subtypes were identified using 150 pM ^{125}I PD151242 for ET_A or ^{125}I BQ3020 for ET_B (specific activity 2000 Ci/mM for both (Nycomed Amersham)).

GROWTH UNDER THE INFLUENCE OF ET-1 AND ENDOTHELIN RECEPTOR ANTAGONISTS

Twenty thousand cells per well were plated into 24 well plates and grown for 24 hours in fully supplemented medium. The cells were washed and incubated in serum free medium containing 10^{-7} – 10^{-12} M ET-1/well for 48 or 72 hours. At each time point the cells were fixed in 10%

formaldehyde and cell number measured using the methylene blue assay.²⁶ The optimum concentrations of ET-1 for stimulation of growth from this experiment were used to investigate receptor antagonism.

ET_A receptor antagonists BQ123 or BQ610 (100 nM), or ET_B receptor antagonist BQ788 (100 nM) were added to each well with or without ET-1 at 10^{-8} M (LIM1215) or 10^{-9} M (HT29). The plates were fixed with 10% formaldehyde at 48 and 72 hours; cell number was measured using the methylene blue assay.

Results

PRODUCTION OF ET-1 IN HUMAN COLORECTAL CANCER CELLS

The production of ET-1 by the colorectal cell lines LIM1215 and HT29 was measured in media conditioned for 48 or 72 hours. Values were corrected for a baseline reading of 0.6 fmol/ml (medium only). For LIM1215 there was only a small increase with time from 21.3 fmol/ml/ 10^6 cells at 24 hours to 22.5 fmol/ml/ 10^6 cells at 48 hours. In contrast, HT29 secreted a higher concentration of ET-1 at 24 hours (41.7 fmol/ml/ 10^6 cells) which further increased to 71.7 fmol/ml/ 10^6 cells at 48 hours.

ENDOTHELIN RECEPTOR EXPRESSION IN HT29 AND LIM1215 COLORECTAL CANCER CELLS

Binding for ET-1, ET_A antagonist (PD151242), and ET_B agonist (BQ3020) in both cell lines was demonstrated by autoradiography. Total binding was clearly in excess of non-specific binding for ET-1 and its receptor subtypes for both LIM1215 and HT29 colorectal cancer cells, suggesting the presence of both receptors in these cell types (fig 1).

