Endothelins and hypoxia-inducible factor in cancer

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Abstract

The endothelin system is a family of three similar small peptides, two G-protein-coupled receptors and two proteinases. Endothelins have several physiological roles, notably in embryonic differentiation and vascular homeostasis. Numerous types of tumour express endothelins and their regulation is often aberrant when compared with the normal tissue from which the tumour arose. However, endothelin expression is tumour-type specific, and in some instances, expression of individual members of the endothelin system will be upregulated, while in other tumour types, they may be downregulated. Endothelins have numerous potential roles in tumours including modulating angiogenesis, inducing mitogenesis and invasion of tumour cells, and protecting cells from apoptosis. Expression of endothelins is controlled by the tumour microenvironment, whilst the endothelins themselves modify that environment; a case in point is that hypoxia stimulates endothelin expression via hypoxia-inducible factor (HIF)-1, while endothelins stabilise HIF-1 leading to expression of, for instance, vascular endothelial growth factor. This review highlights the potential roles of endothelins in various cancers and describes the pre-clinical and clinical progress that has been made in several tumour types – notably prostate, ovary, melanoma and breast cancer. The interactions between the endothelin network and HIF-1 are highlighted.

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Endothelins and cancer

The endothelin network

The endothelin (ET) family (Table 1) consists of three 21 amino acid (aa) peptides (ET-1, ET-2 and ET-3), two G-protein-coupled receptors (ET-RA and ET-RB) and two membrane-bound ET-converting enzymes (ECE-1 and ECE-2; Kedzierski & Yanagisawa 2001). ET-1 was initially found in the conditioned medium of cultured endothelial cells and its activity as a potent vasoconstrictive peptide was described (Itoh *et al.* 1988); ET-2 and ET-3 were rapidly described following discovery of ET-1 (Inoue *et al.* 1989), and further potential roles in a variety of tissues have been described (Kedzierski & Yanagisawa 2001). The three ET isoforms, which are highly conserved in human, rat and mouse (Saida *et al.* 2000), derive from three separately regulated genes yet have a similar structure (Inoue *et al.* 1989): 21 aa

peptides with a hydrophobic C-terminus and two cysteine bridges at the N-terminus (Fig. 1). The peptide sequences of ET-2 and ET-3 differ from ET-1 by two and six residues respectively.

Human ET-1 derives from a 212 aa precursor, preproendothelin-1, which is intracellularly cleaved by ECE-1 and ECE-2 (Opgenorth *et al.* 1992); further enzymes, yet to be identified, may be involved (Lambert *et al.* 2000). Removal of the signal sequence generates the 195 aa proendothelin-1, which is further processed to release the intermediate 38 aa 'big ET-1'. ECEs hydrolyse big ET-1 to yield the active 21 aa ET-1.

A novel endothelin peptide, ET-1(1–31), comprises 31 aa and is derived from hydrolysis of big ET-1 by chymase. Although ET-1(1–31) has been reported to have biological activity via direct or indirect mechanisms, the role of ET-1(1–31) is as yet relatively unknown. It is of note, however, that ET-1(1–31) may be chemotactic for both neutrophils and monocytes (Cui *et al.* 2001).

Table 1 Genes and peptide sequences of the ET network. Amino acids that differ in ET-2 and/or ET-3 from ET-1 are marked in bold/underlined

Gene	Mapping position	Peptide or protein	ET isoform peptide sequence
EDN1	6p24	ET-1	CSCSSLMDKECVYFCHLDIIW
EDN2	1p34	ET-2	CSCSS W L D K E CVV F CHLDIIW
EDN3	20q13	ET-3	C T C F T Y K D K E CV Y Y CHLDIIW
EDNRA	4q31	ET-RA	—
EDNRB	13q22	ET-RA	—
ECE1	1p36	ECE-1	—
ECE2	3q28-29	ECE-2	—

The gene for each endothelin has a distinct pattern of tissue expression: ET-1 is expressed by endothelial cells of many organs (Kedzierski & Yanagisawa 2001); ET-2 in the ovary (Ko *et al.* 2006) and intestine (Uchide *et al.* 1999) and ET-3 is found in the brain (Shinmi *et al.* 1989). Endothelins and their receptors are also expressed by ‘mobile’ inflammatory cells such as monocytes and macrophages (Ehrenreich *et al.* 1990, Grimshaw *et al.* 2002a). There is a relatively low basal level of synthesis of endothelins but these genes are readily inducible by inflammatory stimuli.

