Journal of Cellular Biochemistry 40:431–437 (1989)

Effects of Bombesin on Human Small Cell Lung Cancer Cells: Evidence for a Subset of Bombesin Non-Responsive Cell Lines

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The effects of bombesin on three human small cell lung carcinoma cell (SCLC) lines (NCI-H69, NCI-H128, and NCI-H345) have been examined and compared to the effects of the peptide on the mouse fibroblast cell line Swiss 3T3, and the rat pituitary tumor cell line GH3W5. While all three SCLC lines expressed messenger RNA encoding pro-gastrin releasing peptide (GRP), only the NCI-H345 cells expressed detectable membrane receptors for GRP and responded to nanomolar concentrations of bombesin as shown by ^{125}I-GRP binding, total inositol phosphate accumulation, and increased clonal growth in soft agarose. These data show that some SCLC lines are insensitive to bombesin and do not express detectable membrane receptors for GRP.

Key words: growth factor, gastrin releasing peptide, GRP receptor, inositol phosphate, clonal growth

Bombesin, a tetradecapeptide isolated from frog skin [1], exhibits a wide array of biological activities [2]. In particular, it displays mitogenic properties on mouse fibroblasts and human bronchial epithelial cells in vitro [3,4]. This effect of bombesin on fibroblasts is thought to involve binding of bombesin to high-affinity membrane receptors followed by an increase in intracellular phosphatidylinositol (PI) turnover [5,6]. Mammalian counterparts of bombesin have been identified and characterized. These bombesin-like peptides, fragments of a 27-amino-acid-long peptide, gastrin-releasing peptide (GRP), all share with bombesin the same carboxy terminal heptapeptide. This carboxy terminal portion has been shown to be necessary for biological activity of GRP [7–9].

Peptides from the GRP family are produced in human small cell lung cancer (SCLC) cells [10–12]. These peptides have been shown to have mitogenic effects on some SCLC cells in culture [13–15]. Furthermore, monoclonal antibodies against the carboxy terminus of GRP were found to inhibit the in vitro growth of SCLC cells in culture, and to reduce tumor growth in nude mice implanted with SCLC cells [15]. From these data, it has been suggested that bombesin-like peptides from the GRP family

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Received December 27, 1988; accepted March 14, 1989.

can function as autocrine growth factors in SCLC cells [15] through a loop involving release of endogenous bombesin-like peptides that then stimulate cell surface receptors.

In an attempt to characterize further the mechanisms of action of bombesin on SCLC cells, we have examined three SCLC cell lines for expression of pro-GRP mRNA, effects of bombesin on PI turnover, receptor binding, and effects of bombesin on growth of these cells in soft agarose. We show that, while all three cell lines examined express the pro-GRP messenger RNA, only one is responsive to bombesin with respect to PI turnover and clonal growth. Our results suggest that the bombesin-insensitivity of the other two SCLC cell lines may be due to their lack of cell surface receptors.

MATERIALS AND METHODS

Cell Lines

Small cell lung carcinoma (SCLC) lines NCI-H345 (kindly provided by Dr. E. Sausville, NCI–Navy Medical Oncology Branch), NCI-H69, and NCI-H128 (obtained from the American Type Culture Collection, Rockville, MD), were maintained in RPMI 1640 medium supplemented with 30 nM selenium, 5 μg/ml bovine insulin, 10 μg/ml human transferrin, 2 mM glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin (SIT medium). The rat pituitary tumor cell line GH3W5, a subclone from the GH3 cell line (obtained from Dr. Ralph Schwall, Genentech, Inc.), and mouse fibroblast cell line Swiss 3T3 (obtained from the American Type Culture Collection) were maintained in DMEM medium supplemented with fetal calf serum (10%) or calf serum (5%) for GH3W5 or 3T3 cells, respectively; 2 mM glutamine and penicillin and streptomycin as above. All cell lines were maintained at 37°C in 95% air/5% CO_2, in a humidified atmosphere.

