Targeting the Neurophysin-related Cell Surface Antigen on Small Cell Lung Cancer Cells Using a Monoclonal Antibody against the Glycopeptide Region (MAG-1) of Provasopressin

Brendan P. Keegan, Vincent A. Memoli, and William G. North

Departments of Physiology [B. P. K., W. G. N.] and Pathology [V. A. M.], Dartmouth Medical School, Lebanon, New Hampshire 03756

Abstract
The vasopressin (VP) gene is largely expressed in hypothalamic neurons, where the resultant pro-VP protein is enzymatically cleaved into its peptide hormone components, which include the neuropeptide VP, VP-associated neurophysin, and VP-associated glycopeptide (VAG). Small cell lung cancer (SCLC) tumors also express the VP gene, but the tumor pro-VP protein can remain intact and localize to the cell surface membrane. Previous studies have shown that polyclonal antibodies directed against different regions of the pro-VP protein bind specifically to the surface of cultured SCLC cells and recognize proteins of ~20 and ~40 kDa in cultured SCLC whole-cell lysate. Thus, these proteins have been designated neurophysin-related cell surface antigen (NRSA). A monoclonal antibody (mAb) designated MAG-1 was raised in this laboratory using a synthetic peptide representing the COOH-terminal sequence of VAG. The MAG-1 mAb recognizes NRSA in SCLC cell and tissue lysates by Western analysis, whereas immunofluorescent cytometric and microscopic analyses indicate that MAG-1 reacts specifically with NRSA on the surface of viable SCLC cells of both the classical and the variant subtype. Immunohistochemical analysis demonstrates that MAG-1 reacts with human SCLC tumor, but not with normal pulmonary epithelial cells in lung tissue. Additionally, a MAG-1 Fab fragment was generated that was also able to recognize NRSA. This is the first study to demonstrate that a mAb directed to the VAG region of the pro-VP protein has the potential for development into an in vivo diagnostic and therapeutic tool that targets plasma membrane-incorporated NRSA.

Introduction
Lung cancer is the leading cause of cancer-related deaths worldwide, and SCLC comprises about 16% of all lung cancer cases in the United States (1). Currently, SCLC is diagnosed on the basis of gross morphological and histological data and is too often identified after the disease has reached its advanced stages (2). Although there is a high response rate to present treatments consisting of high-dose chemotherapy with or without radiotherapy, disease recurrence is frequent, and tumors become resistant to these approaches, resulting in 2-year survival rates of only 6–12% (3). Considerable toxicity is also associated with these therapies. The pressing need for effective screening and non-toxic treatment methods has spawned a search for new approaches to combat the disease, taking advantage of the numerous molecular and genetic abnormalities that have been described for SCLC (2, 4, 5). The prospect of antibodies directed against cell surface tumor-specific antigens is attractive not only for use in the differential diagnosis of SCLC but also for localizing and eradicating tumors because they have the potential to elicit minimal side effects (6). This strategy is most effective when directed against tumor-specific antigens that are not lost or modulated, and products of the VP gene may provide for such an antigen.

The expression of the VP gene is largely restricted to hypothalamic neurons, and it encodes for a protein product of ~17 kDa, to which an N-glycosidic side chain of ~4 kDa is added, resulting in the ~20-kDa pro-VP precursor. This protein is normally packaged into secretory vesicles, where it undergoes enzymatic cleavage to generate VP, VP-NP, and VAG (7). These components are then secreted into the circulation. SCLC tumors and cultured cells also express the VP gene; however, intact pro-VP protein can become localized to the cell surface plasma membrane (8, 9). Polyclonal antibodies raised against VP-NP bind specifically to the surface of cultured SCLC cells, as determined by immunofluorescence analysis (10–12). Thus, the target of these antibodies has been termed NRSA (13). Polyclonal anti-VP-NP recognizes proteins of ~20 and ~40 kDa in total protein extracts from SCLC cultured cells by Western analysis (13). The ~20-kDa protein corresponds in size to the pro-VP protein, and the ~40-kDa protein is believed to be a related form (14–19). Polyclonal antibodies that have been raised...
against the VP, VP-NP, or VAG regions of the pro-VP protein display specific staining of SCLC tumor sections, whereas they exhibit a very low incidence of immunoreactivity with the non-neuroendocrine tumors examined (8, 20).

