Antagonists of Growth Hormone-Releasing Hormone Inhibit the Proliferation of Experimental Non-Small Cell Lung Carcinoma

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ABSTRACT

Recent studies show that antagonists of growth hormone-releasing hormone (GH-RH) inhibit proliferation of various cancers indirectly through blockade of the endocrine GH-insulin-like growth factor (IGF) I axis and directly by an action on tumor cells involving the suppression of autocrine/paracrine IGF-I, IGF-II, or GH-RH. The effectiveness of therapy with GH-RH antagonist JV-1–38 and its mechanisms of action were investigated in NCI-H838 non-small cell lung carcinoma (NSCLC) xenografted s.c. into nude mice and in vitro. Treatment with GH-RH antagonist JV-1–38 significantly (P < 0.05–0.001) inhibited tumor growth as demonstrated by a 58% decrease in final tumor volume, 54% reduction in tumor weight, and the extension of tumor doubling time from 8.5 ± 1.38 to 12 ± 1.07 days as compared with controls. Using ligand competition assays with 125I-labeled GH-RH antagonist JV-1–42, specific high-affinity binding sites for GH-RH were found on tumor membranes. Reverse transcription-PCR revealed the expression of mRNA for GH-RH and splice variant 1 (SV1) of GH-RH receptor in H838 tumors. Reverse transcription-PCR analysis also demonstrated that H838 tumors express IGF-I and IGF-I receptors. Tumoral concentration of IGF-I and its mRNA expression were significantly decreased by 25% (P = 0.05) and 65% (P < 0.001), respectively, in animals receiving JV-1–38, whereas serum IGF-I levels remained unchanged. In vitro studies showed that H838 cells secreted GH-RH and IGF-I into the medium. The growth of tumor cells in vitro was stimulated by IGF-I and inhibited by GH-RH antagonist JV-1–38 and a GH-RH antisera. Our results extend the findings on the involvement of IGF-I in NSCLC and suggest that GH-RH antagonist JV-1–38 and a GH-RH antiserum. Our results extend the findings on the involvement of IGF-I in NSCLC and suggest that GH-RH antagonists could be partly direct and mediated by SV1 of tumoral GH-RH receptors. The finding of GH-RH and SV1 of GH-RH receptors in NSCLC provides a new approach to the treatment of this malignancy based on the use of antagonistic analogues of GH-RH.

INTRODUCTION

Lung cancer is the leading cause of cancer-related deaths in the Western world (1). About 155,000 Americans die of lung tumors each year. This represents 25–30% of all cancer deaths in the United States (2). The estimated annual number of lung cancer deaths worldwide is nearly 1 million (3). Consequently, it is necessary to explore novel therapeutic approaches to lung carcinomas.

NSCLC1 account for ~80% of all lung cancer cases (4). NSCLC is considered an aggressive tumor type with poor prognosis that typically develops resistance to the various treatment modalities (5, 6).

Several series of antagonistic analogues of GH-RH were synthesized in our laboratory in the past 10 years in an endeavor to develop a new class of antitumor agents (7–9). We have shown that GH-RH antagonists inhibit the growth of diverse cancers such as pancreatic (10), colorectal (11), prostatic (12, 13), breast (14), and renal (15) cancers; glioblastomas (16); osteosarcomas (17, 18); SCLC; and NSCLC (19, 20). GH-RH antagonists block the secretion of GH from the pituitary by inhibiting the binding of hypothalamic GH-RH to pituitary GH-RH receptors. Thus, the antineoplastic effect of GH-RH antagonists is caused by the suppression of the GH release from the pituitary, which in turn results in the inhibition of the hepatic production of IGF-I (8, 9). An inhibition of experimental SCLC and NSCLC cancers, achieved with relatively high doses of early GH-RH antagonists, was associated with reduced serum IGF-I levels (20). IGF-I is a well-known mitogen for various cancers including NSCLC (21, 22).

