Growth hormone-releasing hormone: An autocrine growth factor for small cell lung carcinoma

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Antagonists of growth hormone-releasing hormone (GHRH) inhibit the growth of various cancers in vivo. This effect is thought to be exerted through suppression of the pituitary growth hormone–hepatic insulin-like growth factor I (IGF-I) axis and direct inhibition of autocrine/paracrine production of IGF-I and -II in tumors. However, other evidence points to a direct effect of GHRH antagonists on tumor growth that may not implicate IGFs, although an involvement of GHRH in the proliferation of cancer cells has not yet been established. In the present study we investigated whether GHRH can function as an autocrine/paracrine growth factor in small cell lung carcinoma (SCLC). H-69 and H-510A SCLC lines cultured in vitro express mRNA for GHRH, which apparently is translated into peptide GHRH and then secreted by the cells, as shown by the detection of GHRH-like immunoreactivity in conditioned media from the cells cultured in vitro. In addition, the levels of GHRH-like immunoreactivity in serum from nude mice bearing H-69 xenografts were higher than in tumor-free mice. GHRH(1–29)NH2 stimulated the proliferation of H-69 and H-510A SCLCs in vitro, and GHRH antagonist JV-1–36 inhibited it. JV-1–36 administered s.c. into nude mice bearing xenografts of H-69 SCLC reduced significantly (P < 0.05) tumor volume and weight, after 31 days of therapy, as compared with controls. Collectively, our results suggest that GHRH can function as an autocrine growth factor in SCLCs. Treatment with antagonistic analogs of GHRH may offer a new approach to the treatment of SCLC and other cancers.

Lung cancer is the leading cause of cancer-related deaths in the Western world. Small cell lung carcinoma (SCLC) is a subset of lung cancer that accounts for about 20% of cases and is characterized by poor prognosis resulting from the limited therapeutic options available when the disease is diagnosed (1).

Antagonistic analogs of growth hormone-releasing hormone (GHRH) inhibit the growth of various cancers, such as osteosarcomas (2), glioblastomas (3), SCLC and non-SCLC (4), prostatic (5, 6), renal (7), pancreatic, colorectal (8, 9), and breast cancers (10). This effect is exerted in part by endocrine mechanisms through the inhibition of growth hormone (GH) release from the pituitary, which, in turn, results in the reduction of the hepatic production of insulin-like growth factor I (IGF-I) (11). IGF-I is a potent mitogen for various cancers, including SCLC (12). Other evidence, based on the significant reduction in concentrations of IGF-I and/or IGF-II in osteosarcomas (2) and non-SCLC (4), renal (7), prostatic (5, 6), pancreatic, and colorectal cancers (8, 9) and a decrease in expression of mRNA for IGF-II in tumors after treatment of nude mice with GHRH antagonists, suggests that the inhibition of tumor growth may be the result of direct effects of GHRH antagonists on autocrine/paracrine production of IGFs in tumors (2, 5, 6, 11). This view is supported by the observation that antagonists of GHRH inhibit the proliferation of various human cancer cell lines cultured in vitro, suppress the production of IGF-II, and decrease the telomerase activity (13, 14). GHRH antagonists also could inhibit tumor growth directly by blocking the action of tumoral GHRH by mechanisms independent of IGFs.

Although the expression of GHRH in primary tumors and increased GHRH-like immunoreactivity in the serum of lung cancer patients have been demonstrated (15–17), the function of GHRH in tumorigenesis remains obscure. The aim of our study was to investigate the role of GHRH in the growth of H-69 and H-510A human SCLC. First, we investigated whether H-69 and H-510A cells express and secrete GHRH. Then, we evaluated the effects of GHRH(1–29)NH2 and potent antagonist analog of GHRH JV-1–36 (18) on cell proliferation in vitro. We also studied the antitumor activity of GHRH antagonist JV-1–36 in nude mice bearing xenografted H-69 cells. The effects of the treatment with GHRH antagonists on serum levels of GHRH, IGF-I, and IGF-II also were evaluated.

Materials and Methods

Peptides. GHRH(1–29)NH2 and GHRH antagonist JV-1–36 ([PhAc-Tyr1,D-Arg2,Phe(4-Cl)6,Arg9,Abu15,Nle27,D-Arg28,Har29]hGH-RH(1–29)NH2) (where PhAc is phenylacetyl, Abu is α-amino butyric acid, Nle is norleucine, and Har is homoarginine) were synthesized by solid-phase methods and purified as described (18). For daily injections, peptides were dissolved in 0.1% DMSO in sterile 10% propylene-glycol/water solution.