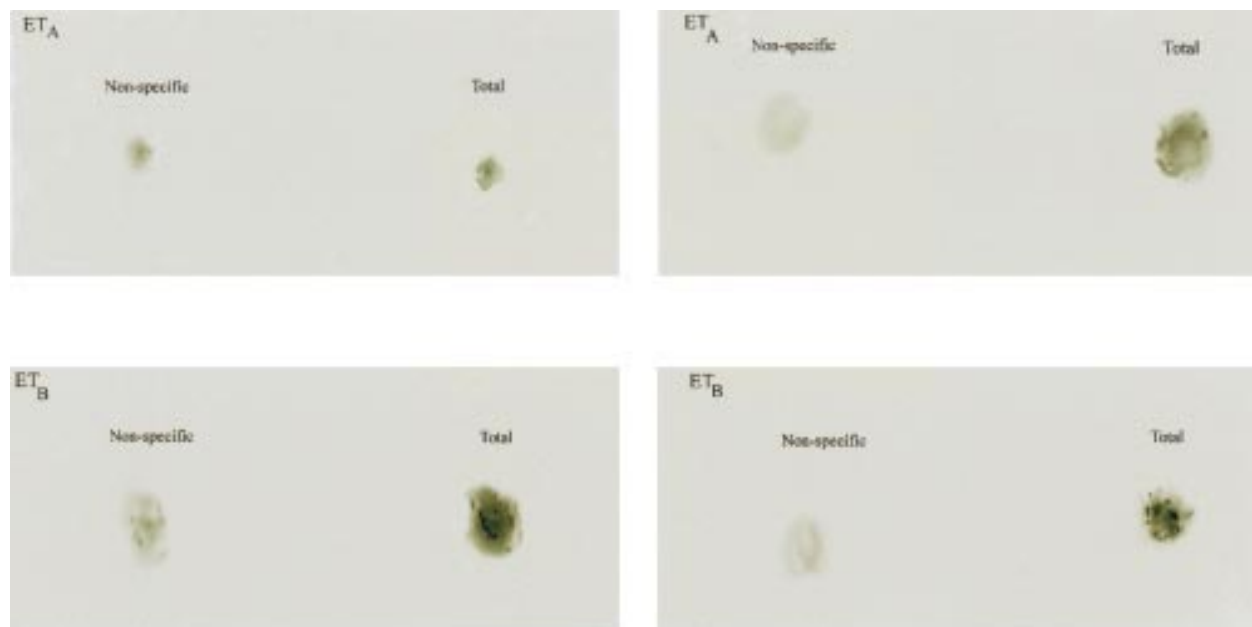


Figure 1 Binding for endothelin receptors (ET_A and ET_B) on LIM1215 (left) and HT29 (right) cell cytopspins was demonstrated by autoradiography. Slides were incubated with ET_A antagonist (^{125}I PD151242) or ET_B agonist (^{125}I BQ3020) for total binding. Non-specific binding was determined by incubation with excess unlabelled ligand. Total binding was clearly in excess of non-specific binding, suggesting the presence of both receptors in these cell types.

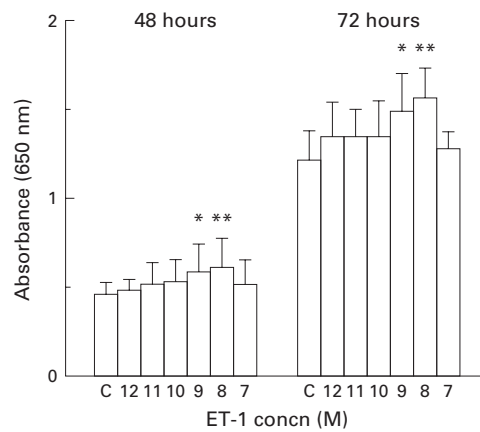


Figure 2 Effect of addition of endothelin 1 (ET-1) on LIM1215 cell growth. Cells were grown for 48 or 72 hours in the presence of increasing concentrations of ET-1 (10^{-12} M to 10^{-7} M; shown on the x axis as 12 to 7). Cell growth was measured using the methylene blue assay and read as absorbance (equivalent to cell numbers) at 650 nm. Results are shown as mean (SD). Statistically significant growth is shown as * $p < 0.05$ or ** $p < 0.01$ (Student's paired t test). C, control.

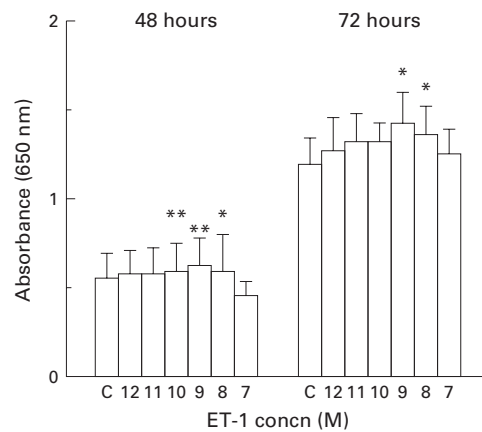


Figure 3 Effect of addition of endothelin 1 (ET-1) on HT29 cell growth. Cells were grown for 48 or 72 hours in the presence of increasing concentrations of ET-1 (10^{-12} M to 10^{-7} M; shown on the x axis as 12 to 7). Cell growth was measured using the methylene blue assay and read as absorbance (equivalent to cell numbers) at 650 nm. Results are shown as mean (SD). Statistically significant cell growth is shown as * $p < 0.05$ or ** $p < 0.01$ (Student's paired t test). C, control.

EFFECT OF EXOGENOUS ADDITION OF ET-1 AND RESPONSE TO ET_A AND ET_B ANTAGONISTS

Addition of ET-1 to both LIM1215 and HT29 resulted in a dose dependent increase in cell number (figs 2, 3). For LIM1215 this was maximal at 10^{-8} M with a rise of 32.7% and 28.4% above controls at 48 and 72 hours, respectively. In contrast, the maximal number of cells for HT29 occurred with 10^{-8} M ET-1 when there was an increase of 13.4% and 15.7% above control numbers at 48 and 72 hours, respectively.

Competitive ET_A antagonists BQ123 and BQ610 significantly inhibited ET-1 induced proliferation of both LIM1215 and HT29 at 48 and 72 hours (figs 4, 5). ET_B antagonists had no effect on ET-1 stimulated proliferation.

