Inhibition of Neuropeptide-stimulated Tyrosine Phosphorylation and Tyrosine Kinase Activity Stimulates Apoptosis in Small Cell Lung Cancer Cells

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ABSTRACT

Small cell lung cancer (SCLC) cell growth is sustained by multiple autocrine and paracrine growth loops involving neuropeptides. The bombesin family of peptides are autocrine growth factors in H345 SCLC cells and provide a paradigm for the study of growth factors and mitogenic signaling in SCLC cells. We show that bombesin (and other neuropeptides) stimulates protein tyrosine phosphorylation (particularly focal adhesion kinase) and protein tyrosine kinase (PTK) activity in intact SCLC cells. Furthermore, the broad spectrum neuropeptide receptor antagonist [D-Arg', D-Phe5, D-Trp7',9, Leu'4]substance P inhibits all neuropeptide-mediated signals (including PTK activation), SCLC cell growth in vivo and in vitro, and also increases the natural rate of apoptosis seen in growing SCLC cell lines. Hence the effect of selective PTK inhibition on SCLC cell growth and apoptosis was examined. We show that selective inhibition of PTK activity, with genistein and (3,4,5-tri-hydroxyphenyl)-methylene(-propanedinitrile) tyrphostin-25 inhibits basal and neuropeptide-stimulated SCLC cell growth. Genistein and tyrphostin-25 also stimulate apoptosis in SCLC cells. Inhibition of proliferation in these cells is intimately linked to apoptosis, because these changes occurred without any effect on SCLC cell cycle kinetics, suggesting that apoptosis occurs independently of the cell cycle and that failure to progress through the cell cycle results in apoptosis. Because tyrphostin-25 fails to influence p53 or Bcl-2 expression in these cells, this mode of programmed cell death appears to be via a p53- and Bcl-2-independent mechanism. These results provide evidence that tyrosine phosphorylation is a mitogenic signal in SCLC cells and suggest that regulation of the level of protein tyrosine phosphorylation represents a critical determinant of whether SCLC cells survive and proliferate or die by apoptosis. Thus, PTK inhibition may provide a novel therapeutic option in SCLC that has become resistant to conventional chemotherapeutic agents.

INTRODUCTION

Lung cancer is the most common fatal malignancy in the developed world. SCLC constitutes 25% of all lung cancers and follows an aggressive clinical course. In spite of initial sensitivity to radio- and chemotherapy, the 2-year survival of patients with SCLC remains less than 5% (1). Thus, novel therapeutic strategies are needed, and it is most likely that these strategies will arise from a better understanding of the cell biology of SCLC cells.

One of the most striking manifestations of the neuroendocrine phenotype of SCLC cells is the elaboration of a large number of peptide hormones (2). The first of these to be identified in SCLC was GRP (3). GRP is the principal mammalian counterpart of bombesin, a 14-amino acid peptide, that serves as the prototype for a large family of naturally occurring bombesin-like peptides containing a highly conserved NH2-terminal heptapeptide region. Bombesin has a near identical COOH-terminal structure to GRP and binds with high affinity to the GRP receptor, with both peptides displaying identical biological activity (4). Demonstration of pre-pro GRP mRNA, protobombesin COOH-terminal peptide, and multiple GRP gene-associated peptides in SCLC confirms that GRP and its related peptides originate in these tumors (5—7). In addition to secreting bombesin-like peptides, SCLC also exhibit cell surface receptors (1—3 × 107/cell) for GRP, as demonstrated by the specific binding of [125I-Tyr4]bombesin to SCLC, suggesting that GRP could also act as an autocrine growth factor (8).

The hypothesis of autocrine growth stimulation by GRP in SCLC was first tested by Cutitta et al. (9), using a monoclonal antibody to [Lys3]bombesin (2A11). Under serum-free conditions, this antibody inhibited the clonal growth of two SCLC cell lines (including H345 cells) and retarded the growth of SCLC xenographs in nude mice. The specific bombesin/GRP receptor antagonist, [Leu'4-α(CH2)NH-Leu'5]bombesin, has been shown to inhibit basal colony growth of H345 SCLC cells grown in semisolid medium (10). These results strengthened the hypothesis that GRP is an autocrine growth factor for SCLC cells.

Our previous work has shown that in addition to GRP, a variety of other neuropeptides, including bradykinin, gastrin, vasopressin, cholecystokinin, and neurotensin, also promote a rise in intracellular calcium (Ca2+) and clonal growth in SCLC cells (11). Consequently, it appears that the autocrine growth loop of bombesin-like peptides may be only part of an extensive network of autocrine and paracrine interactions involving a variety of Ca2+-mobilizing neuropeptides in many SCLC cells. This conclusion is supported by the demonstration that the “broad-spectrum” neuropeptide receptor antagonist [D-Arg', D-Phe5, D-Trp7',9, Leu'4]substance P can inhibit SCLC cell growth in vitro and in vivo (12, 13).

