Studies on the Expression of Endothelin, Its Receptor Subtypes, and Converting Enzymes in Lung Cancer and in Human Bronchial Epithelium

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Lung cancer, particularly small cell lung cancer (SCLC), is characterized by production of numerous peptides and their resulting clinical syndromes. Such peptides can act as autocrine growth factors for these tumors. In this study, we investigated the role of endothelin (ET)-1 in lung cancer. Using reverse transcription/polymerase chain reaction (RT-PCR), enzyme-linked immunosorbent assay, and immunocytochemistry, we screened a panel of lung cancer cell lines for ET-1, its receptors, and endothelin converting enzyme-1 (ECE-1), which generates the active form of ET-1. ET-1 messenger RNA was expressed in five of seven SCLC, four of four non-small cell lung cancer (NSCLC), and human bronchial epithelial (HBE) cells. The intracellular isoform of ECE-1, important in processing ET-1 if an autocrine growth loop is to function, was downregulated in the lung cancer cell lines as compared with expression of the extracellular isoform. Endothelin A receptor (ETAR), which mediates the mitogenic effects of ET-1 in prostate and ovarian cancer, was upregulated in HBE cells compared with expression in three of seven SCLC and two of four NSCLC cell lines. Endothelin B receptor (ETBR) was more widespread, being expressed in seven of seven SCLC, four of four NSCLC, and the HBE cells. We used flow cytometry to measure mobilization of intracellular calcium as a functional assay for the ETAR. These data concurred with the RT-PCR results, indicating that the ETAR was downregulated or was involved in an alternative signal transduction pathway in lung cancer, and no evidence of functional receptor mediating an autocrine growth loop was found. From our study, the data do not support the putative functional autocrine growth role of ET-1 in lung cancer. We propose instead that ET-1 may act as a paracrine growth factor for surrounding epithelial and endothelial cells via alternative pathways, promoting angiogenesis and stromal growth.

The endothelin family comprises three distinct peptides encoded by separate genes located on different chromosomes (1). Endothelin-1 (ET-1) is the most extensively investigated family member. The ET-1 gene encodes a precursor peptide, preproendothelin-1, which is cleaved by a neutral endopeptidase to form proendothelin-1 or big ET-1. This is further cleaved by endothelin converting enzyme-1 (ECE-1) to ET-1(2). This proteolytic cleavage of Trp-Va

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Abbreviations: complementary DNA, cDNA; 4,6-diamidino-2-phenylindole, DAPI; endothelin converting enzyme-1, ECE-1; enzyme-linked immunosorbent assay, ELISA; endothelin, ET; endothelin A receptor, ETAR; endothelin B receptor, ETBR; fluorescein isothiocyanate, FITC; glyceraldehyde-3-phosphate dehydrogenase, GAPDH; human umbilical vein endothelial cell, HUVEC; messenger RNA, mRNA; normal human bronchial epithelial cell, NHBE; non-small cell lung cancer, NSCLC; reverse transcriptase/polymerase chain reaction, RT-PCR; small cell lung cancer, SCLC,

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bond by ECE-1 is critical to the function of ET-1 because the precursors have negligible biologic activity. Three isoforms of ECE-1 exist: ECE-1α, ECE-1β (3), and ECE-1c (4). They differ only in their N-terminal regions and are derived from a single gene through the use of alternative promoters and differential splicing. The main difference between the isoforms is their subcellular distribution. ECE-1b is predominantly intracellular, whereas ECE-1α and ECE-1c are mainly extracellular (4). Intracellular ECE-1b converts endogenously produced ET-1 and may therefore be important for an autocrine growth loop to function. The extracellular forms, ECE-1α and ECE-1c, act as ectoenzymes expressed on the cell surface, capable of cleaving big ET-1 supplied from outside the cell. The abundance of each isoform varies between cell types investigated and, because the three isoforms have similar enzymatic properties and cleave big ET-1 with comparable efficiencies, their differential subcellular localization and expression within different cell types may be of regulatory importance in the production and subsequent actions of ET-1.

