A Comparison of Synaptophysin, Chromogranin, and L-Dopa Decarboxylase as Markers for Neuroendocrine Differentiation in Lung Cancer Cell Lines

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ABSTRACT

Synaptophysin is a Mr 38,000 integral membrane glycoprotein expressed by a variety of normal and neoplastic neuroendocrine cells. We studied synaptophysin as an immunocytochemical marker for neuroendocrine differentiation in lung cancer and compared it to the immunocytochemical expression of chromogranin A, a marker for dense core (endocrine) granules, and the biochemical activity of l-dopa decarboxylase (DDC), the key amine-handling enzyme. Of the 250 cell lines available to us, we selected examples representative of the following cell types: bronchial carcinoids (n = 4), small cell lung cancer (SCLC) (n = 7), extrapolunmonary small cell carcinomas (n = 4), and non-small cell lung cancers (n = 18) whose neuroendocrine status had been previously determined on the basis of electron microscopy and DDC activity. We demonstrated (a) there was a higher incidence of synaptophysin than chromogranin A immunoreactivity in carcinoid (100 versus 75%), classic SCLC (70 versus 50%), and variant SCLC (57 versus 29%) cell lines; (b) 3 of the 4 (75%) extrapolunmonary small cell lung cancer cell lines expressed synaptophysin and chromogranin A; (c) 5 of the 7 (71%) non-small cell lung cancer cell lines previously shown to express multiple neuroendocrine markers were positive for synaptophysin, chromogranin A, and DDC activity; (d) none of the other 11 non-small cell lung cancer cell lines expressed synaptophysin or chromogranin A; and (e) formalin fixation and paraffin embedding reduced synaptophysin immunoreactivity in 11 of 14 (79%) of the cell lines, as compared to freshly prepared specimens fixed in 95% ethanol. Western blot analysis using the synaptophysin antibody (SY38) demonstrated immunoreactive proteins ranging from Mr 43,000 to 45,000 in five representative cell lines. The concordance of expression of all three neuroendocrine markers was statistically significant when values for all cell lines were totalled. Synaptophysin was a more commonly expressed marker for variant SCLC cell lines, which rarely showed DDC activity. We conclude that synaptophysin may be a more sensitive and specific marker for neuroendocrine differentiation, when compared to chromogranin A and DDC in lung cancer cell lines which express only part of the neuroendocrine program.

INTRODUCTION

NE tumors of the lung include bronchial carcinoid, SCLC-C, and SCLC-V cell types. It is important to distinguish SCLC from other lung cancers because of the better therapeutic response to cytotoxic therapy in patients (3). Among SCLC, the variant subtype SCLC-V is associated with worse prognosis than SCLC-C. Although considered NE tumors, SCLC-V cell types typically lack dense core granules and may, therefore, be difficult to diagnose (4–6).

ExPuSC is morphologically similar to SCLC and has been described in a large variety of sites, including esophagus, stomach, pancreas, larynx, hypopharynx, salivary glands, nasopharynx, and skin. A significant number of, but not all, ExPuSC tumors have been shown to contain neurosecretory granules and produce ectopic hormones (which can be demonstrated using immunohistochemical techniques) (7–9). We included four ExPuSC cell lines in our study because of their similarities to NE tumors of the lung, named SCLC.

NSCLC do not express NE features and are usually diagnosed on the basis of histology alone. However, we and others have reported that some NSCLCs, diagnosed by conventional light microscopy, express a variety of NE markers when immunocytochemical techniques are employed. This subset of NSCLCs that express multiple NE markers has been named NSCLC-NE (10, 11).

Specific NE markers in lung cancers consist of a large number of defined hormones and neuropeptides, both eutopic (i.e., gastrin-releasing peptide, calcitonin, and serotonin) and ectopic (adrenocorticotropic hormone, vasopressin, and neurotensin). However, these NE products are seldom very useful for diagnosis, since their expression in tumors is quite variable and may be subtype specific (10).