The cellular and molecular responses elicited by GRP in H345 cells hence provide an excellent model for studying the mechanisms underlying neuropeptide-stimulated SCLC growth. Studies undertaken in metabolically labeled whole-cell and membranous preparations demonstrate that the GRP receptor in SCLC cells is coupled to PLC (14); hence, activation is accompanied by an elevation of intracellular Ca2+ (15). Because non-hydrolyzable GTP analogues could modulate PLC activation in response to GRP, it was concluded that this was a heterotrimeric G-protein-coupled response (14, 16). The GRP receptor has since been cloned from H345 SCLC cells and shown to belong to the family of GTP-binding, protein-linked receptors with seven hydrophobic domains (17) and as such does not display intrinsic tyrosine kinase activity. Despite the importance of tyrosine phosphorylation in the action of many mitogens and oncogenes, the role of tyrosine kinase activation in GRP-mediated SCLC growth remains unknown.

An important consideration in the development of anti-cancer agents that target mitogenic signals is whether their action results in cytostasis or cytotoxicity. Several studies have now shown that withdrawal of growth factors from both normal and tumor cells results in a specific and highly organized form of cell death termed apoptosis (18—22). Cancer may be considered a disease resulting from not only...
abnormal cell proliferation but also from dysfunctional cell death (apoptosis). The mechanisms that regulate apoptosis remain poorly understood. Recently, a preliminary report has indicated that [d-Arg5, d-Phe8, d-Trp7,9, Leu11]substance P can induce apoptosis in a variety of lung cancer cell lines, although the mechanism for any such effects remains unclear (23).

In view of the fact that tyrosine phosphorylation plays a major role in signal transduction through most cell surface receptors and oncogenes, such a protein modification may also play an important role in the mechanism of apoptosis. However, recent studies are contradictory: inhibition of tyrosine kinases leads to apoptosis in HL60 and M07E cells (24). On the other hand, tyrosine phosphorylation appears to be a major trigger for apoptosis following ionizing radiation, and in this model, protein kinase inhibitors are protective (25). In addition, tyrosine kinase activation provides an early and requisite signal for Fas-induced apoptosis in susceptible cell lines (26) and α-IFN-stimulated apoptosis in acute lymphoblastic leukaemia cells (27). Induction of apoptosis by cross-linking of membrane immunoglobulin receptors is dependent on PTK activity (particularly btk) in a B-cell lymphoma model (28).

SCILC cells have been shown to express relatively high levels of bcl-2 (29). High levels of expression of Bcl-2 prevent cell death in response to a wide variety of cellular stress and cytotoxic agents (30). Hence, the P-glycoprotein-independent multidrug resistance of SCILC cells may be, at least in part, due to high levels of Bcl-2. Little is known about the mechanisms regulating Bcl-2 expression. In an interleukin 3-dependent murine myeloid cell line, it has been shown that PTKs can regulate Bcl-2 levels (31). In addition, p53 down-regulates bcl-2 expression (32, 33). Genotoxic agents cause p53 accumulation in the nucleus, leading either to transient cell cycle arrest or apoptosis (34). This may in part involve an enzymatic cascade involving mitogen-activated protein kinases (known to activate c-jun in response to irradiation), which phosphorylate p53 at specific residues (35).

In this study, we have investigated the ability of bombesin (and other neuropeptides growth factors) to stimulate protein tyrosine phosphorylation and PTK activity in SCILC cells and subsequently tested the effect of selective PTK inhibition on SCILC cell growth and apoptosis. The relationship between Bcl-2 and p53 expression, PTK phosphorylation and PTK activity in SCILC cells and subsequently determined using an EPICS Profile II (Coulter Electronics, Luton, United Kingdom). For determination of cell cycle phase, SCILC cells were washed twice in PBS and fixed in 70% ice-cold ethanol; then their DNA content was determined by staining with 50 μg/ml propidium iodide. The proportion of cells in G1, S, and G2-M phases (minimum of 2000 cells analyzed per condition) was calculated using standard methods.

**MATERIALS AND METHODS**

**Cell Culture.** Stock SCILC cells were cultured in RPMI 1640 containing 25 mm HEPES (Life Technologies, Paisley, United Kingdom) and supplemented with 10% (v/v) FCS (heat-inactivated at 56°C for 50 min), 50 units/ml penicillin, 50 μg/ml streptomycin, and 5 μg/ml L-glutamine. Cultures were maintained in SITA medium (25-cm² flasks) in the presence or absence of tyrphostin-25. Cells were centrifuged onto glass slides, fixed with methanol, stained with May-Grunwald-Giemsa stain, and examined using a Olympus BH-2 microscope at a magnification of ×400. Apoptotic cells display typical morphological features, including shrinkage and chromatin condensation. The percentage of SCILC cells undergoing apoptosis was also assessed by acridine orange staining and fluorescent microscopy as described previously (36).

