Role of the endothelin axis in the proliferation of human thyroid cancer cells

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Summary

Objective Endothelin-1 (ET-1) may play a role in carcinogenesis. ET-1 axis is overexpressed in thyroid carcinoma. We investigated the expression and the production of ET-1 by thyroid cancer cells as well as the effect of ET-1 receptor antagonism on cell proliferation.

Design Human papillary and follicular thyroid carcinoma cell lines were cultured.

Measurements (i) Prepro-ET-1, ET-1 receptors (ET\(_{\alpha}\) R and ET\(_{B}\) R) and ET-1 converting enzyme (ECE) by reverse transcriptase polymerase chain reaction (RT-PCR); (ii) the presence of ET\(_{\alpha}\) R by western blot; (iii) ET-1 concentrations in medium by an enzyme immunoassay; (iii) the proliferation of cells by BrdU and tritiated thymidine incorporation.

Results RT-PCR detected the presence of mRNA for prepro-ET-1, ET\(_{\alpha}\) R and ECE in papillary and follicular carcinoma cells. ET\(_{\alpha}\) R was only expressed by follicular cells. ET\(_{\alpha}\) R was also detected in both cell types by western blot. Measurements of ET-1 concentrations demonstrated a secretion of active ET-1 by the cells. ET\(_{\alpha}\) R antagonism with atrasentan reduced cell proliferation by 16% in papillary carcinoma cells (P < 0.05) and by 51% in follicular carcinoma cells (P < 0.001).

Conclusions Papillary and follicular carcinoma cells express all components of the ET-1 axis. ET\(_{\alpha}\) R antagonism exerts antiproliferative effects, which open up new therapeutic perspectives in thyroid carcinoma.

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Introduction

Endothelin-1 (ET-1), an endothelium-derived 21 residue peptide was originally shown to be a potent and long-lasting vasoconstrictor.\(^1\)\(^2\) It belongs to a family of multifunctional peptides (ET-1, -2 and -3) that exerts its effects via two G protein-coupled receptors, ET\(_{\alpha}\) (ET\(_{\alpha}\) R) and ET\(_{B}\) (ET\(_{B}\) R).\(^3\) Whereas ET\(_{B}\) R binds the three peptides with equal affinity, ET\(_{\alpha}\) R binds ET-1 and ET-2 with higher affinity than ET-3.\(^3\) Because of its vasoactive effects as well as its mitogenic properties on vascular smooth muscle cells, ET-1 has since been implicated in the pathophysiology of various cardiopulmonary disorders, including notably arterial and pulmonary hypertension.\(^5\)\(^6\)

More recently, ET-1 has also emerged as an important factor involved in several processes affecting carcinogenesis, such as cellular proliferation and survival, angiogenesis, and bone metastasis.\(^7\)\(^8\) In particular, ET-1 was shown to play a key role through the activation of ET\(_{\alpha}\) R in the development and progression of prostatic and ovarian cancers.\(^9\)\(^–\)\(^12\) Consequently, ET\(_{\alpha}\) R antagonists have proved to have anti-tumour activity in these cancers.\(^10\)\(^–\)\(^12\)

In papillary and medullary thyroid carcinoma, we previously demonstrated an overexpression of the ET-1 axis including the ET-1 precursor, ET\(_{\alpha}\) R, and ET-1 converting enzyme (ECE).\(^13\)\(^–\)\(^15\) The expression of the other ET-1 receptor, ET\(_{B}\) R, was, however, unaltered. More recently, we found that the activation of ET-1 axis in thyroid cancer was interrelated with the nitric oxide pathway, both systems being overexpressed in concert with angiogenic factors.\(^16\)

In view of the above findings obtained from surgical human thyroid samples, it was important to establish whether the ET-1 axis could also be expressed by thyroid cancer cells in culture, and constitute a new potential target for anticancer therapy. To this end, we studied the expression of ET-1 and its receptors in human papillary and follicular cell lines as well as its production by the same cells. In addition, we tested the effect of the specific ET\(_{\alpha}\) R antagonist atrasentan on the proliferation of these cells.

