Selective Stimulation of Small Cell Lung Cancer Clonal Growth by Bombesin and Gastrin-releasing Peptide

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ABSTRACT

Human small cell lung cancers (SCLC) produce and secrete the regulatory peptide bombesin (BN) or its mammalian counterpart gastrin-releasing peptide (GRP). In addition, several SCLC tumor lines have been shown to express high affinity receptors for BN/GRP. On the basis of these findings, we investigated the effect of exogenously added BN and GRP on the soft agarose colony growth of a panel of human cell lines. In serum-free defined medium, colony formation of 9 of 10 SCLC cell lines was stimulated up to 150-fold by BN or GRP, with peak colony stimulation observed at 50 nM BN. In contrast, no stimulatory effect of BN was observed on nine non-SCLC cell lines. Although no stimulation of colony growth by BN was seen in serum-supplemented medium, addition of BN to the serum-free medium increased colony efficiency to that achieved by serum in most of the SCLC cell lines. GRP 1-27, the active mammalian analogue of BN, stimulated colony growth of SCLC cells similar to the manner of BN, while the physiologically inactive BN analogue, des-Leu (13)-Met (14)-BN, had no effect on colony growth. No correlation was observed in SCLC cell lines between the response of these cells to exogenous BN and the amount of cellular BN/GRP produced or the presence of BN receptors. These data suggest that BN/GRP may in some instances function as an autocrine growth factor for SCLC and indicate new ways for modulating SCLC growth in patients with this tumor.

INTRODUCTION

Bombesin is a 14-amino acid peptide which was initially isolated from the skin of the frog Bombina bombina (1). In mammals, bombesin-like immunoreactivity has been found predominantly in the brain, lung, and intestine (2-4). More recently, a mammalian analogue of bombesin, GRP, has been isolated from the porcine gastrointestinal tract (5), and the cDNA cloned from a human pulmonary carcinoid tumor (6). GRP is a 27-amino acid peptide with a carboxy-terminal heptapeptide sequence identical to that of amphibian bombesin. In fact, the homologous carboxy-terminal region in both peptides is responsible for receptor recognition, and such similarity in sequence results in common antigens shared by bombesin and GRP as demonstrated by immune cross-reactivity of respective polyclonal antisera (7, 8).

When injected into laboratory animals, both bombesin and GRP elicit similar pharmacological effects suggesting that these two peptides interact with the same cellular receptors. Thus we refer to these as the BN/GRP peptides. BN/GRP peptides bind to a single class of high affinity, saturable receptors which have been demonstrated in the rat brain (3, 9), in guinea pig pan-

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1 The opinions or assertions herein are the private views of the authors and are not to be construed as official or as reflecting the views of the Department of the Navy or the Department of Defense.

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3 The abbreviations used are: GRP, gastrin-releasing peptide; SCLC, small cell lung cancer; BN, bombesin; SSM, serum-supplemented medium (RPMI 1640 supplemented with 10% fetal bovine serum); HITES, RPMI 1640 supplemented with hydrocortisone (10 nm), bovine insulin (5 µg/ml), human transferrin (10 µg/ml), 17β-estradiol (10 nm), and sodium selenite (30 nm).

creatic acinar cells (10), and in cultured rat pituitary cells (11). BN/GRP stimulates gastric, pancreatic, and pituitary hormone secretion including gastrin, prolactin, growth hormone, and insulin (5, 8, 9, 12).

In the human lung, BN/GRP has been found in cells in the bronchial and bronchiolar epithelium (4). While BN/GRP is readily detected in fetal and newborn lung, significant levels are absent in adult lung suggesting that in the pulmonary tree BN/GRP may have an important role in fetal lung function and/or development (13, 14). More recently Ghatei et al. (14) have shown that in lungs from infants with respiratory distress syndrome, bombesin-like immunoreactivity-positive cells are markedly reduced. Supportive evidence for a role of BN/GRP in cell proliferation has been the demonstration that in vivo administration of bombesin will induce gastric hyperplasia in rats (15) and cause pancreatic hyperplasia in humans (16), while in vitro the addition of bombesin to cultures of mouse 3T3 cells or human bronchial epithelial cells will increase thymidine incorporation and cell number (17, 18).

