CLUSTER-10 LUNG-CANCER ANTIBODIES RECOGNIZE NSPs, NOVEL NEURO-ENDOCRINE PROTEINS ASSOCIATED WITH MEMBRANES OF THE ENDOPLASMIC RETICULUM

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We have identified a novel gene (the NSP gene) encoding 3 transcripts and coding for 3 neuroendocrine-specific proteins (NSPs), by screening a CDNA expression library of the small-cell lung-cancer (SCLC) cell line NCI-H82 with the cluster-10 lung-cancer antibodies RNL2 and RNL3. The 3 transcripts code for NSPs with apparent molecular weights of 135 kDa (NSP-A), 43 to 45 kDa, and 35 kDa (NSP-B), respectively. Two 23 kDa (NSP-C), NSP-A and NSP-B are recognized by antibodies RNL2 and RNL3, while second-generation antibodies, specifically recognizing NSP-A and NSP-C, have been produced after immunization with a hybrid protein obtained after bacterial expression of the largest NSP transcript or with a synthetic peptide specific for NSP-C.

The NSPs exhibit a highly restricted distribution pattern and are expressed mainly in neural and neuro-endocrine cell types, and in neuro-endocrine tumours. Of the different types of lung tumours, main SCLC and carcinoids were positive in immunocytochemical assays using the anti-NSP antibodies, while non-SCLC were in general negative. The subcellular distribution of the NSPs was studied in human SCLC cell lines. They do not co-localize with components typical of neuro-endocrine granules, such as synaptophysin and chromogranin. The use of NSP antibodies in the immunofluorescence technique applied to cultured SCLC cells, made it obvious that these proteins localize in the endoplasmic reticulum. Cell fractionation procedures, monitored by immunoblotting assays, indicated an association of the NSPs with the microsomal fraction, from which they could be solubilized with Triton X-100. Gel filtration studies with this solubilized fraction revealed that NSPs form supramolecular aggregates with a molecular weight of more than 500 kDa.

In our study of the biological behaviour of small-cell lung cancer (SCLC), and in particular of its most aggressive form, the variant type of SCLC (Carney et al., 1985; Gazdar, 1989), we have searched for differences in the molecular composition between the classic and variant type of SCLC. The approach that was initially chosen included the preparation of variant-type SCLC-specific monoclonal antibodies (MAbs). In this way 2 MAbs, designated RNL2 and RNL3, were obtained and tested for reactivity using a large panel of lung-cancer cell lines, as well as normal and malignant human tissues. Special emphasis was placed on tissues and tumours with neuro-endocrine features. From these studies it became obvious that the antibodies RNL2 and RNL3, which have been grouped as cluster-10 antibodies during this 3rd Workshop on Lung Tumours and Differentiation Antigens, recognized neuro-endocrine-related cellular constituents, which could be identified in immunoblotting as a group of 3 to 4 proteins with molecular weights varying from 35 to 135 kDa. After detailed molecular analysis, these proteins were found to be derived from one gene, which we designated the neuro-endocrine-specific-protein (NSP) gene. Here we present a review of our studies on the products of this NSP gene (Broers et al., 1991; Roebroek et al., 1993; Van de Velde et al., 1994).

The NSP gene, its transcripts and neuro-endocrine-specific expression pattern

Screening of a prokaryotic CDNA expression library of the variant SCLC cell line NCI-H82, using a mixture of RNL2 and RNL3 MAbs, revealed 5 cDNAs. Using the largest cDNA as molecular probe in Northern blot analysis of SCLC cells and carcinoid tissue, we identified 3 transcripts of different length. These 3 transcripts with an estimated size of 3.4, 2.3 and 1.9 kbp (Fig. 1a) showed identical 3' sequences, but unique 5' sequences. Comparison of the cDNA sequences of these transcripts revealed that the unique 5' sequences of the 2.3-kb transcript comprised 41 nucleotides and those of the 1.8-kb transcript at least 88 nucleotides. Southern blot analysis, sequencing and YAC cloning experiments indicated that the various transcripts originate from a single gene. The NSP gene has recently been mapped by fluorescence in situ hybridization analysis to human chromosome 14q21-q22 (Kools et al., 1994).