Since its isolation from porcine aortic endothelial cells (5), ET-1 has been found in epithelial, mesangial, neuronal, and glial cells. Its physiologic actions, especially vasconstriction, have been well characterized. The mitogenic action of ET-1 has been shown in several cell types, including fibroblasts (6), vascular smooth muscle (7), renal mesangial cells (8), and airway smooth muscle (9).

ET-1 is hydrophilic and unable to cross the plasma membrane; it binds to specific cell-surface receptors, which regulate its effect within the cell. There are at least two receptor subtypes, endothelin A (10) and endothelin B (11) receptors. These belong to the family of G-protein–linked receptors with seven transmembrane–spanning domains. The vasoconstrictor effects of ET-1 are mediated via endothelin A receptors (ETAR) (12), which are typically found on vascular smooth muscle. Stimulation of endothelin B receptors (ETBR), mainly found in vascular endothelium, causes vasodilatation mediated through the production of nitric oxide (13). Both these receptors are present on many cell types other than vascular endothelium and smooth muscle (14). Human lung is particularly rich in both ETAR and ETBR (15), suggesting that ET-1 plays an influential role in the normal physiology of the lung.

Growth promotion by ET-1 involves activation of several signal transduction pathways. The mitogenic effects are mediated through the ETAR by activation of phospholipase C, hydrolysis of phosphatidylinositol (16), and mobilization of the second messengers inositol triphosphate and diacylglycerol (17, 18). These second messengers then cause a biphasic increase in intracellular calcium. There is an initial transient peak derived from intracellular
It is of interest that ET-1 is produced by several human cancer cell lines, including breast, colon, pancreas (20), cervix and larynx (21), endometrium (22), prostate (23), and ovary (24). Production of ET-1 by these cancer cells suggests that it may be involved in their development and progression. For example, Nelson and coworkers (25) demonstrated that an ETAR-specific antagonist could block the mitogenic actions of ET-1 on prostate cancer cell lines in culture, thus implying that the ETAR mediates the mitogenic effects of ET-1 in prostate cancer. The same investigators proposed that the ETBR subtype was downregulated in prostate cancer because there was little expression of ETBR in the prostate cell lines investigated by reverse transcription/polymerase chain reaction (RT-PCR) and Southern blot analyses.

Lung tumors, particularly small cell lung cancer (SCLC), produce a host of peptides, which act as autocrine growth factors for these cells (26). Of these, the bombesin-like peptides (gastrin-releasing peptide) and vasopressin are the most extensively studied, and their role as an autocrine growth factor for SCLC is well established (27–33). A nonautocrine growth loop requires active peptide production together with expression and activation of its receptor by the peptide in the same cell. Involvement of ET-1 in the biology of lung cancer was first suggested when ET-1 messenger RNA (mRNA) was demonstrated in a variety of lung cancers by in situ hybridization (34). More recently, Cohen and colleagues (35, 36) demonstrated expression of ET-1, its receptors, and ECE-1 in several lung cancer cell lines and proposed that ET-1 may act as an autocrine growth factor for these cells.

In this study we investigate the expression of ET-1, ETAR, ETBR, and the ECE-1 isoforms in a panel of lung cancer cell lines and discuss the possible role of ET-1 in the progression of these tumors.

Materials and Methods

Culture of Cell Lines

All cancer cell lines were maintained in RPMI medium (GIBCO, Paisley, UK) supplemented with 10% bovine calf serum (Sigma, Dorset, UK), at 37°C in a humidified atmosphere and 5% CO2.