Several laboratories, including our own, have demonstrated that the majority of human SCLC produce BN/GRP (19-24). In addition to immunohistochemical studies, biochemical characterization shows the bombesin-like immunoreactivity to be similar to GRP (19, 24). We have also demonstrated that some SCLC cell lines express a single class of high affinity, saturable binding receptors for BN/GRP that are similar if not identical to those previously demonstrated on nontumorous tissue (25). Finally, depolarizing stimuli will cause the release of BN/GRP from these SCLC tumor cells in vitro (26). In contrast to SCLC cells, BN/GRP or BN/GRP receptors have not been demonstrated in cell lines of other forms of lung cancers or in a variety of other non-amine precursor uptake decarboxylation human tumor cell lines (25). Because of the production of BN/GRP, the identification of BN/GRP receptors in SCLC cells, and the role of BN/GRP as a putative growth-stimulatory agent of the normal fetal lung and gut, it is reasonable to test the hypothesis that BN/GRP can serve as a growth factor for SCLC. To this end, we evaluated the effects of BN/GRP on the in vitro growth of SCLC and other human tumor cells using a soft agarose clonogenic assay. We conclude from these studies that BN/GRP is a potent stimulator of the clonal growth of human SCLC.

MATERIALS AND METHODS

Cell Lines. Methods used for the isolation, growth, and characterization of the tumor cell lines used in this study have been described previously (27-33). In culture SCLC cells grow as floating cells aggregated in contrast to non-SCLC cell lines which grow as adherent monolayer cultures. SCLC cells express elevated levels of L-dopa decarboxylase (29), neuron specific enolase (30), the BB isozyme of creatine kinase (31), and BN/GRP. Bombesin/GRP and BN/GRP receptor assays on cell line lysates were performed as described previously (19, 25). All cell lines tested express human isozymes, form colonies in soft agarose, and cause tumors in athymic nude mice with a histology similar to that of the tumor specimen of origin. The cell
lines have been in continuous culture for periods ranging from 6 months to 6 years and were free of Mycoplasma contamination as determined by Microbiological Associates, Bethesda, MD.

Growth Studies. The influence of hormones on the growth of cell lines was tested using a soft agarose clonogenic assay as described previously (28, 32). Briefly, a single cell suspension of cells harvested in log-phase growth and washed twice in serum-free medium immediately prior to assay to remove any residual serum was mixed with 0.3% (w/v) agarose (SeaKem, LE; FMC Corporation, Marine Colloids Div., Rockland, ME) in culture medium with or without hormone addition and plated over a base layer of 0.5% agarose and culture medium that had hardened in 35-mm Petri dishes. Plates were initially checked to determine that only single cells had been plated and were observed biweekly for colony formation. The number of cells plated (10⁴) was chosen so that in serum-supplemented medium between 200 and 500 colonies would be obtained in each dish. After 14 days, when colony size and viability was optimal, plates were scored for colony growth (cell aggregates of greater than 50 cells) using an inverted phase microscope. All studies were done in triplicate and each point represents the mean colony count. For all studies the standard error for each test was ±10%.

Studies for the effects of hormones on growth were carried out in both SSM and serum-free chemically defined HITES medium (28, 33). These factors were obtained from Sigma and prepared for use as described previously (29, 33, 34). Synthetic BN and GRP were obtained from Peninsula Laboratories (Belmont, CA). Des-Leu¹⁵-Met¹⁶-BN was supplied by Dr. J. Rivier, Salk Institute. The peptides were dissolved directly in HITES medium immediately before use and sterilized by filtration (0.22-μM Nalgene filter).

RESULTS

Previously we have shown that SCLC cell lines grow in mass liquid culture and clone (with an efficiency of 1–5%) in soft agarose in SSM (27). In addition, we have shown that many SCLC cell lines grew in liquid mass culture in defined HITES medium at a rate comparable to that observed in SSM and could be maintained continuously in this medium (28, 33). However, in HITES medium, the tumor cells either failed to clone or cloned at efficiencies 10–30-fold less than that observed in SSM (Table 1) (28). When synthetic BN¹¹–¹⁴ was added to the HITES medium dramatic stimulation of tumor cell colony growth for SCLC was seen (Fig. 1). A series of dose-response curves for different SCLC lines showed that optimal colony stimulatory effects were seen at an exogenously added BN concentration of 50 nM (Fig. 2). At a 10-fold higher concentration of BN (500 nM) significant reduction of SCLC growth was noted. In contrast, a lung adenocarcinoma cell line (NCI-H125) that does not produce BN did not have clonal growth stimulated over a similar concentration range of exogenous BN (Fig. 2). For subsequent studies test concentrations of 10–50 nM BN were used.