**DNA Fragmentation Analysis.** SCILC cells were cultured in SITA medium for 12—72 h at 37°C in the presence or absence of 25 μM tyrphostin-25. Cells were centrifuged at 13,000 × g for 1 min, the supernatant was removed, and the pellet was lysed in 0.5 ml 6 M guanidinium hydrochloride, 25 mM Tris-HCl (pH 8.0) and 0.5% (v/v) sodium dodecyl sulfate (SDS), and DNA was extracted with an equal volume of phenol/chloroform (5:1 v/v) buffer with an equal volume of 100 mM Tris-HCl, pH 8.0, and the resulting emulsion was centrifuged at 13,000 × g for 2 min. The upper DNA-containing phase was removed, and 0.1 volume of 3 M sodium acetate was added with an equal volume of 2-propanol to precipitate the DNA. The DNA was then pelleted by centrifugation at 13,000 × g for 10 min, and the resulting pellet was washed twice with 2-propanol. The remaining 2-propanol was allowed to evaporate at room temperature. The pellet was resuspended in 50 μl 10 mM Tris-HCl and 1 mM EDTA (pH 8.0) containing 50 μg DNA-free RNase A for 30 min at 37°C. The final samples were mixed with 0.2 volume of loading buffer [10% (v/v) Ficoll 400, 10% (v/v) glycerol, and 0.1% (v/v) bromophenol blue in 89 mM Tris-HCl, 89 mM NaCl, and 10 mM EDTA, pH 8.0]. The DNA fragments were resolved in a 1.8% agarose gel containing 5 μg/ml (v/v) ethidium bromide and viewed under UV light.

**Immunoprecipitation.** SCILC cells (2 × 10⁶ cells) in 0.5 ml were quiesced in serum-free medium for 18—30 h prior to use. Cells were centrifuged (5 × 10⁵) were cultured in SITA in the presence or absence of tyrphostin-25 (25 μM) for 48 h. Cells were centrifuged onto glass slides, fixed with methanol, stained with May-Grunwald-Giemsa stain, and examined using an Olympus BH-2 microscope at a magnification of ×400. Apoptotic cells display typical morphological features, including shrinkage and chromatin condensation. The percentage of SCILC cells undergoing apoptosis was also assessed by acridine orange staining and fluorescent microscopy as described previously (36).

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**Cell lysis.** Cell lysates were incubated with 2 μg anti-p125 Estr or anti-phosphotyrosine antibody to phosphotyrosine PY 20 mA for 3 h at 4°C. Protein A-agarose conjugated to rabbit antiserum IgG was added [40 μl/fraction of 50% (w/v) slurry] and samples incubated for a further 1—2 h at 4°C. Samples were then washed three times with ice-cold lysis buffer and were extracted for 10 min in boiling SDS-PAGE sample buffer [63 mM Tris-HCl (pH 6.8), 2 mM Na₂P₂O₇, 100 mM DTT, 5 μM urea, 5 mM EDTA, 5% (v/v) glycerol, and 0.1% (v/v) SDS] and analyzed by SDS-PAGE and Western blotting.

**Western Blotting.** Cell lysates were run in SDS-PAGE sample buffer containing 2 mM Na₂P₂O₇. Samples were boiled for 3 min, and DNA was sheared by several passes through a 26-gauge needle. Polyepitides were resolved by 10% SDS-PAGE and then transferred to nitrocellulose (Hybond C; Amersham Corp., Buckinghamshire, United Kingdom).

Membranes were blocked using 3% (v/v) BSA in TBST-Were 20 (20 mM Tris-HCl (pH 7.4), 150 mM NaCl, 0.05% (v/v) Tween 20) for 3 h at room temperature and incubated with PY 20 (1:5000). Immunoreactive bands were identified using horseradish peroxidase-conjugated goat antimouse IgG.
specific interaction was visualized using ECL (Amersham) as per manufacturer’s instructions.

**Tyrosine Kinase Assay**

Phosphorylation of a broad specificity peptide substrate was performed using a nonradioactive tyrosine kinase assay kit (Boehringer Mannheim, Lewes, Sussex, United Kingdom). The peptide sequence corresponds to the amino acid sequence 1–17 of gastrin 2 (Bi-EGPWL (E8), AYGWMDF-NH2) biotinylated at the NH2-terminal glutamate and blocked at the COOH-terminal by an amido group.

**Immunocytochemistry**

SCLC cells, 3–5 days after passage, were washed and resuspended in SITA at a density of 5 × 10⁶ cells/ml in the presence 25 μM tyrphostin-25 or 50 μM genistein as indicated. At 48 h, cells (1 × 10⁶) were transferred into round-bottomed 96-well polypropylene plates, permeabilized and fixed with 0.01% (v/v) paraformaldehyde in PBS containing 3.7% (v/v) formaldehyde (50 μl) for 15 min at 37°C (37). The cells were washed twice with PBS containing 0.5% (w/v) BSA and then labeled with saturating concentrations of primary antibody for 1 h at 4°C. The cells were washed with PBS/0.5% (w/v) BSA before the addition of FITC-conjugated secondary antibody (1 h at 4°C). The cells were then analyzed using an EPICS Profile II.

Saturating concentrations of antibodies were determined by titration. The mouse antihuman Bcl-2 mAb was titrated against HL60 cells, and the mouse antihuman p53 mAb was titrated against irradiated embryonic stem cells. Controls included a mouse mAb isotype control (MOPC) and omission of the primary antibody (negative controls).

