Coexpression of Transcripts Encoding EPHB Receptor Protein Tyrosine Kinases and Their Ephrin-B Ligands in Human Small Cell Lung Carcinoma

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ABSTRACT

The EPH family is the largest subfamily of receptor protein tyrosine kinases, consisting of the EPHA and EPHB subgroups. Ephrin-B1, ephrin-B2, and ephrin-B3 are ligands of the EPHB subgroup and are encoded by the EFNB1, EFNB2, and EFNB3 genes, respectively. We have shown previously that EPHB2 transcripts are expressed in six small cell lung carcinoma (SCLC) cell lines. In this study, we examined the expression of EPHB1, EPHB2, EPHB3, EPHB4, and EPHB6 in 4 SCLC tumor specimens and 14 cell lines including 3 cell lines derived from these tumor specimens. To investigate whether potential autocrine loops of EPH receptor and ephrin-B ligands exist in SCLC, the expression of EFNB1, EFNB2, and EFNB3 was also examined. Our data show that transcripts encoding multiple members of the EPHB subgroup and the ephrin-B subgroup are coexpressed in SCLC cell lines and tumors. These results suggest that the EPHB subgroup receptor kinases may modulate the biological behavior of SCLC through autocrine and/or juxtacrine activation by ephrin-B ligands that are expressed in the same or neighboring cells.

INTRODUCTION

EPH family receptor kinases and their ligands (ephrins) are involved in fundamental developmental processes in the nervous system, including axon guidance (1), axon fasciculation (2), neural crest cell migration (3), acquisition of brain subregional identities (4), and neuronal cell survival (5). Evidence also suggests that some members of the EPH family and their ligands are involved in vascularization as well as oncogenesis (6–9). The EPH family is the largest subfamily of protein tyrosine kinases. To date, 14 members of the EPH gene family have been identified in various species (10). Similarly, ephrin ligands constitute a large family, which includes eight members.

Based on the sequence relationships and structures, ephrin ligands are divided into two subgroups: (a) ephrin-A; and (b) ephrin-B (11). The ephrin-A subgroup, which includes five members, is anchored to the cell membrane by the glycosylphosphatidylinositol link, whereas three members of the ephrin-B subgroup are transmembrane proteins. EFNA and EFNB genes encode for the ephrin-A and ephrin-B ligands, respectively. Ephrin family receptors can also be divided into two subgroups based on the relatedness of their extracellular domain sequences and on their ability to bind to the two subgroups of ephrins. The EPHA subgroup, which includes eight receptors, interacts preferentially with ephrin-A ligands, whereas the EPHB subgroup, which includes six receptors, interacts preferentially with ephrin-B ligands (11).

It is known that growth/differentiation factor receptors and their cognate ligands can modulate the biological behavior of various types of tumors through autocrine and/or paracrine activation mechanisms. In SCLC, it was originally shown that bone-mesin-like peptides were involved in the growth stimulation of SCLC cells in culture and xenografts in nude mice through an autocrine activation of their receptors (12). More recently, Krystal et al. (13) have shown that there is an autocrine stimulation of small cell lung cancer growth mediated through coexpression of c-kit and its ligand, the stem cell factor (13). We previously reported that EPHB2 transcripts, which encode a member of the EPHB subgroup, are highly expressed in several SCLC cell lines (14). In this study, we examined the expression of transcripts encoding five members of the EPHB subgroup as well as their ligands, ephrin-Bs, in SCLC tumor specimens and additional cell lines. We have found that transcripts encoding multiple members of the EPHB and ephrin-B subgroups are expressed together in SCLC tumors and cell lines, suggesting that EPHB receptors and their ligands may modulate the biological behavior of SCLC through an autocrine and/or juxtacrine activation mechanisms.

MATERIALS AND METHODS

SCLC Cell Lines. NCIH and NCIN series of SCLC cell lines were obtained from Dr. John D. Minna (University of Texas, Dallas, TX) and from the American Type Culture Col-
lection (Manassas, VA). These cell lines were maintained in RPMI 1640 supplemented with 5% fetal bovine serum.