Two receptors for endothelin have been characterised: ET-RA (also known as EDNRA or ET_AR) and ET-RB (EDNRB, ET_BR). Each receptor contains transmembrane domains comprising seven stretches of 20–27 aa hydrophobic residues and has an N-terminal signal sequence and long extracellular domain. Endothelins bind these receptors with varying affinity: ET-RA binds ET-1 ≥ ET-2 > ET-3, but ET-RB shows no selective affinity for any ET subtype. Binding of the ligands to these g-protein-coupled receptors may modulate several overlapping signalling pathways resulting in the activation of phospholipase C and MAPK (mitogen-activated protein kinase) pathways, an increase in intracellular calcium and the induction of immediate early genes (Masaki *et al.* 1999, Nelson *et al.* 2003).

Physiological roles of endothelins

Endothelins have a number of physiological roles including: i) blood vessels: they maintain vasoconstriction, ii) heart: they affect the force and rate of contraction of the heart, iii) lungs: they regulate the tone of airways and blood vessels, iv) kidney: they control water and sodium excretion, and acid–base balance and v) brain: they modulate cardio-respiratory centres and hormone release.

Roles of endothelins in cancer

Numerous tumours produce one or more of the endothelins and their receptors, and there are many

potential roles in cancer: i) mitogenesis: endothelins have a mitogenic effect on both tumour and stromal cells and enhance tumour growth, ii) angiogenesis: endothelins modulate tumour angiogenesis, both directly through stimulation of endothelial cells, and indirectly through the induction of vascular endothelial growth factor (VEGF), iii) invasion and metastasis: stimulation of tumour cells with endothelins leads to an invasive phenotype via several autocrine and paracrine mechanisms including activation of matrix metalloproteinases (MMPs), iv) protection from apoptosis: endothelins can protect several cell types – including tumour cells, macrophages and endothelial cells – from apoptosis induced by cellular stresses including hypoxia and serum starvation and v) immune modulation: trafficking, differentiation and activation of tumour-infiltrating immune cells are all modulated by endothelins.

However, the expression and actions of endothelins in cancer are incompletely described and are tumour-type specific. Endothelin expression is increased in many types of tumour, yet in several types of tumour, expression of the endothelin receptors is decreased in neoplastic tissue. For instance, in carcinomas of the breast both ET-RA and ET-RB are increased, yet in prostate cancer ET-RB is decreased (Kopetz *et al.* 2002) and EDNRB is often methylated (Nelson *et al.* 1997),

Figure 1 Structure of endothelin-1. ET-1 is a 21 amino acid peptide with a hydrophobic C-terminus and two disulphide bonds at the N-terminus. ET-2 and ET-3 are structurally similar to ET-1, differing by two and six amino acids respectively. The amino acids which differ in the ET-2 sequence are indicated by ‘+’, while those which differ in ET-3 marked by ‘*’.

while in lung cancer, ET-RA is downregulated (Ahmed *et al.* 2000). The function of the ET-RB receptor in tumours is particularly enigmatic; in some cases, such as breast cancer, ligand-binding to ET-RB initiates several pro-tumour actions, such as promoting invasion (Grimshaw *et al.* 2004), yet in prostate cancer, the loss of ET-RB expression is postulated to increase ET-1 peptide in the tumour due to the loss of endothelin clearance function of ET-RB (Nelson *et al.* 1997).

Several different cell types within solid tumours may contribute to endothelin expression including not only the tumour cells but also fibroblasts, tumour-associated macrophages (TAMs) and endothelial cells. The tumour microenvironment, particularly hypoxia and inflammatory cytokines, may further influence endothelin expression by the tumour and stromal cells.

Endothelin receptor antagonists

The role of endothelins in vasoconstriction has led to the development of several antagonists of the endothelin receptors which are currently under investigation for the treatment of hypertension, heart failure and renal disease (Wessale *et al.* 2002). Bosentan, a mixed ET-RA/RB antagonist, is approved for treatment of pulmonary hypertension and is in clinical trials for malignancies including metastatic melanoma (Kefford *et al.* 2006). Of the antagonists available, it is the modified peptide-based ET-RA antagonist BQ123 (Ihara *et al.* 1992a,b) and ET-RB antagonist BQ788 (Ishikawa *et al.* 1994) that have been used extensively both *in vitro* and *in vivo* to study cancer, although these are not in clinical development. Atrasentan, a highly selective ET-RA antagonist, has been given to cancer patients in phase II trials for prostate cancer and delayed time to progression (Norman 2002). Phase III trials for hormone-refractory prostate cancer are underway (Jimeno & Carducci 2005).

