ABSTRACT

Gastrin-releasing peptide (GRP) is known to be a bombesin-like peptide present in mammalian tissues. Using GRP radioimmunoassay specific for carboxyl-terminal portion, the immunoreactive GRP (IR-GRP) content of 5 fetal lungs, 38 adult lungs, and 131 primary lung tumors was determined. All fetal lung extracts contained IR-GRP ranging from 31 to 140 ng/g, wet weight. IR-GRP was present in 7 to 21% of normal adult lungs and lung carcinomas other than small-cell carcinoma; the amount was not very large except in two cases of adenocarcinoma, in which 110 and 140 ng/g of IR-GRP were detected. In the case of small-cell carcinoma, IR-GRP was found in 23 of the 31 cases examined (74%), and nine (29%) of these contained large amounts of IR-GRP (100 to 14,000 ng/g). As for carcinoid tumors, IR-GRP was found in five of the 12 cases examined (42%), and large amounts of IR-GRP were detected in two cases (5,100 to 130,000 ng/g). Immunohistochemically, IR-GRP was found in the neuroendocrine cells of fetal lungs and in the tumor cells of primary lung tumors. When these tissue extracts were examined by bombesin radioimmunoassay that recognizes bombesin but not GRP, they did not contain immunoreactive bombesin, suggesting that IR-GRP in these tissues is more similar to GRP than to bombesin.

Sephadex G-50 gel filtration always revealed two peaks of IR-GRP in both fetal lungs and IR-GRP-producing tumors. One was eluted at the position corresponding to that of porcine GRP (Peak 1) and the other, at the position just behind that of porcine GRP (14–27) (Peak 2). In the five fetal lungs, Peak 2 comprised more than 83% of the total IR-GRP. In the 12 IR-GRP-producing tumors examined, the ratio of these two peaks differed from case to case.

Our data indicate that IR-GRP, which is present in fetal lung, is often produced by primary lung tumors, especially by small-cell carcinoma and carcinoid tumor, with molecular size heterogeneity.

INTRODUCTION

It is well established that the most common nonendocrine tumor associated with peptide hormone production is lung carcinoma. Wharton et al. (17) recently demonstrated the presence of IR3-bombesin in the bronchial epithelium of human fetal and neonatal lungs, and there have been a few reports suggesting the production of IR-bombesin by primary lung tumors (4, 11, 14, 18).

Meanwhile, McDonald et al. (9, 10) isolated a peptide with gastrin-releasing activity from porcine nonantral gastric tissue. This peptide was termed PGRP and, because of its similarity in pharmacological effects and homologous amino acid sequence to bombesin (Chart 1), GRP is now considered to be a mammalian counterpart of bombesin (9, 21, 24).

Using a PGRP RIA specific for carboxyl-terminal portion, we have demonstrated that IR-GRP was present in fetal and adult lungs and primary lung tumors. When these tissues were examined by bombesin RIA that recognized bombesin but not PGRP and its fragments, they did not contain IR-bombesin, suggesting that IR-GRP in these tissues is more similar to PGRP than to bombesin. In addition, we determined the incidence of IR-GRP production and its molecular size heterogeneity in fetal and adult lungs and primary lung tumors.

MATERIALS AND METHODS

Five human fetal lungs were obtained from legally aborted fetuses with gestational ages of 16 to 34 weeks. Thirty-eight macroscopically normal adult lungs were lobectomy or pneumonectomy specimens which were obtained at the time of operations for primary lung tumors. One hundred thirty-one primary lung tumor tissues (30 squamous cell carcinomas, 44 adenocarcinomas, 14 large-cell carcinomas, 31 small-cell carcinomas, and 12 carcinoid tumors) were all obtained at surgery, except for 14 of the small-cell carcinomas which were obtained at autopsy. Immediately after removal, the tissues were stored at −80°C until extraction.