SCLC cell lines were cultured as suspension aggregates and passed by disaggregation every 3 to 5 d. All cell lines were harvested by trypsinization every 4 to 5 d. NSCLC cell lines used in this study were NCI-H69, NCI-H345, NCI-H711 (generous gift from Dr. T. Subkowski, Queens Medical Centre, Nottingham, UK) cultured in RPMI medium (GIBCO, Paisley, UK) supplemented with 10% bovine calf serum (Sigma, Dorset, UK), at 37°C in a humidified atmosphere and 5% CO2.

Total RNA was extracted from cell lines using a Purescript RNA isolation kit (Gentra Systems Inc., Minneapolis, MN) according to the manufacturer’s protocol. The quantity of RNA was determined by ultraviolet spectrophotometry. Complementary DNA (cDNA) was made using an oligo dT primer (Reverse Transcription System; Promega, Southampton, UK). A total of 3 µl of diluted cDNA template was used for PCR, which was optimized using the Hybrid Touchdown thermal cycler. A total of 35 cycles of PCR was used for reactions unless otherwise stated. Primers were designed to amplify across exon/intron boundaries to distinguish any contaminating genomic DNA amplification. The position of the primers used, in relation to gene structure, is shown in Figure 1. Forward primers for ECE-1 were designed to amplify regions exclusive to each isoform. The same reverse primer was used for all ECE-1 PCRs. The 5’ region of exon 3 is only specific for ECE-1a; exons 1b and 2 are specific for ECE-1b mRNA, and the ECE-1c isoform shares the ECE-1b-specific region in exon 2 but differs in sequence upstream from this, within exon 1 (Figure 1). RT-PCR for glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (39) was used to semiquantify the technique (28 cycles) and test integrity of cDNA. The same cDNA template was used in all PCRs; representative amplification of GAPDH is shown in Figure 2B. PCR products were analyzed on 2% agarose gel electrophoresis. The identity of RT-PCR products was confirmed by automated sequencing (DNA sequencing facility, Queens Medical Centre, Nottingham, UK) using an ABI Prism 373 sequencer (PE Biosystems, Warrington, UK).

Blotting and hybridization of the PCR products from the ECE-1 reaction was performed by transferring the DNA, electroblotted on a 2% agarose gel, to a nylon membrane by capillary blotting. The membrane was hybridized at 65°C, overnight with an α-[32P]deoxyctidine triphosphate–labeled DNA probe, sequence-specific for ECE-1b. This was then washed with increasing stringency washes and subjected to autoradiograph.

Immunocytochemistry

For SCLC cell lines, cytopsins were produced by centrifugation at a concentration of 5 × 104 cells/ml. For the adherent NSCLC cell lines, cells were seeded at 1 × 105/ml in four-well chamber slides and incubated overnight at 37°C. Cells were fixed in 1% paraformaldehyde and, where required, permeabilized in 70% ethanol for 10 min. Cells were incubated with primary antibody at room temperature for 1 h. Primary antibodies used were B61 (detecting all isoforms of ECE-1) at a 1:50 dilution (40), anti-ECE-1a (detecting only the ECE-1a isoform), and anti-ECE-1b (detecting both ECE-1b and ECE-1c isoforms), both used at a 1:100 dilution. All ECE-1 antibodies were a generous gift from Dr. T. Subkowski (BA SF, Ludwigshafen, Germany). An antibody to actin (Sigma) was used as a control for intracellular staining (1:100). Secondary antibodies were used at 1:50 dilution: fluorescein isothiocyanate (FITC)–conjugated antio1mune to detect B61 antibody and FITC–conjugated antirabbit to detect anti-ECE-1a, anti-ECE-1b, and antiaxin primary antibodies. These antibodies were incubated at room temperature for 1 h. 4,6-Diamidino-2-phenylindole (DAPI) (Sigma) nuclear stain was mixed with mountant at 0.1 µg/ml, and slides were viewed under fluorescence with green filter (DM 400) for DAPI and blue filter (DM 510) for FITC.