Northern blot analysis of mRNA from NCI-H82 cells revealed very high levels of the 3.4-kb transcript and low levels of the 3.4-kb transcript (Fig. 1b, lane 1). As shown in Figure 1b, 2 out of 4 other SCLC cell lines and a primary carcinoid lung tumour showed the presence of one or both of the 3.4- and 1.8-kb transcripts (Fig. 1b, lanes 2 to 6). Expression of the 3.4- and 1.8-kb transcript was also found in 2 human neuroblastoma cell lines, in 2 mouse pituitary-adenoma cell lines, in a rat medullary-thyroid-carcinoma cell line and a rat pheochromocytoma cell line. In human parathyroid and thyroid, weak expression of only the 3.4-kb transcript could be detected. No NSP mRNA has been observed in 3 non-neuro-endocrine human cell lines, i.e. a larynx-carcinoma cell line, an oral-carcinoma cell line and a pharynx-carcinoma cell line. In mouse brain, testis and ovary expression of 2 NSP transcripts were found, corresponding to human 3.4- and 1.8-kb mRNA.

Mouse liver, spleen, heart, kidney and lung were negative. In rat pituitary, transcripts of 3.4 kb and 1.8 kb were detected.

NSPs and anti-NSP antibodies

Nucleotide sequence analysis of the 3 different NSP transcripts revealed open reading frames for proteins of 776 (calculated molecular weight of 84 kDa), 356 (calculated molecular weight of 39 kDa) and 298 (calculated molecular weight of 25 kDa) amino acids. Although the calculated molecular weight of the protein deduced from the 3.4-kb sequence (84 kDa) differs significantly from the molecular weight based on the observed electrophoretic mobility (135 kDa), transformation of the complete coding sequences of the 3.4-kb transcript into COS-1 cells, followed by immunoprecipitation of the produced protein, revealed the production of a 135-kDa protein. Similarly, immunoprecipitation of proteins from COS-1 cells transfected with the middle transcript, using RNL2, resulted in protein bands ranging from 45 to 35 kDa. An explanation for the apparent discrepancy in calculated and estimated molecular weight of NSP-A in particular can be found in the fact that the NSPs each contain an amino-terminal region with a relatively high number of negatively charged residues. These properties may lead to lower mobility in SDS gels and also explain the low isoelectric pH of NSPs, i.e., a calculated pI of 4.93 for NSP-B and a calculated pI of 7.04 for NSP-A.
4.35 for NSP-A. Also, NSP-A and NSP-B appeared to be highly phosphorylated, preferentially on serine residues. NSP-A was shown not to be glycosylated. The smallest transcript codes for a 23-kDa protein, designated NSP-C. NSP-C was found not to be phosphorylated. A typical feature found in all 3 NSPs is that the common C-terminal domain contains 2 large hydrophobic regions, probably representing membrane spanning domains. Although the NSPs do not exhibit any apparent amino-acid-sequence homology to known proteins, they share some structural features with other neuro-endocrine proteins, for instance the synaptic-vesicle-associated protein amphiphysin (Lichte et al., 1992) and chromogranin A and B (Wiedemann and Hutten, 1989).