Discussion

The effect of ET-1 on colorectal cancer cell growth was investigated in vitro. The two

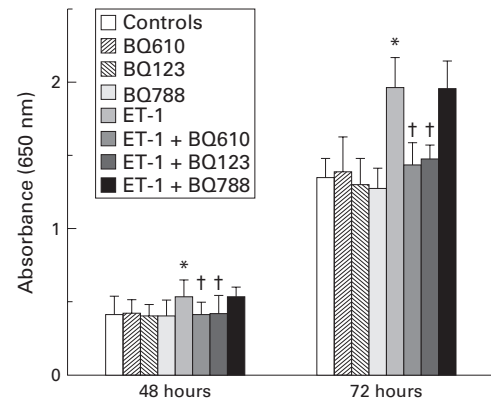


Figure 4 Effect of endothelin 1 (ET-1) and/or endothelin receptor antagonists on LIM1215 cell growth. Cells were grown for 48 or 72 hours in the presence of 10^{-8} M ET-1 and/or ET_A receptor antagonist BQ123 or BQ610, or ET_B receptor antagonist BQ788, all at 100 nM. Cell growth was measured using the methylene blue assay and read as absorbance (equivalent to cell numbers) at 650 nm. Results are shown as mean (SD). Cell numbers were significantly higher in the ET-1 group compared with control cells (* $p < 0.05$, Student's t test); cell numbers from groups treated with both ET-1 and ET_A antagonists were similar to control levels, and were significantly lower than in the ET-1 group († $p < 0.05$, Student's t test).

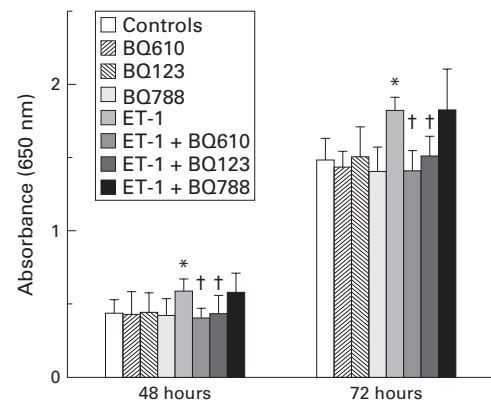


Figure 5 Effect of endothelin 1 (ET-1) and/or endothelin receptor antagonists on HT29 cell growth. Cells were grown for 48 or 72 hours in the presence of 10^{-7} M ET-1 and/or ET_A receptor antagonist BQ123 or BQ610, or ET_B receptor antagonist BQ788, all at 100 nM. Cell growth was measured using the methylene blue assay and read as absorbance (equivalent to cell numbers) at 650 nm. Results are shown as mean (SD). Cell numbers were significantly higher in the ET-1 group compared with control cells (* $p < 0.05$, Student's t-test); cell numbers from groups treated with both ET-1 and ET_A antagonists were similar to control levels, and were significantly lower than in the ET-1 group († $p < 0.05$, Student's t test).

colorectal cancer cell lines used in this study secreted ET-1, as has been demonstrated for other cancers. Levels of ET-1 measured in the medium were similar to those previously reported for HT29 of 23 fmol/ml/ 10^6 cells.¹⁵

ET-1 acts via two receptors, ET_A and ET_B, which were found to be present on LIM1215 and HT29 by autoradiography. This technique, by utilising radiolabelled agonists and antagonists which display high affinity for receptors, not only demonstrated the presence of the receptors but also indicated that they occurred in a functional state.²⁷ Ovarian cancer cell lines have also been shown to produce ET-1 and express ET_A and ET_B receptors.^{18 28 29}

The mitogenic potential of exogenous ET-1 on these cell lines was assessed. This peptide

was found to significantly increase cell number for both cell lines with a greater increase for LIM1215 compared with HT29. The difference may be related to the greater endogenous production of ET-1 by HT29: if the mitogenic effect occurs via one or both of the receptors and these receptors are partially occupied by endogenous ET-1, then addition of further ET-1 will have less of an effect than in a cell line where the receptors are available for binding. The concentrations of ET-1 (10^{-9} M and 10^{-8} M) which resulted in an increase in cell growth are 10 000 times higher than circulating plasma levels of ET-1 in patients with colorectal cancer. However, locally (in tissues), much higher levels may occur.³⁰ For other cell lines, for example Swiss 3T3 fibroblasts, vascular smooth muscle, and ovarian cancer cell lines, a mitogenic effect of ET-1 was produced at 10^{-10} M, only 10-fold lower than that required to produce an effect on LIM1215 and HT29.^{5-7 18}

The effect of ET-1 on LIM1215 and HT29 was mediated via the ET_A and not the ET_B receptor, as demonstrated by the ability of BQ123 and BQ610 to prevent an increase in cell number on addition of ET-1. ET_A has also been demonstrated to be the receptor through which the mitogenic effect of ET-1 is mediated for ovarian cancer cell lines¹⁸ and for a number of other cell lines.^{5 6 9 30} In contrast, BQ788, the ET_B antagonist, had no effect on cell number.