Enzyme-Linked Immunosorbent Assay (ELISA) to Detect Active ET-1

The Biotrak ET-1 ELISA system (Amersham Pharmacia Biotech, Buckinghamshire, UK) was used to detect ET-1 production.
by cultured cells. ET-1 in the samples tested was captured by microtiter plates precoated with ET-1 antibody and detected by a peroxidase-labeled Fab' fragment of ET-1 antibody conjugate. HUVECs, A549, and NCI-H69 were cultured in the same medium for 48 h and the supernatants from these cells were processed according to the manufacturer's instructions.

**Calcium Mobilization Assay**

Cells were harvested at a concentration of $2 \times 10^7$ /ml and resuspended in serum-free RPMI. The method was adapted from Woll and Rozengurt (41) as described by Coulson and coworkers (33). The cells were loaded with Fluo-3 acetoxyethyl ester (Alexis, Nottingham, U.K.) (42, 43), a fluorescent Ca$^{2+}$ chelator, and incubated at 37°C in the dark for 30 min. Cells were then resuspended in a solution containing 1 mg/ml dextrose, 3.6 mM CaCl$_2$, and 0.9 mM MgCl$_2$ in phosphate-buffered saline-A (phosphate-buffered saline with 1 mg/ml bovine serum albumin). Flow cytometric assays were performed using a fluorescence-activated cell sorter (FACS) scan (Becton Dickenson, Plymouth, U.K.). Cells were stimulated with ET-1 at concentrations between $10^{-10}$ to $10^{-2}$ M, and fluorescence (FL1 channel) measured over time. A n increase in fluorescence indicated intracellular calcium mobilization and was calculated relative to the fluorescence of unstimulated cells. Serum was used as a nonspecific stimulus for calcium mobilization to validate the assay for each sample.

**Results**

In this study we investigated the expression of ET-1, ECE-1, and ET-1 receptors (ETAR and ETBR) by RT-PCR in lung cancer cell lines and bronchial epithelial cells. The presence and cellular location of ECE-1 and its isoforms were investigated by immunocytochemistry, and the activation of functional receptor was evaluated by flow cytometry. HUVECs were used as a positive control because ET-1 (1) and ECE-1 (3) were both identified and characterized from these cells. Figure 2A shows ET-1 mRNA expression by RT-PCR in five of seven SCLC and four of four NSCLC cell lines, and in the human bronchial epithelial cells NHBE and SV40-HBE. RT-PCR with GAPDH primers amplified comparable products from the cDNAs.
used, thus semiquantifying the PCR for other templates (Figure 2B). The expression of ET-1 mRNA (Figure 2A) was high in the NHBE and SV 40-HBE cells (lanes 13 and 14) and comparable to that in HUVECs (lane 15). The NSCLC adenocarcinoma line A 549 (lane 11) and the SCLC cell line COR-L88 (lane 6) also showed high expression comparable to the HUVECs, whereas lower levels of expression were seen in the other NSCLC cell lines. Multiple PCR products were seen in GLC-19 SCLC cell line (lane 1) and the HL-60 leukemic cell line (lane 12). The latter cell type represents a nonepithelial malignancy and was used as a negative control. In the absence of low abundance of the transcript, nonspecific amplification can occur, producing multiple products. NCI-H 69 cell line (lane 2) was negative for ET-1.

