Pro-Gastrin-Releasing Peptide (31–98) Is a Specific Tumor Marker in Patients with Small Cell Lung Carcinoma

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ABSTRACT

Gastrin-releasing peptide (GRP) is a specific and an actively secreted product of small cell lung carcinoma (SCLC) cells. Based on this observation, we attempted to develop a new approach for early detection of SCLC and for monitoring therapeutic response in SCLC patients. Recombinant human ProGRP (31–98), a region common to three types of human ProGRP molecules, was synthesized, and a convenient radioimmunoassay system was developed; in this assay, the minimum detectable amount in serum was 10 pg when 0.1 ml of unextracted serum was used. Serum levels of immunoreactive ProGRP (31–98) were measured in 247 normal subjects, 180 patients with nonmalignant pulmonary diseases, 231 patients with non-SCLC, and 140 patients with SCLC. The percentages of subjects with the level greater than 10 pg in normal subjects and patients with nonmalignant pulmonary diseases and with non-SCLC were 1.2, 2.2, and 3.8%, respectively. In contrast, 76% of patients with SCLC had elevated levels; the positive rates in SCLC patients with limited and extensive diseases were 73 and 79%, respectively, indicating that the serum ProGRP (31–98) level could serve as a reliable tumor marker in SCLC patients, even at a relatively early stage of this disease. Moreover, changes in the serum ProGRP (31–98) showed excellent correlation with the therapeutic responses in SCLC patients. These results indicate that the determination of serum ProGRP (31–98) levels plays an important role in the diagnosis and treatment of SCLC patients.

INTRODUCTION

SCLC is an aggressive and rapidly growing neoplasm which tends to be disseminated by the time the diagnosis is made. This cancer, however, has the property of being highly sensitive to systemic chemotherapy; the clinical response rate to combination chemotherapy in untreated SCLC patients is 75–80%, and these modalities can produce up to a 10% 3-year disease-free survival among all SCLC patients (1). Accordingly, a reliable tumor marker could yield valuable information for the treatment of these patients. In some countries, including Japan and some European countries, neuron-specific enolase has been approved for this purpose, but clinical experience has revealed several disadvantages of neuron-specific enolase; these include a lower positive rate in patients with limited disease (defined as tumor confined to the hemithorax or ipsilateral mediastinum), unreliability due to a small difference in values between normal subjects and SCLC patients, and frequent positivity in non-SCLC patients, etc. Therefore, a specific and reliable tumor marker for SCLC patients has long been sought.

GRP is a gut hormone, originally isolated from porcine stomach, with an ability to increase plasma gastrin levels in the dog (2), and now it was revealed that GRP is present in nerve fibers in nonneuronal tissues, brain, and neuroendocrine cells in fetal lung (3–5). We reported previously that SCLC cells produced a large amount of GRP and that plasma GRP levels determined after affinity chromatographic extraction are frequently elevated in SCLC patients (6, 7). However, the method for extracting authentic GRP from plasma required much time, and it was thought to be difficult to use authentic GRP as a routine tumor marker clinically. Holst et al. (8) reported that ProGRP fragment (42–53) immunoreactivity was elevated in the plasma of SCLC patients, but it also required plasma extraction for assay. In the present study, recombinant ProGRP (31–98), a region common to three types of previously cloned human ProGRP molecules (Fig. 1; Ref. 9), was synthesized, and a RIA for this molecule was developed. We found that the determination of ProGRP (31–98) levels in unextracted sera could serve as a specific and reliable tumor marker for SCLC patients.