In addition, it was observed that GH-RH antagonists can inhibit the proliferation of various cancer lines including H-69 human SCLC by a direct action in vitro, under conditions in which the contribution of the hypothalamic GH-RH/pituitary growth hormone/hepatic IGF-I axis is clearly excluded (9, 14, 19, 23–27). This finding may be accounted for by the presence of GH-RH and GH-RH receptors on tumors. Thus, the endogenous production of GH-RH was detected in human SCLC (19), prostate cancer (28), N-methyl-N'-nitro-N-nitrosoguanidine/human osteosarcoma, and SK-ES-1 Ewing sarcoma (27) cell lines. The presence of mRNA for GH-RH was also demonstrated in cell lines of prostatic, breast, ovarian, and endometrial cancers; SCLC; osteosarcomas; Ewing sarcomas (19, 27, 29, 30); and specimens of human prostatic, breast, ovarian, and endometrial cancers (30–32). The proliferation of some cancer lines such as H-69 SCLC, N-methyl-N'-nitro-N-nitrosoguanidine/human osteosarcoma, SK-ES-1 Ewing sarcomas could be stimulated by administration of GH-RH and its agonistic analogues or, conversely, inhibited by GH-RH antagonists (19, 27). SVs of GH-RH receptors, which could mediate the direct effects of tumoral GH-RH and its synthetic antagonists, have been recently identified on various human cancers (33, 34). These observations suggest that GH-RH may be an autocrine growth factor for some cancers, and the direct antiproliferative action of GH-RH antagonists could be exerted through the disruption of an autocrine/paracrine-stimulatory loop (19, 27–29, 35).

In contrast to the extensive findings available on other cancers, no studies have been reported thus far regarding the possible involvement of GH-RH and its tumoral receptors in the pathogenesis of NSCLC. Such investigations could open new venues toward the management of this malignancy, which represents a major national and international health problem. In this study, we have evaluated the effects and the mechanism of action of a new potent antagonistic analogue of GH-RH, JV-1–38, in NCI-H838 human NSCLC cell line. This antagonist, JV-1–38, was selected for the present study because in previous oncological investigations in prostate and breast cancers, it appeared to be the most potent among the analogues tested (8, 9).
INHIBITION OF LUNG CARCINOMAS BY GH-RH ANTAGONIST

MATERIALS AND METHODS

Peptides. hGH-RH_{1–38}NH_{2} and GH-RH antagonist JV-1–38 [PhAc-Tyr^{1}, d-Arg^{2}, Phe(4-Cl)^{3}, Ha^{4}, Tyr(Me)^{5}, Abu^{6}, Nle^{7}, Ha^{29}] human GH-RH_{1–38}NH_{2} were synthesized in our laboratory by solid phase methods as described previously (7). For daily injections, the peptide was dissolved in 1% DMSO in sterile aqueous 10% propylene glycol (vehicle solution).

Cell Line, Animals, and Tumors. Human NSCLC cell line NCI-H838, obtained from American Type Culture Collection (Manassas, VA), was derived from a primary adenocarcinoma of the lungs of a 59-year-old Caucasian man who was a smoker. The tumor was metastatic to the lymph nodes. This cell line was maintained in culture using DMEM with 100 units/ml penicillin, 100 μg/ml streptomycin, 0.25 μg/ml amphotericin B, 2 mM-glutamine (all from Invitrogen), Carlsbad, CA), and 1% fetal bovine serum (Atlanta Biologicals, Norcross, GA).

Male athymic (Ncr-nu/nu) nude mice, ~6 weeks old on arrival, were obtained from the National Cancer Institute (Frederick Cancer Research and Development Center, Frederick, MD), housed in laminar air-flow cabinets under pathogen-free conditions with a 12-h light/12-h dark schedule, and fed autoclaved standard chow and water ad libitum. Xenografts were initiated by s.c. injection of 15 × 10^6 H838 cells into the right flanks of five male nude mice. Tumors resulting after 5 weeks were aseptically dissected and mechanically minced, and 3-mm³ pieces of tumor tissue were transplanted s.c. with a trocar needle. All experiments were conducted in accordance with institutional guidelines for animal care.

