Endothelin-2 Is a Hypoxia-induced Autocrine Survival Factor for Breast Tumor Cells

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Abstract
Endothelins (ETs) are a group of vasoactive peptides (ET-1, ET-2 and ET-3) produced by many cell types that bind to G-protein-linked transmembrane receptors, ET-A receptors (ET-RAs) and ET-B receptors (ET-RBs). These peptides are expressed in several human tumors, including carcinomas of the breast, and have a mitogenic effect in ovarian cancer cell lines. We investigated ET expression in infiltrating ductal carcinomas (IDCs) of the breast and the relationship between ET and hypoxia. ET staining was increased in human grade II IDC samples compared with normal breast tissue. ET-2 and ET-RB mRNA expression were absent in the majority of normal human breast samples (1 of 5 and 0 of 5, respectively) but was present in the majority of IDC tested (13 of 15 and 12 of 15, respectively). In a murine breast cancer model, HTH-K, ET-2, and ET-RB mRNA were detected in tumor but not normal breast tissue, and ET expression colocalized with areas of hypoxia. In vitro, ET-2, ET-RA, and ET-RB mRNA were increased by incubating HTH-K cells in hypoxia (0.1% oxygen) for 24 h. Hypoxia also up-regulated ET-2 mRNA in several human breast tumor cell lines. ET-2 mRNA increased within 3 h in a hypoxia-inducible factor 1-dependent manner. The ET-RB antagonist BQ-788 increased in hypoxia-associated apoptosis of breast tumor cells in vitro. These effects could be reversed by addition of ET-2 peptide. Intratumoral injection of BQ-788 led to an increase in the development and extent of necrosis within the HTH-K tumor and a decrease in the rate of tumor growth. The ET-RA antagonist, BQ-123, also led to a decrease in tumor growth but without a concomitant increase in necrosis. We propose that modulation of ET-2 production via the hypoxia-inducible factor 1 transcription factor and autocrine signaling via ET-RB is a novel mechanism by which tumor cells can withstand hypoxic stress. Treatment of breast carcinomas with ET receptor antagonists may have a therapeutic benefit.

Introduction
ETs are small (21 amino acid) vasoactive peptides produced by endothelial and epithelial cells, macrophages, fibroblasts, and many other cell types (1). ETs have physiological and pathological roles in many organs (2), including the heart, liver, lungs, kidney, and brain. As well as affecting vasconstriction and dilation, ETs play a role in regulating growth in several cell types, including ovarian cancer cell lines and tumors (1, 3), and may also affect differentiation (4), inflammation (5), and angiogenesis (6). There are at least three ET isoforms (ET-1, ET-2, and ET-3) that are encoded by three distinct, independently expressed genes, and at least two receptor subtypes: ET-RAs and ET-RBs (7, 8).

Human tumors, including carcinomas of the breast (9), ovary (3), prostate (10) and lung (11), may express ETs. Most reports focus on ET-1, which may act as an autocrine and paracrine growth factor in ovarian tumors (3, 12, 13). Treatment of human melanoma tumors grown in nude mice with an ET-RB antagonist slows tumor growth (14), whereas ET-RA antagonists slow growth of murine colorectal tumors (15).

Solid tumors contain regions of hypoxia caused either by failure of the tumor vasculature to develop or destruction of the vasculature when necrosis occurs, and this may modulate the expression of a variety of genes (16). Hypoxia modulates ET expression in several cell types e.g., ET-1 is regulated by hypoxia, either positively or negatively, in endothelial cells depending upon source (17, 18). ET receptor expression may also be regulated by hypoxia e.g., the number of ET-RBs on cultured astrocytes increases during transient hypoxia, but the ET-RA does not increase (19). Tumor hypoxia may affect ET expression: ET-2 mRNA is increased by hypoxia in squamous carcinoma cells (20). However, ET-1 production is decreased by hypoxia in colon adenocarcinoma and prostate carcinoma cells (21).

