Effects of Recombinant Neutral Endopeptidase (EC 3.4.24.11) on the Growth of Lung Cancer Cell Lines in Vitro and in Vivo

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ABSTRACT

Many lung cancers are stimulated by an autocrine/paracrine system of neuroendocrine peptide hormones. Attempts to block this autocrine growth pathway by interactions with specific ligand-receptor binding using monoclonal antibodies and peptide-specific antagonists have been largely unsuccessful because of the heterogeneity of hormone production and receptor expression. In the normal lung, neutral endopeptidase (NEP; CD10, CALLA, enkephalinase, and EC 3.4.24.11) plays a physiological role in degrading biologically active peptides, including all peptides implicated in autocrine growth stimulation of lung cancer. Cigarette smoke decreases the activity of NEP, indicating that the lack of NEP contributes to the dysregulation of the peptide autocrine system. The cloning of the human NEP gene allowed for production of sufficient quantities of recombinant NEP (rNEP) to evaluate its role in inhibiting the growth of lung cancer cells. In this study, we evaluated the ability of rNEP to inactivate the peptides involved in lung cancer signal transduction and to inhibit the growth of lung cancer cells as well as normal lung cells in vitro and in vivo in athymic nude mice. We showed that the growth inhibition of lung cancer cells by rNEP was related to the dose and schedule. Continuous exposure to high doses was required for growth inhibition. These studies confirm the importance of NEP in this autocrine pathway.

INTRODUCTION

SCLC and 20% of NSCLCs have neuroendocrine features including autocrine/paracrine growth stimulation by a variety of neuropeptides (1–5). The lung cancer cells express receptors specific for each peptide (5, 6). These peptides bind to their specific receptors, which share similar seven membrane-spanning regions coupled to heterotrimeric G-proteins (7, 8). Peptide-receptor binding activates the G-proteins, which then activate an intracellular signal pathway, which culminates in gene transcription and cell proliferation (9–11). Attempts to inhibit the pathway by blocking peptide-receptor interactions have been largely unsuccessful because of heterogeneity of peptide production and receptor expression (1, 4–6).

NEP (CD10, CALLA, enkephalinase, and EC 3.4.24.11) is a cell surface peptidase whose physiological role is to inactivate bioactive peptides such as gastrin-releasing peptide, BK, gastrin, tachykinins, and other peptides (12–15). NEP plays a major role in pulmonary development, physiology, inflammation, and injury (15–17). NEP is expressed at high levels in the adult lung, especially in pulmonary epithelial cells where it regulates broncho-constriction and tissue responses to peptides (14, 18). Cigarette smoke was shown to inactivate NEP in the lung (19). Carcinogens found in tobacco increase peptide production in the lung, and smokers have increased levels of neuropeptides in their bronchoalveolar lavage fluid (20). Thus, smokers have increased production and decreased destruction of neuropeptides, which serve as autocrine/paracrine growth factors in the pathogenesis of lung cancer.

Most lung cancers and lung cancer cell lines have decreased or absent mRNA and protein expression of NEP compared with bronchial epithelial cells, normal lung fibroblasts, and mesotheliomas (18, 21, 22). This increases their susceptibility to the growth stimulatory effects of peptides. In one study, exogenous rat kidney NEP was able to inhibit the in vitro growth of a single human SCLC line, NCI-H345, that is growth stimulated by exogenous bombesin (21). As there is increased peptide production and decreased peptide destruction in lung cancer, we sought to determine whether human NEP would inhibit the in vitro and in vivo growth of human lung cancers.

MATERIALS AND METHODS

Cell Lines. Human SCLC lines NCI-H187, NCI-H1048, NCI-H345, NCI-H740, NCI-H82 and NCI-H209 and human NSCLC lines NCI-H2122 and NCI-H157 were kindly provided...
Effects of rNEP on the Growth of Lung Cancer Cell Lines

by Drs. Bruce Johnson (National Cancer Institute, Bethesda, MD) or John Minna and Adi Gadzar (Simmons Cancer Center, Dallas, TX). The SCLC line NCI-H69 and the NSCLC line A549 were obtained from American Type Culture Collection (Manassas, VA). The SCLC line GLC-20 was obtained from Dr. Karen Kelly (University of Colorado Cancer Center, Amsterdam, the Netherlands), and Dr. A. Koros (University of Pittsburgh, School of Medicine, Pittsburgh, PA) kindly provided the SCLC line SHP77. The normal lung bronchial epithelial line BEAS-2B transformed with SV40 was obtained from Dr. Karen Kelly (University of Colorado Cancer Center). The normal lung fibroblast lines MRC-9 and CCD-111u were purchased from American Type Culture Collection.