Among the currently available general NE markers are NSE, Leu-7, CgA, and DDC (2, 10). Immunocytochemical and enzymatic techniques have demonstrated these general NE markers to be useful investigative and diagnostic tools, but not without some limitations. NSE, the γ-subunit of the glycolytic enzyme enolase, was originally thought to be expressed exclusively in neurons and NE cells (12). It is now known that NSE is expressed in a variety of non-NE cells and tumors, which has led many investigators to question its specificity (13–15). Leu-7, a differentiation antigen shown to be selectively expressed on human natural killer cells (16), is also expressed on SCLC cells and other benign and malignant NE and nerve cells, although the precise antigenic determinant of the antibody has yet to be determined (17, 18). CgA was originally isolated from the chromaffin granules of the adrenal medulla (19) and has been found to be expressed by a variety of normal and neoplastic human polypeptide hormone-producing tissues (20–22). Although its function is not known, CgA is associated with the co-secretion of a wide variety of hormones, suggesting some role in the secretory process (22). Because SCLCs have a paucity...
of secretory granules, the use of CgA as a general NE marker may be limited only to those neoplasms containing more abundant dense core granules. These facts demonstrate the need for additional NE markers with greater sensitivity and specificity.

Syn was originally isolated from bovine neurons and is a M, 38,000 membrane-integrated glycosylated polypeptide to which a monoclonal antibody (SY 38) has been raised. Syn has been demonstrated in a variety of normal and neoplastic NE cells of neural and epithelial type and has been localized within normal and neoplastic cells of the bronchopulmonary tract. While some results suggest that Syn immunoreactivity is maintained in formalin-fixed paraffin-embedded specimens, others have reported variable immunoreactivity. Our goal in the present study was to define the sensitivity and specificity of Syn as a marker for NE differentiation in lung cancer cell lines and to compare it to other general NE markers.

MATERIALS AND METHODS

Cell Lines. Four bronchial carcinoid, 4 ExPuSC, 10 SCLC-C, 7 SCLC-V, 11 NSCLC, and 7 NSCLC-NE cell lines were established, maintained, and characterized in our laboratory, as described previously. Cell lines demonstrating substrate adherence were trypsinized by adding 10 mL of warm PBS to the flask and 5 mL of 0.05% trypsin to stop cell adherence. Once trypsinization was complete (5–10 min), 10 mL of fresh serum-containing medium were added to neutralize the trypsin. The cell suspension was centrifuged at 1200 rpm for 5 min and resuspended in fresh medium. Trypsinized cells were allowed to recover for 2–3 h prior to immunocytochemical staining. All cell lines were washed twice in 10 mL of ice-cold PBS and cytospinnzied on poly-l-lysine (Sigma Chemical Company, St. Louis, MO)-coated slides.

Immunocytochemical Staining. Immunocytochemical staining was performed by the avidin-biotin-peroxidase technique, using Vectastain ABC staining kits (Vector Laboratories, Burlingame, CA), following the vendor's instructions with a few modifications. Mouse monoclonal anti-Syn (SY 38, lot 10644532-01) was purchased from Boehringer Mannheim (Indianapolis, IN) and used at a 1:10 dilution. Mouse monoclonal anti-CgA (LK2H10) was a gift from Dr. Barry S. Wilson (University of Michigan Medical Center, Ann Arbor, MI) and was used at a dilution of 1:100.

Immunoreactivity for Syn and CgA was tested following the fixation of freshly made cytospin preparations in methanol, acetone, and 95% ethanol. Fixation for 10 min in ice-cold PBS and cytospin preparation onto poly-l-lysine (Sigma Chemical Company, St. Louis, MO)-coated slides. Immunocytochemical staining was performed by the avidin-biotin-peroxidase technique, using Vector Laboratories ABC staining kits (Vector Laboratories, Burlingame, CA), following the vendor's instructions with a few modifications. Mouse monoclonal anti-Syn (SY 38, lot 10644532-01) was purchased from Boehringer Mannheim (Indianapolis, IN) and used at a 1:10 dilution. Mouse monoclonal anti-CgA (LK2H10) was a gift from Dr. Barry S. Wilson (University of Michigan Medical Center, Ann Arbor, MI) and was used at a dilution of 1:100.

Immunoreactivity for Syn and CgA was tested following the fixation of freshly made cytospin preparations in methanol, acetone, and 95% ethanol. Fixation for 10 min in ice-cold 95% ethanol appeared to give better results. The 5 Syn-positive NSCLC cell lines all belonged to the NSCLC-NE subtype.