In this paper, we have studied ET expression in human breast carcinomas, which contain regions of hypoxia (22), and the links between hypoxia and ETs in a murine breast cancer model. HTH-K is a murine model of breast carcinoma derived from a spontaneous tumor in a colony of BALB/c.
Tumor Hypoxia and Endothelin-2

c-neu transgenic mice and established as a transplantable tumor and cell line (23, 24). HTH-K forms comedo-type, metastatic, and highly angiogenic breast carcinomas that share several features with the equivalent human malignancy.

Materials and Methods

Human Tumors. Liquid nitrogen frozen samples and paraffin sections of human breast tissues (normal, benign, and grade II carcinomas) were kindly supplied by Dr. Cheryl Gillett, Headly Atkins Breast Pathology Laboratory, Guy’s Hospital (London, United Kingdom).

Cell Culture. HTH-K cells were cultured as described previously (23). Several human breast tumor cell lines (MCF-7, BT20, BT474, and MDAMB468) were cultured similarly. For hypoxic experiments, cells were grown to semi-confluence, and the medium was replaced before hypoxic culture. Cells were incubated in a Heto-Holten (Camberley, United Kingdom) hypoxic incubator (0.1% O₂, 5% CO₂, 37°C).

HTH-K Tumor and NITP Treatment. The tumor was grown in female BALB/c mice as described previously (23). The tumor was excised when ~1 cm³. Normal breast tissue was taken at the same time from the tumor-bearing mouse. The tissue was snap frozen in liquid nitrogen or placed in 10% formol saline.

NITP [supplied by Oxford BioMedica (United Kingdom) Ltd.] is a marker of tumor hypoxia. Two h before the tumors were excised, sonicated NITP mixture (7 mg of NITP, 50 µl of DMSO, and 0.45 ml peanut oil) was injected i.p. (0.2 ml/mouse).

Treatment of HTH-K Tumors with ET Receptor Antagonists. HTH-K cells were injected s.c. into 24 mice. After 3 days, tumors were injected intratumorally with an ET-RA-specific antagonist (25–27) or an ET-RB-specific antagonist BO-788 (28) from American Peptide Co., Inc., (50 µg/mouse/day in 0.1 ml of PBS; eight mice/group) for 5 days. Control groups were injected with PBS only. Tumor size was measured using calipers. Twenty-four h after the final injection, tumors were removed and fixed in 10% formal saline.

IHC. Paraffin sections of tumors were examined for reduced NITP using a rabbit antithyophiline antibody (Sigma-Aldrich Co. Ltd., St. Louis, MO) or for mature ET using a rabbit antihuman ET antibody (Oncogene Research Products, Cambridge, MA), which binds all ET isoforms (ET-1, ET-2, and ET-3). An appropriate biotinylated secondary antibody was used. The sections were probed with streptavidin-peroxidase and developed using 3,3’-diaminobenzidine substrate followed by counterstaining with Harris’s hematoxylin.

RNA Extraction. Frozen tissue was homogenized in a mill under liquid nitrogen (Glen Creston, Stanmore, United Kingdom). Tri-Reagent (Sigma-Aldrich Co. Ltd.) was added to the homogenized tissue. For cell lines, Tri-Reagent was added to the adherent cells. Total RNA was prepared according to the manufacturer’s instructions, and the isolated RNA was DNase treated.

RT-PCR. Total RNA was reverse transcribed using the Ready-To-Go kit from Amersham Pharmacia Biotech, Inc.

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Statistical Analysis. Results were tested for statistical significance using ANOVA or Student’s t test with InStat Version 2.01 software.
Results

ET Peptide Staining Is Increased in Human Breast Carcinomas. We compared staining of mature ETs in paraffin sections of human normal breast tissue and benign fibroadenoma with c-neu-positive and -negative grade II IDC. There was no positive staining of ET peptides in the normal breast (0 of 5 samples) or benign tissue (0 of 5 samples). However, in both the c-neu-positive (5 of 5 samples) and -negative IDC (5 of 5 samples), there was strong staining of ET expression by tumor cells (Fig. 1A). In the c-neu-positive tumors, tumor cells were widespread and strongest ET staining could be seen in prenecrotic areas or areas bordering necrotic lesions. In contrast, in c-neu-negative tumors, tumor cells appeared to be in discreet, avascular tumor islands surrounded by stroma. Tumor cells in these islands stained strongly for ET.