Methods

Cell lines

The human papillary thyroid carcinoma cell line TPC-1 was a generous gift from Prof D. Vitagliano (University of Napoli ‘Federico II’, Italy) while the human follicular thyroid cell line FTC133 was purchased from ECCC (European Collection of Cell Culture). TPC-1 cells were grown in Dulbecco’s modified Eagle’s medium (DMEM) and FTC133 cells were grown in DMEM-Ham’s F12 (1 : 1) containing 2 mM glutamine. The two media were supplemented with 10% foetal calf serum (FCS). The cells were maintained at 37 °C in a 5% carbon dioxide incubator. All the experiments including cell proliferation measurements were performed in presence of foetal calf serum. Human keratinocytes were used as positive controls for detecting ET\(_{\alpha}\) R.
RNA extraction and reverse transcriptase polymerase chain reaction (RT-PCR)

RT-PCR was used to detect prepro-ET-1, ET$_A$ R, ET$_B$ R, and ECE-1 transcripts. Total RNA was extracted using RNAeasy® Minikit (Qiagen, Venlo, Holland) following the manufacturer’s instructions and was quantified by spectrophotometry ($\lambda$, 260 nm). Reverse transcription (RT) was performed with 1 µg of RNA, 7.5 µM random hexamers, and M-MLV reverse transcriptase (Inviogen, Carlsbad, CA). The PCR reaction was performed in 20 µl buffer containing 1/20 of RT final volume, 250 µM each dNTP, and 0.5-µl DNA polymerase Taq Go Taq (Promega, Madison, WI). PCR conditions were 95 °C for 5 min, followed by 30 cycles (95 °C for 30 s, 59 °C for 30 s, and 72 °C for 1 min) and a final extension at 72 °C for 7 min. The primer sets obtained from Eurogentec (Belgium) were as follows: (1) ET$_A$ R, 5’ CCTGCTCCTTTATCCTGGCCAG3’ and 5’AGATCGCATGCAACCAAG3’; (2) ET$_B$ R, 5’AGATCAAGGAGACTTTCTAAATAC3’ and 5’CAATCTCAAGACCATAGA3’; (3) prepro-ET-1, 5’TCAGTTGAACGGGAGGTTTTT3’ and 5’CCGCTCTCGAGGGATTGCC3’; (4) ECE-1, 5’TGGGGGACCTTCAGCAACCT3’ and 5’GGGGTTCGAGGTGTTGC3’; and (5) glyceraldehyde-3-phosphate dehydrogenase (GAPDH), 5’CCATCAACCATCTTCCAGGAG3’ and 5’GTGTCATGGAATGACCTTG3’.

The PCR products were separated by 1% agarose gel electrophoresis in the presence of ethidium bromide for UV fluorescence detection. GAPDH was used as an internal control. No detectable PCR products were present in water controls or in controls amplified without prior reverse transcription.

Cell proliferation assay

BrdU and tritiated thymidine incorporation was used to assess the proliferation of TPC-1 and FTC133 cells, respectively. TPC-1 cells were plated in 96-well plates for 24 h and then incubated with different concentrations of clinical grade atrasentan (Abbott Laboratories, Abbott Park, IL) for 20 h. Two hours before the end of treatment, BrdU was added to the medium. Cells were then fixed and stained for BrdU incorporation (“Cell proliferation Elisa BrdU” kit, Roche Applied Science, Indianapolis, IN). FTC133 cells were seeded in six-well plates and incubated for 24 h. Atrasentan at different concentrations was then added to the medium. Six hours before the end of the 20-h treatment, [$^3$H]-thymidine (GE Healthcare) at 1 µCi/ml was added. At the end of the exposure period, medium was removed by aspiration, washed twice with phosphate-buffered saline and exposed to 10% trichloracetic acid (TCA) for 1 h; TCA-precipitated material was washed twice with 5% TCA and solubilized with 500 µl of 1 N NaOH. Radioactivity was determined using liquid scintillation counting. Proteins were determined by a Protein Assay kit (Bio-Rad Laboratories GmbH, Hercules, CA).

Western blot analysis

Total cell lysates were obtained from homogenized TPC-1 and FTC133 cell lines. The protein extracts were resolved by 10% SDS-PAGE and afterwards probed with a goat anti-ET$_A$ R polyclonal antibody (Santa Cruz Biotechnology Inc., Santa Cruz, CA). Immunoreactive proteins were visualized by enhanced chemoluminescence (Perkin Elmer, Waltham, MA) according to the manufacturer’s instructions.

Evaluation of ET-1 secretion

Extraction of ET-1. The media were acidified with 0-1% trifluoracetic acid and centrifuged at 3000 g for 10 min. The supernatant was applied to Sep-Pak C18 cartridges (Waters Associates, Milford, MA). After elution with 3 ml of 60% acetonitrile/0-1% trifluoracetic acid, the lyophilized material was analysed for ET-1 content.