RESULTS

The results for Syn staining distribution are summarized in Table 1. All carcinoids were positive for Syn and 3 of the 4 cell lines had over 50% cells positive. Syn staining in small cell carcinoma cell lines was more heterogeneous, with 14 of the 21 cell lines positive. The 5 Syn-positive NSCLC cell lines all belonged to the NSCLC-NE subtype.

Western blot analysis was performed to demonstrate antibody specificity on representative cell lines including 1 carcinoid, 1 SCLC-C, 2 SCLC-V, and 1 ExPuSC (Fig. 1). Physical characterization of these cell lines by SDS-PAGE revealed a distinct cellular component which expressed Syn immunoreactivity. This component migrated as a single band in Fig. 1, lanes 1 and 2, and as a series of bands in Fig. 1, lanes 3–5, with a determined molecular weight ranging from 43,000 to 45,000 under the reduced conditions. The reactivity was specific for antibody SY38, since no immunoreactive bands were observed in any of the cell lines when indifferent mouse monoclonal antibody (MOPC 21) was substituted for SY38 (Fig. 1).

Syn, CgA, and DDC expression in all cell lines is summarized in Table 1. All carcinoids were positive for Syn and 3 of the 4 cell lines had over 50% cells positive. Syn staining in small cell carcinoma cell lines was more heterogeneous, with 14 of the 21 cell lines positive. The 5 Syn-positive NSCLC cell lines all belonged to the NSCLC-NE subtype.

Table 1 Expression of synaptophysin staining distribution in all cell types

<table>
<thead>
<tr>
<th>Cell line (no.)</th>
<th>Syn staining no. of cell lines (percentage)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Carcinoid (4)</td>
<td>0 (0) 1 (25) 2 (33) 3 (75)</td>
</tr>
<tr>
<td>Small cell carcinoma (21)</td>
<td>7 (33) 1 (5) 7 (33) 6 (29)</td>
</tr>
<tr>
<td>SCLC-C (10)</td>
<td>3 (30) 1 (10) 3 (30) 3 (30)</td>
</tr>
<tr>
<td>SCLC-V (7)</td>
<td>3 (43) 0 (0) 3 (43) 1 (14)</td>
</tr>
<tr>
<td>ExPuSC (4)</td>
<td>1 (25) 0 (0) 1 (25) 2 (50)</td>
</tr>
<tr>
<td>Non-small cell lung cancer (18)</td>
<td>13 (72) 1 (5) 3 (17) 1 (5)</td>
</tr>
<tr>
<td>NSCLC-NE (7)</td>
<td>2 (29) 1 (14) 3 (43) 1 (14)</td>
</tr>
<tr>
<td>NSCLC, other (11)</td>
<td>11 (100) 0 (0) 0 (0) 0 (0)</td>
</tr>
<tr>
<td>Total (43)</td>
<td>20 (47) 2 (5) 11 (26) 10 (23)</td>
</tr>
</tbody>
</table>

h. DDC values of ≥4 units/mg of soluble protein were scored positive, since minimal DDC activity can occur in most tissues, including normal lung.

Western Blot Analysis. Tumor cell lines were evaluated by Western blot analysis for expression of Syn, using a modified method of the Towbin and Gordon technique. Whole-cell lysates were adjusted to equal protein concentrations and applied to the gel at 10–20 μg of protein/well. SDS-PAGE was performed under reduced conditions and fractionated products were electroblotted onto nitrocellulose membranes. The membranes were then treated with anti-Syn antisemur (SY38, 0.1 mg/ml) at a dilution of 1:100, followed by exposure to rabbit anti-mouse IgG (lot 17529; Cappel Laboratories, Inc., Cochranville, PA) at a dilution of 1:1000. Selective antibody deposition was identified with labeled 125I-Protein A. To confirm the specificity and competency of our immunostaining technique with the anti-Syn reagent, a parallel experiment was performed on identical cell lines, using an indifferent isotypically matched monoclonal antibody, MOPC 21, at a concentration of 0.1 mg/ml.