ET-2 and ET-RB mRNA Expression Is Increased in Breast Carcinomas. Using RT-PCR, we compared the ET and ET receptor mRNA expression in normal breast tissue, benign fibroadenoma, and grade II IDC. In contrast to the IHC staining, ET could be detected in all tissues. ET-1 and ET-RA mRNA was expressed in all tissues (Fig. 1B and Table 2), but ET-3 was rarely expressed. The number of tumor samples showing expression of ET-2 and ET-RB mRNA was increased compared with normal or benign tissue samples. There was no difference between c-neu-positive or -negative IDC.

ET Expression by the HTH-K Tumor Model. ET-2 and ET-RB mRNAs were detected in HTH-K tumor tissue but not normal murine breast (Fig. 2A). However, ET-1 and ET-RA mRNAs could be detected in both normal and malignant breast tissue. ET-3 mRNA was found in the tumor tissue but only occasionally in the normal breast.

The HTH-K cell line, grown in vitro, expressed mRNA for all three ET. Both ET-RA and ET-RB mRNA expression could be detected in the cell line.

The HTH-K Tumor Model Has Hypoxic Regions That Colocalize with ET Expression. Because the murine breast and HTH-K tumor tissues had similar mRNA expression profiles of ET compared with equivalent human tissues, we used the HTH-K model to analyze the relationship between hypoxia and ETs in breast carcinomas. IHC was used to analyze the distribution of hypoxia and ET peptides in paraffin sections of HTH-K tumors from mice that had been treated with the hypoxia marker NITP. Serial sections were stained

Table 2 RT-PCR screen of ET and receptor mRNA expression by human breast tissue (also see Fig. 1)

<table>
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<th>c-neu-negative IDC</th>
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<td>0/4</td>
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for reduced-NITP and for ET; the ET antibody recognizes all ET isoforms. Similar to human tumors (22), staining for reduced-NITP showed the presence of focal areas of hypoxia within the HTH-K tumor, particularly in areas adjacent to necrotic lesions (Fig. 2B); mature ET colocalized with these areas of hypoxia.

**HTH-K Cell Line mRNA Expression of ET-2 and ET-RAs and ET-RBs Is Increased by Hypoxia.** ET-2 mRNA was tumor specific and ET expression was associated with areas of hypoxia in the HTH-K tumor. Using Northern blotting, we analyzed the effect of in vitro hypoxia (0.1% oxygen) on the HTH-K cell line expression of ET-2 and its receptors. mRNA levels were normalized to rRNA levels (18S and 28S) as hypoxia can affect housekeeping gene expression, and indeed, HTH-K/actin mRNA was reduced compared with rRNA during hypoxia (Fig. 3a).

Expression of ET-2, ET-RA, and ET-RB mRNAs was all significantly up-regulated by incubating HTH-K cells for 24 h in 0.1% oxygen (Fig. 3, a and b). Hypoxia induced a similar up-regulation in several human breast tumor cell lines (MCF-7, BT20, BT474, and MDAMB468). Of these, MDAMB468 cells showed the greatest and most consistent increase in ET-2 mRNA (Fig. 3c).

ET-1 and ET-3 mRNA expression by HTH-K cells were low, and hypoxia did not increase these mRNAs to levels detectable by Northern blotting (data not shown).