NSP-A and NSP-B are recognized by antibodies RNL2 and RNL3, as concluded from the fact that both antibodies revealed 4 35- to 45-kDa proteins (including a 43 to 45 triplet) and the 135-kDa protein in Western blot analysis of the SCLC cell line NCI-H82 (Fig. 2). Competition binding assays showed that the 2 antibodies recognize different epitopes of these proteins. In another SCLC cell line, SCLC 21H, only the 135-kDa protein, but not the 43- to 45-, 35- or 23-kDa NSPs, could be detected. RNL2 and RNL3 showed neither reactivity with the NSP-C protein nor with the COS-1 cells transfected with the NSP-C transcript, indicating that these 2 reagents do not recognize NSP-C epitopes. Second-generation mouse MAbs MON 160, MON 161 and MON 162 were produced after immunization with a partially purified, bacterially expressed NSP-A hybrid protein. Because of their exclusive reactivity with the 135-kDa protein in immunoblots of NCI-H82 and reactivity with different fusion proteins, these second-generation MAbs apparently recognize different epitopes located at the unique amino-terminal part of NSP-A. These antibodies also detected the 135-kDa protein in other SCLC cell lines and showed tissue-distribution patterns similar to those observed for RNL2 and RNL3. Using the NSP-A hybrid protein, we produced a polyclonal rabbit anti-serum, POL 1, that was shown to react with NSP-A and NSP-B in NCI-H82. A rabbit NSP-C anti-serum POL-8 was raised against a synthetic peptide consisting of the 20 amino-terminal residues of deduced NSP-C. This anti-serum reacted strongly with the NSP-C fusion protein in immunoblotting, in COS-1 cells expressing the 18-kDa NSP-C transcript, in SCLC 21H cells and in rat brain. On basis of the results described above, the epitopes for the different antibodies can be mapped to regions of the different NSPs as shown in Figure 3.
Localization and supramolecular organization of NSPs.

To define their subcellular localization in lung-cancer cells, we studied the distribution of NSPs in different soluble and membranous fractions of SCLC cell lines NCI-H522, NCI-H524 and SCLC 21-H. A minor amount of NSPs was found in the PBS-soluble fraction of these cells, but the main NSP fraction was found in the 3500 g pellet and in the 100,000 g microsomal fraction. These results indicated that the NSPs are associated with membranous components. They could be solubilized from these membranous fractions with Triton X-100. These experiments revealed also a co-distribution of the individual NSPs in these subcellular compartments.

Gel filtration experiments, using the PBS-soluble or Triton X-100-soluble fraction of SCLC cell homogenates, showed that the NSPs are aggregated into large proteinaceous complexes with a molecular weight of more than 300 kDa.

Immunofluorescence studies of cell cultures were performed to elucidate the subcellular localization of these NSP aggregates. In adherent cells of SCLC cell lines NCI-H52 and NCI-H524, strong perinuclear staining and a uniform staining of a fibrogranular network, spanning the whole cytoplasm, was observed (Fig. 4a). A similar staining pattern of a lace-like network, seen most clearly in the cellular periphery, could be demonstrated in COS-1 cells transfected with NSP-A, NSP-B and NSP-C. CDNA constructs using antibodies RNL2, RN13 (Fig. 4b), a mixture of MON 100, MON 101, MON 102 or POL 8. This typical staining pattern suggested that the NSPs might be associated with cytoskeletal or membranous structures in the cell. Co-localization studies with antibodies to microtubules, microfilaments, intermediate filaments such as cytokeratin, vimentin, neurofilaments, showed a partial overlap in the staining pattern of microtubules and NSP. The results of double immunofluorescence studies with antibodies to proteins known to occur in the endoplasmic reticulum (such as Ca²⁺-ATPase) clearly showed that NSPs are localized on this membranous structure, in accordance with the observation that the NSPs are associated with the microsomal cell fraction. From analysis of deletion mutants it appeared that the hydrophilic cytosolic portion of the NSP is critical for the observed membrane binding. Double-label immunofluorescence studies with antibodies to synaptophysin excluded an association of NSPs with small neuroendocrine vesicles. Staining with chromogranin antibodies revealed that the cell tested do not contain dense-cored secretory granules. We conclude that the NSPs form membrane-associated aggregates in the endoplasmic reticulum of neuroendocrine cells.

Tissue distribution of NSPs.