PGRP and its fragments, PGRP(14–27), HPP-PGRP(18–27), PGRP(19–27), PGRP(21–27), and PGRP(1–13), were synthesized by the conventional method for peptide synthesis (24). Bombesin, tyrosylated bombesin, bombesin fragments, bombesin(4–14), bombesin(6–14), bombesin(7–14), bombesin(8–14) [≡ PGRP(21–27)], Substance P, and ovine cyclic somatostatin were also synthesized by the methods described previously (21–23).

The other materials purchased were: synthetic AVP from Peptide Research Foundation Co. (Osaka, Japan); bovine albumin Cohn Fraction V from Dai-Ichi Pure Chemicals Co. (Tokyo, Japan); Trasylol (aprotinin) from Bayer Co. (Leverkusen, West Germany); CPG-10 (mesh size, 120/200) from Electro-Nucleonics, Inc. (Fairfield, N. J.); Sephadex G-50 superfine and G-10 from Pharmacia Fine Chemicals AB (Uppsala, Sweden); Bio-Gel p-4 from Bio-Rad Laboratories (Richmond, Va.); and Na125I and 125I-human albumin from New England Nuclear (Boston, Mass.).

Generously supplied as gifts were synthetic ACTH from the
National Pituitary Agency (Baltimore, Md.) and synthetic human calcitonin from Teikoku Hormone Manufacturing Co. (Tokyo, Japan).

RIAs. RIAs used in this study were RIA-specific for the carboxy-terminal portion of PGRP(1-27) (PGRP-C RIA) and RIA-specific for bombesin (bombesin RIA). The standard diluent used in these systems was a solution of 0.1 M phosphate buffer, pH 7.4, containing 0.1 M NaCl, 0.06% (v/v) monochloroacetic acid, and 1% bovine albumin Cohn Fraction V, and Trasylol (250 IU/ml). Monochloroacetic acid was used to minimize the adsorption of peptides to the test tube.

PGRP-C RIA was performed with a rabbit antiserum (R-6903) raised against synthetic PGRP(1-27) (24). Synthetic HPP-PGRP(18-27) was iodinated by the chloramine-T method (7). The labeled peptide was purified by gel filtration on a Sephadex G-10 column, 0.9 x 15 cm, eluted with 0.1 M phosphate buffer, pH 7.4, containing 0.1 M NaCl, 0.06% (v/v) monoethanolamine, 1% bovine albumin Cohn Fraction V, and Trasylol (250 IU/ml). Monoethanolamine was used to minimize the adsorption of peptides to the test tube.

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The specificity of the RIAs was examined with synthetic PGRP, synthetic bombesin, and their fragments. Cross-reaction with the other polypeptides known to be produced often by lung carcinoma was also investigated. These polypeptides included ACTH, AVP, calcitonin, somatostatin, and Substance P.

Tissue Extraction. The frozen tissue (0.2 to 1.0 g) was cut into small pieces with a razor, transferred into 10 ml of boiling water in a glass homogenizer, and kept at 100° C for 5 min in a water bath. After being cooled in an ice bath, the boiled tissue was homogenized. The homogenate was acidified by addition of 1 ml of 5 M acetic acid, and the supernatant was collected after centrifugation at 1500 x g for 30 min at 4° C.

The column was also calibrated with PGRP(18-27), bombesin, and their carboxyl-terminal fragments of PGRP(1-27) and bombesin exhibited significant cross-reactivity. In the bombesin RIA, bombesin(7-14) exhibited 100% cross-reactivity, and bombesin(1-14) and bombesin(6-14) exhibited 2.0% cross-reactivity. In the bombesin RIA, bombesin(1-14) and bombesin(6-14) exhibited 2.0% cross-reactivity. In the bombesin RIA, bombesin(1-14) and bombesin(6-14) exhibited 2.0% cross-reactivity. In the bombesin RIA, bombesin(1-14) and bombesin(6-14) exhibited 2.0% cross-reactivity. In the bombesin RIA, bombesin(1-14) and bombesin(6-14) exhibited 2.0% cross-reactivity. In the bombesin RIA, bombesin(1-14) and bombesin(6-14) exhibited 2.0% cross-reactivity. In the bombesin RIA, bombesin(1-14) and bombesin(6-14) exhibited 2.0% cross-reactivity. 