Cell lines were maintained in RPMI 1640 with 10% heat-inactivated FBS or serum-free HITES (hydrocortisone, insulin, transferrin, β-estradiol, sodium selenium) medium (23). All cell lines were grown in 5% CO2 at 37°C with 100% humidity.

dRNA. Human NEP was cloned and sequenced by Malfroy et al. (24) in 1988. The full-length cDNA for human NEP was inserted into a mammalian expression vector in front of the human cytomegalovirus immediate early promoter yielding the recombinant plasmid pCISHENK (25). Membrane-bound rNEP is expressed in Chinese hamster ovary cells (CHO) stably transfected with this plasmid. Electroporetically active rNEP is obtained from solubilized extracts of the transfected CHO cells after column chromatography. Khepri Pharmaceutical, Inc. kindly provided the rNEP.

3HTdR Uptake. The effect of rNEP on the spontaneous proliferation of SCLC and NSCLC tumor cell lines was measured using a 3HTdR-incorporation assay. Cells were plated in 100 μl of growth medium at a concentration of 1,000–5,000 viable cells/well for NSCLC and 10,000 viable cells/well for SCLC in 96-well plates (Corning Glass, Corning, NY). After plating, cells were allowed to recover and settle overnight. rNEP or control proteins were added daily. After a 7-day incubation with various concentrations of rNEP or control proteins, 0.4 μCi [methyl-3H] thymidine, 6.6 Ci/mmol (ICN Biochemicals, Aurora, OH), was added in a 20-μl volume of RPMI 1640. Cells were incubated for an additional 24 h before harvesting, using a Titertek Cell Harvester (Flow Laboratories, Rockville, MD). Using a Beckman LS1801 scintillation counter (Beckman Scientific, Fullerton, CA), 1-min sample counts were performed. Each concentration of rNEP was added to triplicate wells and the mean ± SE values were compared with control values of counts in growth medium without rNEP.

MTT Growth Assay. Cell viability was assayed using a modified tetrazolium salt MTT assay (26). Briefly, 1,000 normal lung fibroblasts or BEAS2-B cells, 1,000 or 5,000 NSCLC cells, and 10,000 viable SCLC cells were plated in a 100-μl volume in 96-well plates (Corning Glass). The cells were allowed to recover overnight. rNEP or control protein was added in bulk, and the plates were incubated as above. After an 8-day incubation, the tetrazolium salt MTT assay was added at a final concentration of 0.4 mg/ml to each well. The microtiter plates were incubated for 4 h at 37°C. At the end of the 4-h incubation, the medium was aspirated off leaving the dark blue formazan product in the bottom of the wells. The reduced MTT product was solubilized by adding 100 μl of 0.2N HCl in 75% isopropanol, 23% MilliQ water to each well. Thorough mixing was done using a Titertek multichannel pipetman. The absorbency of each well was measured using an automated plate reader. In a second set of experiments, rNEP or control was added in bulk on day 1 and on day 8. On day 16, MTT was added, and the plates were harvested as above.

Intracellular Calcium Assay. Intracellular calcium levels were assayed in a flow cytometric assay using Indo-1 AM as described previously (4, 5). After incubation of the cell lines with Indo-1 AM (Molecular Probes, Eugene, OR), flow cytometric analyses were performed with an EPICS 752-cell sorter (Coulter Electronics, Hialeah, FL). A model INNOVA 09/5-argon ion laser (Coherent, Palo Alto, CA) provided the UV excitation (80mW at 360 nm). Calcium-bound Indo-1 AM emits a violet fluorescence (397–415 nm), the intensity of which was measured using a 410 nm-band filter (Oriel, Stanford, CT). The blue emission fluorescence of the free Indo-1 AM (480–500 nm) was measured with a 490 nm-band pass filter (Oriel). Viably loaded cells were gated from debris, dead cells, and unloaded cells by forward angle and 90-degree light scatter and 490 nm fluorescence. The ratio of the 410:490 (violet:blue) fluorescence emission was calculated digitally for each cell by the MDADS hardware and displayed on a linear scale. Data were collected by the MDADS computer, including 410 and 490 fluorescence and the 410:490 ratio versus time. Measurements were performed on the unstimulated cells followed by the addition of the neuropeptide stimulus. Cells exhibiting a fluorescence ratio of at least 10% greater than that of the unstimulated population were scored as positive.

Determination of Apoptosis. The SCLC line H345 was plated at a density of 10,000 cells/well in a 96-well plate and incubated for 8, 12, or 16 days with rNEP or growth medium alone. The cells were harvested using a pipetman, washed once, and resuspended in 2.5% Rnase A in 10 mM EDTA buffer. After a 2-h incubation, the cells were analyzed by an EPICS XL-MCL (Coulter Electronics) for a sub-G1 area of apoptotic cells (Modfit Verity Software Topsham, MA). The presence of apoptotic cells was confirmed by fluorescent microscopy.