MATERIALS AND METHODS

Materials. Recombinant human ProGRP (31–98) was synthesized by Tonen Co., Ltd. (Saitama, Japan); the methods of synthesizing this recombinant peptide will be reported elsewhere. Briefly, the chemically synthesized gene encoding the fusion protein of the amino-terminal, 17 amino acids of TrpE protein, and ProGRP (31–98) was expressed in Escherichia coli. The bacterial cells were disrupted enzymatically with lysosome and solubilized in 6 M guanidine-HCl. The soluble fraction was dialyzed against 6 M urea, followed by ion exchange chromatography on a column of Q-Sepharose (Pharmacia P-L Biochemicals, Uppsala, Sweden). The fusion protein was cleaved with cyanogen bromide in formic acid, and ProGRP (31–98) was purified by gel filtration chromatography on a Superdex 75 pg column (Pharmacia P-L Biochemicals). The purity of ProGRP (31–98) was higher than 95% as determined by densitometric analysis after Coomassie blue staining and sodium dodecyl sulfate polyacrylamide gel electrophoresis. After purification of the recombinant protein to homogeneity, peptide sequence analysis revealed that at least the 35 amino-terminal amino acid residues were identical to those of human ProGRP (31–98). A homology search of ProGRP (31–98) for similar sequences was performed by FASTA server (SWISS-PROT protein sequence database, Release 27.0; PIR sequence database, Release 38.0; PRF sequence database, Release 931; translated protein sequence from GenBank, Release 80.0) at the Supercomputer Laboratory, Institute for Chemical Research, Kyoto University (10); the amino acid sequences of ProGRP (31–98) were not homologous to other proteins, except for the rat ProGRP precursor (67% identity in 60 amino acids overlap) and the amphibian (Bombina orientalis) ProGRP precursor (44% identity in 25 amino acids overlap). The other materials purchased were: bovine serum albumin (Cohn Fraction V) from Dai-ichi Pure Chemicals (Osaka, Japan); Sephadex G-75 fine from Pharmacia P-L Biochemicals; and [125I] and [125I]-labeled human albumin from New England Nuclear (Boston, MA).

Subjects. A serum sample file collected at the National Cancer Center Hospital East from 140 consecutive patients with previously untreated SCLC was analysed. In these patients, the stage of disease (limited versus extensive) was decided according to the Veterans Administration Lung Study Group criteria (11). The responses to therapy were evaluated by imaging diagnostic techniques, including plain and tomographic chest X-ray and computed tomographic scan, and then the overall response to systemic chemotherapy was determined by a panel of experts at the National Cancer Center Hospital East.
analyzed according to the World Health Organization criteria (12). From the same institution, serum samples were also obtained from 180 patients with nonmalignant pulmonary diseases and 231 patients with non-SCLC. The nonmalignant pulmonary diseases were pneumonia, pulmonary suppuration, interstitial pneumonia, pulmonary mycotic diseases, pulmonary tuberculosis, pulmonary embolism, bronchial asthma, chronic bronchitis, bronchiectasis, pulmonary sequestration, and sarcoidosis. The non-SCLC patients consisted of 116 with adenocarcinoma, 94 with squamous cell carcinoma, and 21 with large cell carcinoma. Serum samples of 247 normal control subjects were collected at the Health Science Center Foundation (Sagamihara, Kanagawa 228, Japan). Venous blood samples were drawn into tubes and centrifuged at 1,500 × g for 10 min. After centrifugation, the serum samples were stored at −20°C until extraction. Thirteen frozen tissues of SCLC obtained at autopsy were extracted by the boiling water method (6), reconstituted in 1 ml of 1 M acetic acid, and stored at −20°C.

Radioimmunoassay. Recombinant human ProGRP(31–98) was emulsified with an equal volume of complete Freund's adjuvant and used for immunization. Ten guinea pigs were immunized, and one of the antisera (GP- ProGRP-6–2) was used at a final dilution of 1:140,000 in this study. The assay was performed in 0.1 M phosphate buffer (pH 7.4) containing 0.1 M NaCl, 0.06% (v/v) monoethanolamine, and 1.0% bovine serum albumin. Monoethanolamine was used to minimize the adsorption of peptides to the test tubes. Recombinant human ProGRP(31–98) was used as the assay standard, and the results were expressed as the mol equivalent of human ProGRP(31–98). The recombinant human ProGRP(31–98) was radioiodinated by the chloramine-T method (6). The specific activity was 1.9 MBq/nmol (13). One-tenth ml of standard solution containing synthetic ProGRP(31–98) or serum sample was added to 0.4 ml of properly diluted anti-ProGRP(31–98) antisera, followed by 0.2 ml of 125I-ProGRP(31–98) (total count; approximately 2,000 cpm). The mixture incubation was carried out for 48 h at 4°C. After addition of properly diluted goat anti-guinea pig γ globulin antibody (Antibodies, Inc., Davis, CA) without any precipitating agent, the assay tubes were further incubated for 24 h at 4°C. For separating the bound from the free 125I-ProGRP(31–98), assay tubes were centrifuged at 1,500 × g for 30 min at 4°C; then the supernatants were decanted, and the precipitates were counted by a γ counter. The trace bound/total was always ranged from 0.3 to 0.4. The peptide was examined for cross-reaction with other peptides.