RESULTS

RIAs. A displacement of more than 2 S.D. of the trace bound/total was always observed when 6.25 pg of PGRP(1-27) (1 pg = 0.356 fmol) were added to the incubation tube for PGRP-C RIA, and 6.25 pg of bombesin (1 pg = 0.617 fmol) were added for bombesin RIA.

Cross-reactions with PGRP(1-27), PGRP(14-27), PGRP(19-27), PGRP(21-27), bombesin(1-14), bombesin(6-14), bombesin(8-14) [= PGRP(21-27)], PGRP(1-13), ACTH, AVP, calcitonin, somatostatin, and Substance P on a molar basis are shown in Chart 2. In the PGRP-C RIA, carboxyl-terminal fragments of PGRP(1-27) and bombesin exhibited significant cross-reactivity. In the bombesin RIA, bombesin(1-14) and bombesin(6-14) reacted equally, but bombesin(8-14), PGRP(1-27), and carboxyl-terminal fragments of PGRP(1-27) cross-reacted only slightly.

The relative immunoreactivity of the peptides presented in Chart 2 and that of HPP-PGRP(18-27), bombesin(4-14), and bombesin(7-14) in these 2 RIAs, determined by the amount of each peptide needed to inhibit the binding of labeled antigen to antiserum by 50%, are shown in Table 1. Considerable cross-reactivity of carboxyl-terminal fragments of PGRP and bombesin was observed in PGRP-C RIA. In the bombesin RIA, bombesin(6-14) and bombesin(7-14) exhibited 100 and 2.2% cross-reactivity, respectively, but bombesin(8-14), PGRP(1-27), and carboxyl-terminal fragments of PGRP(1-27) did not cross-react significantly (less than 0.03%).

Cross-reactions with PGRP(1-13) and with the other peptide hormones at concentrations of up to 100 pmol/tube were not observed in these 2 assay systems.

When PGRP-C RIA was used, the coefficient variations of inter- and intraassay at 50 pg/tube were 14.7% (n = 19) and 7.2% (n = 10), respectively.

Dose-Response and IR-GRP Content of Normal Lungs and
Primary Lung Tumors. The dose-response curves of the extracts of 3 small-cell carcinomas, 2 carcinoid tumors, and 2 fetal lungs in PGRP-C RIA are shown in Chart 3. Since they were parallel to the dose-response curve of PGRP(1-27) in logit-log scale graph, the active materials present in the extracts had the structure immunologically indistinguishable from the carboxyl-terminal portion of PGRP(1-27).

The incidence of IR-GRP and its amount in fetal lungs, adult lungs, and primary lung tumors assayed by PGRP-C RIA are shown in Chart 4. The limit of detectability by PGRP-C RIA was 6.25 pg/tube for PGRP(1-27), so that the minimum amount that could be detected at 2 points was 2.5 ng/g, wet weight, when 1 g of tumor tissue was used as a starting material.

Immunoreactive GRP was present in all fetal lung extracts examined. The quantities were 120 to 140 ng/g, wet weight, in 4 lungs from fetuses of gestational ages of 16 to 24 weeks, and 31 ng/g in the lungs of a 34-week-fetus.

In 34 of the 38 adult lungs, IR-GRP was undetectable. In the remaining 4 cases, the amounts detected were smaller than those in the fetal lungs (2.6 to 20 ng/g, wet weight).

In 73 of the 88 cases of squamous cell carcinoma, adenocarcinoma, and large-cell carcinoma, there was no detectable IR-GRP. In 13 of the other 15 cases, the amount of IR-GRP was less than 100 ng per g, wet weight, and the remaining 2 tumors, which were adenocarcinomas, contained 110 and 140 ng of IR-GRP per g, wet weight, respectively.

In the cases of small-cell carcinoma, IR-GRP was found in 23 of the 31 cases examined (74%). Three of the tumors contained 1,200 to 14,000 ng/g, wet weight, and 6 contained 100 to 960 ng/g, wet weight. In the other 14 cases, the IR-GRP content was 6.9 to 93 ng/g, wet weight, and in only 8 cases was the content less than 100 ng/g, wet weight.
peptide undetectable.