The influence of 50 nM BN on the colony growth of 10 SCLC cell lines from different patients and 9 non-SCLC cell lines including 7 lung cancer lines and 2 malignant melanoma lines was evaluated. Tests were carried out in both SSM and serum-free HITES (Table 1). Although all tumor cell lines formed colonies in soft agarose in SSM with a colony-forming efficiency ranging from 1 to 10%, the addition of BN in the presence of serum had little or no effect on colony growth for any cell type. Similar observations were made using BN over a concentration range of 1–1000 nM (data not shown). However, in serum-free HITES medium, colony formation of 9 of 10 SCLC lines was stimulated 7–150-fold over that observed in control cultures (Table 1). The number of colonies formed in HITES medium supplemented with 50 nM BN was in most cases 0.4 to 1.2 times the number scored in serum-supplemented medium. In one case no BN stimulation was observed (NCI-H82) while in another (NCI-N691) colony formation was observed only in HITES medium with BN supplementation (Table 1). No obvious correlation was observed in these SCLC cell lines between in vitro responses to BN, the production of BN by the cell lines, and the presence of cell surface receptors for BN. In contrast to SCLC cultures, no increase in colony formation was observed for 9 non-SCLC cell lines when tested in either SSM or serum-free HITES medium (Table 1).

The effect of GRP¹¹–²⁷ on the cloning of SCLC cells was tested at a concentration of 10 nM and compared to BN at an equimolar concentration of 50 nM (Fig. 2). At a 10-fold higher concentration of BN (500 nM) significant reduction of SCLC growth was noted. In contrast, a lung adenocarcinoma cell line (NCI-H125) that does not produce BN did not have clonal growth stimulated over a similar concentration range of exogenous BN (Fig. 2). For subsequent studies test concentrations of 10–50 nM BN were used.

DISCUSSION

In this paper we report that bombesin and GRP are potent stimulators, up to several hundred fold, for the in vitro clonal growth of established SCLC cell lines. The peptides stimulated SCLC at peak concentrations of 10–50 nM with effects observed in the presence of serum-free chemically defined medium. Under the same growth conditions, BN had little or no effect on the clonal growth of 9 non-SCLC cell lines, including other lung cancer cell lines. GRP, a 27-amino acid peptide with an
SCLC STIMULATION BY BOMBESIN AND GRP

Fig. 1. In vitro soft agarose colony growth of cell line NCI-H249 in serum-free HITES medium without (A or C) and with (B or D) 50 nM BN supplementation.

MO
ÜJ 200
U
100
S
3
8
8
Small Cell Lung Cancer
o—D NCI-N417
a a NCI-N691
o o NCI-H148
IMCI-H249
Decarciinoma of the Lung
. . NCI-H128
A
5 10 50 100
BOMBESIN CONCENTRATION InMI
243
500 1000
40
Fig. 2. Dose-response curve of BN effects on the clonal growth of four SCLC cell lines and a single non-SCLC cell line.

Table 2 Influence of BN, GRP, and des-Leu13-Met14-BN on the clonal growth of SCLC cell lines

<table>
<thead>
<tr>
<th>Growth media</th>
<th>NCI-H510</th>
<th>NCI-H345</th>
<th>NCI-H526</th>
</tr>
</thead>
<tbody>
<tr>
<td>HITES</td>
<td>62 (1)</td>
<td>52 (1)</td>
<td>50 (1)</td>
</tr>
<tr>
<td>HITES + 10 nM BN</td>
<td>720 (12)</td>
<td>380 (7)</td>
<td>640 (13)</td>
</tr>
<tr>
<td>HITES + 10 nM GRP</td>
<td>742 (12)</td>
<td>410 (8)</td>
<td>600 (13)</td>
</tr>
<tr>
<td>HITES + 10 nM BN + 10 nM GRP</td>
<td>820 (13)</td>
<td>450 (9)</td>
<td>664 (13)</td>
</tr>
<tr>
<td>HITES + 10 nM des-Leu13-Met14-BN</td>
<td>44 (0.7)</td>
<td>44 (0.8)</td>
<td>72 (1.4)</td>
</tr>
</tbody>
</table>