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SYNAPTOPHYSIN IN LUNG CANCER CELL LINES

Fig. 1. Western blot analysis for Syn of ExPuSC (NCI-H510), SCLC-C (NCI-H146), SCLC-V (NCI-H446 and NCI-N417), and carcinoid cell line (NCI-H727). Whole cell lysates were subjected to SDS-PAGE fractionation under reducing conditions and electroblotted to nitrocellulose membranes. The membranes were treated with antisynaptophysin antiserum (SY38, 0.1 mg/ml) at a dilution of 1:100. All cell lines contained peptides detected between a range of M, 43,000 to 45,000. When membranes were treated with undiluted indifferent monoclonal antibody MOPC 21 (0.1 mg/ml), no bands were seen, verifying the specificity of the immune reaction.

Table 2 Expression of synaptophysin, chromogranin, and 1-DOPA decarboxylase

<table>
<thead>
<tr>
<th>Cell line (no.)</th>
<th>Syna</th>
<th>Cga</th>
<th>DDa</th>
</tr>
</thead>
<tbody>
<tr>
<td>Carcinoid (4)</td>
<td>4 (100)</td>
<td>3 (75)</td>
<td>3 (75)</td>
</tr>
<tr>
<td>Small cell carcinoma (21)</td>
<td>14 (67)</td>
<td>10 (48)</td>
<td>10 (48)</td>
</tr>
<tr>
<td>SCLC-C (10)</td>
<td>7 (70)</td>
<td>5 (50)</td>
<td>8 (80)</td>
</tr>
<tr>
<td>SCLC-V (7)</td>
<td>4 (57)</td>
<td>2 (29)</td>
<td>0 (0)</td>
</tr>
<tr>
<td>ExPuSC (4)</td>
<td>3 (75)</td>
<td>3 (75)</td>
<td>2 (50)</td>
</tr>
<tr>
<td>Non-small cell lung cancer (18)</td>
<td>5 (28)</td>
<td>5 (28)</td>
<td>6 (33)</td>
</tr>
<tr>
<td>NSCLC-NE (7)</td>
<td>5 (71)</td>
<td>5 (71)</td>
<td>6 (86)</td>
</tr>
<tr>
<td>NSCLC, other (11)</td>
<td>0 (0)</td>
<td>0 (0)</td>
<td>1 (9)</td>
</tr>
<tr>
<td>Total (43)</td>
<td>23 (54)</td>
<td>18 (42)</td>
<td>19 (44)</td>
</tr>
</tbody>
</table>

All lung carcinoma cell lines

| Neuroendocrine' (28) | 20 (71) | 15 (54) | 17 (61) |
| Nonneuroendocrine' (11) | 0 (0) | 0 (0) | 1 (9) |
| Total (39) | 20 (51) | 15 (38) | 18 (46) |

* Staining results of all cell lines were scored on cell distribution (0-3) and staining intensity (0-6). The sum of these values (0-6) was used to tabulate staining results for each antibody. Values of ≥3 were scored positive. Numbers in parentheses, percentage of cell lines scored positive.

§ One unit of enzyme activity is defined as 1 nmol 14CO2/h. DDC values of ≥4 units/mg soluble protein were scored positive. Numbers in parentheses, percentage of cell lines scored positive.

DDC activity, 437 ± 46 (mean ± SE) and 436 ± 112 units/mg, respectively, while none of the SCLC-V showed any DDC activity.

The staining for both Syn and CgA was cytoplasmic, with no staining in nuclei (Figs. 2-4). The Syn immunoreactivity was more diffuse and less intense than that of CgA and this difference was most evident in carcinoids (Fig. 2). Similar but considerably weaker staining was observed in paraffin-embedded cell line pellets, which demonstrated a loss of Syn and CgA immunoreactivity in 11 of 14 (79%) and 8 of 13 (62%) cell lines, respectively.

The concordance for Syn, CgA, and DDC expression in all
SYNAPTOPHYSIN IN LUNG CANCER CELL LINES

Fig. 3. Photomicrographs of immunocytochemical staining in SCLC cell lines. A, positive, mostly cytoplasmic, immunostaining with SY 38 in the SCLC-C cell line NCI-H345. × 900. B, positive cytoplasmic immunostaining in the variant SCLC cell line NCI-N417, using SY 38. This cell line lacked both CgA immunoreactivity and DDC activity but was positive for Syn also in the Western blot analysis. Note the presence of nucleoli (arrows), characteristic of SCLC-V. × 1100.