Northern Blot Analysis

Total cytoplasmic RNA was isolated by the method of Wong et al. [16]. Briefly, 5×10^6 cells were lysed with 0.5% Nonidet P-40 in 10 mM, Tris/HCl buffer pH 7.5 containing 150 mM NaCl, 2 mM $MgCl_2$, and 10 mM vanadyl ribonucleoside complex (BRL, Bethesda, MD), at 4°C for 2–3 min. Nuclei were removed by centrifugation (13,000 *g*, 15 s) and the supernatants were diluted with 1 volume of 10 mM, Tris/HCl buffer pH 7.5 containing 150 mM NaCl, 5 mM EDTA, and 1% SDS. The RNA was extracted three times with phenol/chloroform (50/50 v/v) then precipitated twice with ethanol. RNA (8 μg) was subjected to electrophoresis through 1.2% agarose (Sea Kem high-gelling temperature, FMC, Philadelphia, PA) gels containing 6% formaldehyde [17], and transferred to nitrocellulose (BA 85, Schleicher and Schuell, Keene, NH) [18]. After 4 h prehybridization, the blots were hybridized in 5 × SSC, 50% formamide for 18 h at 42°C to a ^{32}P-end labelled 83 base pair (bp) synthetic oligomer (10^8 cpm/μg) [19] complementary to bases 125 to 207 of the coding region of human GRP [20]. Blots were washed twice at room temperature in 5 × SSC/0.1% SDS, followed by two washes at 42°C in 0.5 × SSC/0.1% SDS, then exposed with XAR (Kodak) film for 96 h at −70°C with two intensifying screens. The blots were then boiled in 0.1 × SSC/0.1% SDS, and the prehybridization-hybridization steps repeated as described above using a ^{32}P-end labelled β-actin probe (45 bp, 10^8 cpm/μg).

Determination of Total Inositol Phosphates

Swiss 3T3 and GH3W5 cells were plated at 10^4 and 10^5 cells per well, respectively, in 12-well plates. After 48 h, cells were prelabelled for 60 h in fresh maintenance media containing 2 μCi/ml of [^{3}H]-myo-inositol (19.0 Ci/mmole, Amersham, Arlington Heights, IL). Labelled cells were washed three times in assay buffer (HBSS phenol red free, 0.1% BSA, 10 mM LiCl, gassed with 95% air/5% CO_2) at 37°C then incubated in 500 μl of this buffer containing bombesin or other compounds, as described. After 10 min at 37°C, cells were extracted by addition of 50 μl of 5 M $HClO_4$ containing 5 mM diethylenetriaminepentaacetic acid and 1 mM EDTA. After 30 min agitation on ice, extracts were neutralized by addition of 500 μl of 1.5% K_2CO_3 [21]. Cell extracts were vigorously triturated, transferred into 12 × 75 mm polystyrene tubes, and centrifuged at 2,000 *g* for 10 min. Total inositol phosphates (IP) in the supernatant were determined by the method of Berridge et al. [22]. Briefly, 800 μl of supernatant were loaded onto a column packed with 1 ml AG-1X8 dowex resin in the formate form (Bio-Rad, Richmond, CA). After an initial wash with 16 ml of H_2O followed by 16 ml of 60 mM sodium formate containing 5 mM sodium tetraborate, total IP were eluted with 2 × 2 ml fractions of 1.2 M ammonium formate/0.1 M formic acid. Radioactivity in each fraction was counted by liquid scintillation counting. The three SCLC cell lines were prelabelled for 60 h as described above, washed three times by successive addition of 25 ml assay buffer followed by centrifugation at 200 *g* for 10 min, and resuspended after the final wash at a concentration of 8–12 × 10^5 cells/ml in assay buffer. Five hundred μl aliquots of the cell suspensions were transferred with swirling into 12 × 75 mm polystyrene tubes containing appropriate amounts of bombesin or other compounds, as described. Incubations were stopped after 10 min at 37°C by acidification as described above. Total IP were determined as described above.

Binding of ^{125}I-GRP

Specific binding was determined by the method of Moody et al. [23]. SCLC cells were washed three times in phosphate-buffered saline (PBS) at room temperature, and resuspended at a concentration of 10^7 cells/ml in SIT medium containing 0.25% bovine serum albumin (BSA). Cells were transferred in 100 μl aliquots to 1.5 ml eppendorf tubes, equilibrated to 37°C, and 100 μl of ^{125}I-labelled gastrin-releasing peptide (GRP) (Amersham 2126 Ci/mmol) containing approximately 30,000 cpm were added without (total binding) or with (non-specific binding) 1 μM bombesin (Peninsula, Belmont, CA). Binding was stopped after 30 min at 37°C by addition of 800 μl ice-cold PBS containing 0.1% BSA. Cells were pelleted by centrifugation at 13,000 *g* for 1 min at 4°C, and the pellets were washed once with 1 ml of the same buffer before pellet-associated radioactivity was determined in a gamma counter.

