Published in final edited form as:

Liver Int. 2009 August ; 29(7): 1031–1042. doi:10.1111/j.1478-3231.2009.01997.x.

Endothelin inhibits cholangiocarcinoma growth by a decrease in the vascular endothelial growth factor expression

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Abstract

Background: Endothelins (ET-1, ET-2, ET-3) are peptides with vasoactive properties interacting with ET_A and ET_B receptors. ET-1 inhibits secretin-stimulated ductal secretion (hallmark of cholangiocyte growth) of cholestatic rats by interaction with ET receptors.

Aim: The aims of the studies were to evaluate (i) the effect of ET-1 on cholangiocarcinoma growth in Mz-ChA-1 cells and nude mice and (ii) whether ET-1 regulation of cholangiocarcinoma growth is associated with changes in the expression of vascular endothelial growth factor-A (VEGF-A), VEGF-C, VEGF receptor-2 (VEGFR-2) and VEGFR-3.

Methods: We determined the expression of ET_A and ET_B receptors on normal and malignant (Mz-ChA-1) cholangiocytes and human cholangiocarcinoma tissue and the effect of ET-1 on the proliferation and expression of VEGF-A, VEGF-C (regulators of tumour angiogenesis) and its receptors, VEGFR-2 and VEGFR-3, in Mz-ChA-1 cells. *In vivo*, Mz-ChA-1 cells were injected into the flanks of athymic mice and injections of ET-1 or saline into the tumours were performed daily.

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The effect of ET-1 on tumour size, cell proliferation, apoptosis, collagen quantity and the expression of VEGF-A and VEGF-C and VEGFR-2 and VEGFR-3 were measured after 73 days.

Results: Higher expression of ET_A and ET_B was observed in malignant compared with normal cholangiocytes. ET-1 inhibited proliferation and VEGF-A, VEGF-C, VEGFR-2 and VEGFR-3 expression of Mz-ChA-1 cells. Chronic ET-1 treatment decreased tumour volume, tumour cell proliferation and VEGF-A and VEGF-C expression but increased apoptosis and collagen tissue deposition compared with controls.

Conclusions: Modulation of VEGF-A and VEGF-C (by ET-1) may be important for managing cholangiocarcinoma growth.

Keywords

biliary cancer; cholangiopathies; neuroendocrine hormones; proliferation; vascular endothelial growth factor

Cholangiocarcinoma, the tumour originating from the biliary epithelium, accounts for about 3% of all gastrointestinal cancers and represents the second most common primary liver tumour after hepatocarcinoma (1). Biliary tumours are extremely aggressive and display a poor prognosis (1-4). The lack of therapeutic tools for such a devastating disease is due, at least in part, to the lack of knowledge regarding the mechanisms regulating cholangiocarcinoma growth (2-7). However, increasing evidence has shown that neuropeptides and neuroendocrine hormones are among those factors that are able to affect cholangiocarcinoma biology, either promoting or inhibiting its growth (1,4,7-11). While oestrogens and insulin-like growth factor 1 stimulate cholangiocarcinoma proliferation (12), gastrin inhibits cholangiocarcinoma growth by activation of protein kinase C- α (10). In Mz-ChA-1 cells, α 2-adrenoreceptor stimulation causes upregulation of cyclic adenosine 3',5'-mono-phosphate, which leads to inhibition of cholangiocarcinoma growth by decreased extracellular-signal-regulated kinase1/2 (ERK1/2) activity (11). A recent study has shown that leptin increased the cholangiocarcinoma growth by phosphorylation of STAT-3 and ERK1/2 (7). The two endocannabinoids, anandamide and 2-arachidonylglycerol, exert differential effects on cholangiocarcinoma growth via receptor-independent mechanisms, with anandamide inhibiting growth and 2-arachidonylglycerol stimulating cholangiocarcinoma cell growth (13).

Endothelin-1 (ET-1), a 21 amino acid vasoconstrictor peptide, regulates the pathogenesis of hypertension and heart failure (14). ET-1 exerts a multitude of effects in liver cells (15-19). ET receptors are expressed by rat hepatic stellate cells (16). ET-1 has been shown in human gallbladder epithelial cells (17) and in bile ducts of human cirrhotic liver (18). The importance of ET-1 in participating to liver homeostasis is supported by the finding that cholangiocytes synthesize hepatic ET-1 during the development of hepatopulmonary syndrome after extrahepatic bile duct obstruction (19). ET-1 inhibits secretin-induced ductal secretion (15) (a hallmark of cholangiocyte proliferation) (20,21) in cholestatic rats by interacting with ET_A receptors on cholangiocytes.