Endothelin receptor antagonists inhibit proliferation of Kaposi's sarcoma cells (Bagnato *et al.* 2001), Ewing's sarcoma and neuroblastoma cells (Berry & Burchill 2002), melanoma cells (Lahav *et al.* 1999) and ovarian carcinoma cells (Bagnato *et al.* 1999).

Endothelins and HIF-1

Transcriptional regulation of numerous hypoxia-responsive genes, including VEGF and ET-1, is via the critical mediator of hypoxia-induced transcription, hypoxia-inducible factor (HIF)-1, which is a dimer of α and β subunits; HIF-1 β is present in all cells and is stable under normoxia, but HIF-1 α is rapidly ubiquitinated and degraded in the presence of oxygen. Hypoxia stabilises the HIF-1 α monomer, leading to

heterodimerisation and the formation of the HIF-1 complex that can then initiate transcription of genes whose promoter contains a hypoxia response element (HRE; Wenger 2000). There is a reciprocal relationship between endothelin expression and HIF activity: not only does HIF mediate transcription of endothelins, but endothelins stabilise the HIF-1 α monomer during normoxia leading to HIF-mediated transcription of angiogenic genes and chemokine receptors.

Hypoxia induces endothelin transcription in several cell types including endothelial cells (Kourembanas *et al.* 1991) and tumour cells (Koong *et al.* 2000, Grimshaw *et al.* 2002b). There is a functioning proximal HRE in the antisense strand of the promoter of ET-1 (Aversa *et al.* 1997, Hu *et al.* 1998) and induction of endothelin expression by hypoxia is via HIF-1 (Minchenko & Caro 2000). ET-2 is stimulated by hypoxia in squamous cell carcinoma cells (Koong *et al.* 2000) and breast carcinoma cells, and *in vivo* endothelin expression co-localises with hypoxic areas of murine breast tumours (Grimshaw *et al.* 2002b). The upregulation of ET-2 bears the characteristics of being HIF-dependent (i.e. it is also induced by the iron chelator cobalt chloride) but HIF-1 α stabilisation and HIF/HRE binding has not yet been conclusively proven.

No HRE has yet been described in the promoter of either endothelin receptor, but in certain cell types, both receptors may be induced by hypoxia (Shibaguchi *et al.* 2000, Grimshaw *et al.* 2002b). This may, however, be a secondary effect due to the increase in endothelin production seen under hypoxic conditions: endothelins themselves induce endothelin receptor production by breast carcinoma cells (Grimshaw *et al.* 2004). It is also possible that one or more of the cytokines induced by hypoxia stimulate receptor production. It has been reported that ET-RA mRNA and protein is induced by cobalt chloride (an iron chelator which inhibits the HIF-degradation pathway) in several breast cancer cell lines (Wulffing *et al.* 2005c).

Although hypoxia is the major factor that stabilises HIF-1 α , other stimuli such as growth factors, hormones and nitric oxide may stabilise HIF-1 α and induce transcription of HIF-dependent genes. One such factor that stabilises HIF-1 α is, in fact, ET-1 which *in vitro* induces VEGF transcription via ET-RA in normoxic ovarian carcinoma cells in a HIF-1-dependent manner by an extent comparable with hypoxia (Spinella *et al.* 2002); these effects are inhibited by BQ123 (Fig. 2). Inhibition of human ovarian tumour growth in nude mice after treatment with Atrasentan is associated with reduced VEGF and microvessel density (Spinella *et al.* 2004b).