This report describes the detection of NRSA in cultured SCLC cells and human SCLC tumor tissue using a mAb designated MAG-1, which was generated using a synthetic peptide representing the COOH-terminal portion of the VAG region of the pro-VP protein. MAG-1 recognizes the −20- and −40-kDa NRSA proteins in cultured SCLC cell lysate by Western analysis, whereas immunofluorescence cytomteric and microscopic analyses indicate that it binds to the surface of these cells. More importantly, the −20- and −40-kDa NRSA proteins were detected in the lysate of human SCLC tumor biopsy samples by Western analysis using MAG-1, but they were not detected in the lysate of nontumor human lung tissue. We also demonstrate by immunohistochemical analysis that MAG-1 reacts with human SCLC tumor, but not with normal pulmonary epithelial cells in lung tissue. Because NRSA is not typically found on the surface of normal cells, it is anticipated that it can serve as an excellent target in a MAG-1-based approach for tumor localization in the diagnosis and therapy of SCLC.

Materials and Methods

Cultured Cell Lines and Human Tissues. Cultured cell lines were maintained at 37°C and 5% CO₂. The NCI-H82 variant-type SCLC cell line was obtained from the American Type Culture Collection (Manassas, VA) and cultured in RPMI 1640 (Mediatech, Herndon, PA) with 10% FBS (Hyclone, Logan, UT). The NCI-H345 classical-type SCLC cell line, a gift from Dr. J-J. Legros (Liege, Belgium), was maintained in RPMI 1640 supplemented with 10% FBS, 10⁻⁵ M β-estradiol, 10⁻⁸ M hydrocortisone, 5 μg/ml insulin, 5 μg/ml transferrin, 5 mg/ml sodium selenite (ITS; Sigma, St. Louis, MO), and 0.01 μM HEPES. The Lu-165 classical-type SCLC cell line (21), a gift from Dr. J. Coulson (Liverpool, United Kingdom), was maintained in RPMI 1640 with 10% FBS. The Beas-2B transformed human bronchial epithelial cell line, a gift from I. Pitha-Rowe in the laboratory of Dr. E. Dmitrovsky (Dartmouth Medical School), was maintained in LHC-8 media with epinephrine. The mouse myeloma/spleen hybrid cell line Sp2/0-Ag14 was obtained from the American Type Culture Collection and maintained in DMEM (Mediatech) with 10% FBS.

mAbs and Fab Fragments. All procedures involving animals were conducted with the approval of the American Association for the Accreditation of Laboratory Animal Care-certified Dartmouth College and Dartmouth-Hitchcock Medical Center Institutional Animal Care and Use Committee. MAG-1 mAb was generated against a synthetic 18-amino acid peptide representing the COOH-terminal VAG region of the pro-VP protein (VAGc18; VQLGAGEPFEPAQPDDAY) coupled to bovine thyroglobulin using glutaraldehyde. This complex was used as a 1 mg/ml solution (peptide equivalent concentration) in 0.05 μm sodium phosphate (pH 7.0) that had been sonicated with an equal volume of complete Freund’s adjuvant to immunize BALB/c mice. A follow-up immunization was performed 21 days later using a mixture of antigen with incomplete Freund’s adjuvant, and spleen cells were harvested after an additional 5 days. The spleen cells were hybridized with Sp2/0-Ag14 cells, and viable hybridomas were selected using DMEM containing 10% FBS and supplemented with hypoxantine-aminopterin-thymidine (Sigma). Clones were screened for the production of antibodies using ¹²⁵I-VAGc18 peptide by displacement RIA (22). The MAG-1-producing clone was isolated and used to generate ascites fluid in BALB/c mice, and the mAb was purified by immunoadfinity chromatography using a column comprised of VAGc18 conjugated to cyanogen bromide-activated Sepharose 4B (Sigma). MAG-1 was determined to be of isotype IgG1 using a Clonotyping System kit (Southern Bio- technology Associates, Birmingham, AL). Fab fragments of MAG-1 were generated using an ImmunoPure IgG1 Fab and (Fab)₂ Preparation Kit (Pierce, Rockford, IL) following the manufacturer’s instructions, and complete ficin digestion of the IgG molecule was confirmed by Western analysis.