**SCLC Tumor Specimens.** All SCLC tumor specimens were effusion samples obtained from patients treated at the Kingston Regional Cancer Center as part of a study approved by the Research Ethics Board of Queen’s University. The samples were prepared as described previously (15), and they all consisted of more than 90% tumor cells.

PT-S65 was a pleural effusion obtained from a patient with extensive stage SCLC with liver metastases. At the time that the pleural fluid was obtained, the patient had been treated with cyclophosphamide, Adriamycin, and vincristine, alternating with etoposide and cisplatin. After an initial partial response to this therapy, the tumor recurred, and the patient developed a large pleural effusion. A cell line was established from the pleural fluid and named WL-E.

PT-S169 was obtained from a pleural effusion of a patient who had presented with extensive stage SCLC with liver metastases. At the time that the pleural fluid was obtained, the patient had been treated with oral etoposide and had a minimal response, but the tumor then started to progress. The patient died shortly after the pleural fluid was obtained. A cell line was established from the pleural fluid and named LV-E.

PT-S228 was a pericardial effusion sample from a patient with extensive stage SCLC with liver metastases. The patient had initially been treated with cyclophosphamide, Adriamycin, and vincristine, alternating with etoposide and cisplatin. The patient had a good partial response to this treatment. After completion of treatment, the tumor recurred, and the patient developed a pericardial effusion. The pericardial effusion was drained, and the PT-S228 tumor sample was isolated from this effusion. A cell line was established from the pleural fluid and named GE-E.

PT-S331 was a pleural fluid sample from a patient with extensive stage SCLC who had not received chemotherapy.

**RNA Extraction and Northern Blot Analysis.** Total cellular RNA preparations and Northern blotting procedures were described in detail elsewhere (16). Briefly, total cellular RNA was prepared by the method described by Auffray and Rougeon (17) or by the method of Chomczynski and Sacchi (18). The expression of **EPHB1**, **EPHB2**, **EFNB1**, **EFNB2**, and **EFNB3** was examined by Northern hybridization using DNA fragments of the corresponding cDNAs as probes (14, 19). The blots were washed in 0.1 × SSC containing 1% SDS at 63°C and exposed to X-ray film overnight at −70°C.

**RT.** Total RNA (1–2 µg) extracted from SCLC tumors or cell lines was mixed with 35 µg of random hexamers and 250 ng of oligo(dT)15 primer, 200 µM deoxynucleotide triphosphates, one-fifteenth of the RT reaction, 50 mM KCl, 2 mM MgCl2, 10 mM Tris-HCl (pH 8.3), and 1 unit of AmpliTaq Gold (PE Applied Biosystems). PCR conditions were as follows: (a) 95°C for 12 min; (b) 18 cycles of 95°C for 30 s, 55°C for 30 s, and 72°C for 5 min; and (c) 72°C for 10 min. Under these conditions, PCR was at the exponential phase of amplification. Nucleotide sequences for the PCR primers are as follows: (a) **EPHB1**, 5′-GAGATGGAAAGCTGAAGGAGCTAGG-3′ (sense primer) and 5′-CCAGCATGAGCTTGTTAGA-3′ (antisense primer); (b) **EPHB2**, 5′-AAAAAGGACGGTGAGC-3′ (sense primer) and 5′-TCACAGGTTGCTCTTGGTC-3′ (antisense primer); (c) **EPHB3**, 5′-AGCAACGCTTGTTGACAAATG-3′ (sense primer) and 5′-TCCATAAGCTGCTAGCCTCCC-3′ (antisense primer); (d) **EPHB4**, 5′-GCTTGTTTGGGTTCCTCCC-3′ (sense primer) and 5′-TGACATCCACCTCCCCATCA-3′ (antisense primer); (e) **EPHB6**, 5′-AGGAATGTTCCCTCCCTTGGTCC-3′ (sense primer) and 5′-CGGAACCTCTGCTCTATTGC-3′ (antisense primer); (f) **EFNB1**, 5′-GAGGACAGAACACTGTCA-3′ (sense primer) and 5′-GAACAATGCCACCTTGGGAGT-3′ (antisense primer); (g) **EFNB2**, 5′-GCAATGGTCTGATGATCTGAC-3′ (sense primer) and 5′-AGGAATGTTCCCTCCCTTGGTCC-3′ (antisense primer); (h) **EFNB3**, 5′-CTGAAATGCGCATGAAAAG-3′ (sense primer) and 5′-ACGCCAGGCAAGAGCCGCAAC-3′ (antisense primer); (i) **IGFIR**, 5′-AGCAAAATGCTTCCGAC-3′ (sense primer) and 5′-TCCATAAGCTGCTAGCCTCCC-3′ (antisense primer); (j) **RET**, 5′-ACCTACTCATATTGTGCTGGG-3′ (sense primer) and 5′-CTGGCTCCTTCCACTAGT-3′ (antisense primer); and (k) **GAPD**, 5′-GAGGTTGAAGGGTGCAGTCA-3′ (sense primer) and 5′-TTGAGGCTCAATGAGGGCTG-3′ (antisense primer).