Fig. 4. Photomicrographs of immunocytochemical staining in a NSCLC-NE cell line. The relatively abundant cytoplasm in scattered cells of the NSCLC-NE cell line NCI-H1570 is positive for synaptophysin (A) and chromogranin A (B). × 900.

cell lines was statistically significant using Fisher's exact test \( P < 0.05 \) (Table 3). Good concordance was observed for all subtypes except SCLC-V. Of the 57% Syn-positive SCLC-V cell lines, 57% demonstrated concordant expression of Syn and CgA, while only 43% demonstrated concordance between Syn and DDC. Syn appears to be a more sensitive marker for SCLC-V, which characteristically shows only partial expression of the NE phenotype and is associated with an aggressive clinical course.
Carcinoma cell lines, 1 bronchioloalveolar and 1 adenosquamous. Light microscopy techniques may prove to be clinically important for NSCLC differentiation in NSCLC tumors diagnosed by conventional methods. None of the SCLC-V cell lines were positive for Syn (57%), while only 2 of 7 (29%) stained positively for CgA (3). Syn was expressed in 70% of the SCLC-C cell lines, which is consistent with our earlier findings, which report excellent concordance of two of the three NE markers (Syn, CgA, DDC). Of interest, 2 of these were large cell NSCLC-NE cell lines in the present study and found that 4 of 7 (57%) were scored positive for Syn and CgA. Presently, the biological or clinical implications of this are not fully understood.

**DISCUSSION**

Our findings indicate that Syn is a specific and sensitive immunocytochemical marker for NE differentiation in lung cancer cell lines and ExPuSC. We demonstrated that Syn immunoreactivity is expressed more often in bronchial carcinoid, SCLC-C, and SCLC-V cell lines, in comparison to immunoreactivity with CgA. These results suggest that Syn may be a more sensitive biochemical marker for NE differentiation than CgA in these subtypes. Antibodies to both Syn and CgA showed positivity with the same frequency in ExPuSC and NSCLC-NE cell lines, while none of the other NSCLC cell lines expressed either Syn or CgA.

When all cell types were considered, the concordant expression of two of the three NE markers (Syn, CgA, DDC) was highest for CgA and DDC (81%). These results are in agreement with our earlier findings, which report excellent concordance between CgA and DDC in both SCLC and NSCLC lung tumors (3). Syn was expressed in 70% of the SCLC-C cell lines, which correlates well with a previous study on tumor specimens (24).

Of the SCLC-V cell lines which are characterized by altered morphology and partial lack of the NE program (4, 5), 4 of 7 (57%) expressed Syn, while only 2 of 7 (29%) stained positively for CgA. The presence of Syn in SCLC-V was also confirmed by Western blot analysis. None of the SCLC-V cell lines were found to express DDC. This is in agreement with previous findings which report a loss of dense core granules and lack of DDC activity in this SCLC subset (4, 5). Because patients with SCLC-V may respond less well to chemotherapy (6), the detection of these tumors is of clinical and biological interest.

We have recently shown that as many as 20% of NSCLC tumors, most commonly adenocarcinomas and large cell carcinomas, contain multiple NE markers (NSCLC-NE) (10). Preliminary results suggest that patients with NSCLC-NE tumors may respond more favorably to chemotherapy than patients with other NSCLC tumors (11). We included a panel of 7 NSCLC-NE cell lines in the present study and found that 4 of 7 (57%) NSCLC-NE cell lines expressed all three NE markers, Syn, CgA, and DDC. Of interest, 2 of these were large cell carcinoma cell lines, 1 bronchioloalveolar and 1 adenosquamous carcinoma. Thus, the addition of Syn as a marker for NE differentiation in NSCLC tumors diagnosed by conventional light microscopy techniques may prove to be clinically important, since it has been suggested that NSCLC-NEs are initially more sensitive to chemotherapy than other NSCLCs (11, 36).