Cell Culture and Cell Proliferation Assay. The human SCLC cell line NCI-H-69 was obtained from American Type Culture Collection (Manassas, VA) and cultured in RPMI 1640 medium (GIBCO) supplemented with 10% FBS, glutamine (2 mM), penicillin (100 units/ml), streptomycin (100 mg/ml), and amphotericin B (100 units/ml) at 37°C in a humidified 95% air/5% carbon dioxide atmosphere. The human SCLC cell line H-510A was a gift from H. Oie (National Cancer Institute–Naval Oncology Unit, Bethesda, MD). Except for the substitution of 10% newborn calf serum for 10% FBS, this cell line was cultured as described above. Cells were passaged weekly and monitored routinely for mycoplasma contamination by using a detection kit (Boehringer Mannheim). All culture media components were purchased from GIBCO. The effect of the peptide analogs on cell proliferation was examined by the MTT [3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl tetrazolium bromide] assay as described (19). Results were calculated as percent T/C, where T = optical density (OD540) of treated cultures [HITES medium (4) plus GHRH analogs] and C = OD540 of untreated cultures (HITES medium alone).

Abbreviations: GH, growth hormone; GHRH, GH-releasing hormone; SCLC, small cell lung carcinoma; IGF-I and IGF-II, insulin-like growth factor I and II, respectively; HITES medium, human glyceraldehyde-3-phosphate dehydrogenase.

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Weighed. Removed and weighed. Tumors were dissected, cleaned, andria. Body weights were recorded and various organs were
blood was collected. The serum was separated and analyzed by
Moore, Mundelein, IL) and sacrificed by decapitation, and trunk
were anesthetized with methoxyflurane (Metofane; Pitman–

animals, when tumors had grown to a volume of approximately 70
resulting after 3 weeks were retransplanted as mentioned above

3

transplanted s.c. by trocar needle into 5 male animals; tumors

3

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3

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3

of GHRH in serum and medium, GHRH(1–29)NH2 was

KCl, 1.7 mM MgCl2, 200 μM of each dNTP, 2.5 units of Taq
DNA polymerase, and 0.4 mM each primer. The primers used were
5’-TCCCTGACCTAAACACGGACAC-3’ and 5’-

Statistical Analyses. Data are expressed as mean ± SE. Statistical
analyses were performed by using the Student two-tailed t test. All P
values are based on two-sided hypothesis testing.

Expression of mRNA for GHRH and Secretion of GHRH by H-69 and
H-510A Cells Cultured in Vitro. Total RNAs isolated from H-69 and
H-510A cells cultured in vitro were subjected to reverse
transcription–PCR analysis for the expression of mRNA for GHRH.
PCR products were electrophoresed in 2% agarose gel and
stained with ethidium bromide. Aliquots of each PCR product were
electrophoresed on a 2% agarose gel and

% of GHRH

Table 1. Production of GHRH in culture medium from H-69 and
H-510A SCLC

<table>
<thead>
<tr>
<th>Time, h</th>
<th>H-69</th>
<th>H-510A</th>
</tr>
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<tbody>
<tr>
<td>0</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>27</td>
<td>2.93</td>
<td>9.47</td>
</tr>
<tr>
<td>72</td>
<td>3.26</td>
<td>3.80</td>
</tr>
<tr>
<td>168</td>
<td>2.23</td>
<td>4.75</td>
</tr>
</tbody>
</table>

Aliquots of medium at the indicated periods of time were subjected to RIA for the detection of GHRH. ND, not detectable.
from H-510A cells, when the incubation was prolonged to 72–168 h, could be due to inactivation.

Effect of GHRH(1–29)NH₂ and GHRH Antagonist JV-1–36 on the Proliferation of H-510A and H-69 SCLC in Vitro. H-69 and H-510A SCLC cells cultured in vitro were exposed to various concentrations of GHRH(1–29)NH₂ or GHRH antagonist JV-1–36, and the effect on the proliferation was followed by the MTT assay. As shown in Fig. 2a, GHRH(1–29)NH₂ at 2 × 10⁻⁷ M stimulated the proliferation of H-69 and H-510A cells by 17% (P < 0.005) and 21% (P < 0.0001), respectively. The growth of H-510A cells also could be stimulated significantly by 2 × 10⁻⁸ M and 2 × 10⁻⁶ M hormone. H-69 cells showed smaller proliferative responses. GHRH antagonist JV-1–36 at 10⁻⁵ M inhibited the proliferation of H-69 and H-510A cells by 18% (P < 0.001) and 75% (P < 0.001), respectively, as compared with controls (Fig. 2b). The inhibition of proliferation was already apparent at 10⁻⁶ M JV-1–36.