RT-PCR designed to detect all three ECE-1 isoforms (Figure 3A) and that designed to detect the ECE-1b and ECE-1c isoforms (Figure 3B) were positive throughout the cell lines tested. The ECE-1 isoforms individually, however, had more heterogenous expression. The extracellular ECE-1a isoform (Figure 3C) was seen by RT-PCR in the HUVECs (lane 15), which was used as a positive control, the NSCLC cell line NCI-H 460 (lane 8), and the NHBE and SV 40-HBE cells (lanes 13 and 14), but the bands were less intense in these lines as compared with that in HUVECs. Similarly, the intracellular ECE-1b isoform was only clearly present by RT-PCR in the HUVECs (Figure 3D, lane 15). The products of this PCR were therefore Southern blotted and hybridized using a probe for ECE-1a isoform. An example of this is shown in the NCI-H 460 cell line (Figure 4A, lane 1). The pattern of fluorescence, seen as a bright ring at the periphery of the cells or uniformly across the cell surface, is characteristic of an SCLC cell line positive for the pan-ECE-1 isoform antibody, is shown in Figure 4A. These cells are characteristically small in size and have relatively large nuclei with prominent nucleoli and scant cytoplasm. Staining with the FITC-conjugated antibody to detect the pan-ECE-1 isoform antibody shows a bright ring staining the cell membrane, which can be seen separate from the nuclei (Figure 4A, top panel). Nuclear staining with DAPI for the same field of view is shown to delineate position of cells and to distinguish location of immunofluorescence (Figure 4A, bottom panel). For comparison, the NCI-H 345 SCLC cell line (Figure 4A, top and bottom), the NCI-H 460 NSCLC cell line (Figure 4B, top and bottom), and the SV 40-HBE cells (Figure 4C, top and bottom) are shown with positive immunofluorescence for the ECE-1 pan-isoform antibody and with DAPI nuclear staining. In contrast, the ECE-1a antibody resulted in positive immunofluorescence only in the NCI-H 460 cell line (Figure 4D, top and bottom) and in the SV 40-HBE (Figure 4E, top and bottom). The HUVECs again were used as positive control (Table 1). The other lung cancer cell lines tested were negative for the ECE-1a isoform. An example of this is shown in the NCI-H 345 cell line (Figure 4F, top and bottom).
Table 1

<table>
<thead>
<tr>
<th>Antibody: Isoforms Detected</th>
<th>ECE-1 (B61) abc</th>
<th>ECE-1a</th>
<th>ECE-1bc bc</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cell Line</td>
<td>SCLC</td>
<td>NSCLC</td>
<td>Normal lung</td>
</tr>
<tr>
<td>NCI-H 345</td>
<td>SCLC</td>
<td>+ + +</td>
<td>+ + +</td>
</tr>
<tr>
<td>GLC-19</td>
<td>SCLC</td>
<td>+ + +</td>
<td>+</td>
</tr>
<tr>
<td>Lu-165</td>
<td>SCLC</td>
<td>+ + +</td>
<td>+ + +</td>
</tr>
<tr>
<td>NCI-H 460</td>
<td>NSCLC</td>
<td>+ + +</td>
<td>+ + +</td>
</tr>
<tr>
<td>COR-L 23</td>
<td>NSCLC</td>
<td>+ + +</td>
<td>+ + +</td>
</tr>
<tr>
<td>SV-40 HBE</td>
<td>Normal lung</td>
<td>+ + +</td>
<td>+ + +</td>
</tr>
<tr>
<td>HUVEC</td>
<td>Endothelial cells</td>
<td>+ + +</td>
<td>+ + +</td>
</tr>
</tbody>
</table>

Definition of abbreviations: NSCLC, non-small cell lung cancer; SCLC, small cell lung cancer; + + + = strong, + + = moderate, + = weak, – = negative immunofluorescence.

Intensity of staining is related to that in HUVECs (positive control).