A number of angiogenic factors including vascular endothelial growth factor (VEGF) are overexpressed by human cholangiocarcinoma tissues and cell lines such as KMC-1, KMC-2, KMBC and KMG-C (22). Overexpression of VEGF has been suggested to contribute to the 'angiogenic switch' of the malignant phenotype in human cholangiocarcinoma (23). Increased expression of VEGF (by oestrogens) plays an important role in regulating human cholangiocarcinoma growth (24). A number of studies have shown that VEGF regulates the function of normal and hyperplastic cholangiocytes (25-27). No information exists regarding the *in vitro* and *in vivo* effects of ET-1 on (i) the growth and apoptosis of cholangiocarcinoma cells and (ii) the expression of the angiogenic factor, VEGF-A and VEGF-C, and its receptors

VEGFR-2 and VEGFR-3 in cholangiocarcinoma. On the basis of these studies, we evaluated (i) the expression of ET_A and ET_B in the human cholangiocarcinoma cell line, Mz-ChA-1 (10,11,13) and the normal cell line, H69 (6), and tissue arrays containing cholangiocarcinoma and normal biopsy samples, (ii) the *in vitro* effects of ET-1 on the growth of H69 and Mz-ChA-1 cells, (iii) the *in vivo* effect of ET-1 on the growth of tumour cell lines implanted in nude mice and (iv) whether the *in vitro* and *in vivo* effects of ET-1 on MzChA-1 cell growth are associated with changes in VEGF-A and VEGF-C expression.

Materials and methods

Materials

Reagents were purchased from Sigma Chemical Company (St. Louis, MO, USA) unless otherwise specified. ET-1 was purchased from Calbiochem (La Jolla, CA, USA). The polyclonal antibodies (sheep antisera) against the rat ET_A and ET_B receptors were purchased from Alexis Co. (San Diego, CA, USA). Dulbecco's phosphate-buffered saline (PBS) was obtained from Celox (Hopkins, MN, USA).

Cholangiocarcinoma cell lines

We used (i) H69 cells that are SV40-transformed normal human cholangiocytes (a gift from Dr Gores, Mayo Clinic, Rochester, MN, USA) (6) and (ii) the human cholangiocarcinoma cell line, Mz-ChA-1, which has been widely used for evaluating the mechanisms of cholangiocarcinoma growth by us and others (5,10,11,13,28-30). Mz-ChA-1 cells from human gallbladder (10,11,13,31) were obtained from Dr Fitz (University of Texas Southwestern Medical Center, Dallas, TX, USA). Mz-ChA-1 cells were cultured as described (5,10,11,13, 28).

Expression of endothelin A and endothelin B receptors

We evaluated the expression of ET_A and ET_B receptors in H69 and Mz-ChA-1 cells by immunofluorescence and real-time polymerase chain reaction (PCR) (13,32). For immunofluorescence, cells were seeded onto coverslips and allowed to adhere overnight. Following washes with cold 1 × PBS, coverslips were fixed with 4% paraformaldehyde (in 1 × PBS) at room temperature for 5 min. Cells were permeabilized in 1 × PBS containing 0.2% triton-X100 (PBST) and blocked in 4% bovine serum albumin (BSA in PBST) for 1 h at room temperature. Appropriate dilutions (in 1% BSA/PBST) of ET_A or ET_B receptor antibodies were added to the coverslips and incubated overnight at 4 °C. Cells were washed three times for 10 min in PBST and a 1:100 dilution (in 1% BSA/PBST) of a cy3-conjugated secondary antibody (Jackson ImmunoResearch Laboratories Inc., West Grove, PA, USA) was added for 2 h at room temperature. Cells were washed three times for 10 min in PBST and mounted onto microscope slides with Antifade gold containing 4,6-diamidino-2-phenylindole as a counterstain (Molecular Probes, Eugene, OR, USA). Negative controls (with pre-immune serum substituted for primary antibody) were also included. Images were taken on an Olympus IX71 fluorescence microscope with a DP70 digital camera (Olympus, Tokyo, Japan).