Human GRP RIA was performed with a guinea pig antiserum (GP-hGRP 7–3) raised against chemically synthesized human GRP(1–27). This peptide was used as the assay standard and the labeled antigen, and the RIA was carried out as reported previously (6).

**Cold Permeation Chromatography.** A tissue extract solution (0.05 ml) prepared from SCLC (ProGRP concentration, 150,000 pm) and 0.2 ml of serum from each of two patients with SCLC (ProGRP concentration, 4,800 and 5,700 pm, respectively) was chromatographed on a Sephadex G-75 fine column (1.0 × 56.0 cm) which had been equilibrated and eluted with 1 M acetic acid. 125I-human albumin and Na125I were added to the samples, which were then eluted from the column at a speed of 6 ml/h by the fraction collector-pump control system (6). Fractions of 0.8 ml each were collected, lyophilized, and reconstituted in the assay buffer. The column was also calibrated with cytochrome C (M, 12,384), human GRP (31–98) (M, 7,726), human GRP (1–27) (M, 2,859), and human GRP (18–27) (M, 1,121).

**Changes in Serum ProGRP Levels following Treatment.** Ten SCLC patients who achieved CR, 12 patients who achieved PR, and 8 with PD were examined for correlations between changes in serum ProGRP(31–98) levels and responses to therapy. The patients who achieved CR and PR had no prior therapy. Serum samples were obtained sequentially from these patients after treatment, and the serum ProGRP(31–98) levels were measured.

**Statistical Analysis.** For evaluating the ability of serum ProGRP(31–98) levels to predict the diagnosis of SCLC, we calculated sensitivity, specificity, efficiency, predictive value of positive results, and predictive value of negative results, expressing the fractions as percentages (14). The following equations were used:

\[ \text{Sensitivity} = \frac{\text{True-positive}}{\text{True-positive} + \text{false-negative}} \times 100 \]
\[ \text{Specificity} = \frac{\text{True-negative}}{\text{True-negative} + \text{false-positive}} \times 100 \]
\[ \text{Efficiency} = \frac{\text{True-positive} + \text{true-negative}}{\text{True-positive} + \text{true-negative} + \text{false-positive} + \text{false-negative}} \]
\[ \text{Predictive value of positive results} = \frac{\text{True-positive}}{\text{True-positive} + \text{false-positive}} \times 100 \]
\[ \text{Predictive value of negative results} = \frac{\text{True-negative}}{\text{True-negative} + \text{false-negative}} \times 100 \]

True-positive was defined as a high serum ProGRP(31–98) level in a patient with SCLC, and false-positive was defined as a high serum ProGRP(31–98) level in a patient with non-SCLC.

The Student's t test was used to determine the statistical significance between the serum ProGRP(31–98) levels in SCLC patients with limited disease and extensive disease. The relationship between the concentration of GRP (1–27) and ProGRP (31–98) immunoreactive products in patients with SCLC was assessed with the Pearson product-moment correlation analysis.

**RESULTS**

**RIA for ProGRP.** The standard curve of ProGRP(31–98) was linear in a log-log plot at the concentration ranging from 0.001 to 0.016 pmol/tube. When 0.1 ml of unextracted serum was used as a sample, the minimum detectable amount was 10 pm. The coefficients of variation in inter- and intraassays at 0.004 pmol/tube were 6.3% (n = 14) and 8.6% (n = 8), respectively. The dose-response curves of three extracts of SCLC tissues and five serum samples obtained from SCLC patients were parallel to the dose-response curve of ProGRP(31–98), indicating that the active materials present in these samples had the structure immunologically indistinguishable from recombinant ProGRP(31–98). Two GRP-related peptides, GRP and bombesin, and 19 other peptides did not show any significant cross-reactivity (less than 0.01%).

**Serum ProGRP Levels in Lung Cancer Patients.** The serum ProGRP(31–98) levels and the frequency of serum ProGRP(31–98) elevation in normal subjects and patients with nonmalignant pulmonary diseases and lung cancers are shown in Fig. 2. Almost all normal subjects and patients with nonmalignant pulmonary diseases and large cell carcinoma had levels less than 10 pm. ProGRP was detected in only 3% of the patients with squamous cell lung carcinoma and lung adenocarcinoma. On the other hand, the ProGRP(31–98) levels were elevated in 107 of the 140 untreated SCLC patients (76%); the mean level was 588 pm, ranging from 11 to 5,700 pm.