Quantification of ET-1. The lyophilized samples were reconstituted in an appropriate medium just before quantification. ET-1 was measured using a commercial enzyme immunoassay kit (TiterZyme®, Assay Designs, Ann Arbor, MI). The per cent cross-reactivities of the antibody are reported as 100% for ET-1 (1–21), < 0-1% for ET-2 (1–21), < 0-1% for ET-3 (1–21), < 0-1% for ET-1 (1–31) and < 0-1% for Big ET-1. The sensitivity of the assay is 0-14 pg/ml. ET-1 concentrations were determined after 48 h in the culture medium with and without thyroid cancer cells.

Statistics

Results are presented as mean ± SEM. ANOVA for repeated measures was used to evaluate the effect of atrasentan on cell proliferation and a Dunnett’s posthoc test for contrast analysis. A P-value of < 0-05 was considered to be statistically significant.

Results

Prepro-ET-1, ET$_A$ R, ET$_B$ R, and ECE mRNA expression in FTC133 and TPC-1 cells

Gene expression of prepro-ET-1, ET$_A$ R, ET$_B$ R, and ECE was examined by RT-PCR. After gel electrophoresis separation of PCR amplification products, bands of the predicted size were clearly detected for prepro-ET-1 (636 bp), ET$_A$ R (309 bp), and ECE but not of ET$_B$ R.

Presence of ET$_A$ R in FTC133 and TPC-1 cells

Having established the expression of the different components of the ET-1 system in the thyroid cancer cells, we used the western blot technique to detect the presence of ET$_A$ R, the receptor mainly involved in the proliferation process. For both FTC133 and TPC-1 cell extracts, as well as for human keratinocytes used as positive controls, a band corresponding to the predicted size of ET$_A$ R (54 kD) was present (Fig. 2).
media containing 10% FCS were subtracted from those measured in the presence of the cells. The results obtained from three different runs revealed that FTC133 cells secreted 0 ± 0, 13·1 ± 0·5, and 16·5 ± 0·7 pg ET-1 into the medium during a 48-h culture of $10^5$ cells. In three other experiments, TPC-1 cells were found to secrete 0 ± 0, 2·2 ± 0·2, and 10·3 ± 0·6 pg ET-1 per $10^5$ cells under the same experimental conditions. Zero pg means that no additional ET-1 was detectable in those experiments.

**Effect of atrasentan on the proliferation of FTC133 and TPC-1 cells (Fig. 3)**

To study the effects of atrasentan on the proliferation of thyroid cancer cells, we used two different methods: tritiated thymidine for FTC133 cells and BrdU incorporation for TPC-1 cells. Seven separate experiments were performed for each cell type. Treatment with atrasentan at concentrations ranging between 1 and 100 µM determined a dose-dependent inhibition of spontaneous growth of both FTC133 cells ($P = 0.0008$ by ANOVA) and TPC-1 cells ($P = 0.042$). The maximal effects obtained at the highest concentration reached 51% ($P < 0.01$) in FTC133 cells and 16% ($P < 0.05$) in TPC-1 cells. In order to assess a possible nonspecific effect of atrasentan vehicle (ethanol 0·2%), the volume of ethanol corresponding to the 100-µM concentration of atrasentan was added to the culture medium in four TPC-1 proliferation experiments. There was no difference in proliferation with or without ethanol.

**Discussion**

In our previous studies performed in human thyroid samples obtained at the time of surgery, we demonstrated an overexpression of ET-1, ECE, and ET$_A$R in papillary and medullary thyroid carcinomas by immunochemistry and real-time quantitative PCR.$^{13-15}$ The results of the present study indicate for the first time that the same elements (i.e. ET-1, ECE, and ET$_A$R mRNA) are also expressed by isolated cultured human thyroid cancer cells of papillary and follicular origins. In addition, the western blot technique shows that, beyond gene expression, ET$_A$R is well present in these cells. As regards ET$_B$R, its expression was not previously found to be overexpressed in papillary thyroid carcinoma.$^{13,15}$ Our current findings that cultured papillary cells do not express ET$_B$R are thus in line with former pathological results. However, follicular cells do express ET$_B$R. Similarly, in ovarian cancer, ET$_A$R was detected in 84% of carcinomas but ET$_B$R in only 50%.$^{11}$ In prostate cancer, ET$_A$R was highly expressed whereas ET$_B$R expression was decreased or absent.$^{9,10}$ In