For real-time PCR analysis of receptor expression, RNA was extracted from Mz-ChA-1 cells using the RNeasy Mini Kit (Qiagen Inc., Valencia, CA, USA) according to the manufacturer's instructions and reverse transcribed using the Reaction Ready™ First Strand cDNA synthesis kit (SuperArray, Frederick, MD, USA). These reactions were used as templates for the PCR assays using a SYBR Green PCR master mix and specific primers designed against human ET_A and ET_B genes (SuperArray) in the real-time thermal cycler Applied Biosystems Inc, Foster City, CA (ABI Prism 7900HT sequence detection system). One microlitre of the cDNA template was added to 12.5 µl of master mix, 10.5 µl of DI water and 1 µl of reverse transcriptase PCR human primers designed specifically for the messages for ET_A and ET_B and

glyceraldehyde-3-phosphate dehydrogenase (GAPDH), the housekeeping gene (32). A delta delta of the threshold cycle ($\Delta\Delta C_T$) analysis was performed using H69 as the control sample. Data are expressed as relative mRNA levels $\pm$ SEM ($n = 3$).

Cholangiocarcinoma tissue array analysis

Immunoreactivity for ET_A and ET_B was assessed in commercially available Accumax tissue arrays (Isu Abxis Co. Ltd, Seoul, Korea) by immunohistochemistry (33) using specific antibodies for ET_A and ET_B. These tissue arrays contain 48 well-characterized cholangiocarcinoma biopsy samples from a variety of tumour differentiation grades as well as four control liver biopsy samples. Semiquantitative analysis was performed using the following parameters. Staining intensity was assessed on a scale from 1 to 4 (1 = no staining, 4 = intense staining) and the abundance of positively stained cells was given a score from 1 to 5 (1 = no cells stained, 5 = 100% stained). The staining index was calculated by the staining intensity multiplied by the staining abundance, which gives a range from 1 to 20.

Effect of endothelin-1 on cholangiocarcinoma cell growth *in vitro*

The growth of Mz-ChA-1 cells was evaluated by a 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulphophenyl)-2H-tetrazolium, inner salt (MTS) proliferation assay (5,13). After trypsinization, Mz-ChA-1 cells were seeded into 96-well plates (10 000 cells/well) in a final volume of 200 μ l medium. In dose-response curve experiments, cells were stimulated for 48 h with 0.2% BSA (basal) or ET-1 (from 0.1 to 10 nM) before evaluating growth by MTS assays (5,13). We also evaluated the effect of ET-1 (10 nM for 48 h) on the growth of H69 cells. In separate experiments, Mz-ChA-1 cells were stimulated for 48 h with 0.2% BSA (basal) or ET-1 (10 nM) in the absence or presence of 1 h pre-incubation with BQ-610 (a specific ET_A receptor antagonist, 100 nM) (15,34) or BQ-788 (a specific ET_B receptor antagonist, 100 nM) (35). Cells were also incubated with only BQ-610 or BQ-788 (100 nM) for 48 h. Cell proliferation was assessed using a colorimetric cell proliferation assay (CellTiter 96Aqueous; Promega Corp., Madison, WI, USA) and absorbance was measured at 490 nm by a microplate spectrophotometer (Versamax; Molecular Devices, Sunnyvale, CA, USA). The proliferation index was calculated as the ratio of the absorbance of cells incubated with ET-1 compared with controls.

Effect of endothelin-1 on the expression of vascular endothelial growth factor-A, -C, vascular endothelial growth factor receptor-2 and -3 in cholangiocarcinoma cells

After trypsinization, Mz-ChA-1 cells (1×10^6) were incubated until they reached 70% confluence and were serum starved overnight. Then, cells were stimulated with (i) 0.2% BSA (basal), (ii) ET-1 (10 nM) for 6 h in the absence or presence of BQ610 and BQ788 (100 nM) or (iii) BQ610 or BQ788 (100 nM). Subsequently, we evaluated the expression of VEGF-A, VEGF-C, VEGFR-2 and VEGFR-3 by real-time PCR using specific primers and the methods described above. The $\Delta\Delta C_T$ analysis was performed and data were expressed as fold change compared with either basal or inhibitor treatment alone.