The relation between serum ProGRP(31–98) level and stage of disease in these 140 SCLC patients was investigated. In the 60 patients with limited disease, 44 had elevated levels (73%), and the mean ± SD was 306 ± 725 pm. In the 80 patients with extensive disease, 63 had elevated levels (79%), and the mean ± SD was 785 ± 1,170 pm. There was a significant difference between ProGRP(31–98) levels in these two groups (P = 0.010).

The clinical usefulness of tumor markers could be evaluated by several indicators including sensitivity (true-positive rate in SCLC patients) and specificity (true-negative rate in non-SCLC patients).
When the value of serum ProGRP(31–98) as a tumor marker for SCLC was examined, sensitivity, specificity, efficiency, predictive value of positive results, and predictive value of negative results were 76, 97, 89, 94, and 87%, respectively.

**Gel Permeation Chromatography.** The gel filtration patterns of a tissue extract prepared from SCLC and sera from two patients with SCLC are presented in Fig. 3. In the case of the tissue extract, immunoreactive GRP yielded two peaks, as was reported previously (6); one was a peak compatible with GRP(1–27), and the other was a peak compatible with GRP(18–27). In contrast, immunoreactive ProGRP yielded one major peak, which eluted at a position slightly ahead of that of cytochrome C. Because the molecular size of this peak seemed to be greater than that of ProGRP(31–98) and because, according to data presented below, most molecules of ProGRP were degraded to GRP(1–27) and the COOH-terminal fragments of ProGRP, it is reasonable to speculate that this peak may represent a mixture of ProGRP(31–125), (31–118), and (31–115) (Fig. 1). It is possible to postulate that a small amount of further degraded molecules are also present; Lebacq-verheyden et al. (15) reported that a number of novel peptides were generated by additional cleavages of ProGRP(31–125) in a SCLC cell line.

With respect to the two sera, immunoreactive ProGRP showed one major molecular form similar to that present in the tissue extract.

**GRP and ProGRP Levels in SCLC Tissue Extracts.** The relationship between GRP(1–27) and ProGRP(31–98) in individual tissue extracts obtained from patients with SCLC is shown in Fig. 4. The levels of ProGRP(31–98) were well correlated with those of GRP(1–27) ($r = 0.995; P = 0.0001$), indicating that most of the ProGRP molecules were processed to GRP(1–27) and the COOH-terminal fragment of ProGRP.

**Changes in Serum ProGRP Levels following Treatment.** As shown in Fig. 5, serum ProGRP(31–98) levels were undetectable in all of these patients when the tumor disappeared, and they remained undetectable for 1 month, when the patients were judged to have achieved CR.

In the PR group, serum ProGRP(31–98) levels had decreased to an undetectable range in 4 of the 12 patients (33%), when the patients...
achieved PR. In the remaining eight patients in that group (67%), the serum ProGRP(31–98) levels had decreased, but they were detectable in all of these patients when they achieved PR. PR was defined as a 50% or greater reduction in the sum of the products of the greater and lesser diameters of all measured lesions lasting at least 1 month and an absence of any new lesions.

In eight PD patients, serum ProGRP(31–98) levels were determined and compared with the levels before treatment. Serum ProGRP(31–98) levels had increased at the time of the PD judgment. PD was defined as a 25% or greater increase in the sum of the products of the greater and lesser diameters of all measured lesions or the appearance of new lesions.

DISCUSSION

Amphibian bombesin is a tetradecapeptide found in 1971 in the skin extract of the European frog, Bombina bombina (16). Bombesin was later found to possess gastrin-releasing activity. In 1978, Wharton et al. (17) demonstrated the presence of bombesin in the neuroendocrine cells of the bronchial epithelium of human fetal and neonatal lungs, and following up on this observation, several investigators found that SCLC cells frequently produced a bombesin-like peptide (18–21). In 1983, we claimed that the bombesin-like peptide produced by SCLC cells is GRP rather than bombesin, based on immunological studies (6). GRP is a gut peptide originally isolated by McDonald (2) in 1978 from porcine nonantral gastric tissue as a peptide with gastrin-releasing activity. In 1984, Spindel et al. (22) cloned human GRP complementary DNA from a pulmonary carcinoid tumor, and we demonstrated frequent expression of GRP mRNA in SCLC tissues (23). Very recently, Nagalla et al. (24) reported that frogs have independent genes for both GRP and bombesin. These facts clearly indicated that the bombesin-like peptide produced by SCLC cells is GRP, not bombesin. The present study confirmed this point by the demonstration that SCLC cells produce authentic GRP and the COOH-terminal fragment of ProGRP in an equimolar fashion, indicating that the bombesin-like peptide produced by SCLC cells is actually the product of the GRP gene.