Immunofluorescence Staining and Flow Cytometry. 5 × 105 cells were stained with the monoclonal antihuman NEP antibody J5 (Coulter Immunology, Hialeah, FL; Refs. 21, 22, and 27) or the isotype-matched control UPC-10 (Sigma Chemical Co., St. Louis, MO). The cells were counterstained with goat antimmunoglobulin G2a-FITC (Southern Biotechnology Assoc., Inc., Birmingham, AL). Flow cytometry was used to determine the percentage of positive cells and the mean fluorescence intensity.

NEP Enzymatic Assay. rNEP-containing samples (100 μl) were added to the wells of a 96-well flat-bottomed plate, and 2-fold serial dilutions were performed. A positive control of rNEP at 1 μg/ml serially diluted 2-fold was included in the assay. The negative control consisted of medium serially diluted. After dilution of the sample, 100 μl of substrate solution was added, and the plates were read at 405 nm on a Thermomax automated plate reader (Molecular Devices, Sunnyvale, CA) set on the kinetic mode for 20 min. The substrate solution contained 60 μl of succinyl-phelylalanyi-alanyl- phenylalanyi-p-nitroanilide and 2.4 units/ml aminopeptidase M in 100 mM Tris (pH 7.5). Activity of the rNEP-containing samples was quantitated.
**RESULTS**

**Expression of NEP on Cultured Cell Lines.** Before evaluating the growth inhibitory effects of rNEP on cultured cell lines, the cell surface expression of NEP protein was determined using flow cytometry. The percentage of cells expressing surface NEP was determined by incubating the cell lines with the J5 monoclonal antibody which is specific for human NEP (21, 22, 27). Table 1 shows the percentage of cells in each cell line reactive with the J5 monoclonal antibody. Of the five classic SCLC lines examined, three lacked NEP expression; one had low expression (<15%) and one had moderate expression (42%). Of the two variant SCLC lines, one had no NEP expression and the other line had moderately high expression (55%). A SCLC line of extrapulmonary origin had high NEP expression (90%). Of the three NSCLC lines, two adenocarcinomas had no expression or low expression of NEP and one squamous line had high expression. The two normal lung fibroblast lines had moderate expression (38%).

**Expression of NEP in Xenograft Studies.** For in vivo studies, NCI-H69, NCI-H209, and NCI-H82 cells were heterotransplanted into the flank of athymic nude mice (NCr-nu females, 22 g, obtained from Taconic Farms, Germantown, NY). The heterotransplanted cells consisted of small tumor fragments microdissected from tumor-bearing athymic mice. These fragments were used because they produced the most consistent tumor takes and growth. The fragments (30 mg/tumor) contained approximately 10^6 viable cells. Two sets of experiments were conducted. In the first set of experiments, treatment was begun 6 days after tumor heterotransplantation for NCI-H82 or 25 days after heterotransplantation for NCI-H209 when tumors were small and staged at ~70 mg. The treatments were injected (i.p.) three times daily for 28 days. The treatments were saline control (100 μl) or rNEP at 0.033 mg/kg q 8H, 0.33 mg/kg q 8H, or 3.33 mg/kg q 8H (0.1 mg/kg/day, 1 mg/kg/day, or 10 mg/kg/day). In the second experiment, rNEP or saline control treatment was initiated in H69 heterotransplants at day 28 when the tumor was well established and staged at 250 mg. The treatments, saline control (100 μl) or rNEP at 1 mg/kg/day, 10 mg/kg/day, or 50 mg/kg/day, were injected i.p. once daily for 14 days. Perpendicular tumor diameters (mm) were measured serially at the indicated time points and the mean tumor weight (mg) was calculated from the formula: weight = length × width (2/2). All experiments were conducted with 10 animals/treatment group and 20 animals/control group. The mean and SE were calculated for each group. Differences between the growth rates of heterotransplanted tumors were conducted using an unpaired t test obtained from Stat View, (Abacus Concepts, Cupertino, CA).

**Degradation of Neuropeptides by rNEP.** The calcium flux assay was used to evaluate the ability of rNEP to degrade neuropeptides to the level where they no longer caused signal transduction in a biological system. A 1-μg/ml solution of rNEP was incubated with 10 μM of BK, GRP (aa 20–27), NT, or CCK (aa 26–33) from Peninsula Laboratories (Belmont, CA) in a 37°C water bath for 0, 15, 30, 45, or 60 min. The incubated samples were then analyzed for the ability of the residual neuropeptide to release intracellular calcium.

**Expression of NEP on Cultured Cell Lines.** Table 1 shows the percentage of cells in each cell line responding to the addition of each neuropeptide at 100 nM. The percentage of NEP+ cells as determined by calcium flux testing is shown in Table 1.