Effect of endothelin-1 on the growth of cholangiocarcinoma cells implanted in nude mice

Male 8-week-old BALB/c nude (nu/nu) mice were purchased from Taconic Farms, Germantown, NY, USA. The mice were kept in a temperature-controlled environment (20–22 °C) with 12-h light-dark cycles and free access to water and mouse chow. All animal experiments were performed in accordance with a protocol approved by the Scott and White Institutional Animal Care and Use Committee and in accordance with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication no. 85-23, revised 1996). Mz-ChA-1 cells (3×10^6 viable cells) were suspended in 0.5 ml of extracellular matrix gel and injected subcutaneously in the left back flank of these

animals. This cell line was chosen for its ability to form solid tumours when injected into nude mice. Mice were divided into two groups: (i) the first ($n = 3$, control) received 0.9% NaCl injections (150 μ l) into the implanted tumour and (ii) the second ($n = 3$) received ET-1 injections (100 ng/kg body weight in 150 μ l of 0.9% NaCl) (36) into the tumour. The same operator performed the injections every day starting from 'day 0', when the tumours were implanted, for 73 days. The size of the tumours was assessed by a tri-axial measurement, twice a week. Tumour parameters (length, width, height) were measured by an electronic calliper and the volume was determined using the following equation: tumour volume (mm^3) = $0.5 \times [\text{length (mm)} \times \text{width (mm)} \times \text{height (mm)}]$. The measurements started from the third week, when the tumour mass was well established. A third operator, in a coded and blinded fashion, evaluated morphometric parameters. Latency of tumour growth was determined as the time needed for the tumour to increase to 150% of the initial volume. At the end of the observation period of 73 days, mice were anaesthetized with sodium pentobarbital (50 mg/kg body weight, IP) and sacrificed according to the institutional guidelines. Heart, liver and kidney were isolated from the two groups of animals, fixed in formalin, embedded in paraffin, processed for histopathology and stained with haematoxylin and eosin (H&E) for the detection of tissue damage.

Morphological analysis of tumour tissues

A blinded operator dissected tumour tissues from each group of mice. Neoplastic tissues were fixed in formalin, embedded in paraffin, processed for histopathology and sections (4 μ m) were stained with H&E for routine examination or with Masson's trichrome for collagen deposit evaluation and examined by light microscopy. Tumour sections ($n = 4$, from each group of animals) were stained for cytokeratin-7 (CK-7) and proliferating cellular nuclear antigen (PCNA) as described (37). Following staining, sections were counterstained with haematoxylin and examined with a microscope (Olympus BX 40; Olympus Optical Co. Ltd, Tokyo, Japan). Data were expressed respectively as percentage of total area occupied by CK-7-positive tumour cells and number of PCNA-positive malignant cholangiocytes per 100 malignant cells counted in seven different tumour fields.

To evaluate the protein expression of VEGF-A and VEGF-C, tumour sections ($n = 4$) from each group of animals were incubated overnight at 4 °C with antibodies specific for VEGFA (1:400) or VEGF-C (1:50). Sections were rinsed with $1 \times$ PBS for 5 min, incubated for 10 min at room temperature with a secondary biotinylated antibody (Dako LSAB2, code K0675; Dako, Milan, Italy), followed by DAKO ABC (Dako LSAB2, code K0675) and developed with 3,3'-diaminobenzidine. Sections were analysed in a coded fashion by an Olympus BX 40 light microscope (Olympus) with a Videocam (Spot Insight Diagnostic Instrument Inc., Sterling Heights, MI, USA) and processed with an Image Analysis System (Delta Sistemi, Rome, Italy). For all the immunoreactions, negative controls (the primary antibody was replaced with pre-immune serum) were included. Data were expressed as the percent of VEGF-A- and VEGF-C-positive malignant cholangiocytes counted in seven different tumour fields. In tumour samples from ET-1- or vehicle-treated mice, for the detection of apoptosis on single cells, the terminal deoxynucleotide transferase end labelling (TUNEL) method (ApopTag; Chemicon Int., Temecula, CA, USA) was used according to the manufacturer's instructions. In each tumour section ($n = 4$), the number of TUNEL-positive tumour cells was assessed in six slides for each group. Positive cells were counted in six non-overlapping fields (magnification $\times 20$) for each slide. Negative controls, obtained by incubating the tumour sections with normal pre-immune serum from the same species and then with the secondary antibody, were used to confirm the specificity of the staining.

Statistical analysis

All data are expressed as mean $\pm$ SEM. Differences between groups were analysed by the Student unpaired *t*-test when two groups were analysed and analysis of variance (ANOVA) when more than two groups were analysed. A *P*-value < 0.05 was used to indicate statistical significance.