In Vivo Studies. The experiment was started when tumors had grown to ~35 mm³. Animals were randomly divided into two treatment groups: group 1 (seven mice), control, vehicle solution; and group 2 (eight mice), GH-RH antagonist JV-1–38 injected s.c. twice a day at a dose of 10 μg/animal. Tumor volume (length × width × height × 0.5236) and body weights were measured twice a week. The experiment was terminated on day 30. Mice were sacrificed, and tumors were dissected, cleaned, and weighed. Tumor specimens were snap-frozen and stored at ~70°C for various analyses. Tumor volume doubling time was calculated between day 1 and day 30 using the formula

\[ \text{days of treatment} = \frac{\text{[Log (final volume)]} - \text{[Log (initial volume)]}}{\text{Log} 2} \]

as described by Smolev et al. (36).

Histological Methods. The methods used in the histological investigations were described (10), including the detection of the NOR in tumor cell nuclei. The argyrophilic NOR count is a good indicator of cell proliferation rate.

In Vitro Studies. The effect of various agents on the rate of cell proliferation in SFM was evaluated by the crystal violet assay described previously (37). Briefly, cells were seeded into 96-well microplates at 5 × 10^3 cells/well and cultured in RPMI 1640 supplemented with 10% fetal bovine serum, 100 units/ml penicillin, 100 μg/ml streptomycin, L-glutamine, 2 m M -glutamine (all from Invitrogen), and 1% fetal bovine serum (Atlantic Biologicals, Norcross, GA). Cells were seeded into 96-well Falcon microplates at 5 × 10^3 cells/well and cultured in RPMI 1640 supplemented with 10% fetal bovine serum, penicillin G (sodium salt; 100 units/ml), and streptomycin sulfate (100 μg/ml) in a humidified atmosphere containing 5% CO_2 at 37°C. After 2 days, the confluent cultures were washed with N_E medium containing 0.3% BSA and maintained in this medium for 2 additional days. The compounds were then added to the cultured cells, incubated for a period of 20 h, and pulsed with 0.25 μCi/well [methyl-^3]H]thymidine (specific activity, 25 Ci/mmol; Amersham Corp.) in a total volume of 175 μl/well for 3–4 h. The cells were fixed with ice-cold 10% trichloroacetic acid, washed twice with trichloroacetic acid, and solubilized in 0.2 % NaOH. The lysate was mixed with scintillation mixture (CytoScint; ICN Biomedicals), and the radioactivity was determined by liquid scintillation counting (LS 380 I; Beckman Instruments, Irvine, CA).

RIA for GH-RH, IGF-I, and IGF-II. The methods used for determination of IGF-I levels in serum as well as IGF-I and IGF-II concentrations in the cytosol fraction of tumors, after acid-ethanol cryoprecipitation have been described in detail (13, 17, 18, 20).

For the measurement of GH-RH, IGF-I, and IGF-II secretion by H838 NSCLC cell line in vitro, 5–6 × 10^6 cells were seeded in 75-cm² flasks and allowed to attach for 24 h when the medium was replaced by serum-free N_E medium. After 0, 24, or 48 h in culture, 10 ml of medium from H838 NSCLC cells were lyophilized and reconstituted in 1 ml of PBS-EDTA buffer containing 1% BSA, 1 mM DTT, and 0.001% pepstatin A. IGF-I and IGF-II in these samples were assayed directly without an acid-ethanol cryoprecipitation step. GH-RH was measured by using 125I-GH-RH_{1–40} (Bachem) as labeled hormone and anti-GH-RH_{1–40} (SV-95) antisera in a 0.1% N_E medium. After 0, 24, or 48 h in culture, 10 ml of medium from H838 NSCLC cells were lyophilized and reconstituted in 1 ml of PBS-EDTA buffer containing 1% BSA, 1 mM DTT, and 0.001% pepstatin A. IGF-I and IGF-II in these samples were assayed directly without an acid-ethanol cryoprecipitation step.

RNA Extraction. Total RNA was extracted from frozen tissue samples using the Micro RNA Isolation Kit (Stratagene, La Jolla, CA) according to the manufacturer’s instructions. The quality and quantity of RNAs were assessed by spectrophotometry at 260 and 280 nm (A260/A280 >1.8) before reverse transcription. RNA to be analyzed for GH-RH was subjected to further isolation of poly(A)RNA using the MicroPoly(A) Pure Small Scale mRNA Purification Kit (Ambion, Austin, TX) according to the manufacturer’s instructions.

