A new possible lung cancer marker: VGF detection from the conditioned medium of pulmonary large cell neuroendocrine carcinoma–derived cells using secretome analysis

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ABSTRACT: The prognosis of malignant neuroendocrine tumors of the lung is known to be very poor. Aiming to identify new markers of pulmonary neuroendocrine tumors in early stages and also differential diagnostic markers between large cell neuroendocrine carcinoma and small cell lung cancer, we comprehensively analyzed peptides which were secreted into conditioned medium by LCN1, a large cell neuroendocrine carcinoma cell line. Specific peaks in conditioned medium but not in used medium alone were detected using matrix-associated laser desorption/ionization time of flight mass spectrometry. Two peptide fragments of 40 and 19 amino acid residues were identified by matrix-associated laser desorption/ionization time of flight mass spectrometry. These two fragments were demonstrated to be parts of VGF nerve growth factor inducible (VGF), which is usually expressed in nerve cells or neuroendocrine cells. RT-PCR analysis of lung cancer cell lines showed that VGF mRNA was expressed only in neuroendocrine carcinoma–derived cells. Our data suggest that VGF can be used as a novel serological diagnostic marker of pulmonary neuroendocrine tumors. (Int J Biol Markers 2009; 24: 282-5)

Key words: Secretome, VGF peptide, VGF mRNA, LCNEC, SCLC

INTRODUCTION

It is widely recognized that neuroendocrine tumors of the lung range from low-grade typical carcinoid (TC), intermediate-grade atypical carcinoid (AC), to high-grade small cell lung carcinoma (SCLC) and large cell neuroendocrine carcinoma (LCNEC) (1). LCNEC was the newest member of the neuroendocrine tumors of the lung in the 1999 WHO classification, categorized as a variant of large cell carcinoma, and considered to have a poor prognosis like SCLC (2). Optimal chemotherapy for LCNEC has yet to be established. Exact differential diagnoses and specific biomarkers of LCNEC are necessary to establish novel therapies for LCNEC. The differential diagnosis between LCNEC and SCLC is based on various histological and cytological criteria, including a larger cell size, abundant cytoplasm, prominent nuclei, vesicular nuclei or coarse chromatin, and a polygonal rather than fusiform shape of the nuclei (3, 4). The presence of neuroendocrine markers such as chromogranin, synaptophysin, neural cell adhesion molecules, and neurosecretory granules identified by electron microscopy or immunohistochemistry is necessary for the diagnosis of LCNEC (4). Progastrin-releasing peptide (proGRP) is well known as a specific serodiagnostic marker of SCLC and LCNEC; however, its positive rate is low in LCNEC and the early stages of SCLC.

Many studies have been carried out to identify potential tumor markers in human body fluids including serum, plasma, urine, cerebrospinal fluid, and nipple aspirate (5-10). However, these approaches are hampered by the marked variability and complexity of both control and patient samples, and by the laborious biochemical analyses. Also, the majority of identified proteins are produced by inflammatory cells due to secondary body defense mechanisms rather than by the tumor cells (11). Recently, a novel approach called “the cell line secretome,” which analyzes proteins in conditioned media from cancer cell lines grown in serum-free medium, has been reported (12, 13). Because there are no markedly abundant proteins such as serum albumin, immunoglobulin, or other major serum proteins, and the environment supports only tumor cells, the proteins identified in the conditioned medium are thought to be of tumor cell origin.

In the present study, we analyzed peptides in
serum-free conditioned medium of LCNEC-derived LCN1 cells by reverse-phase high-performance liquid chromatography (RP-HPLC) and matrix-associated laser desorption/ionization time of flight mass spectrometry (MALDI-TOF MS). The specificity of proteins identified in the conditioned medium of LCN1 cells for LCNEC, but not in used medium alone, was validated by RT-PCR.

MATERIALS AND METHODS

Sample preparation

The LCN1 cell line, derived from LCNEC (4), was grown in serum-free medium (LCN1 SFM). Hybridoma-serum-free-medium (SFM) (Invitrogen, Carlsbad, CA, USA) was established as follows: LCN1 cells were maintained in RPMI-1640 medium with 10% FCS at 37°C, in 5% CO₂/95% air. A 50% volume of SFM was added on each passage, gradually promoting a serum-free condition. When cells showed confluency, they were centrifuged at 700 rpm for 10 minutes, and the supernatants were collected and purified using the following method: The conditioned medium from LCN1 cells and used medium alone underwent solid-phase extraction for desalting and concentration. Solid-phase extraction was performed using an Oasis HLB-column (Waters, Milford, MA, USA). The cartridges were prewashed with 1 mL each of 2% ACNt [acetonitrile (ACN) in 0.1% trifluoroacetic acid (TFA)], 90% ACNt, and 50% ACNt, respectively, and were equilibrated using 2 mL of 2% ACNt. Immediately after conditioned or used medium alone was loaded, the cartridges were washed and equilibrated with 4 mL of 2% ACNt. Proteins and peptides were eluted using 500 µL of 70% ACNt. The extracted solutions were lyophilized.