Immunoreactive GRP was found in 5 of the 12 bronchial carcinoid tumors examined (42%), and large amounts were detected in 2 cases (5,100 to 130,000 ng/g, wet weight).

**IR-GRP and IR-Bombesin in Fetal Lungs and Primary Lung Tumors.** The IR-GRP and IR-bombesin contents of the fetal lung and primary lung tumor extracts examined by both PGRP-C RIA and bombesin RIA are shown in Table 2. The limit of detectability by bombesin RIA was 6.25 pg/tube for bombesin(1-14), so that the minimum amount that could be detected at 2 points was 2.5 ng/g, wet weight, similar to that observed in PGRP-C RIA when 1 g of tissue was used as a starting material.

IR-bombesin was undetectable in all of the fetal lungs and in 6 of the 7 tumors. The one tumor in which IR-bombesin was detected contained the largest amount of IR-GRP, but the amount of IR-bombesin was only 0.0083% of that of IR-GRP. These results indicate that IR-GRP in fetal lungs and primary lung tumors is different from bombesin immunologically, suggesting that the active material found in these tissues has an amino acid sequence similar to that of the carboxyl-terminal portion of PGRP rather than that of bombesin.

**Gel Filtration Studies.** The 5 fetal lung and 12 primary lung tumor extracts were subjected to gel filtration. The IR-GRP content of the fetal lungs is shown in Table 2. The quantities in the 12 tumors ranged from 20 to 130,000 ng/g, wet weight, as indicated in the legends for Charts 6 and 7.

Gel filtration studies revealed that IR-GRP in the fetal lung extracts was composed of 2 peaks (Chart 5). A small but definite peak (Peak 1) was eluted at the position corresponding to that of PGRP(1-27) and comprised 5.8 to 17% of the total IR-GRP. In addition, the major IR-GRP peak (Peak 2) was eluted at the position just behind that of PGRP(14-27) or bombesin.

Gel filtration patterns of the 8 tumor extracts of small-cell carcinoma are illustrated in Chart 6. In all cases, 2 peaks corresponding to Peak 1 and Peak 2 were observed. In Cases 1, 2, and 3, the major peak was Peak 2, and the patterns were similar to those of fetal lung; Peak 1 comprised only 11 to 13% of the total IR-GRP in these 3 cases. In Cases 4, 5, 6, and 7, Peak 1 was larger than in Cases 1 to 3, and this peak comprised 22 to 33% of the total IR-GRP. In Case 8, the major peak was Peak 1, which comprised 53% of the total IR-GRP.

Gel filtration patterns of 2 carcinoid tumors and 2 adenocarcinomas are illustrated in Chart 7. The 2 peaks were also observed in all cases. In Cases 9 and 11, Peak 2 was the major one, and Peak 1 comprised 25 and 25% of the total IR-GRP, respectively. However, Peak 1 comprised 47% of the total IR-GRP in Case 10 and 88% in Case 12.

**Immunohistochemical Studies.** In all fetal lungs examined, the cells located basally in the bronchial and bronchiolar epithelium were occasionally stained for IR-GRP (Fig. 1). They were triangular or elongated with slender processes. These cells were similar to argyrophil or neuroendocrine cells in both their location and shape (16).

In the 2 cases of small-cell carcinoma with 270 and 960 ng/g, wet weight, of IR-GRP, positively stained tumor cells were found (Fig. 2). The distribution of staining was irregular, and the immunoreactivity appeared most often as dots and fibrils. In the case of carcinoid tumor with the largest amount of IR-GRP (130,000 ng/g, wet weight), most of the tumor cells were stained (Fig. 3), although the staining intensity varied from cell to cell.

When antigen-preabsorbed antisera and nonimmune serum were used, no positively stained cells were observed in the fetal lung tissues or the tumor tissues.