Figure 2 Promotion of tumour angiogenesis by endothelins via HIF1. ET-1 binds to ET-R activating multiple signalling pathways. Similar to hypoxia, these pathways result in the stabilisation and translocation of HIF1 α , allowing the formation of the HIF1 complex in the nucleus. The HIF1 complex binds to the HRE in the promoter of 'hypoxia-responsive' genes such as VEGF and the endothelins themselves, and it gives rise to expression of the target gene. Protein products (e.g. VEGF and endothelins, and potentially several further pro-angiogenic genes which have an HRE in their promoter) act in a paracrine manner on VSMCs and endothelial cells and promote proliferation, migration of such cells and tubule formation resulting in neovascularisation of the tumour. BQ123 (or other ET-R inhibitors) block binding of endothelin to ET-R and prevent the ET-mediated stabilisation of HIF1 α . BQ123 also inhibits the direct angiogenic effects of endothelins on VSMCs, while BQ788 inhibits pro-angiogenic effects of endothelins on endothelial cells.

In primary and metastatic melanoma cell lines in normoxic conditions, ET-1 or ET-3 binding to ET-RB increases HIF-1 α protein and subsequently upregulates VEGF, COX-1/COX-2 (cyclo-oxygenase) protein expression and COX-2 promoter activity, PGE₂

production, and does so to a greater extent under hypoxia (Spinella *et al.* 2007). Silencing HIF-1 α by siRNA prevents ET-mediated COX-2 transcriptional activity, PGE₂ and VEGF production, and MMP activation. COX-1/COX-2 inhibitors block

ET-induced PGE₂ and VEGF secretion, MMP activation and cell invasion, indicating that both enzymes function as downstream mediators of ET-induced invasive properties. In melanoma xenografts, the ET-RB antagonist A-192621 suppresses HIF-1 α accumulation, tumour growth, neovascularisation, VEGF expression and MMP-2.

In human breast tumour cells, CCR7 (C-C chemokine receptor 7) is upregulated by ET-1 stimulation via ET-RA and this is HIF-dependent (Wilson *et al.* 2006). Endothelin-mediated induction of CCR7 leads to increased chemotaxis and invasion towards the CCR7 ligands CCL19 and CCL21. Release of CCL21 (C-C chemokine 21) by the lymph nodes in conjunction with CCR7 expression by tumour cells is thought to modulate the organ specificity of breast cancer metastases (Muller *et al.* 2001). Expression of ET-1 and CCR7 correlate in primary breast tumours and are associated with the presence of lymph node metastases (Wilson *et al.* 2006). A high level of HIF-1 α is a poor prognostic factor in several malignancies including breast cancer (Bos *et al.* 2001) as is expression of the endothelin system (Wulfig *et al.* 2003).

Advances in endothelin-based therapeutic strategies

Prostate cancer

The role of endothelins has been studied in both the normal and transformed prostate. Endothelins are produced in the prostate gland by epithelial cells and are found in high concentrations in seminal fluid (up to 5 μ g/l; Casey *et al.* 1992). ET-RA and ET-RB are found in the normal tissue, but in the malignant prostate, there is a loss of ET-RB and increased levels of ET-1 (Nelson *et al.* 1997). Proposed roles include growth promotion, apoptosis inhibition, bone formation and stimulation of nociceptive receptors. ET-1 can act alone as a mitogen, but its effects are the greatest as a co-mitogen with a variety of growth factors, including bFGF, IGFs and PDGF (basic fibroblast growth factor, insulin-like growth factor, platelet-derived growth factor; Kopetz *et al.* 2002). ET-1 also alters the balance of osteoblasts and osteoclasts to favour new bone formation, which is a characteristic of the metastatic disease (Guise *et al.* 2003). In the PC3 human prostate cancer cell line, ET-1 is upregulated by IL-1 β (interleukin-1 β), tumour necrosis factor- α and transforming growth factor- β (Le Brun *et al.* 1999), all of which may be found in the tumour microenvironment.

Phase III clinical trials indicate that ET-RA antagonists used in prostate cancer are well tolerated but with

mild side effects related to vasoconstrictive effects of ET-1 (Lassiter & Carducci 2003). Atrasentan delays time to progression in prostate cancer in phase III clinical trials (Jimeno & Carducci 2005). A randomised phase II study of Atrasentan alone or in combination with zoledronic acid in men with metastatic prostate cancer showed no evidence for additive or synergistic effects of combination therapy with Atrasentan and zoledronic acid on bone turnover markers (Michaelson *et al.* 2006). However, ET-RA blockade enhances taxane effects in prostate cancer *in vitro* and *in vivo* (Akhavan *et al.* 2006). Endothelins modulate nociception and have been implicated in pain associated with prostate carcinoma; peripheral ET-RA antagonism attenuates carcinoma-induced pain (Schmidt *et al.* 2007).