Effect of JV-1–36 on Growth of H-69 SCLC Xenografted into Nude Mice. Nude mice bearing xenografts of H-69 SCLC were treated with daily s.c. injections of JV-1–36 at two different dose levels. After 31 days of treatment with JV-1–36 at the dose of 20 µg/day the mean tumor volume was significantly (P < 0.05) reduced to 461 ± 91 mm³, corresponding to a decrease of 80%, as compared with that of the control group (2,254 ± 584 mm³) (Table 2 and Fig. 3). JV-1–36 administered at 10 µg/day per animal also inhibited tumor growth by 54% but this decrease was not significant. The final tumor weights were reduced by 73% (P < 0.05) and 45% (not significant) in the groups treated with JV-1–36 at 20 µg/day and 10 µg/day, respectively, as compared with the control group (Table 2). At the end of the experiment, no significant differences in body weights and the weight of various organs such as lung, heart, liver, and kidneys were observed between the groups, indicating that treatment with JV-1–36 was not toxic for the tumor-bearing animals (data not shown).

Effect of JV-1–36 on Serum Levels of GHRH in Nude Mice Bearing H-69 SCLC. RIA for GHRH showed that serum levels of GHRH in nude mice bearing H-69 tumors were about 90% higher than the concentrations in serum of tumor-free animals (Table 3). Treatment of H-69 tumor-bearing animals with GHRH antagonist JV-1–36, at the dose of 20 µg/day per animal, resulted in a 40% (P < 0.05) decrease in serum levels of GHRH compared with the controls receiving vehicle. JV-1–36 administered at 10 µg/day per animal had no effect on the levels of GHRH in the serum.

Effect of JV-1–36 on GH, IGF-I, and IGF-II Levels in Serum of Nude Mice Bearing H-69 SCLC. No significant differences were found in the serum levels of GH, IGF-I, and IGF-II in nude mice bearing...
H-69 SCLC and treated with GHRH antagonist JV-1–36 at 10 μg/day or 20 μg/day per animal, as compared with the controls.

Discussion

The prognosis for patients with SCLC is poor because of the limited therapeutic options available at the late stages during which the disease is usually diagnosed. The identification of factors that regulate the growth of SCLC would be valuable because it could allow the development of assays for screening patients with SCLC and provide the basis for novel therapeutic approaches.

The paracrine hypothesis for tumor growth postulates that cancer cells can produce hormone-like substances, usually peptides, that are released into the extracellular fluid, act upon the same cells, and stimulate their growth. A classical paracrine agent for SCLC is bombesin (24, 25). The link between bombesin/gastrin-releasing peptide and SCLC was discovered by Cuttitta et al., who found that SCLC cells both secrete and respond to bombesin-like peptides (24). Many SCLC lines also express neuromedin B (25). Our work shows that human SCLC cells produce GHRH, which stimulates their growth by GHRH. This may fulfill the autocrine requirements for oncogenesis of events that result in tumor inhibition, which may or may not involve the IGFs (8). The reduction in IGF-I and IGF-II levels observed in many tumors after therapy with GHRH antagonists (8) could be the consequence of an initial blocking of autocrine/paracrine GHRH. The subsequent inhibition of tumor growth then could result from a suppression of IGF-I or IGF-II production. A therapy based on GHRH antagonists could be applied for the treatment of SCLCs and other cancers that appear to depend on the production of GHRH, not only as a mechanism to manipulate the GH/IGF-I axis or suppress tumoral IGF-I and IGF-II, but also to inhibit the autocrine/paracrine stimulation by GHRH. To establish whether this autocrine stimulation is a general feature of primary SCLCs and other cancers, the extension of these investigations to additional cell lines and the screening of primary tumors for the production of biologically active GHRH are required. In addition, epidemiological studies on the levels of circulating GHRH could reveal whether elevated levels of GHRH are associated with an increased risk for lung cancer and other cancers.

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Table 3. Serum levels of GHRH in tumor-free nude mice and nude mice bearing xenografts of H-69 SCLC treated with GHRH antagonist JV-1–36 at doses of 20 μg/day or 10 μg/day per animal

<table>
<thead>
<tr>
<th>Mice</th>
<th>GHRH, ng/ml</th>
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<tr>
<td>Tumor-free</td>
<td></td>
</tr>
<tr>
<td>JV-1–36 (10 μg/day)</td>
<td>10.63 ± 1.05</td>
</tr>
<tr>
<td>JV-1–36 (20 μg/day)</td>
<td>5.49 ± 0.61</td>
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