**Plasma Hormone Levels and Clinical Observations.** In 10
normal subjects, plasma IR-GRP levels were undetectable (less than 62.5 pg/ml). In 5 patients with small-cell carcinoma, whose tumor tissue contained appreciable amounts of IR-GRP (48, 93, 130, 270, and 1200 ng/g, wet weight), plasma IR-GRP levels were also undetectable. Gastric acid secretion studies were not performed in these patients, but none of them complained of symptoms suggesting peptic ulcer.

In addition, the clinical features in 2 patients with small-cell carcinoma and 2 with carcinoid tumor that had been shown to contain more than 1000 ng of IR-GRP per g, wet weight, were examined retrospectively. Although plasma IR-GRP levels were not determined, these patients also did not complain of any symptoms suggesting peptic ulcer.

**DISCUSSION**

In this study, we have demonstrated, by use of PGRP RIA specific for carboxyl-terminal portion, that IR-GRP is present in fetal and adult lungs as well as in primary lung tumors. Gel filtration studies revealed that IR-GRP in these tissues was composed of 2 peaks. Immunohistochemically, IR-GRP was found in the neuroendocrine cells of fetal lung and in the tumor cells of primary lung tumors.

PGRP has a striking structural similarity to bombesin in its carboxyl-terminal decapptide sequence, as shown in Chart 1, and has been considered a mammalian counterpart of bombesin (9, 21, 24). Recently, several studies demonstrated the presence of IR-bombesin in fetal lungs and primary lung tumors (4, 11, 14, 18). We also examined tissue extracts by our own bombesin RIA, but these tissues did not contain any appreciable amounts of IR-bombesin. In the PGRP-C RIA, the carboxyl-terminal fragments of both PGRP and bombesin exhibited significant cross-reactivity, indicating that the antibody recognized the common sequences of these peptides and that the antibody could not discriminate PGRP from bombesin. In the bombesin RIA, however, bombesin(6-14) and bombesin(7-14) exhibited 100 and 2.2% cross-reactivity, but bombesin(8-14), PGRP(1-27), and carboxyl-terminal fragments of PGRP cross-reacted at less than 0.03%, indicating that the asparagine-glutamine residues of bombesin (Positions 6 and 7) are the important immunological determinants. Furthermore, PGRP(19-27) has an amino acid sequence similar to that of bombesin(8-14), except for the glutamine residue (Position 7), but it does not cross-react in this bombesin RIA (less than 0.01%). These results indicate that our bombesin RIA mainly recognizes the glutamine residue at Position 7 of bombesin and that PGRP(1-27) and its carboxyl-terminal fragments did not cross-react in this assay, because the glutamine residue is replaced by histidine in the case of PGRP. Therefore, the fact
that tissue extracts contain appreciable amounts of IR-GRP without detectable amounts of IR-bombesin indicates that the active material found in fetal lungs and primary lung tumors has an amino acid sequence similar to that of PGRP rather than of bombesin.

The presence of IR-bombesin in fetal lungs was first reported by Wharton et al. (17), but its molecular size heterogeneity has not yet been reported. Our data demonstrate that IR-GRP in fetal lung is composed of 2 peaks; Peak 1 was eluted at the position corresponding to that of PGRP(1-27) and Peak 2, at the position just behind that of PGRP(14-27). More than 83% of the total IR-GRP in fetal lung was at Peak 2, and the proportion of these 2 peaks was almost identical in all fetal lungs. We examined the molecular size heterogeneity of IR-GRP in the porcine gastrointestinal tract (stomach mucosa, pancreas, and duodenal muscles) and porcine nerve tissues (cerebral cortex, hypothalamus, and medulla oblongata) (24) and found that the gel filtration patterns of IR-GRP in human fetal lungs are similar to those in the porcine medulla oblongata.

The high incidence and the large amounts of IR-GRP indicate that IR-GRP production is a very common phenomenon in small-cell carcinoma. In addition, our results also indicate that carcinoid tumors have the ability to produce IR-GRP, a fact that was not reported previously. It has been reported that various polypeptide hormones such as ACTH-lipotropins-melanotropins-endorphins, AVP-neurophysin II, somatostatin, and calcitonin are produced by primary lung tumors, especially small-cell carcinoma and carcinoid tumor (12, 13); IR-GRP can be added to these hormones as another marker for characterizing the endocrine nature of these tumors.