**Materials**

SCLC cell lines NCI-H345 and -HS10 were purchased from the American Type Culture Collection (Rockville, MD). RPMI 1640 and culture medium was obtained from Life Technologies, Inc. Bombesin, gastrin, [Leu]3-, ψ(CH3-NH)-Leu14 bombesin and [D-Arg1, D-Phe5, D-Trp7,9, Leu11]substance P were all obtained from Sigma Chemical Co. (St. Louis, MO). Genistein and tyrphostin-25 were purchased from Calbiochem (Nottingham, United Kingdom). Agarose-linked and horseradish peroxidase-conjugated anti-Tyr (P) mAb PY 20 and anti-p125FAK mAb were purchased from Affiniti Research Products (Nottingham, United Kingdom). Bcl-2 and p53 mAbs were obtained from DAKO (Buckinghamshire, United Kingdom). Enhanced chemiluminescence (ECL) assay kit was purchased from Amersham. All other reagents were of the purest grade available.

**RESULTS**

**Bombesin Stimulates Tyrosine Phosphorylation, FAK Phosphorylation, and Tyrosine Kinase Activity in Intact H345 SCLC Cells via Specific Bombesin Receptors.** Bombesin-like peptides act as autocrine growth factors in H345 SCLC cells. To further define the mitogenic signal transduction pathways involved, we tested whether bombesin could stimulate tyrosine phosphorylation and PTK activity in H345 SCLC cells. H345 SCLC cells placed in quiescent serum-free medium for 18–30 h were stimulated with bombesin at the appropriate concentration for 10 min and lysed; then phosphorylated tyrosine proteins were immunoprecipitated and analyzed by Western blotting. In three independent experiments, a number of proteins showed an increase in tyrosine phosphorylation, particularly those with apparent molecular weights of M, 140,000, M, 130,000, and M, 120,000. p120 (Fig. IA) showed an increase in tyrosine phosphorylation. Bombesin-stimulated increases in tyrosine phosphorylation was concentration dependent, evident at 0.1 nM and maximal at 100 nM (Fig. 1A).

Bombesin (100 nM) caused a rapid (30 s) and sustained increase (60 min) in the tyrosine phosphorylation of p120 (results not shown). Tyrosine phosphorylation is, therefore, one of the earliest events stimulated by bombesin in intact H345 SCLC cells and comparable to its effect on Ca²⁺ mobilization. The very similar concentration-dependence relationships for bombesin-stimulated tyrosine phosphorylation and clonal growth (EC₅₀ 10 and 7 nM, respectively) support the hypothesis that this event may play a role in the mitogenic action of this growth factor.

As noted, analysis of anti-Tyr(P) immunoprecipitates showed that a major substrate of bombesin-stimulated tyrosine phosphorylation in SCLC cells is a protein migrating with an apparent molecular weight of M, 120,000. To investigate whether this protein represented p125 focal activation kinase (FAK), we immunoblotted anti-Tyr(P) immunoprecipitates from bombesin-treated cells (100 nM; 10 min) with...
Tyrosine Kinase Inhibitors Stimulate Apoptosis in SCLC

Fig. 2. [d-Arg¹, d-Phe⁵, d-Trp⁷-⁹, Leu¹⁴]Substance P stimulates apoptosis in SCLC cells. Two × 10⁵ H345 SCLC cells/ml were grown in SITA medium in the presence of 50 µM [d-Arg¹, d-Phe⁵, d-Trp⁷-⁹, Leu¹⁴]Substance P or diluent (control). Cells were counted at the times indicated. Apoptosis was judged morphologically using acridine orange staining and visualized under fluorescence microscopy. Each column represents the mean of four experiments (bars, SEM) using acridine orange staining, visualized under fluorescence microscopy.

specific anti-p125FAK mAb. Fig. 1B (upper panel) shows that bombesin causes a dramatic increase in the phosphorylation of p125FAK. This effect was concentration dependent with an EC₅₀ of 1.02 ± 0.16 nm (mean ± SEM, n = 5), as determined by scanning densitometry. FAK is itself a tyrosine kinase that is activated by phosphorylation on tyrosine residues. Bombesin also increased total cellular tyrosine kinase activity in H345 SCLC cells (0.18 ± 0.02 to 0.31 ± 0.03 pmol phosphate/min, n = 4 ± SD; Fig. 1C), which may in part relate to the activation of FAK. Similar results were seen for bradykinin and gastrin in H69 and H510 cells, respectively (H69 control, 0.18 ± 0.01 bradykinin-stimulated 0.36 ± 0.04 pmol/min; H510 control, 0.17 ± 0.03, gastrin-stimulated 0.40 ± 0.04 pmol/min; n = 2, experiments performed in duplicate ± SD).