Soft Agarose Assay

SCLC cells maintained in SIT medium were washed twice in this medium, and cell density was adjusted to 10^5 cells/ml. One hundred μl of the cell suspensions were added to 2 ml of 0.4% (w/v) agarose (Sea Plaque, FMC Corporation) in SIT medium with or without bombesin, and plated over a hardened baselayer of 3 ml 0.6% agarose in 35 mm wells of 6-well plates. After 3 to 4 weeks, colonies were fixed with 50% methanol for 10 min, allowed to air-dry for 30 min, and stained with Camco Quick Stain

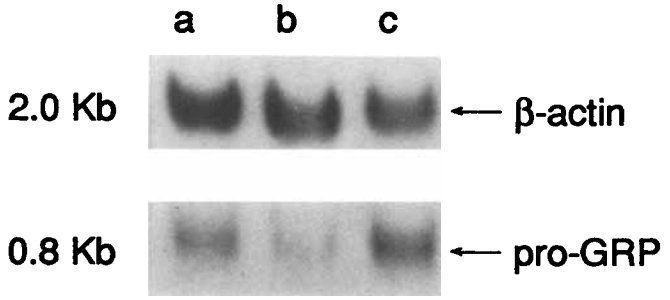

Fig. 1. Northern blot analysis of SCLC cells for pro-GRP messenger RNA. Total cytoplasmic RNA from NCI-H69 (**lane a**), -H128 (**lane b**), and -H345 cells (**lane c**) was extracted from cells lysed with NP-40. Eight μg of RNA was electrophoresed, then blotted onto a nitrocellulose filter. The filter was hybridized sequentially with a pro-GRP and a β-actin radiolabelled oligonucleotide probe.

II (American Scientific Products, Chicago, IL) for 20 s. After destaining with water, colonies were counted using an ARTEK colony counter.

RESULTS

To determine if the messenger RNA encoding pro-GRP is present in the three SCLC cell lines NCI-H69, -H128, and -H345, we conducted northern blot analysis of total cytoplasmic RNA using an 83 bp nucleotide probe for the pro-GRP mRNA [20]. Under high stringency, the pro-GRP probe hybridized to a 0.8 kb RNA band, thus confirming the presence of messenger encoding pro-GRP in all three cell lines (Fig. 1).We did not attempt to quantitate the amounts of pro-GRP mRNA detected. However, the intensity of the hybridization bands as compared to those obtained using a control β-actin probe suggests that NCI-H345 cells transcribe slightly more pro-GRP RNA than do the other two SCLC cell lines (Fig. 1).

In agreement with earlier reports [5], bombesin induced a concentration-dependent stimulation of PI turnover in Swiss 3T3 cells, as demonstrated by an approximately twofold increase in accumulation of total inositol phosphates in these cells in the presence of lithium [24] (Fig. 2). Under the same conditions, bombesin also stimulated PI turnover in GH3W5 cells as well as in the SCLC cells NCI-H345, but did not have any effect on the other two SCLC cells, NCI-H69 and NCI-H128 (Fig. 2). The half-maximal stimulation of PI turnover in Swiss 3T3, GH3W5, and NCI-H345 cells was achieved with concentrations of bombesin in the nanomolar range (Fig. 2), consistent with the known affinity of bombesin for its receptors [23,25,26]. The bombesin receptor antagonist (D-Arg^{1}, D-Pro^{2}, D-Trp^{7,9}, Leu^{11})-substance P [27] prevented this stimulation in the three cell lines (Fig. 3), further suggesting that this effect of bombesin is receptor mediated.

To determine if bombesin receptors are indeed present on the SCLC cells, we studied the binding of ^{125}I-GRP. As shown in Figure 4, specific ^{125}I-GRP binding (i.e., displaceable by 1 μM non-labelled bombesin) could be demonstrated in NCI-H345 cells. In contrast, such specific ^{125}I-GRP binding could not be demonstrated in NCI-H69 cells or NCI-H128 cells (Fig. 4).