### Table 1: Nep expression: measured by immunofluorescence staining and neuropeptide responsiveness of cell lines (measured by calcium flux)\(^a\)

<table>
<thead>
<tr>
<th>Cell line</th>
<th>BK</th>
<th>GRP</th>
<th>CCK</th>
<th>NT</th>
<th>No. positive/No. tested(^b)</th>
<th>NEP positive(^c)</th>
</tr>
</thead>
<tbody>
<tr>
<td>SCLC: classic</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>NCI-H345</td>
<td>22%</td>
<td>56%</td>
<td>49%</td>
<td>25%</td>
<td>4/4</td>
<td>0%</td>
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<tr>
<td>NCI-H69</td>
<td>34%</td>
<td>10%</td>
<td>49%</td>
<td>22%</td>
<td>4/4</td>
<td>0%</td>
</tr>
<tr>
<td>NCI-H209</td>
<td>0%</td>
<td>15%</td>
<td>58%</td>
<td>39%</td>
<td>3/4</td>
<td>0%</td>
</tr>
<tr>
<td>NCI-H187</td>
<td>26%</td>
<td>0%</td>
<td>0%</td>
<td>53%</td>
<td>2/4</td>
<td>13%</td>
</tr>
<tr>
<td>NCI-H740</td>
<td>23%</td>
<td>0%</td>
<td>20%</td>
<td>0%</td>
<td>2/4</td>
<td>42%</td>
</tr>
<tr>
<td>SCLC: variant</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>GLC-20</td>
<td>30%</td>
<td>0%</td>
<td>0%</td>
<td>0%</td>
<td>1/4</td>
<td>55%</td>
</tr>
<tr>
<td>HCl-H82</td>
<td>0%</td>
<td>0%</td>
<td>0%</td>
<td>0%</td>
<td>0/4</td>
<td>0%</td>
</tr>
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<td>SCLC: extrapulmonary</td>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>NCI-H1048</td>
<td>25%</td>
<td>31%</td>
<td>38%</td>
<td>14%</td>
<td>4/4</td>
<td>90%</td>
</tr>
<tr>
<td>NSCLC</td>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>NCI-H157</td>
<td>46%</td>
<td>0%</td>
<td>0%</td>
<td>71%</td>
<td>2/4</td>
<td>90%</td>
</tr>
<tr>
<td>A549</td>
<td>67%</td>
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<td>1/4</td>
<td>0%</td>
</tr>
<tr>
<td>NCI-H2122</td>
<td>56%</td>
<td>0%</td>
<td>0%</td>
<td>0%</td>
<td>1/4</td>
<td>16%</td>
</tr>
<tr>
<td>Normal lung fibroblast</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MRC-9</td>
<td>89%</td>
<td>20%</td>
<td>ND(^d)</td>
<td>0%</td>
<td>2/3</td>
<td>59%</td>
</tr>
<tr>
<td>CCD-11LU</td>
<td>84%</td>
<td>22%</td>
<td>34%</td>
<td>0%</td>
<td>3/4</td>
<td>70%</td>
</tr>
<tr>
<td>Bronchial epithelial cells</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>BEAS-2B</td>
<td>80%</td>
<td>34%</td>
<td>0%</td>
<td>38%</td>
<td>3/4</td>
<td>21%</td>
</tr>
</tbody>
</table>

\(^a\) Additions of each neuropeptide at 100 nM.
\(^b\) Number of peptides producing ≥10% of cells with a calcium flux/number peptides tested.
\(^c\) Percentage of NEP+ cells as determined by flow cytometry using the J5 monoclonal antibody.
\(^d\) ND, not done.
Effects of rNEP on the Growth of Lung Cancer Cell Lines

Table 2: Degradation of neuropeptides by rNEP at 37°C to biologically inactive fragments (measured by calcium flux)

<table>
<thead>
<tr>
<th>Peptide</th>
<th>Activity remaining after incubation of peptide + rNEP at 37°C for</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0 Min</td>
</tr>
<tr>
<td>BK</td>
<td>100%</td>
</tr>
<tr>
<td>GRP</td>
<td>100%</td>
</tr>
<tr>
<td>CCK</td>
<td>100%</td>
</tr>
<tr>
<td>NT</td>
<td>100%</td>
</tr>
</tbody>
</table>

* Peptides incubated for 60 minutes in buffer alone at 37°C remained completely active. Cell lines used to assay activity: SHP77/BK; SWISS 3T3/GRP(aa 20–27); H345/CCK(aa 26–33); H157/NT.
* BK response also 0% at 15 minutes after rNEP incubated for 1 week in RPMI-15% FBS at 37°C. See text.

Response of Normal Lung Fibroblasts, Bronchial Epithelial Cells, SCLC, and NSCLC Cell Lines to Exogenous Neuropeptides. We evaluated the cell lines in Table 1 for functional neuropeptide receptors by their ability to release intracellular calcium (an early transient event in signal transduction) in response to the addition of exogenous neuropeptides. The flow cytometric method used in this study allowed for the rapid analysis of intracellular calcium changes within individual cells. The classic and extrapulmonary SCLC lines had the highest responsiveness. At least two of four exogenously added peptides produced a response in all SCLC lines. The response of the NSCLC and variant SCLC lines was lower with the average response being to only one of the four added peptides. BK was the peptide that most frequently produced a response by the NSCLC and variant SCLC lines (5, 6, 28). The lung fibroblast and bronchial epithelial cell lines had high responsiveness to the exogenous peptides, especially BK. However, the duration of the response of the cell lines from normal lung tissue was significantly shorter than the response of the lung cancer cell lines (data not shown). There was no relationship between NEP cell surface expression and the responsiveness of the cell lines to various peptides (Table 1).