In the only in vitro cancer model in which ET-1 mitogenic signalling has been studied, ovarian cancer cells were used. Binding of ET-1 to the ET_A G protein coupled receptor resulted in activation of phospholipase C activity and Ca²⁺/PKC signalling, which are the classical effectors of G protein signalling. Furthermore, other intracellular targets activated included: tyrosine kinases (for example, focal adhesion kinase p125^{FAK}), p42 mitogen activated protein kinase (MAPkinase), and immediate early response genes (for example, fos).²⁸ This suggests that ET-1 not only uses phospholipase C/PKC pathways but cross talks with tyrosine kinase cascades. These intracellular steps have been implicated in mitogenic signalling via ET_A in a variety of cell types, including fibroblasts and vascular smooth muscle cells.³⁰⁻³⁴

In conclusion, ET-1 can stimulate net cell growth of LIM1215 and HT29 colorectal cancer cell lines via the ET_A receptor. Whether this effect is mediated via a mitogenic stimulus as in ovarian cancer cells and other non-cancer cells, or an anti-apoptotic signal, or a combination of the two, has not yet been demonstrated in this model. However, the findings of Asham and colleagues¹⁹ and Shankar and colleagues²⁰ that ET-1 is produced by colorectal cancers combined with the data from this study are consistent with the notion that ET-1 may act as a mitogen in colorectal cancer and that there may be some therapeutic potential in the use of ET_A antagonists.

- 1 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**:2863-7.
- 2 Arai H, Nori S, Aramori I, et al. Cloning and expression of a cDNA encoding an endothelin receptor. *Nature* 1990;**348**:730-2.