The “broad spectrum” receptor antagonist [d-Arg¹, d-Phe⁵, d-Trp⁷-⁹, Leu¹⁴]Substance P (1–50 µM) inhibited the ability of bombesin to stimulate both tyrosine phosphorylation and PTK activity (Fig. 1C, lower panel). The bombesin receptor antagonists [Leu¹³,-NH₂(CH₂)₉NH]Leu¹⁴]bombesin reduced the bombesin-mediated stimulation of tyrosine phosphorylation and PTK activity in H345 SCLC cells (results not shown). These effects were observed in the same concentration range that has been shown previously to inhibit receptor-mediated mitogenic responses to bombesin through its receptor (38, 39).

[d-Arg¹, d-Phe⁵, d-Trp⁷-⁹, Leu¹⁴]Substance P Stimulates Apoptosis in SCLC Cells. An important consideration in the application of antitumor agents that target mitogenic signals is whether their action results in cytostasis or cytotoxicity. We, therefore, examined the effect of [d-Arg¹, d-Phe⁵, d-Trp⁷-⁹, Leu¹⁴]Substance P on apoptosis in SCLC cell lines. In established SCLC cell lines, a “natural” background rate of apoptosis was seen. When H345 cells (5 × 10⁴) were grown in SITA medium in the presence of 50 µM [d-Arg¹, d-Phe⁵, d-Trp⁷-⁹, Leu¹⁴]Substance P, the proportion of SCLC cells showing the characteristic features of apoptosis (see “Materials and Methods”) increased markedly compared to untreated controls (Fig. 2). These changes were both concentration and time dependent; at 50 µM [d-Arg¹, d-Phe⁵, d-Trp⁷-⁹, Leu¹⁴]Substance P, an increased rate of apoptosis was first evident at around 5 h, and the IC₅₀ of 25 µM was similar to that seen for growth inhibition (39). These results indicate that for growing SCLC cells, neuropeptide receptors may provide signals that prevent apoptosis.

Genistein and Tyrophostin-25 Inhibit Bombesin-stimulated Increases in Tyrosine Phosphorylation, p125FAK Phosphorylation, and Tyrosine Kinase Activity in H345 SCLC Cells. To determine whether specific PTK inhibition could mimic the growth inhibition and pro-apoptotic effects of [d-Arg¹, d-Phe⁵, d-Trp⁷-⁹, Leu¹⁴]Substance P, we first examined whether genistein and tyrophostin-25 could specifically inhibit bombesin-induced protein tyrosine phosphorylation in SCLC cells. Quiescent H345 cells were pretreated with genistein or tyrophostin-25 for 4 h, stimulated with bombesin 100 nM for 10 min, lysed, and then immunoblotted with anti-Tyr(P) antibody. Fig. 3A (upper panel) shows that 4 h pre-treatment with either genistein (left) or tyrophostin-25 (right) caused a concentration-dependent inhibition of bombesin-stimulated tyrosine phosphorylation. These results were confirmed by anti-Tyr(P) immunoblotting of anti-Tyr(P) immunoprecipitates from H345 cells pretreated with either genistein or tyrophostin-25 (Fig. 3A, lower panel). Further analysis of anti-Tyr(P) immunoprecipitates from bombesin-treated H345 cells, with anti-p125FAK mAb 2A7, showed that pretreatment of cells for 4 h with 25 µM tyrophostin-25 or 50 µM genistein markedly reduced bombesin-stimulated tyrosine phosphorylation of p125FAK (Fig. 3B, upper panel). Pretreatment with either genistein or tyrophostin-25 also completely blocked the bombesin-stimulated increase in tyrosine kinase activity in H345 cell extracts (Fig. 3B, lower panel). Our own observations that 25 µM tyrophostin-25 and 50 µM genistein do not affect bombesin-mediated Ca⁺⁺ mobilization in H345 SCLC cells (data not shown) and that data in Swiss 3T3 cells indicating that tyrophostin-25 in the µM range has no effect on protein kinase C, protein kinase A, or PLC (40) would imply that in the concentration range used in this study, the dominant effect of tyrophostin-25 and genistein is to inhibit tyrosine kinase activity. However, because of the different specificities and mode of action of PTK inhibitors, we have used both genistein and tyrophostin-25 throughout to ensure that observed effects seen were evident with both inhibitors and hence solely due to their action on PTKs.

Tyrophostin-25 and Genistein Inhibit SCLC Growth. This result prompted us to test the effect of tyrophostin-25 on neuroepitope-stimulated colony formation. Tumor and transformed cells including SCLC cells are able to form colonies in agarose medium. There is a positive correlation between cloning efficiency and the histological involvement and invasiveness of the tumor in specimens taken from SCLC (41). SCLC cells were grown in 1 ml SITA containing 10⁴ viable cells and 0.3% agarose in the presence of diluent or 25 µM tyrophostin-25 with or without neuropeptide agonist. Fig. 4 (upper panel) shows that tyrophostin-25 inhibits basal colony formation in H345 SCLC cells (data not shown) and that data in Swiss 3T3 cells indicating that tyrophostin-25 in the µM range has no effect on protein kinase C, protein kinase A, or PLC (40) would imply that in the concentration range used in this study, the dominant effect of tyrophostin-25 and genistein is to inhibit tyrosine kinase activity. However, because of the different specificities and mode of action of PTK inhibitors, we have used both genistein and tyrophostin-25 throughout to ensure that observed effects seen were evident with both inhibitors and hence solely due to their action on PTKs.