The Ability of rNEP to Inactivate Neuropeptides. To determine whether rNEP would degrade the neuropeptides known to stimulate neuroendocrine lung cancer into biologically inactive fragments, a 1-μg/ml concentration of rNEP was incubated with 10 μM concentrations of neuropeptides for periods of 15–60 min. After the incubation, calcium assays were conducted to determine whether the rNEP-treated peptides were still capable of initiating signal transduction. The results are shown in Table 2. In the SCLC line SHP77, a 10-nm solution of BK saturates the BK receptors on the cell surface, giving a maximum response of 90% of the cells. With an 0.05-nM solution of BK there is still a measurable release of calcium in 25% of the cell population. A 0.01-nM solution of BK results in no measurable release of intracellular calcium. After 15 min of incubation with rNEP, the 10 μM solution of BK failed to elicit any response, indicating that the BK was inactivated to a concentration of <0.05 nm. GRP was more slowly inactivated by rNEP, taking 60 min to inhibit 96% of its activity. The ability of CCK to elicit a calcium response was completely inhibited in 30 min, and the NT response was inhibited in 45 min.

Due to the duration of the cell proliferation and viability experiments, the stability of rNEP was assayed in serum-free HITES media, in RPMI 1640+10%FBS, and in cell culture supernatant by testing its enzymatic activity on the artificial substrate succinyl-phenylalanyl-alanlyl-phenylalanyl-p-nitroanilide. Furthermore, a 1 μg/ml solution of rNEP, incubated for 8 days at 37°C in 10%FBS-RPMI 1640, was evaluated by calcium flux assay to inactivate a 10-nM solution of the neuropeptide BK. The rNEP remained fully active for greater than one week in all of the above conditions (data not shown).

Effect of rNEP on Cell Proliferation and Viability. The effects of varying concentrations of rNEP on 3HThdR incorporation in SCLC and NSCLC cancer cell lines are shown in Fig. 1. Because SCLC cell lines are known to continually produce peptides, these initial experiments were conducted with daily additions of rNEP. In these experiments, the indicated concentration of rNEP was added each day for 7 days, and the cells were harvested on day 8. As shown in Fig. 1A and Table 3, a dose-dependent inhibition of thymidine incorporation occurred with daily additions of rNEP. The H345 line (which had the greatest peptide responsiveness) was the most sensitive SCLC line with complete inhibition of proliferation noted with the daily addition of 5 μg/well rNEP (35 μg/well total). At this concentration, marked inhibition was also observed in five of the other six SCLC cell lines (65–95%). rNEP had no effect on the SCLC line H740, which was relatively unresponsive to exogenous peptide stimulation compared with the other classic SCLC lines. The effect of rNEP on 3HThdR incorporation of NSCLC cancer cell lines is shown in Fig. 1B and Table 3. The NSCLC cell lines were also inhibited in a dose-dependent manner. This inhibition was 74–86% at the highest concentration of rNEP added (35 μg). The degree of inhibition of proliferation was not due to differences in the doubling time of the cell lines. The proliferation of the SCLC line H345, which has a doubling time of ~72 h, was just as inhibited as the SCLC line H187, which has a doubling time of ~24 h.

We next tested the ability of rNEP to inhibit in vitro growth as assayed in the MTT assay. In these experiments, the rNEP was added in bulk on the 1st day, and the cells were harvested on day 8. Fig. 2A shows the results of the seven SCLC lines. The results were similar to those observed in the 3HThdR assays. Again, the rNEP produced the greatest growth inhibition on H345 and there was little inhibition of the growth of H740 cells. The rNEP produced 63–94% growth inhibition of the three NSCLC cell lines at the highest concentration. The effect of rNEP on the growth of normal lung fibroblasts and bronchial epithelial cells in the MTT assay is shown in Fig. 2C. The rNEP produced no inhibition of the growth of the CCD-11Lu or MRC-9 cells. Moderate (20%) inhibition of the BEAS2-B cells was observed at the highest rNEP concentration.

In the studies described above, at 8 days 30–70% of lung cancer cells in several of the lines remained viable after the single administration of rNEP. To determine whether these cells were resistant and not dependent on peptide growth factors, a second administration at the same concentration of rNEP was added on day 8 and the cells were harvested on day 16. The results of these experiments are shown in Fig. 3, A-F. Incubation of the SCLC lines H69, H1048, and GLC-20 (Fig. 3, A-C)
Fig. 1 Effect of varying concentrations of rNEP on ³HtdR incorporation in SCLC and NSCLC cell lines. The rNEP (0, 0.1, 1, 2, and 5 µg) was added daily for 7 days. ³HtdR was added on day 7, and the cells were harvested on day 8 as described in “Materials and Methods.” A, results in the seven SCLC cell lines. The rNEP inhibited the proliferation of six of seven cell lines in a dose-dependent manner. B, results in two NSCLC lines. The rNEP also inhibited the proliferation of the two NSCLC lines in a dose-dependent manner.