Reverse Transcription. Reverse transcription was performed using the GeneAmp RNA Core Kit (Perkin-Elmer, Foster City, CA). Two μg of total RNA (for IGF-I, IGF-II, IGF-IR, and β-actin) or 4 μg of total RNA (for GH-RH receptors) or 2 μg of poly(A)^+RNA (for GH-RH) were reverse transcribed into cDNA with Moloney murine leukemia virus reverse transcriptase according to the manufacturer’s instructions.

PCR Amplification. The PCR amplifications of the cDNAs for human β-actin, IGF-I, IGF-IR, and GH-RH receptors were performed using the GeneAmp Gold RNA PCR Core Kit (Perkin-Elmer) and GH-RH and β-actin were performed using the GeneAmp Gold RNA PCR Reagent Kit (Perkin-Elmer) with 3 μl of cDNA (for IGF-I and β-actin), 14 μl of cDNA (for GH-RH), 2.5 μl of cDNA (for IGF-II and IGF-IR), or 5 μl of cDNA (for GH-RH receptors) was amplified in a 25 μl solution containing 2 mM MgCl_2 (for IGF-I, GH-RH receptors, IGF-IR, and β-actin) or 2.5 mM MgCl_2 (for GH-RH and GH-RH), 1X PCR buffer, 200 μM concentrations of each deoxynucleotide triphosphate, 2.5 μl of AmpliTaq DNA polymerase (for IGF-I, IGF-IR, GH-RH receptors, and β-actin), 1 unit of AmpliTaq Gold DNA Polymerase and 0.2 μM concentrations of each primer (for IGF-I and β-actin), 0.15 μM concentrations of each primer (for IGF-II and IGF-IR), 0.75 μM concentrations of each primer (GH-RH), or 0.3 μM concentrations of each primer (GH-RH receptors). The primers used were 5'-ATC TGG CAC CAC ACC TTC TAC AGC GGG GAA CCG CTC ACC GCC AAT GGT GAT-3' for human β-actin; 5'-ACA TCT TCC ATT ATC CTG GAT TTT CTT TTG C-3' and 5'-CCCC TCT ACT TGC GTT CTT CAA ATG TTC TCC C-3' for IGF-I; 5'-AGT CTA TGG AGT GTC TGG TGG TGC TCT CACT TCT GGC-3' and 5'-TGG AGG ACG GAT TGT CCA GTG TTG CG-3' for GH-RH; 5'-AAC CAC GAG CCT GAG GAA CT-3' and 5'-CAG CAT AAT CAC CAA CCC TCT-3' for IGF-IR; 5'-ATT TGA CGA GTG CCT CCG AG-3' and 5'-TTT GTT CTG CCT ACA TGC TGG TGG-3' for GH-RH; 5'-CCT ACT GCC CTT AGG ATG CTG G-3' and 5'-ATC TCA CGG AAG TGG CAT GCC G-3' for the first PCR; and 5'-GCC CCT TCT TTG AGG AAG G-3' and 5'-CAG GTG CCA TGG GTA GAG CAC G-3' for the second PCR for GH-RH receptors. For β-actin, samples were denatured for 1 min at 94°C for 2 min, followed by 35 cycles of 94°C for 30 s, 58°C for 30 s, and 68°C for 45 s. PCR fragments were analyzed by electrophoresis on 2% agarose gels and stained with ethidium bromide before quantification by densitometry.