Tyrosine Kinase Inhibitors Genistein and Tyrophostin-25 Stimulate Apoptosis in SCLC Cells. To examine whether inhibition of PTK activity by [d-Arg¹, d-Phe⁵, d-Trp⁷-⁹, Leu¹⁴]Substance P could explain the ability of this neuropeptide antagonist to induce apoptosis in these cells, the effect of tyrosine kinase inhibitors on the rate of apoptosis in SCLC cell lines H345, H69, and H510 was examined. Flow cytometric analysis of control H345 cells at time 0 (Fig. 5A, upper left panel) shows essentially a single population of cells with high forward scatter. This population represents normal SCLC cells. Flow cytometric analysis of control H345 cells at time 0 (Fig. 5A, upper left panel) shows essentially a single population of cells with high forward scatter. This population represents normal SCLC cells. Flow cytometric analysis of control H345 cells at time 0 (Fig. 5A, upper left panel) shows essentially a single population of cells with high forward scatter. This population represents normal SCLC cells. Flow cytometric analysis of control H345 cells at time 0 (Fig. 5A, upper left panel) shows essentially a single population of cells with high forward scatter. This population represents normal SCLC cells. Flow cytometric analysis of control H345 cells at time 0 (Fig. 5A, upper left panel) shows essentially a single population of cells with high forward scatter. This population represents normal SCLC cells. Flow cytometric analysis of control H345 cells at time 0 (Fig. 5A, upper left panel) shows essentially a single population of cells with high forward scatter. This population represents normal SCLC cells. Flow cytometric analysis of control H345 cells at time 0 (Fig. 5A, upper left panel) shows essentially a single population of cells with high forward scatter. This population represents normal SCLC cells. Flow cytometric analysis of control H345 cells at time 0 (Fig. 5A, upper left panel) shows essentially a single population of cells with high forward scatter. This population represents normal SCLC cells. Flow cytometric analysis of control H345 cells at time 0 (Fig. 5A, upper left panel) shows essentially a single population of cells with high forward scatter. This population represents normal SCLC cells. Flow cytometric analysis of control H345 cells at time 0 (Fig. 5A, upper left panel) shows essentially a single population of cells with high forward scatter. This population represents normal SCLC cells. Flow cytometric analysis of control H345 cells at time 0 (Fig. 5A, upper left panel) shows essentially a single population of cells with high forward scatter. This population represents normal SCLC cells. Flow cytometric analysis of control H345 cells at time 0 (Fig. 5A, upper left panel) shows essentially a single population of cells with high forward scatter. This population represents normal SCLC cells. Flow cytometric analysis of control H345 cells at time 0 (Fig. 5A, upper left panel) shows essentially a single population of cells with high forward scatter. This population represents normal SCLC cells. Flow cytometric analysis of control H345 cells at time 0 (Fig. 5A, upper left panel) shows essentially a single population of cells with high forward scatter. This population represents normal SCLC cells. Flow cytometric analysis of control H345 cells at time 0 (Fig. 5A, upper left panel) shows essentially a single population of cells with high forward scatter. This population represents normal SCLC cells. Flow cytometric analysis of control H345 cells at time 0 (Fig. 5A, upper left panel) shows essentially a single population of cells with high forward scatter. This population represents normal SCLC cells. Flow cytometric analysis of control H345 cells at time 0 (Fig. 5A, upper left panel) shows essentially a single population of cells with high forward scatter. This population represents normal SCLC cells. Flow cytometric analysis of control H345 cells at time 0 (Fig. 5A, upper left panel) shows essentially a single population of cells with high forward scatter. This population represents normal SCLC cells.
Apoptosis was confirmed by both acridine orange staining and electron microscopy. Flow cytometric analysis was used to examine the non-apoptotic population appear to be trypan blue positive at any one time [similar to exclusions at 2.4 to 7.8 x 10^4]. It can be seen that the percentage of cells able to exclude trypan blue falls sharply with tyrphostin-25 treatment; from 2.4 to 7.8 x 10^4. The effect of tyrphostin-25 on the dynamics of H345 SCLC cell culture growth was examined (Table 1). Tyrphostin-25 caused a marked decrease in cell number, reflecting an increase in the rate of apoptosis (3.8 to 27.7%). With the total number of apoptotic cells rising from 2.4 to 7.8 x 10^4. It can be seen that the percentage of cells able to exclude trypan blue falls sharply with tyrphostin-25 treatment; however, the percentage of cells that are trypan blue positive is consistently lower than the sum of cells that are apoptotic and necrotic (Table 1). Hence, approximately one-third of the apoptotic SCLC cell population appear to be trypan blue positive at any one time [similar results were seen with genistein (results not shown)].