**Chemiluminescent Detection of Biotinylated PCR Products.** PCR products (10 µl of a total of 20 µl of PCR products) were subjected to 6% PAGE. DNA bands were electrotransferred onto nylon membrane (Hybond N, Amersham) and immobilized to the membrane by baking the filter for 30 min at 80°C, followed by 1 min of UV irradiation. The biotinylated PCR products were then detected using the Southern Light chemiluminescent detection procedure (Tropix, Inc.). Quantification of mRNA expression was performed by densitometric analysis on X-ray films. Relative levels of the expression of a given transcript were then determined by taking the ratio between the densitometric unit of the sample and that of the internal control, **GAPD**.

**RESULTS**

**Expression of Transcripts Encoding EPHB Receptor Tyrosine Kinases in SCLC Cell Lines.** We previously reported that **EPHB2** transcripts were expressed at high levels in six SCLC cell lines (14). This observation prompted us to examine the expression of transcripts encoding five members of the EPHB receptor kinase subgroup (**EPHB1**, **EPHB2**, **EPHB3**, **EPHB4**, and **EPHB6**).
EPHB6) in additional SCLC cell lines and tumor specimens. The expression of EPHB5 transcripts was not examined in this study because the human EPHB5 gene has not been identified.

We first examined the expression of EPHB2 transcripts in 11 SCLC cell lines by (A) Northern blot analysis and by (B) semiquantitative RT-PCR analysis. EPHB2 is expressed into three transcripts of 4, 5, and 11 kb in size (arrows) that are generated by alternative splicing and by alternative use of polyadenylation signals (14, 26). Ethidium bromide staining of 18S rRNA shows the loading and integrity of RNA used in Northern analysis. The same RNA samples were used for the RT-PCR analysis, in which GAPD transcripts were used as an internal control.

Fig. 1 Expression of EPHB2 in SCLC cell lines. The expression of EPHB2 transcripts was examined in 11 SCLC cell lines by (A) Northern blot analysis and by (B) semiquantitative RT-PCR analysis. EPHB2 is expressed into three transcripts of 4, 5, and 11 kb in size (arrows) that are generated by alternative splicing and by alternative use of polyadenylation signals (14, 26). Ethidium bromide staining of 18S rRNA shows the loading and integrity of RNA used in Northern analysis. The same RNA samples were used for the RT-PCR analysis, in which GAPD transcripts were used as an internal control.

RT-PCR. The method was also advantageous because it allowed us to examine the expression of at least 10 transcripts in the SCLC tumor samples in which the amount of tumor RNA available was very limited (see below). However, when scarcity of RNA was not a problem, Northern blot analysis was also performed to examine the expression of EPHB and EFNB transcripts in SCLC. For example, the expression of EPHB1, EFNB1, EFNB2, and EFNB3 transcripts was investigated in SCLC cell lines by both methods.