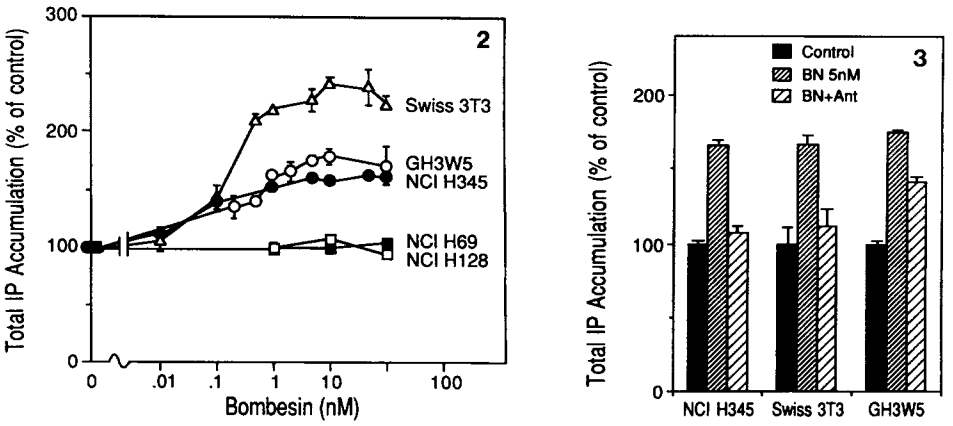

Fig. 2. Effect of bombesin on phosphatidylinositol turnover in mouse 3T3 cells, rat GH3W5 cells, and various SCLC cells. Cells were treated for 10 min with the indicated dose of bombesin in the presence of 10 mM LiCl. Accumulation of total inositol phosphates was measured by ion-exchange chromatography as described in Materials and Methods. Data points (expressed as percent of control) are means +/− s.e.m. of three determinations.

Fig. 3. Effect of a bombesin-receptor antagonist on bombesin-induced increase in phosphatidylinositol in various cells. The bombesin-receptor antagonist (D-Arg^1, D-Pro^2, D-Trp^{7,9}, Leu^{11})-substance P was used at a concentration of 80 μM. Bombesin concentration was 5 nM. Data points (expressed as percent of control) are means +/− s.e.m. of three determinations. Total IP accumulation induced by 5 nM bombesin was significantly different ($P < 0.05$ or better) from control and bombesin + antagonist for all three cell lines.

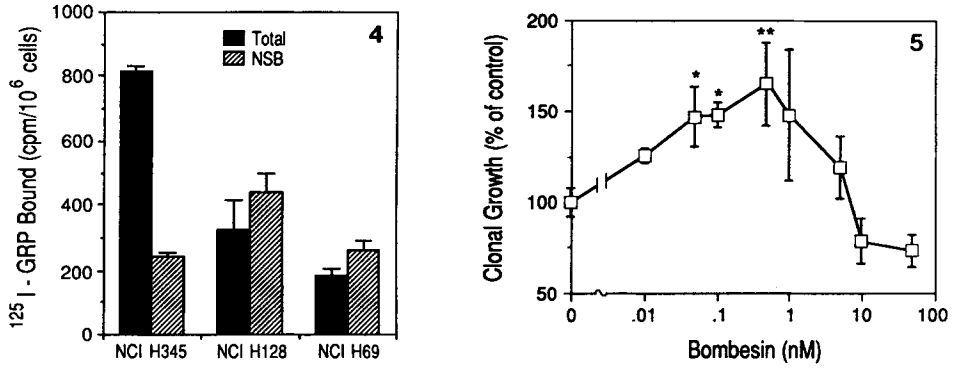

Fig. 4. Binding of ^{125}I-GRP to SCLC cells. Cells were incubated with ^{125}I-GRP in the presence or absence of 1 μM bombesin. After 30 min at 37°C, binding was stopped by addition of 1 ml ice-cold PBS containing 0.1% BSA as described in Materials and Methods. Data are means +/− s.e.m. of triplicate determinations. Bombesin significantly decreased ^{125}I-GRP binding ($P < 0.005$) on NCI-H345 cells only.