We also examined the relationship of apoptosis to the cell cycle. Flow cytometric analysis was used to examine the non-apoptotic H345 SCLC cell population in relationship to their progression through the cell cycle during tyrphostin-25 treatment. Apoptosis was not preceded by growth arrest and may indicate that either apoptosis is not restricted to one particular point in the cell cycle or that failure to progress through the cell cycle leads to apoptosis (results not shown). Similar results were obtained in the H69 and H510 SCLC cell lines (results not shown).

The Stimulation of SCLC Cell Apoptosis by Genistein and Tyrphostin-25 Is Independent of Bcl-2 and p53 Expression. SCLC cell lines H69, H345, and H510 all express high levels of Bcl-2 (29). High levels of bcl-2 expression prevent apoptosis in response to a wide variety of cell stress and cytotoxic drugs (30). p53 is frequently mutated in lung cancers, resulting in p53 protein of increased stability. Inactivation of normal p53 protein in lung cells is important in lung tumor development (42). p53 can act as an intracellular sensor of genotoxic stress (34) and can regulate the function of Bcl-2 (32, 33). We, therefore, examined the relationship between Bcl-2 and p53 expression, PTK activity, and apoptosis. SCLC cell lines H69 and H345, 3-5 days after passage, were washed and resuspended in SITA at a density of 5 x 10^5 cells/ml in the absence or presence of 25 μM tyrphostin-25 or 50 μM genistein for 48 h. The cells were fixed and permeabilized, and the level of p53 and Bcl-2 proteins were analyzed by flow cytometry, using indirect immunofluorescence. In four independent experiments, the levels of p53 and Bcl-2 remained unchanged following tyrphostin-25 treatment, despite the induction of apoptosis by these PTK inhibitors (Fig. 7); similar results were seen with genistein 50 μM (results not shown). In contrast, the chemotherapeutic agent etoposide (a topoisomerase II inhibitor), which induces apoptosis of SCLC cells, caused a marked increase (460%) in detectable

Fig. 3. Genistein or tyrphostin-25 inhibit bombesin-induced tyrosine phosphorylation and tyrosine kinase activation in intact H345 SCLC cells. Exponentially growing cultures of H345 SCLC in SITA medium were washed and resuspended in serum-free quiescent medium for 18-30 h prior to experimentation. A. effect of genistein or tyrphostin on bombesin-induced tyrosine phosphorylation. Upper panel: 2 x 10^6 cells were pretreated for 4 h with diluent (—) or μM concentrations of genistein (left) or tyrphostin-25 (right) as indicated and then were stimulated with 100 nM bombesin for 10 min. The cells were lysed and analyzed by Western blotting with anti-Tyr(P) mAb, as described in “Materials and Methods.” The autoradiograph shown is representative of three independent experiments. Lower panel: 2 x 10^6 cells were pretreated for 4 h with diluent (—) or 25 μM tyrphostin-25 (TP) or 50 μM genistein (Gen) as indicated and then stimulated with 100 nM bombesin for 10 min. The cells were lysed, and anti-Tyr(P) immunoprecipitates were analyzed by Western blotting with anti-Tyr(P) mAb as described in “Materials and Methods.” The autoradiograph shown is representative of three independent experiments. B. upper panel: effect of genistein and tyrphostin-25 on bombesin-induced p125FAK tyrosine phosphorylation. Two x 10^6 H345 cells were incubated in the presence of diluent (—), 25 μM tyrphostin-25 (TP), or 50 μM genistein (Gen) for 4 h and then stimulated with 100 nM bombesin. After 10 min, the cultures were lysed and immunoprecipitated with anti-p125FAK mAb. Immunoprecipitates were analyzed by Western blotting with an anti-Tyr(P) mAb as described in “Materials and Methods.” The autoradiograph shown is representative of two independent experiments. Lower panel: 2 x 10^6 cells were pretreated for 4 h with diluent (—) or 25 μM tyrphostin-25 (TP) and 50 μM genistein (Gen) and stimulated with 100 nM bombesin for 10 min. Tyrosine kinase activity was determined by measuring the phosphorylation of a broad specificity peptide, per the manufacturer’s instructions (see “Materials and Methods”). Results are expressed as the percentage of maximum stimulation. Each column represents the mean of two experiments in duplicate (bars, SD).
p53 protein (results not shown). Hence, it appears that PTK inhibition induces apoptosis in a p53- and Bcl-2-independent fashion.

DISCUSSION

The cellular and molecular responses elicited by bombesin-like peptides (in particular, the GRP-stimulated H345 SCLC cell line) provide a paradigm for the study of neuropeptide growth factors in SCLC cells. We show that bombesin induces tyrosine phosphorylation of a number of cellular proteins, with a major substrate identified as p125FAK. The induction of p125FAK tyrosine phosphorylation is rapid and occurs at bombesin concentrations known to induce growth. It has been suggested that tyrosine phosphorylation of p125FAK is a point of convergence in the action of integrins, oncogenic forms of p60src, and mitogenic neuropeptides and thus may be a critical element in mitogenic signaling (43). Phosphorylated p125FAK functions as a cytosolic tyrosine kinase, and consequently a marked increase in tyrosine kinase activity is seen following bombesin stimulation of H345 SCLC cells.