Figure 3. ECE-1 isoform mRNA expression detected by RT-PCR in a panel of cell lines. Ethidium bromide–stained agarose gels showing (A) ECE-1 mRNA using ECE-1abc/ECE-1rev primers, detecting all isoforms of ECE-1. (B) ECE-1bc mRNA using ECE-1bc/ECE-1rev primers, detecting the ECE-1bc and c isoforms only. (C) ECE-1a mRNA using ECE-1a/ECE-1rev primers, detecting the ECE-1a isoform only. (D) ECE-1b mRNA using ECE-1b/ECE-1rev primers, detecting the ECE-1b isoform only. (E) Blotting and hybridization of PCR products with probes for ECE-1b. (F) ECE-1c mRNA using ECE-1c/ECE-1rev primers, detecting the ECE-1c isoform only. Counting from left to right, lanes representing SCLC cell lines: lane 1, GLC-19; lane 2, NCI-H 69; lane 3, NCI-H 345; lane 4, NCI-H 711; lane 5, Lu-165; lane 6, COR-L 88; lane 7, COR-L 24. NSCLC cell lines: lane 8, NCI-H 460; lane 9, COR-L 23; lane 10, MOR/P; lane 11, A 549. Control cell lines: lane 12, HL 60; lane 13, NHBE; lane 14, SV-40 HBE; lane 15, HUVECs. Lane 16, no cDNA.

The positive ECE-1bc antibody staining seen throughout the cell lines is again likely to be due to high levels of the ECE-1c isoform present. This corresponds to expression of ECE-1c throughout the cell lines by RT-PCR and compar-
Figure 4. Expression and subcellular distribution of ECE-1 isoforms by immunocytochemistry. (A–C) Pan-isofrom staining (top panels) and corresponding nuclear staining with DAPI (lower panels). (A) NCI-H345 SCLC cell line, (B) NCI-H460 NSCLC cell line, (C) SV40-HBE bronchial epithelial cell line. (D–F) ECE-1a isoform staining (top panels) and corresponding nuclear staining with DAPI (lower panels). (D) NCI-H460 NSCLC cell line, showing pattern of extracellular staining. (E) SV40-HBE bronchial epithelial cell line. (F) Negative staining in NCI-H345 SCLC cell line. (G) ECE-1bc staining in permeabilized HUVECs. Intense staining in the permeabilized cells suggests a contribution by the intracellular ECE-1b isoform because only the permeabilized cells would also be able to stain for both the intra- and extracellular isoforms. (H) Nonpermeabilized NCI-H460. (I) Permeabilized NCI-H460. No difference is seen in the differential cell preparation, suggesting the main isoform is the ectoenzyme ICE-1c.
atively reduced expression of ECE-1a and ECE-1b isoforms in the lung cancer cell lines.

Having demonstrated that lung cancer cells have the machinery to activate proendothelin, it was important to show that these cells actually produce active ET-1. A n ELISA system with negligible cross-reactivity with proendothelin was used to detect active ET-1 in the supernatants of cultured cells. HUVECs were used as positive controls. The A549 NSCLC cell line showed high expression of preproendothelin by RT-PCR (Figure 2A), comparable to the HUVECs, and thus was selected for this assay. The NCI-H69 SCLC cell line was negative for preproendothelin and thus was selected. Figure 5 shows the levels of ET-1 detected in the supernatants of these cells. The A549 cells showed high levels of ET-1 production, comparable to the HUVECs as shown by the RT-PCR data. The NCI-H69 cells produced negligible amounts of ET-1, also concurring with the RT-PCR data. This would suggest that cells co-expressing the precursor and activating enzyme transcripts by RT-PCR all have the potential of producing active ET-1.

Expression of the ET-1 receptors (ETAR and ETBR) was investigated by RT-PCR. This showed that ETAR mRNA was most abundant in the bronchial epithelial cells, with little detected in the lung cancer cell lines (Figure 6A). Only five of 11 of the lung cancer cell lines were positive for ETAR, and the PCR product was less intense in each instance compared with that present in the NHBE and SV40-HBE cells. In contrast, the ETBR was expressed throughout the cell panel (Figure 6B), with the most product amplified in the GLC-19 (lane 1) and COR-L24 (lane 7), both SCLC cell lines. It is of note that two PCR products are seen close together for the COR-L23, NSCLC cell line (lane 9). This may represent a variant or aberrant form of the ETBR. The same cDNA s were used as in the previous experiments (Figure 2), and RT-PCR for GAPDH was as represented in Figure 2B.