Table 3 Effects of rNEP on cell proliferation and growth: relation to peptide response and NEP expression

<table>
<thead>
<tr>
<th>Cell Line</th>
<th>Inhibition ³HtdR</th>
<th>Inhibition MTT*</th>
<th>Mean cells responding‡</th>
<th>NEP Positive</th>
</tr>
</thead>
<tbody>
<tr>
<td>SCLC</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>NCI-H345</td>
<td>99%</td>
<td>94%</td>
<td>38%</td>
<td>0%</td>
</tr>
<tr>
<td>NCI-H69</td>
<td>80%</td>
<td>64%</td>
<td>29%</td>
<td>0%</td>
</tr>
<tr>
<td>NCI-H209</td>
<td>75%</td>
<td>94%</td>
<td>28%</td>
<td>0%</td>
</tr>
<tr>
<td>NCI-H187</td>
<td>95%</td>
<td>86%</td>
<td>20%</td>
<td>13%</td>
</tr>
<tr>
<td>NCI-H740</td>
<td>0%</td>
<td>30%</td>
<td>11%</td>
<td>42%</td>
</tr>
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<td>GLC-20</td>
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<td>50%</td>
<td>8%</td>
<td>55%</td>
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<td>NCI-H1048</td>
<td>95%</td>
<td>38%</td>
<td>27%</td>
<td>93%</td>
</tr>
<tr>
<td>NCI-H82</td>
<td>ND*</td>
<td>82%</td>
<td>0%</td>
<td>0%</td>
</tr>
<tr>
<td>NSCLC</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>NCI-H157</td>
<td>86%</td>
<td>94%</td>
<td>29%</td>
<td>90%</td>
</tr>
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<td>16%</td>
</tr>
<tr>
<td>Normal lung cells</td>
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<td></td>
</tr>
<tr>
<td>CCD-11LU</td>
<td>ND</td>
<td>0%</td>
<td>35%</td>
<td>70%</td>
</tr>
<tr>
<td>MRC-9</td>
<td>ND</td>
<td>0%</td>
<td>36%</td>
<td>59%</td>
</tr>
<tr>
<td>BEAS-2B</td>
<td>ND</td>
<td>20%</td>
<td>38%</td>
<td>21%</td>
</tr>
</tbody>
</table>

* From 8 Day MTT Assay.
‡ From Table 1.
ND, not done.

resulted in complete inhibition of growth at the highest concentration. The H740 (Fig. 3D) cell line remained the least sensitive of the SCLC lines with an 82% reduction compared with control at the highest concentration and a 48% reduction at the second highest concentration of rNEP. As shown in Fig. 3, E and F, there was little increase in the growth inhibition of rNEP on the NSCLC cell lines H2122 and A549 at day 16. Even at the highest rNEP concentration there was only a 70% reduction in viability of A549 cells at day 16, indicating that a considerable fraction of A549 cells may not be dependent on peptide growth factors.

The rNEP produced growth inhibition in the MTT assay in all of the lung cancer cell lines that lacked or had low NEP expression. However, many cell lines that expressed cell surface NEP had growth inhibition also. The rNEP inhibited the growth of the high-expressing cell lines H1048 and H157. The bronchial epithelial cells and the lung fibroblast respond to exogenous peptides and have high expression of surface NEP. The lack of growth inhibition by exogenous rNEP may result from the fact that the endogenous NEP is able to neutralize the low levels of peptides produced by the cells.

A likely mechanism of growth inhibition from deprivation of growth factor would be programmed cell death after a reduction in proliferation. To determine the mechanism of growth inhibition, serial apoptosis assays were conducted over time. As shown in Fig. 4, a small, but constant, minority of SCLC H345 undergo apoptosis when cultured in growth medium for 16 days. However, when cultured in the presence of 10 µg rNEP/well, the fraction of apoptotic cells increased by day 8 and reached peak levels of 45% on days 12 and 16 (Fig. 4B). These results indicate that the decrease in proliferation, as assessed by the ³HtdR incorporation, is followed by an increase in apoptosis.