In normal tissues, reactivity with RNL2 and RN13 antibodies was observed in brain Purkinje cells (Fig. 5a), pancreatic islet cells (Fig. 5b), some cells in the pituitary gland and some peripheral nerve fibres. Spleen reticular fibres appear to be stained, while the prostatic epithelium and some suprabasal cells of the tongue epithelium were also positive with RNL2 and RN13. In lung, only positive staining was observed in the bronchial glands. No reactivity was seen in the gastrointestinal tract, or in the thyroid and adrenal glands. Analysis of lung carcinomas showed a positive reaction in part of the neuroendocrine tumour types i.e. in 5 out of 14 SCLC cases (Fig. 5c) and in 6 out of 8 carcinosarcoma cases (Fig. 5d). In general, no positive reaction was seen in squamous-cell carcinomas, adenocarcinomas or large-cell carcinomas of the lung. However, in a few cases of poorly differentiated squamous carcinomas and in one case of a large-cell carcinoma, local positivity
was seen. In non-pulmonary human tumours a staining reaction was observed in a sub-set of neuro-endocrine tumours, such as pituitary adenomas (Fig. 5e) and insulinomas (Fig. 5f). Some other malignancies, such as medullary Schwannomas, prostate carcinomas, thyroid carcinomas and melanomas, were positive. From these data, we conclude that the antibodies recognize a sub-set of neuro-endocrine tissues and tumours. Altogether, the neuro-endocrine-specific expression pattern found in the Northern and Western blot studies are in accordance with the results of the immunohistochemical studies.

Species cross-reactivity of the antibodies RNL2 and RNL3 was restricted to rabbit brain and rhesus monkey. They were negative in rat, chicken and hamster tissues. Second-generation antibodies MON 160, MON 161 and MON 162 showed a broader species cross-reactivity, and appeared to cross-react with hamster, mouse and rat cell lines.

DISCUSSION

We have identified a novel neuro-endocrine-specific protein gene (NSP gene) encoding a family of proteins with NSP-A (135 kDa), NSP-B (35, 43-45 kDa) and NSP-C (23 kDa) as its main, known members. The NSP gene products showed prevalent expression in neuro-endocrine and neural tissues and tumours. The various antibodies to the NSPs, in particular, may therefore become useful for the immunocytochemical recognition of neuro-endocrine cancers. Within the group of SCLC, however, only some of the tumours were found to react with the different NSP antibodies. Our initial aim was directed
towards the production of MAbs specific for the variant type of SCLC. Although our selection procedures were designed to obtain such antibodies, we must conclude that in fact none of the first- or second-generation antibodies makes a clear-cut separation between the classic and variant type of SCLC in cell cultures. It remains to be established whether the positively reacting sub-set of SCLC behaves differently, either clinically or biologically, from NSP-negative SCLC. Also, NSP antibodies can detect the presence of small populations of neuroendocrine cells in non-neuroendocrine tumours. In the group of NSCLC about 10 to 20% of the tumours exhibit neuroendocrine characteristics, which appears to correlate with poor prognosis (Linnola et al., 1988).

In addition to their application in immunocytochemical assays, the NSP antibodies may well prove useful in serum assays of patients with neuro-endocrine neoplasia. Our preliminary results with RNL2 and RNL3, when tested on sera from such patients using a sandwich assay, were negative (data not shown). However, the fact that these 2 antibodies react most strongly with NSP-B, which has not been found in human solid neuro-endocrine tumours and to a lesser extent with NSP-A, might explain these negative results. Future studies will focus on the usefulness of the second-generation NSP-A and NSP-C antibodies.

SCLC cell lines and COS-1 cells transfected with the different cDNA constructs were used to elucidate the localization and supramolecular organization of the newly identified neuroendocrine proteins. Immunofluorescence and differential-centrifugation studies revealed that NSP aggregates are associated with the endoplasmic reticulum. Cell fractionation revealed a strong association of the NSPs with the microsomal fraction, to which they are bound by the hydrophobic carboxy-terminal portion of the NSPs. Despite their neuroendocrine appearance, NSPs have not been found to be associated with neuroendocrine vesicles. However, NSP show some structural similarities with chromogranin A and B, such as abundant acidic-amino-acid regions (Wiedenmann and Huttner, 1989). The NSPs exhibit even more structural resemblance with the synaptic-vesicle-associated protein amphiphysin (Lichte et al., 1992). Like amphiphysin, the NSPs are very acidic, rich in proline residues, contain potential sites for phosphorylation, lack a signal peptide and possess a hydrophobic stretch in the carboxyl-terminal half.

Our findings indicate that the NSPs are novel, membrane-anchored components of the endoplasmic reticulum, particularly expressed in cell types with neuro-endocrine differentiation. Whether the NSP aggregates, observed after gel filtration studies, are homogeneous or heterogeneous in composition remains to be established.

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