We next examined the expression of EPHB1, EPHB3, EPHB4, and EPHB6 transcripts in the same set of 11 SCLC cell lines. As shown in Fig. 2, the expression of EPHB1 transcripts was found in 8 of 11 SCLC cell lines at low levels. EPHB3 transcripts were expressed in nine cell lines at moderate to low levels, with the exception of NCIH526, which expressed EPHB3 at an even higher level than that in the fetal brain. EPHB4 transcripts were expressed in three cell lines at levels similar to that in the fetal brain, and seven cell lines expressed EPHB4 transcripts at very low levels. EPHB6 transcripts were expressed in NCIH345 at a high level, and three cell lines expressed EPHB6 transcripts at very low levels. Thus, these data showed that 10 of 11 SCLC cell lines examined expressed one or more EPHB subgroup transcripts at levels similar to that detected in the fetal brain. An
exception to this was the NCI-H899 cell line, which happened to express high levels of RET (see Fig. 5).

Expression of Transcripts Encoding EPHB Receptor Tyrosine Kinases in SCLC Tumors. Any biological finding made in cell lines that have been in culture for some time might not truly reflect the status of the original tumors. We therefore examined whether SCLC tumor specimens expressed EPHB transcripts as well. Although fresh SCLC tumor specimens are difficult to obtain, we have included four SCLC tumor specimens and three cell lines derived from these tumors in our analysis.

Fig. 3 shows the expression pattern of EPHB transcripts in four SCLC tumor specimens and three corresponding cell lines. EPHB1 expression in these samples was either very low or absent, and none of the tumor specimens expressed EPHB1 transcripts. EPHB2 expression was detected in all of the SCLC cell lines and tumors examined at levels ranging from high (a similar level found in the fetal brain) to moderate. Three of four tumors expressed EPHB3 at moderate to low levels. Tumor PT-S169 did not express EPHB3, but its derivative cell line LV-E expressed detectable levels of EPHB3. All four tumors expressed EPHB4 at moderate levels, and it appeared that tumor specimens expressed higher levels of EPHB4 transcripts than the corresponding cell lines. It was noticed that although tumor PT-S65 expressed EPHB4, its derivative cell line WL-E showed no EPHB4 expression. Finally, EPHB6 expression was found in three of the four tumor specimens, but none of the corresponding cell lines expressed EPHB6 transcripts. Taken together, these data show that multiple EPHB subgroup transcripts are expressed in SCLC tumors.

Expression of EFNB Transcripts Encoding Ephrin-B Ligands in SCLC Cell Lines and Tumor Specimens. Coexpression of receptor protein tyrosine kinases and their cognate ligands, which potentially leads to auto-activation of the receptor kinases, is a common phenomenon in malignant cells, especially SCLC (13, 20). Thus, we next examined whether transcripts encoding ligands of the EPHB subgroup, namely EFNB1, EFNB2, and EFNB3, were also expressed in the SCLC cell lines and tumor specimens.

The expression of EFNB transcripts was first examined in the same set of 11 SCLC cell lines shown in Figs. 1 and 2. As shown in Fig. 4A, EFNB1 expression was detected in all 11 cell lines examined: (a) 6 cell lines expressed EFNB1 transcripts at moderate levels; and (b) others expressed low levels of EFNB1. Similarly, EFNB2 transcripts were detected in all 11 cell lines, ranging from high to low levels: (a) 2 cell lines expressed EFNB2 transcripts at high levels; (b) 3 cell lines expressed EFNB2 transcripts at moderate levels; and (c) 6 cell lines expressed EFNB2 at low levels. Finally, EFNB3 transcripts were detected in 10 of the 11 cell lines examined: (a) 5 cell lines expressed EFNB3 transcripts at moderate levels; and (b) the others expressed low levels of EFNB3. These data were consistent with those obtained by Northern blot analysis (data not shown).

To further confirm this observation, the expression of EFNB transcripts was examined in the four SCLC tumors and cell lines corresponding to three of these samples. As shown in Fig. 4B, EFNB1 expression was found in all of the tumors and cell lines examined at moderate to high levels. EFNB2 expression was detected in all of the samples examined, but its relative expression levels were generally lower than those of EFNB1 transcripts. EFNB2 expression also seems to be higher in the SCLC cell lines than in the corresponding tumors.
RT-PCR analysis was carried out as described in the Fig. 1.