RT-PCR. Total RNA was isolated from cultured cells or human tissues using Trizol (Life Technologies, Inc., Rockville, MD), and 1 μg was used together with oligo(dT) primers and RNase H⁻ reverse transcriptase (Life Technologies, Inc.) in a standard reverse transcription reaction following the manufacturer’s instructions. PCR was performed using primers AVPfwd (5’-aggatgcctgacaccatgctg-3’) and AVPRev (5’-gttcggaggtattgacman-3’) in a reaction using MasterTaq enzyme, TaqMaster PCR Enhancer buffer, and a Mastercycler Gradient thermocycler (Eppendorf, Westbury, NY). These primers are designed to span the 2 introns of the VP gene and amplify the entire coding sequence of the pro-VP protein. Cycle conditions were as follows: 1 × 95°C for 5 min; 4 × 95°C for 1 min, 62°C for 1 min minus 1°C/cycle, and 72°C for 1 min; 30 × 95°C for 1 min, 57°C for 1 min, and 72°C for 1 min; and 1 × 72°C for 10 min.

Western Analysis. Total protein lysates were prepared from cell or lung tissue samples by extraction in 0.1 M HCl containing 0.1% Tween 20, and protein concentrations were determined using differential absorance measurements taken at 215 and 225 nm (23). Lysates (40 μg) were separated on 14% gels by SDS-PAGE using Tris/glycine/SDS buffer (25 mM Tris, 192 mM glycine, 0.1% SDS (pH 8.3)), and the proteins were transferred onto Immobilon-P polyvinylidene difluoride membrane (Millipore, Bedford, MA) in Tris/glycine/SDS buffer with 20% methanol added, using the MiniProtean 3 system (Bio-Rad, Hercules, CA). To block the membranes, they were dried using a Model S83 Gel Dryer (Bio-Rad), and NRSA was detected by sequential incubation with MAG-1 and horseradish peroxidase-conjugated protein L (Pierce). For detection of VP-NP, a rabbit polyclonal antibody produced in this laboratory was used (13), followed by a horseradish peroxidase-labeled goat antirabbit antibody (Life Technologies, Inc.). Signal was generated using Lumi-
Light Western blotting substrate (Roche, Indianapolis, IL), and the membranes were exposed to autoradiography film.

**Immunofluorescence Cytometric and Microscopic Analyses.** Approximately $10^6$ cultured SCLC cells were incubated with varying dilutions of MAG-1 mAb or Fab in PBS with 0.1% BSA and 0.01% sodium azide, followed by FITC-conjugated Fab-specific goat antimouse antibody (Sigma). Each step was performed at 4°C, and the cells were washed in the interim. The cells were then fixed in 1% paraformaldehyde at 4°C and washed, and the fluorescence was measured on a FACStar flow cytometer (Becton Dickinson, Mountain View CA). An aliquot of the cells was removed and resuspended in SlowFade Light (Molecular Probes, Eugene, OR) and mounted for visualization using an Axioskop microscope (Zeiss, Thornwood, NY) with Plan NeoFluar optics connected to a Bio-Rad MRC 1024. An IgG1 isotype control (Hybridoma Library, Dartmouth Medical School) was used to assess nonspecific binding.

**Immunohistochemistry.** Sections of 4–6 µm from each of the formalin-fixed paraffin-embedded specimens of human SCLC, normal lung, or hypothalamus tissue were stained for NRSA with MAG-1 mAb. All steps were performed at ambient temperature unless otherwise stated. The sections were deparaffinized by heat exposure (60°C for 10 min) followed by xylene washes (2 × 5 min), and tissues were rehydrated by washes (2 × 5 min) in descending concentrations of ethanol (100%, 95%, and 70%). After washing with PBS (2 × 5 min), the tissues were subjected to antigen retrieval by incubation in 0.01 M sodium citrate (pH 8.5) for 30 min at 80°C. Slides were washed in PBS (2 × 5 min) and then incubated in Power Block Universal Blocking Reagent (BioGenex, San Ramon, CA) for 5 min. MAG-1 mAb was diluted (1 µg/ml) in PBS containing 0.1% BSA and 0.02% Tween 20 and reacted with the tissue sections for 1 h. After washes with PBS (3 × 5 min), the slides were incubated with MultiLink biotinylated goat anti-immunoglobulins solution (BioGenex) for 30 min. After washing in PBS (3 × 5 min), incubated with Label Peroxidase-Conjugated Streptavidin Solution (BioGenex) for 30 min. After washing in PBS (3 × 5 min), staining was achieved using 3,3′-diaminobenzidine substrate solution (BioGenex) for 3 min. Tissues were then counterstained with hematoxylin, dehydrated in ascending concentrations of ethanol, washed in xylene, and coverslipped using SuperMount mounting medium (BioGenex).