Results

Expression of endothelin receptors

Immunofluorescence showed a positive reaction for ET_A and ET_B receptors in H69 and Mz-ChA-1 cells (Fig. 1A). By real-time PCR, ET_A and ET_B receptor mRNA levels were significantly higher in Mz-ChA-1 cells compared with H69 cells (Fig. 1B and C). Array analysis on human cholangiocarcinoma tissues confirmed that ET_A and ET_B receptors are expressed more in these neoplasias with respect to normal tissues (Fig. 1D).

In vitro effect of endothelin-1 on cholangiocarcinoma cell growth

In vitro studies have shown that ET-1 (at 1 and 10 nM for 48 h) significantly decreased the proliferation of Mz-ChA-1 cells (Fig. 2A). The rationale for using Mz-ChA-1 cells to begin to evaluate the mechanisms by which ET-1 inhibits both *In vitro* and *in vivo* is based on the fact that these cells are able to form tumours when injected into nude mice (5,10). The inhibitory effect of ET-1 (10 nM for 48 h) on Mz-ChA-1 growth was blocked by both BQ-610 and BQ-788 (Fig. 2B), suggesting that ET-1 exerts its inhibitory effects on cholangiocarcinoma growth by interaction with both ET_A and ET_B receptors. ET-1 did not affect the growth of the normal human cholangiocytes, H69 (not shown).

Effect of endothelin-1 on the expression of vascular endothelial growth factor-A, -C, vascular endothelial growth factor receptor-2 and -3 in Mz-ChA-1 cells

By real-time PCR, treatment with ET-1 (10 nM) induced a significant decrease in the message expression of VEGF-A and VEGF-C (measured as ratio to GAPDH mRNA) (Fig. 3A and B). Furthermore, ET-1 inhibited the expression of the message for VEGFR-2 (to which VEGF-A binds) (26) and VEGFR-3 (to which VEGF-C binds) (26) (Fig. 3C and D). Consistent with the concept that both ET_A and ET_B receptors regulate the effects of ET-1 on cholangiocarcinoma growth, ET-1 inhibition of VEGF-A, VEGF-C, VEGFR-2 and VEGFR-3 expression was blocked by BQ610 (an ET_A receptor inhibitor) (15) and BQ788 (an ET_B receptor inhibitor) (15) (Fig. 3A–D).

Effect of endothelin-1 on the growth of tumours implanted in nude mice

At 73 days (the time when mice were sacrificed), a significant difference in tumour size was found in ET-1-treated mice compared with mice injected with vehicle only ($431.47 \pm 55.45 \text{ mm}^3$, ET-1-treated mice vs. $904.08 \pm 194 \text{ mm}^3$, vehicle-treated mice; $P < 0.02$) (Fig. 4A and B). No significant difference in body weight was detected between the two groups of animals following 73 days of treatment (Table 1). There was a significant decrease in the tumour latency of control mice (12.3 ± 4.1 days) vs. mice treated with ET-1 (31.25 ± 8.6 days) ($P < 0.02$) (Table 1).

Immunohistochemistry for PCNA shows a decrease in the proliferative activity of the tumour cells from ET-1-treated mice compared with tumour cells from vehicle-treated mice (Fig. 5A). By immunohistochemistry, a decrease of area occupied by CK-7-positive malignant cholangiocytes in tumour mass of ET-1-treated vs. vehicle-treated animals is shown (Fig. 5A). Immunohistochemistry for VEGF-A and VEGF-C (Fig. 5B), trophic angiogenic factors involved in tumorigenesis and highly expressed in neoplastic tissue (5,38), shows a decrease

of both VEGF-A and VEGF-C in tumours obtained from ET-1-treated mice compared with vehicle-treated mice (Fig. 5B). TUNEL staining analysis (Fig. 5B) showed an increase in apoptosis in tumour cells of ET-1-treated mice compared with vehicle-treated mice. H&E and Masson's trichrome staining (Fig. 6) showed a more expressed fibrotic tissue in ET-1-treated mice compared with vehicle-treated mice (Fig. 6). No organ damage was found by histological analysis in the two groups of animals (not shown).