Flow cytometric assay of calcium mobilization was used to investigate the activation of functional receptors after ET-1 binding. No response to stimulation by ET-1 at concentrations between $10^{-2}$ to $10^{-3}$ M (experiments performed in triplicate) was seen in any of the lung cancer cell lines (three SCLC and three NSCLC) or the human bronchial cells representing normal lung. Serum, acting as a nonspecific stimulus and an internal control for our assay, produced a peak in fluorescence after stimulation, indicating calcium mobilization and confirming functionality of our assay (data not shown). These experiments thus support the finding that the ETAR is downregulated in the lung cancer cell lines, as shown by RT-PCR.

Discussion

SCLC is characterized by the production of numerous peptides that can act as autocrine growth factors for these tumors. Vasopressin was the first neuropeptide found to be mitogenic (44); since then many neuropeptides have been shown to be growth factors for a wide range of cell types (45). ET-1 is one such peptide known to act as a mitogen in several tissues. The finding of ET-1 and its receptors in several tumor cell lines has led investigators to propose that it may act as a growth factor for these tumors (24, 25). In the present study, we have shown by RT-PCR that ET-1 was expressed in over 80% of the lung cancer cell lines tested, including 100% of NSCLC cell lines, as well as bronchial epithelial cells. This suggests that it plays
a role in the maintenance of both the tumor and normal epithelial cells in the lung.

However, because active ET-1 is synthesized by cleavage from a precursor peptide, big ET-1, it was important to investigate the expression of ET-1 together with its activating enzyme, ECE-1. The primary structure of ECE-1 shows that it is a member of the zinc metalloprotease family (46, 47) and is a key site of regulation of ET-1 activity. To understand the importance of this enzyme and its isoforms in lung cancer, it was important to determine the differential abundance and location of the ECE-1 isoforms in lung tumor cells because this may be of regulatory importance. To our knowledge, this is the first study investigating the distribution of ECE-1 isoforms in lung cancer.

The intracellular localization of the ECE-1b isoform makes its presence important for a proposed autocrine growth loop to function, so that big ET-1, produced by the cell, can be processed to its active form before its release. Expression of the intracellular ECE-1b isoform was downregulated compared with the extracellular ECE-1c and required blotting and hybridization of the PCR products to detect its expression accurately. This was interesting in that levels of expression of ECE-1b correlated with differential expression of preproendothelin in the lung cancer cell lines, by RT-PCR. This was most notable in the A549 NSCLC cell line, which exhibited high levels of preproendothelin by RT-PCR and ET-1 by ELISA, together with high levels of expression of ECE-1b. Interestingly, the NCI-H69 SCLC cell line was negative for both ET-1 (by RT-PCR and ELISA) and ECE-1b. This would indicate that coexpression of preproendothelin with the activating enzyme suggests that lung cancer cells produce active ET-1.

However, the ectoenzyme ECE-1c was the most commonly expressed isoform in lung cancer and normal bronchial epithelial cells, being present in all the cell lines investigated. This is in agreement with the findings of other investigators for a range of tissues types (4). Because this is the most abundant isoform, its involvement in lung cancer is likely to be the most important. Therefore, perhaps preproendothelin produced by tumor cells could be activated by ECE-1c expressed on the surface of that cell, causing autocrine growth stimulation. Alternatively, ET-1 produced by tumor cells could be activated by the abundant ECE-1c, and active ET-1 could then act on nearby cells, for example, endothelial cells, to promote angiogenesis. The effects of ET-1 on endothelial cells are well established. Its motogenic and angiogenic activities are mediated via ETBR in the presence of functional endothelial nitric oxide synthase (48, 49). These potential models are illustrated diagrammatically in Figure 7.