Ovarian carcinoma

Ovarian carcinomas secrete ET-1, which acts as an autocrine growth factor for ovarian carcinoma cells via ET-RA (Bagnato *et al.* 1995, Moraitis *et al.* 1997) and also has a paracrine growth effect on the fibroblastic cells of ovarian cancer (Moraitis *et al.* 1999). Such fibroblasts express both receptors, and antagonism of either inhibits endothelin-stimulated growth. However, these fibroblast cell lines do not secrete ET-1, and mitogenesis requires a source of endothelin; co-culture of fibroblasts with carcinoma cells increases growth of both populations of cells when compared with either grown in isolation (Moraitis *et al.* 1999).

ET-1 promotes epithelial-to-mesenchymal transition (EMT) in human ovarian cancer cells (Rosano *et al.* 2005). An ET-1/ET-RA autocrine pathway drives the EMT in ovarian tumour cells by inducing a fibroblastoid mesenchymal phenotype via an integrin-linked kinase (ILK)-mediated signalling pathway leading to glycogen synthase kinase-3 β inhibition, downregulation of E-cadherin, increased levels of β -catenin and Snail and suppression of E-cadherin promoter activity.

As well as affecting growth, endothelins – acting through ET-RA – promote invasion of ovarian tumour cells by upregulating secretion and activation of MMP-2, MMP-9, MMP-3, MMP-7, MMP-13 and MMP-14 (Rosano *et al.* 2001). In addition, ET-1 increases expression of urokinase-type plasminogen activator, its receptor and plasminogen activator inhibitor type-1 and type-2. ILK functions as a downstream mediator of ET-1 to promote invasive behaviour in ovarian carcinoma (Rosano *et al.* 2006).

ET-RA is found in both tumour cells and intratumoural vessels, whereas ET-RB is expressed mainly in endothelial cells; a further action of endothelins in

ovarian cancer is the induction of angiogenesis via HIF-1 α and VEGF (Salani *et al.* 2000, Spinella *et al.* 2002). In primary and metastatic ovarian tumours, ET-1 expression correlates with neovascularisation and VEGF expression, while high levels of ET-1 are detected in the ascitic fluids and correlate with VEGF ascitic concentration (Salani *et al.* 2000). Atrasentan decreases growth of ovarian xenografts in mice and this is associated with decreased VEGF and MMP-2 expression, decreased microvessel density and increased apoptotic tumour cells (Rosano *et al.* 2003b). Combined treatment of Atrasentan/paclitaxel produces additive apoptotic and anti-angiogenic effects.

COX-1 and COX-2 are involved in the production of prostaglandins and play a role in the regulation of tumour progression in several malignancies, including ovarian carcinomas. ET-1 significantly increases the expression of COX-1 and COX-2, the activity of the COX-2 promoter, and the release of PGE₂ from ovarian carcinoma cell lines. A COX-2 inhibitor, NS-398, decreases the endothelin-induced PGE₂ production and VEGF upregulation. Endothelin-induced COX-2 and PGE₂ release are dependent upon the activation of ET-RA and multiple MAPK signal pathways, including ERK1/2 kinase, p38 MAPK and the transactivation of the epidermal growth factor receptor. In human ovarian xenografts, levels of COX-2 are reduced following treatment with Atrasentan (Spinella *et al.* 2004c). As well as inducing PGE₂ in ovarian carcinoma cells, ET-1 increases the expression of PGE₂ receptor type 2 (EP2) and type 4 (EP4) via ET-RA (Spinella *et al.* 2004a); ET-1 and PGE₂ stimulate VEGF production principally through EP2 and EP4 receptors.

Breast cancer

In keeping with the polyfunctional nature of endothelins, there are numerous potential consequences of endothelin expression in breast tumours that may lead to a more aggressive tumour cell phenotype. There is increased expression of several members of the endothelin network in invasive ductal carcinoma (IDC) of the breast when compared with the normal breast or non-invasive ductal carcinoma *in situ*; lymph node metastases of breast cancer have higher a higher degree of endothelin staining still (Alanen *et al.* 2000, Grimshaw *et al.* 2002b). Elevated expression of ET-1 is more common in IDCs with larger size, high histological grade and the presence of lymphovascular invasion (Wulfig *et al.* 2003), and there is increased endothelin in the serum of breast cancer patients with lymph node metastases when compared with those with no lymph node involvement (Hagemann *et al.* 2005).