In contrast, \textit{EFNB3} expression was found to be either low or absent in these SCLC samples, and differential expression of \textit{EFNB3} in tumor/cell line pairs was also noticed. Tumor PT-S169 did not express \textit{EFNB3}, but its derivative cell line (LV-E) expressed \textit{EFNB3} at low levels. In contrast, \textit{EFNB3} expression was detected in tumor PT-S228, but its corresponding cell line, GL-E, showed no \textit{EFNB3} expression. Nonetheless, our data showed that one or more \textit{EFNB} transcripts were expressed in SCLC tumor specimens and their derivative cell lines. Taken together, our results indicate that transcripts encoding multiple members of EPHB and ephrin-B subgroups are coexpressed in SCLC.

Expression of \textit{IGF1R} and \textit{RET} Transcripts in SCLC Cell Lines and Tumors. In addition to EPHB and \textit{EFNB} transcripts, we have examined the expression of \textit{IGF1R} and \textit{RET} transcripts in SCLC cell lines and tumor specimens as controls. \textit{IGF1R} encodes a protein tyrosine kinase receptor for IGF-1 and IGF-2 and is ubiquitously expressed in various tumor cell lines. As shown in Fig. 5, this was also the case for SCLC. \textit{IGF1R} expression was found at relatively high levels across the tumors and cell lines examined, with the exception of one cell line (NCH510) that expressed low levels of \textit{IGF1R} transcripts.

\textit{RET} was included in this study as a control for a receptor protein tyrosine kinase with preferential expression in the nervous system. As shown in Fig. 5, \textit{RET} expression was found in 5 of 14 SCLC cell lines. This observation is similar to the recent finding (21) in which \textit{RET} expression was detected in 12 of 21 SCLC cell lines. In contrast, no detectable \textit{RET} expression was found in SCLC tumor specimens. Because the number of SCLC tumor specimens examined is limited, it remains to be seen whether \textit{RET} expression in SCLC is a cell culture-related phenomenon or whether it has certain contributions to the pathogenesis of SCLC in vivo.

DISCUSSION

Autocrine growth stimulation of SCLC has been recognized for some time. It was first reported that bombesin-like peptides such as gastrin-releasing peptide were involved in growth stimulation of SCLC cells in culture and xenografts in nude mice through an autocrine activation of their receptors (12). Similarly, an autocrine loop mediated by insulin-like growth factor 1 and its receptor has been found in SCLC (20).

The frequent coexpression of the stem cell factor and its receptor, the gene product of the c-kit proto-oncogene, was then reported in SCLC cell lines and tumors (22). Subsequently, an autocrine stimulation of SCLC growth mediated by stem cell factor and c-kit was demonstrated experimentally (13). Furthermore, selective inhibition of protein tyrosine kinase activity in SCLC cells by genistein and a tyrphostin derivative was shown to stimulate apoptosis of the cells (23). Taken together, these studies suggest that auto-activation of tyrosine kinases plays an important role in the growth and survival of SCLC cells.

In this study, we report that transcripts encoding EPHB receptor kinases and their ephrin-B ligands are expressed together in 13 of the 14 SCLC cell lines and all 4 of the tumors examined. These data suggest that there are multiple autocrine loops mediated by EPHB receptors and ephrin-B ligands in SCLC. These data also point to a possibility that additional autocrine loops mediated by EPHA receptor kinases and ephrin-A ligands may exist in SCLC. In fact, our preliminary data showed that \textit{EPHA2} transcripts were expressed in several SCLC cell lines, including NCH1688, which expressed only low levels of \textit{EPHB3}, \textit{EFNB1}, and \textit{EFNB2}. Additional analysis of the expression of eight EPHA receptor kinases and five ephrin-A ligands will be required to further address this question.

Accumulating evidence suggests that overexpression or coexpression of EPH family receptor tyrosine kinases and their ligands could promote tumor progression. For example, \textit{EPHA2} was found to be expressed at high levels in metastatic melanoma cells as compared to normal melanocytes (24). Similarly, \textit{EFNB2} transcripts encoding the ephrin-B2 ligand were highly expressed in primary and metastatic melanomas compared to benign melanocytic nevi (25). Furthermore, the expression of ephrin-A1 and the up-regulation of its receptor, \textit{EPHA2}, were found during the course of melanoma progression (24). In our study, all four SCLC tumor specimens, which coexpressed
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