The ETAR, thought to be the receptor involved in promoting growth in other tumor types (25, 50), was expressed in 45% of the lung cancer cell lines investigated. This corresponds to the findings of Cohen and colleagues (35), who found 57% of lung cancer cell lines expressing ETAR. Flow cytometry was used to demonstrate intracellular calcium mobilization after receptor activation by ET-1. We have previously validated this technique to show intracellular Ca^{2+} mobilization in SCLC cell lines in response to stimulation by the peptide vasopressin, shown to act as an autocrine growth factor for SCLC (33). In a similar manner, the ETAR is thought to use Ca^{2+} as an intracellular secondary messenger in mediating its mitogenic actions in a variety of cell lines. ET-1 binding to ETAR is reported to cause a biphasic response, an initial rapid transient phase followed by a sustained plateau phase (51). In our study, this assay for ET-1 receptor activation did not demonstrate a functional transduction pathway involving Ca^{2+} mobilization, following stimulation by ET-1. However, alternative signaling pathways may exist in lung cancer cells because coupling of receptors to differing signal pathways has been noted between cell types (52). In particular, the transduction pathway mediating the actions of the ETBR subtype, which was found to be expressed throughout the lung cancer cell lines, is less well characterized than pathways mediating effects of ETAR. Receptor-signal transduction coupling may be tissue specific and influence subsequent actions following receptor stimulation in different cells (52).

Figure 7. Diagrammatic representation of ET-1 autocrine and paracrine models in lung cancer. (A) Autocrine growth loop for lung cancer similar to that proposed in ovarian carcinoma. (B) Proposed paracrine loop, with ET-1 from lung cancer cells affecting endothelial motogenesis and angiogenesis, involving ETBR and ECE-1c. Possible autocrine loop via ETBR is shown, but signaling pathways have not been clearly characterized.
ET-1 was downregulated in lung cancer relative to normal bronchial epithelial cells by RT-PCR. ETBR was present throughout the panel of cell lines and may be the more important receptor subtype in lung cancer. Aα has been found in rat mesangial cells (53), stimulation of the ETBR may induce further production of ET-1 by lung cancer cells. Because it has a short half-life, autodestruction of further ET-1 release from the same or surrounding cells via the ETBR may be responsible for the profound and sustained effects of ET-1. Mitogenic actions mediated by the ETBR via an alternative pathway in lung tumors requires further study. Thus, our findings, showing a lack of active ETA R, an abundance of ETBR, and the relative excess of extracellular ECE-1c, would suggest that instead of an autocrine growth loop, paracrine effects of ET-1 are likely to be more important in lung cancer.

In summary, we have demonstrated by RT-PCR that ET-1, ETBR, and the extracellular ECE-1c isoform mRNA expression are high in the lung cancer cell lines investigated. Coexpression of preproendothelin and its activating enzyme results in active ET-1 production in these cells. In contrast, the ETAR was downregulated, and no stimulation of ETAR by ET-1, resulting in Ca2+ mobilization, could be detected. A uterine growth mediated through the ETAR has been shown in ovarian tumors (50). ET-1 has also been shown to influence growth of prostate cancer cells via the ETAR (25). In both tumor types, ETBR was shown to be downregulated. In addition, ET-1 was proposed as an autocrine growth factor for lung cancer by Cohen and coworkers (35, 36) when they found expression of ET-1 and both its receptors in a number of lung tumors and cell lines derived from lung cancers and bronchial epithelium. Our more detailed study into the ECE-1 isoforms and receptor subtypes would indicate that the ETAR is not involved in an autocrine growth loop in lung cancer as in the other tumor types. Instead, we propose that ET-1 may be involved in complex paracrine circuits involving stimulation of epithelial and endothelial cells surrounding the tumor, influencing its growth perhaps through promoting angiogenesis, and these effects of ET-1 on the vasculature have been well established (48, 49). However, an autocrine loop involving the ETBR may be a line of further investigation in view of its abundance in lung cancer cells and normal lung, and because its signal transduction pathways are less well identified.

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