* Numbers in parentheses, fold stimulation observed above HITES medium alone.

affinity for BN receptors similar to that for BN alone (11), also had a similar stimulatory affect for SCLC. In contrast, des-Leu13-Met14-BN, a BN analogue with poor affinity for BN receptors, had little or no effect on the clonal growth of SCLC cells. The addition of both BN and GRP at equimolar concentrations together did not significantly stimulate clonal growth above that observed with either BN or GRP alone suggesting that both BN and GRP mediate their effects in an identical manner. Independently, Weber et al. (34) have shown that tritiated thymidine incorporation and cell number in mass culture are stimulated in two SCLC lines by GRP1_27 and GRP1_4_27 but not by GRP1_16 while no effect was seen on two non-SCLC lines. Taken together these data are consistent with the hypothesis that BN/GRP, a peptide produced and secreted by the majority of SCLC cell lines, and for which some SCLC cells have high affinity binding receptors, may function as an autocrine growth factor for the tumor in vivo.

In recent years considerable advances have been made in the establishment of SCLC cell lines using serum-free defined HITES medium (28, 33). The use of this medium, in a recent study, supported the establishment of continuous SCLC lines.
in 72% of 41 fresh clinical specimens. As the mitogenic effect of BN was observed only in serum-free medium it is possible that the endogenous production of BN and its constant release into culture medium, coupled with its growth promoting effects, may contribute to the relative ease at which continuous SCLC cell lines can be established directly from patient biopsies in serum-free medium (27). The lack of a BN/GRP clonal stimulation in serum-supplemented medium could result because BN/GRP are destroyed by peptidases in serum, bind to serum proteins, and become pharmacologically unreactive or that surface receptors for BN are masked or bypassed by other factors in serum (e.g., all possible clonogenic tumor cells were stimulated by other serum factors). No obvious correlation was observed between the in vitro response to BN and the amount of BN production by the cells or the presence of specific BN receptors on these SCLC cells. It is possible that the growth-promoting effects of BN may be mediated through mechanisms other than binding to specific receptors. It is also possible that only a small fraction of these cells possess BN receptors and are thus responsive to exogenous BN but would not be detected in the receptor assay. In addition, the sensitivity of the BN receptor assay may be such that when the whole cell population is assayed, the small fraction of BN receptor-positive cells would not be detected. Finally, like other receptors for polypeptide hormones, the BN receptor may exhibit ligand-induced “down regulation” of cell surface BN binding capacity through the constant release of BN into the culture medium of these cell lines (5). In studies of isolated pancreatic acini, preincubation of cells with BN induced desensitization of enzyme secretion by reducing the number of active receptors available to interact with BN and stimulate enzyme secretion (10). BN has been detected in mammalian cells of the brain, lung, and intestine (2, 3, 4, 13). The many physiological effects observed after the systemic injection of BN or the injection of BN into the central nervous system are thought to be mediated through the binding of BN with high affinity to specific surface membrane receptors (2, 9, 10). In fetal lung, using immunohistochemical staining techniques, abundant BN-positive cells can be demonstrated which persist into early childhood and then decrease in number in the adult (14). In infants with respiratory distress syndrome, the number of BN-positive cells is significantly diminished (14). These data in conjunction with our results and the in vivo rat models (15) suggest a role for BN in the proliferation and maturation of mammalian cells. However, because BN/GRP stimulate the release of many hormones including gastrin, insulin, growth hormone, glucagon, prolactin, and thyrotropin-releasing hormone, it is possible that some of the observed in vivo effects of BN are due to secondary release of these hormones following binding of BN to its respective cell surface receptors (8).

In conclusion this study demonstrated that BN/GRP is a potent stimulator of the clonal growth of SCLC in vitro and implicates BN/GRP as having an important role in the growth of SCLC cells. Because the data suggest that in some instances BN may function as an “autocrine growth factor” role for BN/GRP in SCLC cells, the development of BN antagonists or antibodies to BN may have important therapeutic application in the treatment of patients with small cell lung cancer. In fact, in a study reported elsewhere (35), a monoclonal antibody specific for the carboxy-terminal region of BN markedly inhibited both the in vitro clonal growth of SCLC lines and the growth of xenografts of these cells in nude mice. Such data further support the role for BN as an autocrine growth factor for SCLC.

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