**ET-2 Peptide and Receptor Synthesis Is Increased by Hypoxia.** We next studied whether mature ET-2 peptide synthesis and release were increased by hypoxia using an enzyme immunoassay. Hypoxia consistently increased the level of ET-2 peptide in the medium of cultured HTH-K cells. In one representative experiment, ET-2 peptide increased 12-fold in the hypoxic medium compared with the normoxic medium (normoxia 4 ± 2.7 pg/ml, hypoxia 48 ± 11.3, n = 8, P < 0.0005; Fig. 3d).

Hypoxia also resulted in increased ET receptor protein synthesis. HTH-K cells were incubated either normoxically or hypoxically for 24 h, and Western blotting of the cell extracts showed that protein levels of ET-RA and ET-RB were increased by hypoxia (Fig. 3e).

**Up-Regulation of ET-2 mRNA Is in a HIF-dependent Manner.** To further characterize ET-2 induction by hypoxia, we analyzed the time course of ET-2 mRNA up-regulation by HTH-K cells and incubated cells with inhibitors or activators of the transcription factor HIF-1.

HTH-K cells were incubated from 0 to 24 h in 0.1% oxygen. By 3 h, ET-2 mRNA had increased significantly compared with the normoxic control (Fig. 4, a and b).

Incubation of normoxic HTH-K cells for 24 h with desferrioxamine (100 μM) or cobalt chloride (50 μM), which mimic hypoxia by inducing transcription from HIF-1-dependent genes (30), increased ET-2 mRNA to a similar level as that found during hypoxia (Fig. 4, c and d). DPI may inhibit hypoxic expression of HIF-1-dependent genes (31). Addition of DPI (10 μM) to hypoxic HTH-K cells partially inhibited the hypoxic induction of ET-2 mRNA (Fig. 4, c and d).

**ET-2 Signals via ET-RB to Increase HTH-K Cell Survival during Hypoxia.** We next analyzed the effects of increased ET-2 synthesis on hypoxic survival of HTH-K cells using antagonists of the ET receptors. Semiconfluent HTH-K cells (~40,000 cells/10 cm² well) were incubated in low-serum medium (2% FCS) under hypoxic conditions for 72 h. During hypoxia in low-serum conditions, HTH-K cells are viable for ~3-4 days but there is little, if any, proliferation (our unpublished data).
During hypoxic incubation, cells were treated with the ET-RA-specific antagonist BQ-123 (Refs. 25–27; 100 ng/ml) or the ET-RB-specific antagonist BQ-788 (Ref. 28; 100 ng/ml). The number of viable cells recovered was significantly decreased by the presence of ET-RB inhibitor BQ-788 (Fig. 5a). Microscopically, cells treated with BQ-788 were smaller and had condensed nuclei (data not shown). The ET-RA antagonist BQ-123 had no significant effect on hypoxic cell survival. The effects of BQ-788 were concentration dependent, showing a typical dose-response curve between 0 and 1000 ng/ml (Fig. 5b).

Addition of 100 ng/ml ET-2 peptide to the culture medium led to the recovery of an increased number of viable cells after hypoxic treatment (Fig. 5c), and this effect could be reversed by the addition of 100 ng/ml BQ-788.

Addition of ET-2 peptide also increased survival of the human breast tumor cell lines during hypoxia, whereas BQ-788 decreased the number of viable cells recovered. Of all of the cell lines tested, MCF-7 cells showed the greatest and most consistent response to ET-2 or BQ-788 (Fig. 5d). All cells treated with BQ-788 were smaller and had condensed nuclei (data not shown).

To analyze whether the effect of ET-2 was hypoxia-specific, we repeated the experiments under normoxic conditions. Neither ET-2 peptide nor receptor antagonists had any significant effect on growth or viability of HTH-K cells grown in normoxic conditions (data not shown).