**Results**

Detection of NRSA in SCLC Tumor Tissue and Cultured Cells. Total RNA was extracted from human lung SCLC tumor and nontumor tissue samples and analyzed for the presence of the VP message by RT-PCR. The PCR reaction was carried out with sequence-specific primers that spanned the 2 introns of the VP gene. Only one product was detected in the reactions using RNA extracted from the SCLC tumor, SCLC cultured cells, and human hypothalamus tissue (Fig. 1). This band corresponds in size to that predicted (570 bp) for the VP message using these particular amplification primers. There was no VP message detected in total RNA extract from the Beas-2B cells, whereas there was a faint band at ~570 bp detected in the nontumor lung tissue extract. It is possible that this represents VP expression by small, undetected SCLC tumor cells embedded in the lung tissue sample or expression by pulmonary neuroendocrine cells (24). However, when SDS-PAGE and Western analysis were performed using the MAG-1 mAb and Fab fragment, NRSA was detected in protein extracts from the cultured SCLC cells and tumor tissue, but not in protein extract from the nontumor lung tissue (Fig. 2A). When cultured SCLC cell protein extract was examined using a polyclonal antibody raised against VP-NP (13), a banding pattern identical to that produced using the MAG-1 mAb was observed (Fig. 2B). The MAG-1 mAb and Fab, as well as the polyclonal anti-VP-NP antibody, recognize proteins with molecular masses of ~20 and ~40 kDa, along with what appear to be degradation products and/or deglycosylated forms of the pro-VP protein. Whereas the ~20-kDa protein corresponds to the expected size for the pro-VP protein, the identity of the ~40-kDa protein is not yet known.

Detection of NRSA at the Surface of Cultured SCLC Cells. NCI-H82 cells were reacted with MAG-1 mAb or Fab, followed by FITC-labeled goat antimouse Fab-specific antibody, and fluorescence was measured on a FACStar apparatus (Fig. 3). A similar level of staining was observed using a 1 µg/ml concentration of MAG-1 mAb or Fab; however, the mean fluorescence measured was increased only ~2-fold when the concentration of Fab used was 100 µg/ml, whereas it increased ~10-fold when using the mAb at that concentration. Because reactions were performed at 4°C in the presence of sodium azide to inhibit internalization of proteins from the plasma membrane, these results indicate that the MAG-1 mAb has a higher binding capacity than MAG-1 Fab for NRSA on the surface of cultured SCLC cells. The mAb was also used to detect NRSA on the surface of Lu-165 and NCI-H345 cultured SCLC cells. The intensity of the fluorescence measured after staining with the isotype control mouse mAb was equivalent to that measured in unstained
The binding of the MAG-1 mAb to NRSA on the surface of cultured SCLC cells was also assessed by fluorescence microscopy. In all cases, a nonuniform pattern of staining of the cell surface was observed on SCLC cells, whereas almost no staining was present when the isotype control antibody was used (Fig. 4). Propidium iodide was used to stain the nuclei of the NCI-H82 cells for contrast, after the cells had been incubated with MAG-1, FITC-conjugated antibody against VP-NP. Approximate molecular mass is indicated to the left of each figure. The blots were probed with antibodies to β-actin and glyceraldehyde-3-phosphate dehydrogenase to evaluate for proper loading (data not shown).

Discussion

We have developed a mAb (MAG-1) directed to the COOH-terminal region of the glycopeptide component of the pro-VP protein, and we have demonstrated that the two major immunoreactive forms of the tumor antigen NRSA are detected using MAG-1 in protein extracts from cultured SCLC cells and in a protein extract from human SCLC tumor. However, these proteins were not detected in protein extract from nontumor human lung tissue by Western analysis using MAG-1. Some cancers, including SCLC, are known to express the VP gene, although it appears that not all of the precursor protein is enzymatically processed and secreted into the circulation as in the hypothalamus. Polyclonal anti-VP-NP antibody has been shown to recognizes proteins of 20 and 40 kDa in SCLC cell extracts (13). The predicted size of the glycosylated pro-VP protein product of the normal VP message is 20 kDa, and this has been demonstrated using a cell-free translation assay (25–27). Previous studies identified an extended VP message, and it was thought that this might account for the 40-kDa protein (13, 28). However, only one form of the predicted size for the normal VP message was detected by RT-PCR in the SCLC cell lines and tumor tissue used in this study, and this corresponded in size to that detected in human hypothalamus. The nature of the 40-kDa protein is currently being studied, and in fact, stable higher molecular mass forms of neurophysin-related proteins have been described previously (14–19).