Cells expressing endothelins and their receptors in IDC include the tumour cells (Grimshaw *et al.* 2002b), the CD68⁺ macrophage infiltrate (Grimshaw *et al.* 2002a) and the endothelial cells (Bagnato & Spinella 2002).

Endothelins have a role in recruiting TAMs: macrophages express both endothelin receptors and chemotaxis towards endothelins via ET-RB and a MAPK-mediated signalling pathway (Grimshaw *et al.* 2002a). As with 'classical' chemokines, migration towards endothelins is inhibited by hypoxia and pertussis toxin. Exposure of macrophages to endothelins *in vitro* leads to increased cell surface HLA-ABC (human leukocyte antigen) indicating an activated phenotype, whilst in patient breast cancer biopsies, foamy activated macrophages accumulate in regions containing tumour cells that express endothelins (Grimshaw *et al.* 2002a,b). Macrophages not only react to endothelins but also produce endothelins themselves and the TAMs contribute to the endothelins in the breast tumour microenvironment; in contrast, no immunoreactive endothelin can be detected in cell extracts from human neutrophils and lymphocytes (Ehrenreich *et al.* 1990).

Exposure of tumour cells to endothelins leads to an invasive breast tumour cell phenotype *in vitro* via both ET-RA and ET-RB (Grimshaw *et al.* 2004). *In vitro*, the invasive capacity of breast tumour cell lines correlates with the level of expression of the endothelins and receptors (Hagemann *et al.* 2005). However, expression of the endothelins and their receptors by benign mammary epithelial cells is not sufficient to elicit an invasive phenotype and it is likely that the endothelins are acting in concert with other factors to induce invasion in tumour cells.

The signalling pathways involved in endothelin-mediated induction of invasion of breast tumour cells are yet to be fully described. However, the induction of invasion involves both receptors and JNK (cJun N-terminal kinase) inhibitors abolish endothelin-mediated invasion of human breast cancer cell line MCF7 cells; however, in co-culture of tumour cells with macrophages, JNK inhibition has only a partial effect (Hagemann *et al.* 2005). Other inhibitors such as PD98059 (MAPKK inhibitor) and pertussis toxin (G-protein inhibitor) only partially inhibit endothelin-mediated invasion. This indicates that multiple overlapping pathways are activated and that factors in co-culture cooperate with endothelin stimulation to induce invasion.

The increase in invasion stimulated by endothelins involves increased activity of MMPs: endothelins induce MMP-1, MMP-2, MMP-9 and MMP-14 activity in macrophage culture and MMP-14 activity

in MCF7 culture. Tissue inhibitor of matrix metalloproteinases-1 release by macrophages and MCF7 cells is also reduced by endothelins. Induction of macrophage MMP activity is modulated via both receptors and can be inhibited by either BQ123 or BQ788, while MMP-14 is induced via ET-RA in MCF7 cells. The non-selective MMP inhibitor FN439 blocks endothelin-mediated invasion (Grimshaw *et al.* 2004).

The spread and trafficking of tumour cells to potentially metastatic sites is controlled by the expression of chemokines by organs and chemokine receptors by tumour cells (Muller *et al.* 2001). ET-1 induces CCR7 mRNA and protein expression by breast tumour cells via ET-RA and HIF-1 leading to increased invasion towards the CCR7 ligands CCL19 and CCL21 (Wilson *et al.* 2006). Further, not only do endothelins induce chemotaxis of tumour cells towards chemokines by upregulating the chemokine receptor, but also they increase chemotaxis towards the C-X-C chemokine CXCL12, which is involved in breast cancer metastasis, without increasing expression of the C-X-C chemokine receptor CXCR4 (Grimshaw *et al.* 2004). The mechanism by which endothelins potentiate the response to chemokines is yet unknown.

In biopsies of invasive breast cancer, the expression of ET-1, ET-RA and ET-RB is associated with increased VEGF expression and vascularity (Wulfig *et al.* 2004a). Bosentan inhibits tumour vascularisation and bone metastasis in an immunocompetent skinfold chamber model of breast carcinoma cell metastasis (Dreau *et al.* 2006). In patients with locally advanced breast cancer receiving high-dose neoadjuvant chemotherapy of epirubicin and cyclophosphamide, increased expression of ET-RA in breast carcinomas is associated with resistance to chemotherapy (Wulfig *et al.* 2004b). ET-RA status may serve as a predictive marker for identifying patients less likely to be responsive to conventional chemotherapy.