- 3 Sakurai T, Yanagisawa M, Takuwa Y, et al. Cloning of a cDNA encoding a non-isopeptide selective subtype of the endothelin receptor. *Nature* 1990;**348**:732-5.
- 4 Yanagisawa M, Kurihara H, Kimura S, et al. A novel potent vasoconstrictor peptide produced by vascular endothelial cells. *Nature* 1989;**332**:411-15.
- 5 Kusuha M, Yamaguchi K, Ohnishi A, et al. Endothelin potentiates growth factor stimulated DNA synthesis in Swiss 3T3 cells. *J Cancer Res* 1989;**80**:302-5.
- 6 Komuro I, Kurihara H, Sugiyama T, et al. Endothelin stimulates c-fos and c-myc expression and proliferation of vascular smooth muscle cells. *FEBS Lett* 1988;**238**:249-52.
- 7 Nakaki T, Nakayama M, Yamamoto S, et al. Endothelin mediated stimulation of DNA synthesis in vascular smooth muscle cells. *Biochem Biophys Res Commun* 1989;**158**:880-3.
- 8 Giaid A, Hamid QA, Springall DR. Detection of endothelin immunoreactivity and mRNA in pulmonary tumours. *J Pathol* 1990;**162**:15-22.
- 9 Yamashita J, Ogawa M, Egami H, et al. Abundant expression of immunoreactive endothelin-1 in the growth of stromal cells in phylloides tumour. *Cancer Res* 1992;**52**:406-9.
- 10 Kojima K, Nihei Z. Expression of endothelin-1 immunoreactivity in breast cancer. *Surg Oncol* 1995;**4**:309-15.
- 11 Ishibashi M, Fujita M, Nagai K, et al. Production and secretion of endothelin by hepatocellular carcinoma. *J Clin Endocrinol Metab* 1993;**76**:378-83.
- 12 Nelson BJ, Chan-Tack K, Hedician SP, et al. Endothelin-1 production and decreased endothelin B receptor expression in advanced prostate cancer. *Cancer Res* 1996;**56**:663-8.
- 13 Kusuha M, Yamaguchi K, Nagasaki K, et al. Production of endothelin in human cancer cell lines. *Cancer Res* 1990;**50**:3257-61.
- 14 Oikawa T, Kusuha M, Ishikawa S, et al. Production of endothelin-1 and thrombomodulin by human pancreatic cancer cells. *J Cancer* 1994;**69**:1059-64.
- 15 Baley PA, Resink TJ, Eppinger U, et al. Endothelin messenger RNA and receptors are differentially expressed in cultured human breast epithelial and stromal cells. *J Clin Invest* 1990;**85**:1320-3.
- 16 Mathieu MN, Chevillard C. Endothelin-1 and subtypes are expressed in the gastric HGT-1 cell line. *J Cardiovasc Pharmacol* 1995;**26**:S508-9.
- 17 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**:1867-71.
- 18 Moraitis S, Langdon SP, Miller WR. Endothelin expression and responsiveness in human ovarian carcinoma cell lines. *Eur J Cancer* 1997;**33**:661-8.
- 19 Asham E, Loizidou M, Lakhani S, et al. Expression of endothelin-1 in 98 patients with colorectal cancer. *Eur J Surg Oncol* 1998;**23**:589.
- 20 Shankar A, Loizidou M, Aliev G, et al. Elevated endothelin-1 levels in patients with colorectal liver metastases. *Br J Surg* 1998;**85**:502-6.
- 21 Asham E, Shankar A, Loizidou M, et al. Production and secretion of endothelin-1 in colorectal cancer. *Br J Surg* 1997;**84**:1596.
- 22 Inagaki H, Bishop AE, Eimoto T, et al. Localisation of endothelin-like immunoreactivity and endothelin binding sites in human colon. *Gastroenterology* 1991;**101**:47-54.
- 23 Inagaki H, Bishop AE, Eimoto T, et al. Autoradiographic localisation of endothelin-1 binding sites in human colonic cancer tissue. *J Pathol* 1992;**168**:263-7.
- 24 Inagaki H, Bishop AE, Yura J, et al. Localisation of endothelin-1 and its binding sites in the nervous system of the human colon. *J Cardiovasc Pharmacol* 1991;**17**:S455-7.
- 25 Dashwood MR, Timm M, Muddle JR, et al. Regional variations in endothelin-1 and its receptor subtypes in human coronary vasculature: pathophysiological implications in coronary disease. *Endothelium* 1998;**6**:61-70.
- 26 Oliver MH, Harrison NK, Bishop JE, et al. A rapid and convenient assay for counting cells cultured in microwell plates: Application for assessment of growth factors. *J Cell Sci* 1989;**92**:513-18.
- 27 Dashwood M, Timm M, Kaski JC. Regional variations in ET_A/ET_B binding sites in human coronary vasculature. *J Cardiovasc Pharmacol* 1995;**26**:S351-4.
- 28 Bagnato A, Tecce R, Di Castro V, et al. Activation of mitogenic signaling by endothelin-1 in ovarian carcinoma cells. *Cancer Res* 1997;**57**:1306-11.
- 29 Bagnato A, Salani D, Di Castro V, et al. Expression of ET-1 and ET_A receptor in ovarian carcinoma: evidence for an autocrine role in tumour growth. *Cancer Res* 1999;**59**:720-7.
- 30 Walden PD, Irtmann M, Monaco ME, et al. Endothelin-1 production and agonist activities in cultured prostate derived cells: implications for regulation of endothelin bioactivity and bioavailability in prostatic hyperplasia. *Prostate* 1998;**34**:241-50.
- 31 Saville MK, Graham A, Malarkey K, et al. Regulation of ET-1 and lysophosphatidic acid stimulated tyrosine phosphorylation of focal adhesion kinase in Rat-1 fibroblasts. *Biochem J* 1994;**301**:407-14.
- 32 Fujitani Y, Ninomiya H, Okada T, et al. Suppression of ET-1 induced mitogenic responses of human aortic smooth muscle cells by interleukin-1 β . *J Clin Invest* 1995;**95**:2474-82.
- 33 Luttrell LM, Daaka Y, Lefkowitz J. Regulation of tyrosine kinase cascades by G-protein-coupled receptors. *Curr Opin Cell Biol* 1999;**11**:177-83.
- 34 Schwartz MA, Baron V. Interactions between mitogenic stimuli, or, a thousand and one connections. *Curr Opin Cell Biol* 1999;**11**:184-9.