Discussion

We have shown that (i) normal and malignant cholangiocyte cell lines and normal and cholangiocarcinoma human tissue of biopsy samples express ET_A and ET_B receptors, (ii) ET_A and ET_B expression is higher in cholangiocarcinoma cells compared with normal cholangiocytes and (iii) ET-1 decreases the growth of the cholangiocarcinoma cell line, Mz-ChA-1 cells. Furthermore, chronic treatment of the Mz-ChA-1 cells, subcutaneously implanted in a nude mouse model, with ET-1 inhibits tumour growth *in vivo*. ET-1 *In vitro* and *in vivo* inhibition of Mz-ChA-1 cell growth was associated with decreased expression of VEGF-A, VEGF-C, VEGFR-2 and VEGFR-3 in Mz-ChA-1 cells and in sections from tumours. The data suggest that modulation of the expression of VEGF and VEGF receptors (by ET-1) may be important for managing cholangiocarcinoma growth.

Endothelin-1, by interacting with its own receptors, regulates the growth and metastasis of several types of tumours by modulating mitogenesis, angiogenesis, invasion and cell metastatic diffusion (39). Specifically, ET-1 enhances the growth and progression of prostatic, ovarian, renal, pulmonary, colorectal, breast, bladder, endometrial carcinomas, brain tumours, melanoma and bone metastases (39). These findings suggest the use of ET-receptor antagonists for the treatment of several types of cancers. Recently, it has been shown that DSL6A pancreatic cancer cells express ET receptors and that the ET-1 receptor antagonist, bosentan, inhibits the proliferation of pancreatic cancer cells (40). A study has demonstrated a beneficial effect of an ET_A receptor antagonist, in combination with paclitaxel and carboplatin, as first-line therapy for advanced non-small cell lung cancer (41). Contrary to these findings (39-42), our study demonstrated an inhibitory effect of ET-1 on cholangiocarcinoma growth by interaction with both ET_A and ET_B receptors. The inhibitory effect of ET-1 on cholangiocarcinoma growth resembles a similar one that is observed for gastrin on hyperplastic and neoplastic cholangiocyte growth (10,20). Gastrin exerts trophic effects on parietal and enterochromaffin-like cells in the gastric mucosa as well as on several gastrointestinal and non-gastrointestinal tumour cell lines (43-46) but does not promote the growth of rat liver and pancreas (47,48). Furthermore, a study has shown that transfected cholecystokinin receptors mediate growth inhibition of human pancreatic cancer cell lines (49). Moreover, we have shown that gastrin inhibits secretin-stimulated ductal secretion (15) (a functional marker of cholangiocyte proliferation) (50-54) and both hyperplastic and neoplastic cholangiocyte growth (10,20,50). Consistent with the concept that ET-1 has inhibitory properties on cholangiocyte growth, we have shown that ET-1 exerts a similar effect seen by gastrin by inhibiting secretin-induced ductal secretion (15).

In our study, ET-1 inhibition of Mz-ChA-1 proliferation was associated with decreased expression of VEGF-A and VEGF-C, two important regulators of hyperplastic and neoplastic cholangiocyte growth (5,24-26,55). Cholangiocarcinoma cells stimulate the development of a rich vascular network, which functions to sustain the metabolic needs and to ensure an adequate support of oxygen and nutrients to the malignant cells (55). In intrahepatic cholangiocarcinoma, high levels of VEGF are required to maintain tumour vascularization and to stimulate malignant cholangiocyte proliferation (55). We propose that the inhibition of cholangiocarcinoma growth by ET-1 is also due to a decreased synthesis or release of VEGF-A and VEGF-C, which (in addition to regulating cholangiocyte functions) (25,26) are potent inducers of vascularization,

development and growth of several cancers (5,55), such as cholangiocarcinoma (5,24). Indeed, VEGF has been shown to stimulate cholangiocyte proliferation by an autocrine mechanism (26) and to prevent cholangiocyte damage by interruption of the blood flow of the hepatic artery (25) that is the main blood supply of the biliary epithelium (27,56). These findings, demonstrating that increased VEGF expression sustains cholangiocyte proliferation (26) and that reduced cholangiocyte VEGF expression induces a decrease in cell growth and an increase in apoptosis (25), support the concept that VEGF is an important player in ET-1 inhibition of cholangiocarcinoma growth. We also speculate that the inhibitory effect on cholangiocarcinoma growth by ET-1 may be due to the vasoconstrictive properties of this peptide, which is able to modify the tumour blood flow (57,58).