As far as the other types of lung carcinoma are concerned, Moody et al. (11) reported that IR-bombesin is present in all cell lines of small-cell carcinoma examined, but not in other types of lung carcinoma cell lines. However, our results show that not only small-cell carcinoma but also other types of lung carcinoma produce IR-GRP, even though the incidence and the amounts produced are not very high. Several polypeptide hormones have been shown to be produced by non-small-cell carcinoma of the lung (1-3, 6), and some investigators consider such a phenomenon to be evidence for the hypothesis that major histological types of lung carcinomas represent neoplastic differentiation within a common cell lineage (2, 3, 5, 25). At any rate, the fact that not only small-cell carcinoma and carcinoid tumor but also non-small-cell carcinoma of the lung can produce IR-GRP suggests that the endocrine nature of the tumor cells does not necessarily correlate exactly with the grade of malignancy or with the morphological differentiation determined by routine histological or histochemical methods.

Gel filtration studies of the tumor tissues as well as of fetal lungs revealed that there were always 2 peaks of IR-GRP. The proportion of these 2 peaks was almost identical in the 5 fetal lungs but, in the tumors, it was different from case to case. In some cases of small-cell carcinoma, carcinoid tumor, and adenocarcinoma, the patterns were similar to those in fetal lungs, but Peak 1 was the main peak in other cases. The gel filtration patterns of peptide hormones produced by tumors were very often different from those in normal tissues, and this molecular size heterogeneity of peptide hormones observed in tumor tissue is considered to be a common feature of peptide hormone-producing tumors (6, 12, 13). We propose that the ratio of Peak 1 to Peak 2 depends on the enzymatic activity that converts Peak 1 to Peak 2, and that the enzyme activity is constant in the neuroendocrine cells of the fetal lung but not in tumor cells. It is also worth noting that Peak 2 is the main peak in the fetal lungs and in many of the tumors; this peak was eluted at a position similar to that of bombesin elution. This finding could be another reason that IR-GRP was thought to be bombesin.

From the clinical standpoint, it is important to examine the relation between clinical symptoms, plasma hormone levels, and hormones produced by the tumor. One of the most important biological activities of PGRP, or of bombesin, is considered to be stimulation of gastrin release with gastric acid hypersecretion (8, 9). However, none of the 4 patients with lung tumors that produced large amounts of IR-GRP (more than 1000 ng/g, wet weight) complained of symptoms due to peptic ulcer. In addition, 5 patients whose lung tumors contained large amounts of IR-GRP had undetectable plasma IR-GRP levels, indicating that plasma IR-GRP levels were not much elevated in these patients. These results suggest that IR-GRP-producing tumors are common among primary lung tumors, but that seldom, if ever, are the IR-GRP-producing tumors associated with elevated plasma levels of IR-GRP with marked clinical symptoms.

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Fig. 1. Immunoperoxidase staining of a fetal lung of the gestational age of 16 weeks. IR-GRP content of the tissue was 140 ng/g, wet weight; 3 cells basally located in the bronchiolar epithelium are stained positively for IR-GRP. × 1040.

Fig. 2. Immunoperoxidase staining of a small-cell carcinoma. IR-GRP content of the tissue was 860 ng/g, wet weight. Cell body of occasional tumor cells is positively stained, but the immunoreactivity appears most often as dots and fibrilis, which may represent the thin cytoplasmic processes of the tumor cells. × 330.

Fig. 3. Immunoperoxidase staining of a peripheral bronchial carcinoid. IR-GRP content of the tissue was 130,000 ng/g, wet weight. Most of the tumor cells are stained positively for IR-GRP, but the staining intensity was very variable. × 530.
GRP Immunoreactivity in Lung Tumors

Fig. 1

Fig. 2

Fig. 3
Production and Molecular Size Heterogeneity of Immunoreactive Gastrinreleasing Peptide in Fetal and Adult Lungs and Primary Lung Tumors

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