Reverse transcription was performed using the GeneAmp RNA Core Kit (Perkin-Elmer, Foster City, CA). Two μg of total RNA (for IGF-I, IGF-II, IGF-IR, and β-actin) or 4 μg of total RNA (for GH-RH receptors) or 2 μg of poly(A)^+RNA (for GH-RH) were reverse transcribed into cDNA with Moloney murine leukemia virus reverse transcriptase according to the manufacturer’s instructions.
94°C and then subjected to 28 cycles of 94°C for 45 s, 60°C for 45 s, and then 72°C for 45 s with a final extension of 10 min at 72°C. For IGF-I, samples were denatured at 97°C for 10 s, then subjected to 5 cycles of 97°C for 20 s, 60°C for 30 s, and 72°C for 30 s, and finally 35 cycles of 95°C for 30 s, 60°C for 30 s, and 72°C for 30 s with a final extension of 7 min at 72°C. For IGF-II, samples were denatured at 95°C for 10 min and the subjected to 35 cycles of 94°C for 40 s and 60°C for 30 s with a final extension of 72°C for 7 min. For IGF-IR, samples were subjected to one cycle of 95°C for 3 min, 56°C for 1 min, and 72°C for 1 min and then 30 cycle of 95°C for 35 s, 56°C for 35 s, and 72°C for 40 s, with a final extension of 7 min at 72°C. For GH-RH, samples were denatured at 95°C for 10 min and subjected to 35 cycles of 94°C for 30 s, 55°C for 30 s, and 72°C for 30 s with a final extension of 7 min at 72°C. For GH-RH receptors, samples were denatured at 95°C for 3 min followed by 35 cycles of 95°C for 30 s, 58°C for 30 s, and 72°C for 2 min with a final extension of 7 min at 72°C; a secondary PCR of 35 cycles of 95°C for 30 s, 60°C for 30 s, 72°C for 1 min with a final extension of 7 min at 72°C was subsequently carried out on 1 μl of the primary PCR product. The number of cycles was determined in preliminary experiments to be within the exponential range of PCR amplification. Negative controls were run in parallel to exclude genomic DNA contamination. PCR products were electrophoresed on a 1.8% agarose gel, stained with ethidium bromide and visualized under UV light.

PCR products were quantitated using the Kodak EDAS 290 imaging system with Kodak 1D Image Analysis Software. Experiments were performed in duplicate and relative mRNA levels of each gene were normalized versus the corresponding levels of human β-actin.

**Radioligand Binding Studies.** Radiiodinated derivative of GH-RH antagonist JV-1–42 was prepared by the chloromamine-T method as described (31). Preparation of the membrane fractions from H-838 human non-SCLCs grown in nude mice was conducted as reported (34). Receptor binding of GH-RH was performed using in vitro ligand competition assays based on the binding of radiolabeled JV-1–42 to tumor membrane fractions described in detail previously (34). The characteristics of the specific ligand binding were determined with the Ligand-PC curve-fitting software and by Scatchard analysis.

**Statistical Analysis.** The SigmaStat Software was used for the statistical analysis of data. Data are presented as mean ± SE. Differences between the groups of the in vivo study were evaluated by one way ANOVA and the Fisher or Mann-Whitney methods. The results of in vitro experiments were compared by one way ANOVA and the Student-Newman-Keuls method.

### RESULTS

**Effect of JV-1–38 on Growth of Xenografts of H838 NSCLC.**

Four weeks after the initiation of the treatment, the tumor volume in the animals given JV-1–38 was significantly (P < 0.001) decreased to 214.65 ± 42.86 mm³, as compared with controls, which measured 509.04 ± 124.48 mm³ (Fig. 1; Table 1) and which corresponded to 42.86 mm³, as compared with controls, which measured 261.9 ± 24.0 mm³ (Table 2). The concentration of IGF-I in control tumor samples was 170.3 ± 14.1 pg/100 μg of protein, and IGF-II was not detectable. Therapy with JV-1–38 significantly decreased the content of IGF-I in tumors to 127.5 ± 6.5 pg/100 μg of protein (25% reduction, P = 0.05; Table 2). Densitometric analysis of the PCR products corresponding to mRNA for IGF-I (Fig. 2A) showed that the expression of IGF-I gene in H838 tumors of animals treated with the GH-RH antagonist was reduced by 65% (P < 0.001) as compared with the controls, which were accepted 100% (Table 2). The expression of IGF-II gene was found in tumor samples, but the level of mRNA for IGF-II did not change after the therapy with JV-1–38 (data not shown). Tumor samples also expressed mRNA for IGF-I receptor (Fig. 2B).

In cultures, 48-h exposure of cell line to 10 μM JV-1–38 did not significantly affect the levels of mRNA for IGF-I and IGF-II, as compared with controls (data not shown).

**Secretion of GH-RH, IGF-I, and IGF-II by H838 Cells Cultured in Vitro.**

The concentrations of GH-RH, IGF-I, and IGF-II in the SFM were measured by RIA. Significant amounts of secreted GH-RH and IGF-I were found in medium from cells after 24 and 48 h of culture as shown in Table 3, whereas the SFM without cells did not contain detectable levels of GH-RH and IGF-I. IGF-II was not detectable.