The inhibitory effect of ET-1 on tumour growth was accompanied by increased apoptosis of cholangiocytes and development of abundant fibrosis. The presence of rich connective tissue into the mass could be explained by the fact that ET-1 represents a key mediator of fibrogenesis and that it is responsible for the differentiation of fibroblasts towards a myofibroblast phenotype (59). Moreover, the fibrogenic mechanism could be activated in response to apoptotic and necrotic processes, more expressed in ET-1-treated tumours with respect to controls (60). In conclusion, our findings raise the possibility of a therapeutic approach for the treatment of biliary tumours by the modulation of the ET axis.

Acknowledgements

We thank Dr Andreea Trache and Anna Webb of the Texas A&M University Health Science Center Microscopy Imaging Center for assistance with confocal microscopy.

This work was supported partly by a grant award from Scott & White to Heather Francis, by an NIH KO1 grant award (DK078532) to Dr DeMorrow, by the Dr Nicholas C. High-tower Centennial Chair of Gastroenterology from Scott & White, the VA Research Scholar Award, a VA Merit Award and the NIH grants DK58411, DK062975 and DK76898 to Dr Alpini, by a MIUR grant 2003060137_004 to the Department of Gastroenterology, Università Politecnica delle Marche, by a MIUR grant PRIN # 2007HPT7BA_003 to Dr Alvaro and by University funds to Dr Onori and PRIN 2007 and Federate Athenaeum funds from the University of Rome 'La Sapienza' to Prof. Gaudio.

Abbreviations

BSA, bovine serum albumin
 CK-7, cytokeratin-7
 $\Delta\Delta\text{CT}$, delta delta analysis of the threshold cycle
 DAPI, 4,6-diamidino-2-phenylindole
 ERK1/2, extracellular-signal-regulated kinase 1/2
 ET-1, endothelin-1
 ET_A, endothelin receptor A
 ET_B, endothelin receptor B
 GAPDH, glyceraldehyde-3-phosphate dehydrogenase
 H&E, haematoxylin and eosin
 MTS, 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulphophenyl)-2H-tetrazolium, inner salt
 PCNA, proliferating cellular nuclear antigen
 PCR, polymerase chain reaction
 PKC, protein kinase C
 TUNEL, terminal deoxynucleotide transferase end labelling
 VEGF, vascular endothelial growth factor

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Fig. 1.

(A) Immunofluorescence for endothelin receptor A (ET_A) and ET_B in H69 cells and the human cholangiocarcinoma cell line, Mz-ChA-1. A positive reaction (green) for ET_A and ET_B receptors is evident in all the cell lines. Nuclei were counterstained with DAPI (blue). Negative controls (with pre-immune serum substituted for primary antibody) were also included. Scale bar = 10 μm. (B and C) Real-time polymerase chain reaction analysis for ET_A and ET_B receptors in total RNA from H69 and Mz-ChA-1 cells. Data are mean ± SEM of three experiments. #*P* < 0.05 vs. H69 cells. (D) Evaluation of protein expression for ET_A and ET_B in tissue arrays containing 48 well-characterized cholangiocarcinoma biopsy samples from a variety of tumour differentiation grades as well as four control liver biopsy samples. Immunoreactivity showed a higher expression of ET_A and ET_B in human cholangiocarcinoma compared with normal tissue. Data are mean ± SEM of four experiments. **P* < 0.05 vs. normal liver.

Fig. 2.

(A) Effect of endothelin-1 (ET-1), by MTS assays, on the growth of the cholangiocarcinoma cell line, Mz-ChA-1. ET-1 (at 0.1–10 nM for 48 h) inhibits the growth of Mz-ChA-1 cells. (B) ET-1-induced decrease of Mz-ChA-1 cell growth at 48 h is blocked by pre-incubation with both BQ610 and BQ788. Data are mean $\pm$ SEM of three experiments. $\&P < 0.05$ vs. corresponding basal value; $*P < 0.02$ vs. other values.

Fig. 3.

Effect of endothelin-1 (ET-1) on the message expression (by real-time polymerase chain reaction) of vascular endothelial growth factor-A (VEGF-A), VEGF-C, VEGF receptor-2 (VEGFR-2) and VEGFR-3 expression in Mz-ChA-1 cells. (A and B) ET-1 (10 nM) decreased the mRNA expression of VEGF-A and VEGF-C of Mz-ChA-1 cells, a decrease that was blocked by pretreatment with BQ-610 and BQ-788. (C and D) ET-1 showed an inhibitory effect on the expression of the message for VEGFR-2 and VEGFR-3, a decrease that was blocked by pretreatment with BQ-610 and BQ-788. Data are mean $\pm$ SEM of three experiments. * $P < 0.05$ vs. corresponding basal values or treatment with inhibitors alone.