Melanoma

Melanoma is an aggressive tumour that can metastasise early in the course of the disease and is resistant to most current therapeutic regimens. Endothelins and ET-RB play a role in melanocyte transformation and melanoma progression. Expression profiling of human melanoma biopsies and cell lines indicates that ET-RB is over-expressed, associated with an aggressive phenotype (Bittner *et al.* 2000) and is a tumour progression marker (Demunter *et al.* 2001). ET-1 promotes melanocyte survival and inhibits u.v.-induced apoptosis by activating the phosphatidylinositol 3-kinase (PI3K)-Akt pathway (Kadekaro *et al.* 2005). Downregulation of

E-cadherin expression by u.v.-induced ET-1 (Jamal & Schneider 2002) results into an enhancement of melanoma invasive capability (Hsu *et al.* 2000). Associated with loss of E-cadherin, activation of ET-RB increases the expression of N-cadherin, MMP-2, MMP-9, and α v β 3 and α 2B1 integrins and inhibits intercellular communication by inducing phosphorylation of the gap junction protein connexin 43 (Bagnato *et al.* 2004). Downstream of ET-RB, activation of focal adhesion kinase and MAPK signalling pathways occurs leading to enhanced cell proliferation, adhesion, migration and MMP-dependent invasion.

In melanoma, upregulation of HIF-1 α is associated with neovascularisation, VEGF expression, poor prognosis and resistance to therapy (Giatromanolaki *et al.* 2003, Postovit *et al.* 2006). Hypoxia and HIF-1 α are essential for melanocyte transformation: only in hypoxic conditions, can the PI3K-Akt pathway transform melanocytes (Bedogni *et al.* 2005, Michaylira & Nakagawa 2006).

Activation of ET-RB in cultured melanocyte precursors promotes cell proliferation, while inhibiting differentiation, and BQ788 inhibits growth of melanoma cell lines, and this is associated with increases in pigmentation and in the dendritic shape that is characteristic of mature melanocytes (Lahav *et al.* 1999). *In vivo*, BQ788 significantly slows growth of human melanoma tumours in nude mice including a complete growth arrest in half of the mice treated. In several melanoma cell lines, inhibition of ET-RB leads to an increase in apoptosis, particularly in metastatic melanoma cells (Lahav *et al.* 2004); microarray analysis showed that BQ788 treatment leads to a reduction in the expression of survival factors including DNA repair enzymes.

In another study, exogenous ET-1 was found to not be a growth factor for human melanoma cells, but blockade of receptors with Bosentan decreased proliferation, induced apoptosis and potentiated the effects of anti-cancer agents, suggesting that combination therapy of endothelin receptor antagonists with alkylating agents may improve their efficacy (Berger *et al.* 2006).

Endothelins are also involved in angiogenesis in mouse models of melanoma; inhibition of ET-RB by BQ788 is accompanied by a strong induction of VEGF expression and repression of the angiogenic suppressor gravin (Lahav *et al.* 2004); these changes correlated with increased angiogenesis in tumours injected with the ET-RB antagonist. ET-1 induces CXCL1 and CXCL8 secretion in melanoma cells via ET-RB (Mangahas *et al.* 2005). In human melanoma xenografts in mice, the ET-RB antagonist A-192621

suppresses HIF-1 α accumulation, tumour growth, neovascularisation, VEGF expression and MMP-2.

A phase II study of Bosentan, a dual endothelin receptor antagonist, as monotherapy in patients with stage IV metastatic melanoma showed disease stabilisation in 6 out of 32 patients (Kefford *et al.* 2006).

Lung cancer

ET-1 has been proposed as a prognostic marker in non-small cell lung carcinoma (NSCLC; Arun *et al.* 2004). There is higher expression of ET-1, ET-RA and ECE-1 in lung tumours when compared with the normal tissue, whilst ET-RB is decreased. ET-1 expression is related to both VEGF expression and poor prognosis in NSCLC (Boldrini *et al.* 2005). Interestingly, ET-1 is increased in the breath condensate of NSCLC patients and this could potentially be used as a non-invasive test for early detection of NSCLC (Carpagnano *et al.* 2004). Whilst the interactions between endothelins and HIF-1 in lung cancer have not yet been studied, HIF-1 has a pivotal role in lung cancer (Swinson *et al.* 2004) and endothelin expression is increased in the lungs during episodes of hypoxia (Earley & Resta 2002, Earley *et al.* 2002).