RP-HPLC

Peptides were analyzed by RP-HPLC using an ODS column (150×2.0 mm, Cadenza CD-C18; Intakt Co., Kyoto, Japan) attached to a Shiseido Nanospace Series HPLC system (Shiseido Co., Tokyo, Japan). The column was maintained at room temperature, and the flow rate of the mobile phase was 100 µL/minute. The composition of the mobile phase was programmed to change over 94 minutes by varying the rates \( r = \frac{[B]}{([A]+[B]×100)\times [A]} \) of solvent A (0.1% TFA) and B (90% ACN, and 50% ACN, respectively, and were equilibrated using 2 mL of 2% ACN. Immediately after conditioned or used medium alone was loaded, the cartridges were washed and equilibrated with 4 mL of 2% ACN. Proteins and peptides were eluted using 500 µL of 70% ACN. The extracted solutions were lyophilized.

MALDI-TOF MS and MALDI-TOF/TOF MS

Externally calibrated mass spectra of the control peptide mixtures and HPLC fractions were measured by employing MALDI-TOF MS (Voyager-DE Pro; Applied Biosystems, Foster City, CA, USA). The matrix was a saturated solution of α-cyano-4-hydroxycinnamic acid (CHCA; Nacalai Tesque, Kyoto, Japan) in 30% ACNt. The peptide mixtures and HPLC fractions (1 µL each) were mixed with 0.5 µL each of saturated CHCA and measured by MALDI-TOF MS. Identification of the peptides was performed using MALDI-TOF/TOF MS (Ultraflex; Bruker Daltonik GmbH, Bremen, Germany) and Mascot search.

RT-PCR

Total RNA from 11 lung cancer cell lines (LCNEC-derived LCN1 and LCN2 cells, SCLC-derived N231, N230, H69, H82, Lu130, and N417 cells, squamous cell carcinoma-derived RERF-LC-AI cells, and adenocarcinoma-derived A549 and LC-2/ad cells) were extracted with Isogen (Nippon Gene, Tokyo, Japan) according to the manufacturer’s instructions. Primers were designed employing Oligo Primer Analysis Software, version 6.0 (Takara Bio, Otsu, Japan) from the VGF nerve growth factor inducible (VGF) mRNA sequence (14). The forward primer was 5’-CACGGGACGCCAGACCTCGAC-3’ (65-85), and the reverse primer was 5’-CTGGGAGCCGGCTTGGTG-3’ (510-526). PCR was performed with pretreatment at 94°C for 2 minutes, followed by 35 cycles of denaturation at 94°C for 30 seconds and annealing/extension at 72°C for 1 minute. Beta-2-microgloblin was used as internal control. PCR products were examined employing 3% agarose gel electrophoresis and stained with ethidium bromide.

Fig. 1 - RP-HPLC elution profile of used medium alone and conditioned medium.

The lower trace is used medium alone and the upper trace is conditioned medium displaced upward for clarity by \( 9 \times 10^{-3} \) absorbance.

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Detection of VGF peptides by secretome analysis

RESULTS

In 18 of 60 samples fractionated by RP-HPLC, specific peptide peaks in conditioned medium but not in used medium alone were detected and measured by MALDI-TOF MS (Figs. 1, 2). Although most peaks were components of the medium, such as insulin and transferrin digests, 2 fragments of VGF, which is a protein existing in nerve cell or endocrine cells, were identified (Fig. 3). To ascertain whether VGF is only expressed in LCNEC, we performed RT-PCR using 11 lung cancer cell lines (Fig. 4). RT-PCR revealed that VGF mRNA was present in 7 out of 8 neuroendocrine carcinoma cell lines, but not in squamous cell carcinoma or adenocarcinoma cell lines.

DISCUSSION

VGF is the non-acronymic name of the gene encoding a 68-kDa protein (VGF) consisting of 615 amino acids in humans and 617 in mice. VGF belongs to the granin family and plays a critical role in regulating body weight, basal metabolism, and the hypothalamus-hypophysis-gonad axis (15). Although information concerning VGF mRNA or protein expression has mainly focused on the nervous system, its expression was also demonstrated in mammalian endocrine cells of the diffuse endocrine system (DES) in the pituitary, thyroid, pancreas, and adrenal medulla (16-18). It was reported recently that proVGF-related peptides are present in endocrine cells during early development and adulthood, and increase under hyperplasia and in tumors (19).

In the present study, we identified VGF fragments in the conditioned medium of cells derived from LCNEC. The same fragments of VGF have been detected in cerebrospinal fluid from patients with psychosis (20). We detected VGF mRNA in all but 1 variant SCLC subtype of
H82 cells of neuroendocrine carcinoma but in no other types of lung carcinoma cell by RT-PCR. No previous report has analyzed human serum, but VGF peptide fragments might be present in sera from patients with neuroendocrine tumors of the lung. Further studies are warranted to clarify the clinical utility of this molecule and the physiological activity of these peptide fragments of VGF.

Conflict of interest statement: None declared.

REFERENCES


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