Although it may not be apparent in Fig. 1, overexposure of the gel indicated that a small amount of VP message was amplified in the RT-PCR reaction using RNA extracted from
the nontumor human lung tissue originating from a patient with emphysema, but NRSA was not detected in a total protein extract of the same tissue sample. It is possible that the presence of VP mRNA indicates that the lung tissue harbored undetected SCLC tumor or tumor progenitor cells. NRSA may also have been present, but at levels that were too low to be detected by the Western blotting assay. Alternatively, the expression of the VP message could represent the presence of pulmonary neuroendocrine cells in the lung tissue sample used (24), but the prohormone may be enzymatically processed and the products secreted, as occurs in the hypothalamus. The latter possibility appears to be supported by the immunohistochemical findings (Fig. 5). MAG-1 reacted with human SCLC tumor, but not with normal pulmonary epithelial cells. Immunoreactivity observed in normal lung sections was restricted to macrophages, which probably represents binding to Fc receptors, and to pulmonary neuroendocrine cells present in bronchioles, suggesting that these cells might also express the VP gene. The level of message detected may be a reflection of the scarcity of these cells. These results indicate that MAG-1 can be used to effectively target NRSA on SCLC tumors, and we are currently undertaking an extensive immunohistochemical evaluation of SCLC and normal lung tissue sections to verify the applicability of MAG-1 for clinical diagnosis.

Previously, polyclonal antibody preparations were shown to specifically stain human SCLC and hypothalamus tissue sections as well as bind to the plasma membrane of cultured SCLC cells in a manner similar to that observed using MAG-1 (8, 10, 12, 20, 29). Therefore it appears the −20- and −40-
kDa NRSA proteins are VP gene-related products that can serve as tumor-specific antigens and can be targeted with antibodies (8,12,13,20,30,31). Although the level of NRSA expression by the different SCLC cell types has not been measured, this study demonstrates that MAG-1 can recognize NRSA on the surface of cultured cell lines derived from SCLC tumors of both the classical and variant subtypes. Fluorescence microscopy revealed that MAG-1 staining was observed with each SCLC cell type examined here. However, it should be noted that individual cells displayed differing levels of immunoreactivity. It is not known whether this is due to assay conditions or to variations in the metabolic status of the individual cells that could effect the expression of NRSA and/or its enzymatic processing. Earlier studies have indicated that the expression of VP message and NRSA can be increased by treatment with catecholamines or glucocorticoids (10,32), and although the key processing enzymes for the pro-VP protein are present in at least the NCI-H82 cultured SCLC cell line (33), the details concerning its regulation in SCLC remain to be fully characterized. Accordingly, further investigation is being performed concerning the conditions that alter the expression of NRSA by cultured SCLC cells.

In principal, mAbs should be of great benefit for use in tumor targeting and therapy (6), and recent studies indicate that certain mAbs display promising potential for therapeutic use, including the IMC-C225 mAb directed against the epidermal growth factor receptor and Herceptin mAb directed against Her2/Neu, which can have significant antitumor effects in cancers that overexpress those receptors (34). The potential uses for the MAG-1 mAb are significant, not only as a tumor-targeting agent for the localization and treatment of SCLC but also for distinguishing SCLC from other forms of lung cancer and aiding its early diagnosis (8,20,30). The ability of the MAG-1 Fab fragment to recognize synthetic antigen as well as NRSA in protein extracts from SCLC tumor and cultured cells was also evaluated because antibody fragments may be better suited for some in vivo applications. The Fab was able to recognize NRSA by Western analysis, but, not surprisingly, it displayed a lower binding affinity for synthetic antigen. However, the localization of antibody molecules to tumor tissue and their ability to penetrate solid tumor depend on a number of factors including size, affinity, rate of clearance, and antigen density (35,36). Variable region fragments (Fv) of this antibody are now being produced by us to assess their potential for in vivo tumor targeting because they may provide additional benefits for use in imaging and therapy (37). The biological activity of VAG is not well characterized, and although a possible function has been described (38), it is clear that further investigation is warranted to assess the potential side effects MAG-1 might have in a therapeutic setting. However, NRSA is not typically found on the surface of normal cells, it is not modulated between classical and variant SCLCs, and there is a low incidence of its expression by non-neuroendocrine lung carcinomas (20). Therefore, NRSA should serve as an excellent target for the localization of SCLC tumors in diagnosis and therapy, using MAG-1 mAb and its fragments.

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