Fig. 4.

(A and B) Malignant xenograft growth was evaluated in mice treated with daily injections of endothelin-1 (ET-1) or NaCl (vehicle) into the tumours. Nude mice were subcutaneously injected with Mz-ChA-1 cells (3×10^6 viable cells) suspended in 0.5 ml extracellular matrix. Mice were injected every day with NaCl or ET-1 inside the implanted tumours, for 73 days. (A) At 73 days, a significant difference in tumour size (arrows) was macroscopically evident in ET-1-treated mice compared with mice injected with vehicle. (B) ET-1 effect on cholangiocarcinoma xenograft growth. At 73 days, the cholangiocarcinoma xenograft volume of mice periodically injected with ET-1 was 431.47 ± 55.45 vs. 904.08 ± 194 mm³ for the control mice. Tumour size is expressed in (mm³) $\pm$ SEM. * $P < 0.05$ vs. corresponding basal value.

Fig. 5.

(A) Evaluation of proliferation [by staining for proliferating cellular nuclear antigen (PCNA)] in liver sections from tumour specimens from nude mice treated with endothelin-1 (ET-1) or vehicle. ET-1 decreased proliferation and increased apoptosis of cholangiocarcinoma cells in an *in vivo* mouse model. Data are mean $\pm$ SEM of three experiments. $*P < 0.05$ vs. PCNA-positive malignant cholangiocytes from vehicle-treated nude mice. Original magnification $\times 20$. (B) Measurement of area occupied by cytokeratin-7 (CK-7)-positive malignant cholangiocytes from mice treated with ET-1 or vehicle. ET-1 administration significantly reduced the area occupied by CK-7-positive malignant cholangiocytes compared with mice treated with control solution. Data are mean $\pm$ SEM of three experiments. $*P < 0.02$ vs. CK-7-

positive cells of tumour mass from vehicle-treated nude mice. Original magnification $\times 20$. (B) Expression of vascular endothelial growth factor-A (VEGF-A) and VEGF-C by immunohistochemistry in tumours from ET-1- or vehicle-treated mice. Both VEGF-A and VEGF-C expression were reduced in malignant cells from ET-1- vs. vehicle-treated mice. Data are mean $\pm$ SEM of three experiments. $*P < 0.05$ vs. VEGF-A- and VEGF-C-positive tumour cells from vehicle-treated nude mice. Original magnification $\times 20$. (B) Evaluation of apoptosis by terminal deoxynucleotide transferase end labelling (TUNEL) staining in tumours from ET-1- or vehicle-treated mice. An increase in the number of TUNEL-positive cholangiocytes demonstrated an increase in apoptosis in ET-1-treated tumours. Data are mean $\pm$ SEM of three experiments. $*P < 0.02$ vs. TUNEL-positive tumour cells from vehicle-treated nude mice. Negative controls (with pre-immune serum substituted for primary antibody) were also included. Original magnification $\times 20$.

Fig. 6.

Photomicrographs of endothelin-1 (ET-1)- or vehicle-treated tumour sections stained with haematoxylin and eosin (H&E) and Masson's trichrome. Cholangiocarcinoma cells forming several acinar or tubular structures are shown in these images (H&E). An amorphous matrix localized between groups of cancer cells was observed in all the tumours. Masson's trichrome analysis showed fibrotic septa (blue) surrounding cholangiocarcinoma nodules. In ET-1-treated tumours, a wide area of fibrotic tissue is evident. Histological analysis demonstrated increased necrosis and damage and a more prominent fibrotic tissue in the ET-1-treated tumours with respect to controls. Original magnification $\times 80$. Data are mean $\pm$ SEM of three experiments. * $P < 0.05$ vs. vehicle-treated tumour.

Table 1

Effect of endothelin-1 treatment on cholangiocarcinoma xenograft growth and nude mice parameters after 73 days of treatment

Parameter	Vehicle	ET-1
Body weight (g)	26 ± 0.47	23.5 ± 0.75 ^{NS}
Tumour latency (days)	12.3 ± 4.1	31.25 ± 8.6 [*]
Tumour size (mm ³)	904.08 ± 194	431.47 ± 55.45 [*]

Data are mean ± SEM of four mice.

ET-1, endothelin-1; NS, not significant; SEM, standard error of mean.

* $P < 0.02$ vs. the corresponding value of vehicle-treated mice.