These results provide direct evidence for the existence of a novel signal transduction pathway in the action of bombesin in SCLC cells, i.e., the stimulation of protein tyrosine phosphorylation and PTK activity. This represents a significant advance in the understanding of mitogenic signal transduction in SCLC cells. Since a number of polypeptide growth factors signaling through receptors with intrinsic tyrosine kinase activity are now known to be important in non-small cell lung cancer cell growth, it may well be that tyrosine phosphorylation and activation of tyrosine kinase activity represents an important mitogenic signal common to all histological cell types of lung cancer.

The ability of the “broad spectrum” neuropeptide receptor antagonist [D-Arg1, D-Phe3, D-Trp79, Leu11] substance P to inhibit bombesin-stimulated tyrosine phosphorylation and PTK activity in the same concentration range in which it inhibits neuropeptide-mediated Ca2+ mobilization and in vivo and in vitro SCLC growth would suggest that both events are receptor mediated (further confirmed for bombesin-stimulated responses by use of the specific bombesin receptor antagonist [Leu13, ψ(CH2NH) Leu14] bombesin; results not shown).

Our results also show that the background rate of apoptosis in growing SCLC cell lines is greatly enhanced by [D-Arg1, D-Phe3, D-Trp79, Leu11] substance P. To determine if specific PTK inhibition affected SCLC growth and could mimic the pro-apoptotic effects of [D-Arg1, D-Phe3, D-Trp79, Leu11] substance P, we first examined whether genistein and tyrphostin-25 could inhibit, in a specific manner, bombesin-induced protein tyrosine phosphorylation in SCLC cells. It was found that genistein and tyrphostin-25 are potent inhibitors of neuropeptide-stimulated tyrosine phosphorylation and PTK activity in SCLC cells. Genistein is a naturally occurring inhibitor that competes with the ATP substrate in PTKs and demonstrates greater selectivity toward this class of kinases compared to serine/threonine kinases (44). Tyrphostins are a series of low molecular weight compounds designed specifically to act as competitive inhibitors at the PTK substrate binding site (45). They have been shown to selectively inhibit both membrane bound (e.g., EGF and PDGF receptors; Refs. 46 and 47) and cytosolic protein tyrosine kinases (including pp60c-Src and c-Abl; Refs. 48 and 49). Although these compounds undoubtedly provide useful tools to analyze the role of tyrosine kinases in signal transduction, it is important that agents with different specificities and modes of action produce similar effects as found in this study. Additional confidence in the specificity of these agents in SCLC is provided by the fact that similar or even higher concentrations of tyrphostin-25 do not inhibit neuropeptide-mediated Ca2+ mobilization. Previous studies in intact Swiss 3T3 cells also indicate that tyrphostin-25 at the concentration used in this study inhibits tyrosine phosphorylation without affecting protein kinase C, protein kinase A, PLC activity, or Ca2+ mobilization (40). This suggests that tyrphostin-25 provides a useful tool with which to evaluate the con-
p53 proteins of increased stability have been detected in primary and failure to progress through the cell cycle results in apoptosis. That apoptosis occurs independently of the cell cycle and suggests that independent multidrug resistance of SCLC cells may, at least in part, prevent cell death in response to a wide variety of cellular stress and oncogenes. In many cell types, high levels of expression of bcl-2 (29). The bcl-2 gene is categorically distinct from classical cytotoxic agents (30). Hence, it is possible that the P-glycoprotein protein in lung cells is an important step in lung tumor development. Evidence is mounting that p53 acts as a molecular policeman to prevent oncogenic mutations in cells exposed to base-damaging agents. On induction of DNA damage, the half-life of p53 is increased, and the protein accumulates in the nucleus. This leads either to transient cell cycle arrest in G1, permitting DNA repair or cell death by apoptosis (34). p53 up-regulates the bax gene but down-regulates lung tumor specimens, and p53 is a very common mutation in SCLC (42). It has become increasingly clear that inactivation of normal p53 protein in lung cells is an important step in lung tumor development. Evidence is mounting that p53 acts as a molecular policeman to prevent oncogenic mutations in cells exposed to base-damaging agents. On induction of DNA damage, the half-life of p53 is increased, and the protein accumulates in the nucleus. This leads either to transient cell cycle arrest in G1, permitting DNA repair or cell death by apoptosis (34). p53 up-regulates the bax gene but down-regulates...
tyrosine phosphorylation controls a balance between activation and inhibition of apoptosis in SCLC cells via p53 and bcl-2-independent mechanisms, unlike standard chemotherapeutic agents. It should now be possible to dissect the early signaling pathways leading to activation of and protection from apoptosis; hence, it may be possible to develop therapeutic agents that induce selective SCLC cell apoptosis. Thus, through a better understanding of signal transduction, we may be able to alter the balance between proliferation and apoptosis in this aggressive cancer.

REFERENCES


Inhibition of Neuropeptide-stimulated Tyrosine Phosphorylation and Tyrosine Kinase Activity Stimulates Apoptosis in Small Cell Lung Cancer Cells

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