BQ-788 Increases Apoptosis of HTH-K Cells during Hypoxia. HTH-K cells cultured in DMEM medium supplemented with 2% FCS were treated with 100 ng/ml BQ-123 or BQ-788 for 24 h under hypoxic conditions. Culture medium

![Figure 3](image-url)

Fig. 3. Normoxic and hypoxic ET and ET receptor expression by HTH-K cell line. a, composite of Northern gel stained with ethidium bromide showing rRNA subunits and Northern blot of ET expression by HTH-K cells. N, normoxic; H, hypoxic. b, graph of mRNA abundance compared with rRNA (n = 4). ET-2 was significantly increased by hypoxia (P < 0.0001), as was ET-RA (P < 0.01) and ET-RB (P < 0.05). ET-1 and ET-3 mRNA were undetectable by Northern blotting. c, Northern blot of ET-2 mRNA expression by MDAMB468 cells under normoxia and hypoxia. d, ET-2 peptide release by HTH-K cells cultured under hypoxic conditions for 24 h. One representative experiment of three is shown. Hypoxia significantly increased the amount of mature ET-2 peptide released into the medium (P < 0.0005). e, Western analysis of ET-RA and ET-RB protein expression by HTH-K cells cultured for 24 h under either normoxia (N) or hypoxia (H). The blots were probed for β-actin protein as a control. Shown is one representative experiment (n = 3).

![Figure 4](image-url)

Fig. 4. ET-2 mRNA is induced in a HIF-dependent manner. a, Northern gel, probed with an ET-2 probe, of RNA from HTH-K cells incubated from 1 to 24 h under 0.1% hypoxia. b, graph of ET-2 mRNA induction by hypoxia. ET-2 mRNA was not up-regulated by 1-h hypoxic incubation but was significantly increased within 3 h (n = 4; P < 0.05) and remains elevated for at least 24 h. c, Northern gel, probed for ET-2 mRNA, of RNA from HTH-K cells incubated normoxically in the presence of 100 μM desferrioxamine (DFO) or 50 μM cobalt chloride (CoCl2) or incubated hypoxically with 10 μM diphenylene iodonium (DPI) for 24 h. d, graph of ET-2 mRNA induction in the presence of HIF-1 inducers or inhibitors. ET-2 mRNA abundance was increased by hypoxia (P < 0.05), DFO (P < 0.005), and CoCl2 (P < 0.005), whereas DPI partially reversed hypoxic ET-2 mRNA induction (P < 0.005).
was removed, and cells were analyzed for apoptosis using a cell death detection ELISA that detects histone-associated DNA fragments. Incubation of HTH-K cells under hypoxia with BQ-788 increased the level of apoptosis compared with untreated hypoxic cells (Fig. 5e). The ET-RA antagonist, however, had no effect on the levels of apoptosis during hypoxia. Neither antagonist affected apoptosis in normoxic cells (data not shown).

**An ET-RB Antagonist Increases HTH-K Tumor Necrosis in Vivo.** Having shown that ET-2 can act as a protective factor in vitro and that BQ-788 reduces hypoxic survival of HTH-K cells, we examined the effect of ET receptor antagonists in vivo. Paraffin sections of tumors treated with either BQ-123 or BQ-788 were stained and analyzed by Gordon Stamp, Professor of Histopathology, Transgenic Pathology Unit, Cancer Research United Kingdom, who was blinded to the treatment and control groups. Treatment of established HTH-K tumors with BQ-788 for 5 days led to an increase in the extent and development of necrosis (Fig. 6a) in all tumors compared with control-treated tumors. The extent of necrosis was calculated by grading 20 random fields/tumor as necrotic or nonnecrotic tumor tissue. Tumors treated with BQ-788 were significantly more necrotic than either control or BQ-123 treated tumors (Fig. 6b). Treatment with the ET-RA antagonist BQ-123 did not produce any gross morphological changes compared with control tumors. However, treatment for 5 days with either the B-receptor antagonist BQ-788 or the A-receptor antagonist BQ-123 led to a significantly ($P < 0.05$) decreased tumor volume compared with control tumors (Fig. 6c).