Bladder cancer

The endothelin system, particularly ET-RB, is over-expressed in bladder cancer. Patients with ET-RB expression tend to have organ-confined tumours and no vascular invasion, and as such are associated with favourable disease-free survival (Wulfing *et al.* 2005a,b). When metastatic bladder carcinoma cells were injected into mice treated with Atrasentan, there was a dramatic reduction of metastases to the lungs (Titus *et al.* 2005). HIF-1 α expression correlates with angiogenesis and unfavourable prognosis in bladder cancer (Theodoropoulos *et al.* 2004).

Nasopharyngeal carcinoma

Elevated plasma big ET-1 is associated with distant failure in patients with advanced-stage nasopharyngeal carcinoma (Mai *et al.* 2006), and there is frequent promoter hypermethylation of the *EDNRB* gene (Lo *et al.* 2002).

Kaposi's sarcoma

Kaposi's sarcoma is a highly angiogenic tumour that expresses endothelins. Both of the endothelin receptors are expressed in the tumour cells and the intratumoural vessels found in Kaposi's sarcoma tissue (Bagnato *et al.* 2001). ET-1 has mitogenic activity for a tumourigenic Kaposi's sarcoma cell line: ET-1 induces a dose-dependent increase in ³H-thymidine

incorporation and addition of either BQ123 or BQ788 blocks the mitogenic response and reduces the basal growth rate of unstimulated cells, suggesting that both receptors mediate the proliferative signal (Bagnato *et al.* 2001). ET-1 induces migration and invasion of Kaposi sarcoma cell lines *in vitro* via both ET-RA and ET-RB, and induction of MMP-2, MMP-3, MMP-7, MMP-9 and MMP-13, as well as MMP-14 (Rosano *et al.* 2003a).

Neuroblastoma

Human neuroblastoma cells express the ECE-1 (Fisk *et al.* 2006), which has been suggested to play an important role in amyloid- β peptide metabolism as one of the amyloid-degrading enzymes. Hypoxia and oxidative stress decrease expression of ECE-1 at the protein level. Serum withdrawal from the incubation medium as well as addition of carbachol or PMA leads to a reduction of the levels of ECE-1 protein in NB7 cells. BQ123 inhibits neuroblastoma cell line proliferation *in vitro* (Berry & Burchill 2002).

Osteosarcoma

ET-1 promotes MMP-2 and MMP-9 induction involving the transcription factor NF- κ B (nuclear factor κ B) in human osteosarcoma (Felx *et al.* 2006).

Cervical cancer

In human papillomavirus-positive cervical cancer cells, ET-RA mediates an ET-1-induced mitogenic effect. Atrasentan inhibits growth and angiogenesis in cervical cancer xenografts (Bagnato & Spinella 2002). Reducing ET-1 in the medium of cervical carcinoma cells by overexpressing neutral endopeptidase, which enzymatically inactivates several bioactive peptides including ET-1, decreases proliferation and invasion of these cells (Terauchi *et al.* 2005).

Conclusions

The mechanism(s) by which endothelins induce angiogenesis, invasion and other potentially 'pro-tumour' effects are as yet incompletely described but potentially include the interaction between the tumour cells, the stroma and the tumour microenvironment. This complex interaction leads to proliferation of endothelial cells, induction of MMP activity, cytokine expression, immune infiltrate activation, inhibition of apoptosis and expression of the endothelins themselves. All these factors may cumulatively facilitate tumour progression and clinical outcome. HIF-1 is

likely to have a pivotal role in the ‘pro-tumour’ role of endothelins. Small molecule inhibitors of the endothelin receptor already exist and the endothelin network may be a suitable therapeutic target for the treatment of several types of cancer that can quickly be exploited. Endothelin receptor antagonists hold the attractive possibility that they will ‘hit’ several different cell types and mechanisms of cancer progression: the antagonists may potentially inhibit angiogenesis by inhibiting endothelial cell mitogenesis, while simultaneously preventing macrophages from producing MMPs, and countering the anti-apoptotic effect of endothelins on the tumour cells themselves.

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