Fig. 5. Effects of bombesin on clonal growth of NCI-H345 SCLC cells. Cells were plated in SIT medium containing 0.4% agarose and the indicated doses of bombesin as described in Materials and Methods. The number of colonies after 25 days were counted on an ARTEK colony counter after staining with Giemsa and Wright's stain. Data are means +/− s.e.m. of three or more determinations. Data were analyzed by Student's paired t-test; *$P < 0.05$; **$P < 0.005$, as compared to control.

To assess the functional significance of the presence of GRP receptors on the NCI-H345 cells, we studied the effects of bombesin on their proliferation. As shown in Figure 5, clonal growth over 25 days in soft agarose was significantly increased by up to 65% at low concentrations of bombesin. Higher concentrations of the peptide (5 nM and higher) were without any effect. Such a biphasic response to bombesin has already been observed on other SCLC cell lines [14] and may be due to bombesin receptor desensitization. Growth of the NCI-H69 and NCI-H128 cells was unaffected by bombesin at all concentrations (not shown). Surprisingly, but consistent with a recent report [28], bombesin did not stimulate the proliferation of any of the SCLC cells in liquid culture (data not shown).

DISCUSSION

We have studied the effects of bombesin on three human SCLC cell lines, NCI-H69, -H128, and -H345, as well as on mouse Swiss 3T3 cells and rat pituitary GH3W5 cells. We have shown that, while the three SCLC cell lines contained messenger RNA encoding the mammalian equivalent of bombesin, GRP, only one (NCI-H345) expressed detectable GRP receptors, as shown by binding studies using ^{125}I-GRP, by measurement of intracellular second messenger systems linked to the GRP receptors, as well as the study of the effects of bombesin on clonal growth of these cells.

SCLC cells are known to express high levels of L-dopa decarboxylase, neuron-specific enolase, creatine kinase brain isozyme, and bombesin-like immunoreactivity that was attributed to peptide products from the pro-GRP gene, in particular GRP 18-27 [29,30]. While the functional significance of the presence of the first three proteins is not clear, it has been suggested that bombesin-like peptides act as autocrine growth factors [15]. Thus, clonal growth of SCLC cells could be increased by addition of bombesin, and inhibited by addition of a specific anti-bombesin monoclonal antibody [15].

The mechanism by which bombesin increases clonal growth of SCLC cells is thought to involve binding of the peptide to specific receptors, triggering an increase in intracellular calcium associated with phosphatidylinositol biphosphate hydrolysis and inositol triphosphate production [31]. A similar chain of events has been proposed to occur in 3T3 cells treated with bombesin [5], a potent mitogen for these cells. In the present study, we confirmed the stimulatory effects of bombesin on PI turnover in 3T3 cells. We also found that GH3W5 cells, which express bombesin receptors [25], respond to bombesin through the same pathway. However, while the PI turnover in NCI-H345 cells could be increased by low (nanomolar) concentrations of bombesin, the peptide had no such effects on two other SCLC cells, NCI-H69 and NCI-H128 (Fig. 2). In parallel with this observation, we could detect specific ^{125}I-GRP binding (Fig. 4) and bombesin-stimulation of clonal growth (Fig. 5) on the NCI-H345, but not on the other two SCLC cell lines. These results suggest that bombesin receptors are not expressed on the surface of NCI-H69 and NCI-H128 cells.

Recently, other groups have reported the identification of subsets of SCLC lines based on bombesin-induced increase in intracellular calcium [31] and on ^{125}I-GRP binding [28]. In the first study [31], while bombesin induced a 65% increase in intracellular calcium on NCI-H345 cells, the peptide was without such effect on NCI-H69 and NCI-H128-L cells. Our present results are fully consistent with these data, and they suggest that the lack of effect of bombesin on some SCLC cells may be due to the absence of cell surface bombesin receptors. As a consequence, an autocrine

growth factor loop involving bombesin-like peptide stimulation of cell surface receptors may not apply to all SCLC cells.

Alternatively, SCLC lines maintained in vitro may not be an appropriate model to study this carcinoma. The inherent differences in growth conditions in vitro as compared to in vivo and possible changes in cell phenotype during adaptation to and maintenance in cell culture conditions may change the sensitivity of the cells to bombesin. Thus, as it has been recently suggested [28], the effects of bombesin on human SCLC cells in vitro may not mimic its effects on the carcinoma in vivo.

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