Effect of rNEP on SCLC Tumor Growth in Athymic Mice. The results on tumor growth in animals treated i.p. for 28 consecutive days with rNEP are shown in Fig. 5. Treatment was begun on day 6 (H82) or on day 25 (H209) after tumor heterotransplantation, when the tumors were staged at ~70 mg. The treatments were 0.1, 1.0, and 10 mg/kg/day (0.033, 0.33, or 3.3 mg/kg/8H). Single daily i.p. injections produced no growth inhibition (data not shown); when administered three times daily, a dose-dependent response to rNEP was observed with both H209 (Fig. 5A) and H82 tumors (Fig. 5B). The maximal
Fig. 2 Effect of varying concentrations on rNEP on cell growth in an 8-day MTT assay. The rNEP (0, 1, 5, 10, and 20 µg) was added in bulk on day 1. A, results in seven SCLC cell lines. As in the 3HTdR assays, rNEP inhibited the growth of six of seven cell lines in a dose-dependent manner. B, rNEP effects on three NSCLC cell lines that were also growth inhibited in a dose-dependent manner. C, effects of rNEP on two normal lung fibroblast cell lines and a bronchial epithelial cell line. There was no growth inhibition except in the bronchial epithelial cell line BEAS-2B, which had a 20% inhibition at the highest concentration of rNEP.

Fig. 3 Effect of varying concentrations of rNEP added twice on in vitro cell growth in a 16-day MTT assay (○). The rNEP (0, 1, 5, 10, and 20 µg) was added in bulk on day 1 and day 8. Fig. A-C, complete inhibition of three SCLC lines in a dose-dependent manner. D, dose-dependent inhibition of the SCLC line H740 at the two highest concentrations tested. E and F, no further significant inhibition of two NSCLC lines at the highest concentration of rNEP. The results of the 8-day assay are shown for comparison (○).

effect on reduction in tumor size was achieved at the highest dose (10 mg/kg/day) on the last day of treatment. The average tumor size on the last day was 49% of control for rNEP-treated H209 tumors and 66% of control for rNEP-treated H82 tumors. The growth inhibition by the highest dose was significant for H209 tumors (P < 0.04), but not for H82 tumors (P = 0.1). In the H209 group, tumor size was followed for an additional 2 weeks after the end of treatment. As shown in Fig. 5A, the rate of regrowth of the rNEP-treated tumors after cessation of treatment was similar to that of the controls. In a separate experiment, the effects of rNEP, on large established tumors was evaluated. In this instance, s.c. xenografts of H69 staged at 250 mg 28 days after heterotransplantation were treated i.p. with 1, 10, or 50 mg/kg/day for 10 days. rNEP had no effect on the growth of these established tumors at any dose tested (data not shown).
DISCUSSION

The neuroendocrine features of SCLC have been recognized for many years. The production and response of SCLC cells to neuropeptides in an autocrine/paracrine growth manner was described in the past decade (1–6). Initial growth factor studies in lung cancer suggested that overproduction of peptides and overexpression receptors deregulated the neuroendocrine pathway (2, 3, 6). It has also been recognized that a portion of NSCLC lung cancers share these pathways (1, 5, 29). Initial studies designed to disrupt the autocrine pathway at the ligand/receptor interaction using specific peptide antagonists or antibodies against specific receptors were largely unsuccessful (1, 3, 8, 30–36). Subsequent studies suggested that the heterogeneity of peptide production and receptor expression, the requirement

Fig. 4  Induction of apoptosis by rNEP in the SCLC line H345. The H345 cells were incubated for 16 days with 10 μg of rNEP. Apoptosis was measured on days 8, 12, and 16 after rNEP addition. Apoptosis was measured by flow cytometry for the sub-G1 peak. A, spontaneous apoptosis occurring in the H345 cells incubated for 16 days in growth medium with no rNEP. B, rNEP-induced apoptosis in the H345 cell line incubated with 10 μg of rNEP.

Fig. 5  Effect of rNEP on the in vivo growth of SCLC xenografts. A, growth of H209 tumors in weight as a function of time after implantation. There were four groups: a saline control (○); and groups treated with rNEP at 0.033 mg/kg q 8 h (●); 0.33 mg/kg q 8 h (▲); or 3.3 mg/kg q 8 h (▼). Treatments were started on day 25 and continued for 28 days. The highest dose significantly inhibited growth compared with the saline control (P < 0.04). After the treatments were discontinued, growth was similar in control and animals treated with the highest rNEP dose. B, growth of H82 tumors in weight as a function of time after tumor implantation. The treatments are the same as in A. The lowest rNEP dose had no effect. The highest dose had some inhibitory effect, which was of borderline significance (P = 0.1).
Effects of rNEP on the Growth of Lung Cancer Cell Lines

Recent studies indicated that there is also decreased degradation of the autocrine peptides in lung cancer. This may be due to low expression of NEP and other peptidases. NEP is an ectoenzyme that degrades biologically active peptides in the extracellular fluid. The amino acid sequence of NEP includes a single sequence of 20 hydrophobic residues near the NH₂ terminus that anchors the enzyme to the plasma membrane (13, 24, 37). The bulk of the protein, including the active site, projects into the extracellular fluid where it degrades peptides at the cell surface by hydrolyzing bonds on the amino side of hydrophilic residues.