A marked loss of Syn immunoreactivity was demonstrated in 11 of 14 (79%) of the formalin-fixed paraffin-embedded cell pellets (previously showing immunoreactivity in freshly prepared ethanol-fixed specimens). These results support previously reported findings that various fixation techniques can alter the immunocytochemical demonstrability of Syn (29). Moreover, it appears that cultured cells are even more likely to lose their immunoreactivity due to formalin fixation and paraffin embedding than routinely processed tissue samples collected in the practice of surgical pathology (28).

Our study on cell lines is in accordance with previously published results showing that immunoreactive Syn is present in vivo in most but not all NE tumors of the lung (25-28, 37, 38). While many studies have included only a few cases, Gould et al. (25) in their study on 270 tumors reported that up to 17 of 19 (88%) pulmonary carcinoids and 6 of 14 (43%) NE carcinomas of the lung were positive for Syn. Higher incidences were reported for lung NE carcinomas (88%) by Lee et al. (27) and for SCLC (79%) by Kayser et al. (28), who also noted that 8 of 74 (10%) NSCLC were positive for Syn. In our in vitro study, 70% of SCLC-C and 67% of all small cell carcinoma cell lines tested positive for Syn. In summary, these studies suggest that, while most lung NE tumors contain Syn, some tumors do not express this protein. Presently, the biological or clinical implications of this are not fully understood.

The gene for human Syn has been previously cloned and was demonstrated to generate a single mRNA transcript which encodes a 30,700-amino acid precursor protein (39). Although the deduced molecular weight of this prepro-Syn molecule is 33,312, multiple forms have been reported in a variety of human tissues, ranging in molecular weight from 38,000 to 40,000 (39). This reported heterogeneity in the calculated molecular weight of the Syn precursor, particularly evident in tumor samples, has been attributed to the degree of glycosylation obtained during posttranslational modification. Studies by Navone et al. (40) and Rehm et al. (41) have shown that chemical deglycosylation or inhibition of de novo glycosylation renders the Syn precursor of 34,000. Our biochemical characterization of Syn-immunoreactive proteins in lung tumor cell lines revealed a slightly higher molecular weight form, ranging from 43,000 to 45,000. This is in accordance with the slightly lower electrophoretic mobility that has been reported for selected tumors, including a bronchial carcinoid, paragangliomas, and retinoblastomas (26, 37, 42). Interestingly, the SCLC-C (NCI-H146) and ExPuSC (NCI-H510) cell lines expressed a higher molecular weight form of M, 45,000, when compared to the SCLC-V (NCI-H446 and NCI-N417) and carcinoid (NCI-H1727) cell lines, in which a M, 43,000 product was noted. These data suggest that, in addition to extensive glycosylation of the Syn molecule, there may be tumor selectivity associated with this posttranslational modification step in lung and extrapulmonary tumor cell lines.

The function of Syn is not known. Syn is a hexameric protein capable of forming transmembrane channels (43) and is one of the major calcium-binding proteins of the synaptic vesicle membrane, with the binding site on its cytoplasmic domain (41). Moreover, Syn is one of the major target proteins of endogenous tyrosine phosphorylation in highly purified synaptic vesicles, as well as in synaptosomes from rat forebrain (44). It can also be phosphorylated by the c-src-encoded protein tyrosine...
kinase. While Syn was initially localized in intracytoplasmic clear vesicles, subsequently smaller amounts were also found in large dense core vesicles (40, 45-49). Thus, it can be speculated that Syn has a role in the intracellular vesicle transport system, in communication with membrane and cytoskeleton, or in exocytosis. In cancer cells, this may involve intracellular trafficking of growth factors and oncogenes.

In summary, we recommend that Syn be added to the panel of general NE markers currently in use for the following reasons: (a) Syn may be a more specific and sensitive biochemical marker for NE differentiation than CgA in carcinoid and classic SCLC cell types; (b) Syn is a more sensitive marker for NE neoplasms which lack part of the NE program (i.e., SCLC-V); (c) Syn may be helpful in identifying differentiating, and classifying small cell carcinomas of extrapulmonary origin or unknown origin; and (d) Syn may be a clinically important tool in identifying patients with NSCLC-NE neoplasms.

REFERENCES


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