**Discussion**

ETs are produced by many tumor cell types (10, 11, 32), including those of the breast (12). Our results show an increase in ET staining in human IDC of the breast compared with normal breast tissue or benign fibroadenomas and that these tumors express ET-2 and ET-RB mRNA. Expression of ET-1 and ET-3 mRNA occurred less often in these tumors. The HTH-K tumor model showed a similar expression profile to equivalent human tumors, and ET-2 and ET-RB could only be detected in the HTH-K tumor and not the normal murine breast. However, it is also possible that detection of these mRNAs in the tumor is possible because of the predomin-
nance of, for example, epithelial cells. Also similar to human tumors, the HTH-K tumor has regions of hypoxia, indicating that much of the vasculature is not functioning and/or not perfused with blood; tumor angiogenesis can result in corrupt vasculature (33). We hypothesized that expression of ET-2 and ET-RB in IDC may be induced by intratumoral hypoxia and that increased expression of these molecules may influence tumor cell behavior during hypoxic episodes.

We used the HTH-K model to address this question and confirmed several key findings with human breast tumor cell lines.

The presence of mature ET peptides colocalized with regions of hypoxia in the HTH-K tumor in vivo, and hypoxia increased ET-2 and ET-RB expression (mRNA and peptide or protein) by HTH-K cells in vitro. In the human c-neu-positive tumors, ET staining was strongest in prenecrotic regions or areas of tumor cells bordering necrotic lesions; such areas are likely to be hypoxic (34). In contrast, in the c-neu-negative carcinomas, discreet tumor islands that stained strongly for ET could be seen. There may be a greater deal of vascularity in c-neu-positive tumors (35), and the tumor islands appear essentially avascular and, hence, are also likely to be hypoxic (22). ET-2 mRNA was consistently found in these tumors, whereas expression of the other ET occurred less often, and so it is likely that the positive staining of tumor cells is because of ET-2 production. However, it cannot be ruled out that some tumors may express ET-1 or ET-3 as well as ET-2.

IHC of the normal breast and benign fibroadenomas showed no positive ET staining in these tissues, whereas all tumors examined produced positive staining. In contrast, ET mRNAs could be detected by RT-PCR in all tissues. This may be because of different rates of ET mRNA/peptide production and degradation but is more likely because of the extreme sensitivity of RT-PCR; similarly, ET-1 mRNA could be detected in HTH-K cells by RT-PCR, but Northern blotting showed that ET-1 mRNA expression was very low.

Induction of ET-2 mRNA by desferrioxamine and cobalt (30) and partial inhibition of hypoxic ET-2 induction by diphenylene iodonium (31) indicate that up-regulation of ET-2 mRNA during hypoxia is HIF-1 dependent. This is supported by the time course of ET-2 mRNA induction: ET-2 mRNA had not increased within 1 h of hypoxia but was induced within 3 h, which is similar to many HIF-1-dependent genes (16).

Fig. 6. BQ-788 increases the development of HTH-K necrosis in vivo. a, H&E-stained sections of HTH-K tumors treated daily with intratumoral injections of PBS (group 1), 50 μg of BQ-123 (group 2) or 50 μg of BQ-788 (group 3) for 5 days. Three representative sections/group of eight are shown. BQ-788 led to an increase in the development and extent of necrosis in all tumors compared with control-treated tumors. BQ-123 had no effect on the development of necrosis. b, the extent of necrosis was calculated by grading 20 random fields/tumor as necrotic or nonnecrotic tissue. Tumors treated with BQ-788 were significantly more necrotic than either control or BQ-123 treated tumors (P < 0.05). c, tumor volume measured after 5 days of treatment with BQ-123 or BQ-788. Both antagonists led to a significantly decreased tumor volume at the end of the treatments (P < 0.05).
This is the first evidence of the involvement of HIF-1 in hypoxic expression of ET-2 mRNA. No hypoxically responsive elements have yet been described in the promoter of murine or human ET-2. The promoter of the ET-1 gene has a HIF-1 binding site, the hypoxia response element, which is bound by HIF-1 during hypoxia in microvascular endothelial cells (36). However, it is worth noting that ET-1 mRNA can be down-regulated during hypoxia (18).