The normal lung and bronchial epithelium express considerable NEP that regulates a number of physiological reactions in the lung (14–17). In the lung, NEP inactivates all of the peptides responsible for the autocrine/paracrine growth of lung cancer cells. NEP activity is reduced by tobacco smoke, and we and others showed that most lung cancer cells have low or absent mRNA and protein expression (14, 18, 19). Shipp et al. (21) showed that exogenous rat NEP inhibited thymidine incorporation by the SCLC cell line H345. This suggested the potential therapeutic use of recombinant human NEP, which would overcome the problem of peptide heterogeneity. The human rNEP is also a nonimmunogenic, naturally occurring human protein that is stable in serum and plasma.

In our studies, rNEP was able to inhibit the in vitro growth of most human SCLC and NSCLC cell lines. Normal lung fibroblasts and bronchial epithelial cells were not growth inhibited despite being sensitive to peptides. This suggests that the growth inhibition was specific for the peptide autocrine pathway such that these normal cells express peptide receptors, but do not produce peptides. Therefore, they are not driven by the malignant autocrine loop. The ability of rNEP to inhibit growth of the lung cancer cells was dependent on the dose and schedule of the rNEP administration. The maximal growth inhibition of the lung cancer cell lines examined required at least 2 weeks of exposure to the highest concentration of the rNEP.

The long exposure and high concentration of rNEP required raised questions about the mechanism of growth inhibition. To evaluate this, we performed apoptosis assays and showed that apoptotic death occurs but is delayed and maximal after 12–16 days of rNEP exposure. The mechanism by which deprivation of autocrine growth factors stimulated apoptotic death was not examined in these studies. However, in other studies we and others showed that biased agonist to neuropeptide receptors stimulate apoptosis while inhibiting proliferation (38, 39).

The neuropeptide receptors have similar structures coupled to the Gαᵣ and Gα₁₂,₁₃ members of the heterotrimeric family of G proteins (40). The binding of neuropeptides to their receptors initiates coordinated signaling through these G proteins. New approaches are being developed to uncouple this coordinate signaling through the G proteins. These approaches, which also circumvent the problem of peptide/receptor heterogeneity, include mutant G proteins (7), substance P derivative (39), and BK antagonist dimers (38). These new biased ligands lead to discordant signaling by selectively activating Gα₁₂,₁₃ while inhibiting Gαᵣ. Gα₁₂,₁₃ stimulation without Gαᵣ stimulation leads to activation of Jun kinases without activating phospholipase C or protein kinase C and this leads to apoptosis. Future studies will evaluate whether autocrine deprivation leads to apoptosis through a similar mechanism.

The in vivo effects of rNEP were also highly dose- and schedule-dependent. The highest in vivo daily dose produced maximal tumor growth inhibition when the therapy was given every 8 h (3.3 mg/kg/8H). It is unlikely that such high doses (~700 mg/day) administered continuously are feasible in humans. Our data suggest that rNEP therapy would be maximally effective when the tumor burden was lowest. Thus, the therapy might be used in an adjuvant setting after surgical resection, with radiotherapy, chemotherapy or a combination of treatments.

An alternative approach would be a gene therapy strategy using the human NEP gene. Gene therapy would anchor the NEP in its native site at the cell surface where it would be more effective at cleaving neuropeptides. To test the feasibility and function of transfected NEP genes, Okamoto et al. (41) transfected the rat NEP gene into epithelial cells that express substance P receptors (substance P is an excellent substrate for NEP) and respond to exogenous substance P. The substance P signal and response was completely abolished in the transfected cells. Furthermore, Papandreou et al. (42) transfected the androgen-independent prostate cell line Tsu-Pr1 with NEP using a tetracycline-inducible vector system. NEP is expressed by androgen-dependent prostate cancer cell lines but not by androgen-independent prostate cell lines. Neuropeptides such as bombesin induce phosphorylation of the focal adhesion kinase p125FAK. Low levels of phosphorylation are found in androgen-dependent prostate cancer cell lines compared with high levels of phosphorylation in androgen-independent lines. Overexpression of cell surface NEP in the Tsu-Pr1 prostate cell line inhibited phosphorylation of p125FAK that resulted in decreased migratory capacity of this androgen-independent prostate cancer cell line. These data suggest that a similar approach might work in lung cancer cells that are peptide responsive and have low NEP expression. Successful transfection would eliminate the need for large amounts of exogenous recombinant protein. Not every cell would need to be successfully transfected for a successful result. Of course, delivery of the gene to the bronchial epithelial cells remains a major obstacle. However, an inhaled method of delivery could be successful in the treatment of dysplasia to prevent progression to malignancy.

In summary, our studies provide additional support for the importance of peptide growth of lung cancer cells. The studies confirm that rNEP can inhibit lung cancer cell growth stimulated by a variety of peptides. The success of rNEP therapy was dependent on the dose and schedule of the rNEP administration and the size of the tumor.

ACKNOWLEDGMENTS

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