The assay system for ProGRP(31–98) developed in the present study, in which 0.1 ml of unextracted serum was used, demonstrated that serum ProGRP(31–98) levels in SCLC patients were elevated at a frequency of 76%. This frequency is consistent with our previous observations that, when tissue GRP concentrations in a large number of SCLC tumors were determined, 74% of the tumors produced GRP (6) and that plasma GRP levels were elevated in 76% of SCLC patients (7). With respect to the stage of disease in SCLC patients, serum ProGRP(31–98) levels were elevated in SCLC patients with limited disease as well as those with extensive disease at almost the same frequency, indicating that the serum ProGRP(31–98) level could serve as a reliable tumor marker in SCLC patients, even at a relatively early stage of this disease. Moreover, it is worth noting that the mean serum ProGRP(31–98) levels in SCLC patients with limited disease and extensive disease were 31 and 79 times higher than the minimum detectable amount, respectively, indicating that the levels in SCLC patients are remarkably high, when compared with other tumor markers such as neuron-specific enolase. This fact may strengthen the reliability of this tumor marker. Furthermore, changes in the serum ProGRP(31–98) levels showed excellent correlation with the therapeutic responses in SCLC patients. In all SCLC patients who achieved CR, the serum ProGRP(31–98) levels had decreased to an undetectable range at the time the patients were judged to have achieved CR; in one-third of the SCLC patients who achieved PR, serum ProGRP(31–98) levels had decreased to an undetectable range, and in the remaining two-thirds, the levels decreased but not to the undetectable range. In SCLC patients with PD, serum ProGRP(31–98) levels were elevated at the time of the PD judgment.

With regard to specificity of this tumor marker in SCLC patients, serum ProGRP(31–98) levels were specifically elevated in SCLC patients. Although our previous studies revealed that GRP(1–27) was detected in tumor tissue extracts of non-SCLC at the frequency of 17% (6), serum ProGRP(31–98) levels were elevated hardly at all in patients with non-SCLC; the frequency was 3%. Moreover, serum ProGRP(31–98) levels were not elevated in patients with chronic obstructive pulmonary diseases, although previous reports demonstrated the increase of GRP-producing neuroendocrine cells in the lungs in these morbid patients (25, 26). These facts may fortify the role of serum ProGRP determination in lung cancer clinics. Meanwhile, we speculate that GRP-producing neuroendocrine tumors other than SCLC reach morbidity with elevated serum ProGRP(31–98) levels; our previous studies revealed that GRP(1–27) was frequently produced by medullary thyroid carcinomas and pulmonary carcinoid tumors (6, 27).

The determination of serum ProGRP(31–98) levels may open a new door for the diagnosis and treatment of SCLC patients: (a) patients with abnormal chest X-ray compatible with a lung mass could be easily evaluated. Based on a predictive value of positive results in lung cancer patients, a probable lung cancer patient with an elevated serum ProGRP(31–98) level has a 94% chance of having SCLC. This might avoid the necessity for potentially dangerous biopsy procedures in severely ill patients. It is worth noting that the rate of positive serum ProGRP(31–98) was almost equal to or greater than that of sputum cytology (28); (b) SCLC is a cancer having a tendency to metastasize to various organs, and in some patients it is difficult to detect distant metastases and to evaluate the therapeutic response by routine X-ray or other imaging diagnostic techniques. In this context, the serum ProGRP(31–98) levels could yield valuable information for reliable assessment of the therapeutic response in SCLC patients; and (c) our preliminary study of serial determination of the serum ProGRP(31–98) levels in SCLC patients revealed that the determination of serum ProGRP(31–98) levels could serve to detect the recurrence of disease in SCLC patients earlier, which would allow earlier treatments. For these purposes, a quick determination of serum ProGRP(31–98) levels is desirable; the assay system used in the present study takes 5 days for completion and requires radiolabeled materials. To overcome these difficulties, we are now developing a ProGRP(31–98) enzyme immunoassay system for clinical use.
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