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melanoma cells, on the other hand, ET\textsubscript{A} R was found to be downregulated and ET\textsubscript{B} R was overexpressed.\textsuperscript{7,18} In colon cancer both receptors were overexpressed.\textsuperscript{7,19} As far as the pathogenic function of both receptors is concerned, there is evidence that ET\textsubscript{A} R activation by ET-1 is able to mediate mitogenic effects in a variety of epithelial tumours such as colorectal, ovarian and prostatic cancers,\textsuperscript{9–12,19} whereas the growth of nonepithelial cancers such as melanoma is purely ET\textsubscript{B} R-dependent.\textsuperscript{8,18}

Once the presence of the ET axis including ET\textsubscript{A} R had been established in thyroid cancer cells, it became important to test whether these cell lines were able to secrete ET-1 and hence influence the cancer cells in a paracrine or autocrine way. Therefore, we measured ET-1 in the medium and found, as had been demonstrated for other cancers, that both papillary and follicular thyroid cells could produce certain amounts of endogenous ET-1, mirroring its mRNA expression. Under these in vitro conditions, secretion was variable. Interestingly, the specificity of the immunometric assay used indicated that ET was either secreted in its active form ET-1 or effectively converted to ET-1 by cell-bound ECE.

The up-regulation of ET\textsubscript{A} R in thyroid cancer cells and its likely implication in cell proliferation prompted us to investigate the effect of the potent and specific ET\textsubscript{A} R antagonist atrasentan (ABT-627) on the spontaneous growth of papillary and follicular cells cultured under optimal conditions (i.e. in the presence of FCS). Atrasentan had a significant inhibitory effect on cell proliferation (i.e. DNA synthesis, in both cell types). Previous studies have investigated the effects of ET-1 receptor antagonism on cell proliferation. In human colon cancer cells, the dual ET\textsubscript{A,B} R antagonist bosentan did not decrease DNA synthesis when cells were serum-deprived.\textsuperscript{19} Bosentan decreased DNA synthesis only in serum-exposed cells and at high concentration. In prostate cancer cell lines, ET\textsubscript{B} R antagonism with atrasentan was found to block proliferation but only under exogenous ET-1 stimulation.\textsuperscript{9} In ovarian carcinoma cells, however, atrasentan inhibited spontaneous cell proliferation both in serum-free and in low-serum media,\textsuperscript{12} at lower concentrations (0.01–2 \textmu M) than in the current study (1–100 \textmu M). The variant results of the above studies probably reflect differences between cancer cell types, proliferation potentials, or experimental protocols. It is noteworthy that the effect of atrasentan on thyroid cancer cells required the presence of serum for sufficient thyroid cell proliferation, similar to colon cancer cell cultures.\textsuperscript{19} Serum-derived growth factors independent of the ET-1 system might have attenuated the effect of atrasentan compared with those observed for ovarian cells in serum-free conditions. Ovarian cells may also be more sensitive to atrasentan than thyroid cells. Furthermore, it is possible that atrasentan would be a weak inhibitor of cellular proliferation in monotherapy or would interfere with other ET-1 functions in carcinogenesis. In human ovarian carcinoma, for instance, atrasentan combined with paclitaxel was shown to produce additive anti-tumour and anti-angiogenic effects.\textsuperscript{12} Further studies are required to assess the effect of atrasentan on other ET\textsubscript{A} R-mediated tumour-promoting activities in thyroid carcinoma, such as escape from apoptosis, angiogenesis, and metastatic spread.

Regarding the clinical effectiveness of ET\textsubscript{A} R antagonism in cancer, further studies are still required to be conclusive. In prostate cancer, for instance, one study\textsuperscript{20} showed that atrasentan could delay the progression of hormone refractory prostate cancer but others failed to demonstrate the benefit of atrasentan in monotherapy, notably vs. a combination therapy with zoledronic acid.\textsuperscript{1,22}

In conclusion, in addition to our previous studies showing an overexpression of the ET-1 axis in thyroid cancers, the present results demonstrate the presence of all ET-1 components in cultured papillary and follicular cells as well as a secretion of ET-1 by cancer cells, suggesting an autocrine or paracrine role for ET-1 in thyroid carcinogenesis. This is supported by the antiproliferative effects of atrasentan demonstrated in the current study. The benefits of ET\textsubscript{A} R blockade in thyroid carcinoma are worth exploring, especially in patients with aggressive inoperable tumours that do not concentrate radioiodine and are refractory to all forms of treatment.

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References