Adding ET-2 peptide to the culture medium modestly reduces the tumor cell death associated with hypoxia. It should be noted that the cells have increased ET-2 production in response to hypoxia, and hence the effect of additional ET-2 might be expected to be small. This effect was mediated via the ET-RB receptor as only treatment with an ET-RB antagonist (28) in vitro led to increased cell death during hypoxia; a similar ET-RA antagonist (25–27) had no effect. Increased cell death caused by the ET-RB antagonist was associated with increased histone-associated DNA fragments indicating increased apoptosis. ET-1 has been shown to protect astrocytes from hypoxic/ischemic injury (37) and has previously been suggested to have a paracrine action in breast tumors (12), but this is the first time that a protective autocrine action of ET-2 has been proposed.

Although ET-2 results in an increased number of cells surviving hypoxic stress and addition of BQ-788 leads to increased apoptosis, the potential mechanisms of ET-2-mediated cell survival are yet to be elucidated. Hypoxia induces caspase-dependent apoptosis in some cell types (38), and ET-2, signaling via ET-RB, may inhibit caspase activation. Several other potential mechanisms of preventing apoptosis exist such as increased production of anti-apoptotic cytokines (39), enhanced glucose uptake (40), and blocking of apoptosis signaling (41). Hypoxia may also cause microsatellite instability in tumor cells (42), which may induce apoptosis (43); ET-2 may modulate mismatch repair genes (45), and ET-2 may modulate mismatch repair genes (44) such that either the damage is repaired or the cell is not prompted to apoptose.

These data indicated that ET-2 is acting via ET-RB to protect cells from hypoxia, therefore we treated the HTH-K tumor in vivo with ET receptor antagonists. BQ-788 led to an increased extent and development of necrosis within the tumor compared with untreated tumors or tumors treated with BQ-123. This increase in tumor necrosis may be because of the ET-RB antagonists inhibiting the tumor cells ability to withstand intratumoral hypoxic stress similar to the situation in vitro. BQ-788 also led to a decreased tumor volume after 5 days of treatment compared with control tumors.

However, although an ET-RA-specific inhibitor had no effect on hypoxic cell survival in vitro and did not increase necrosis in vivo, hypoxia increased the production of ET-RA in vitro and led to decreased tumor volume in vivo. The role of ET-RA up-regulation and presumed signaling is yet to be elucidated but may have a role in another of the ETs’ potential functions distinct from the ET-2/ET-RB protective function. However, it is also likely that the receptor antagonist is affecting cells distinct from the tumor cells, particularly the cells of the vasculature.

Furthermore, it is likely that cells other than the breast epithelial cells or the tumor cells may contribute to ET expression. ET-1 mRNA, which could be detected in the HTH-K tumor but was expressed at very low levels by the HTH-K tumor cells in vitro, may originate from cells distinct from the tumor cells. Indeed, endothelial cells may express all ETs, and ET-1 can be regulated by hypoxia both positively and negatively in these cells (17, 18). It is likely that hypoxia also contributes to the modulation of ET gene expression in these and other cells within the tumor. Moreover, ET-2 released by tumor cells is likely to act on other cells within the tumor further modulating tumor biology, e.g., ETs can stimulate release of proinflammatory cytokines from macrophages (45), and ET-2 may modulate the distribution of macrophages within tumor (46).

In summary, we have shown that ETs are increased in human IDC of the breast compared with normal breast or benign tissue and that these tumors express ET-2 and ET-RB. In our model of breast carcinoma, HTH-K, expression of ET-2 and its receptors is rapidly induced by hypoxia in a HIF-1-dependent manner. ET-2 protects breast tumor cells from hypoxic injury via the ET-RB receptor in an autocrine loop. ET-2 may therefore protect tumor cells against hypoxia within the tumor in vivo. Antagonism of the ET-RB receptor in vivo leads to increased tumor necrosis as well as delayed tumor growth and may therefore be a therapeutic target for the treatment of breast cancer.

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