**Expression of mRNA for GH-RH in H838 NSCLC Tumors.**

Polyadenylated RNAs isolated from tumor samples were subjected to RT-PCR analysis for the expression of mRNA for GH-RH. After PCR products were electrophoresed on agarose gel and stained with ethidium bromide, a 322-bp band, specific for GH-RH, was found in tumor samples as illustrated in Fig. 3A. A band of similar molecular size was also obtained in H-69 SCLC (positive control; Ref. 19).

**RT-PCR Analysis of SV of GH-RH Receptors in H838 NSCLC Tumors.**

The expression of mRNA for GH-RH receptors in H838 NSCLC tumors was evaluated by RT-PCR. Using gene-specific primers for amplifying cDNAs for splice variants (SV) of GH-RH receptors (33), we detected a PCR product of 720 bp in H838 tumors (Fig. 3B). This PCR product corresponded to SV1 of GH-RH receptors described previously (33). No bands representing SV2, SV3, and SV4 were obtained in H838 NSCLC tumors.

**Radioligand Binding Studies.** The characteristics of specific binding of 125I-labeled GH-RH antagonist JV-1–42 to the membrane receptors on H838 tumor cells were determined using ligand competition assays. The computerized nonlinear curve fitting and the Scat-
chard plot analyses of the binding data indicated that in membranes of untreated H838 tumors, radiolabeled JV-1 and JV-1 human NSCLC

TABLE 1

<table>
<thead>
<tr>
<th>Group of treatment</th>
<th>Initial tumor volume (mm³)</th>
<th>Final tumor volume (mm³)</th>
<th>Tumor weight (ng)</th>
<th>Tumor-doubling time (days)</th>
</tr>
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<tbody>
<tr>
<td>Control (n = 7)</td>
<td>34.77 ± 10.39</td>
<td>50.94 ± 124.48</td>
<td>479 ± 146</td>
<td>8.58 ± 1.38</td>
</tr>
<tr>
<td>JV-1-38 (n = 8)</td>
<td>33.43 ± 11.07</td>
<td>214.65 ± 42.36</td>
<td>219 ± 39*</td>
<td>12.00 ± 1.07*</td>
</tr>
</tbody>
</table>

*a, P < 0.05 versus control; ***, P < 0.001 versus control.

**TABLE 2**

<table>
<thead>
<tr>
<th>Animal</th>
<th>Serum IGF-I (ng/ml)</th>
<th>Protein (ng/100 µg)</th>
<th>mRNA (IGF-I/β-actin)</th>
</tr>
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<tbody>
<tr>
<td>Control group</td>
<td>168.3</td>
<td>191</td>
<td>0.184</td>
</tr>
<tr>
<td>2</td>
<td>280.4</td>
<td>n.i.*</td>
<td>0.073</td>
</tr>
<tr>
<td>3</td>
<td>351.7</td>
<td>n.i.</td>
<td>0.212</td>
</tr>
<tr>
<td>4</td>
<td>257.6</td>
<td>154</td>
<td>0.254</td>
</tr>
<tr>
<td>5</td>
<td>324.0</td>
<td>n.i.</td>
<td>0.319</td>
</tr>
<tr>
<td>Mean ± SE</td>
<td>261.9 ± 24.0</td>
<td>170.3 ± 14.1</td>
<td>0.234 ± 0.034</td>
</tr>
</tbody>
</table>

**JV-1-38 treatment**

<table>
<thead>
<tr>
<th>Protein (ng/100 µg)</th>
<th>mRNA (IGF-I/β-actin)</th>
</tr>
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<tbody>
<tr>
<td>255.8</td>
<td>0.139</td>
</tr>
<tr>
<td>331.0</td>
<td>0.074</td>
</tr>
<tr>
<td>419.3</td>
<td>0.132</td>
</tr>
<tr>
<td>245.4</td>
<td>0.120</td>
</tr>
<tr>
<td>224.6</td>
<td>0.059</td>
</tr>
<tr>
<td>351.3</td>
<td>0.018</td>
</tr>
<tr>
<td>213.2</td>
<td>0.073</td>
</tr>
<tr>
<td>342.2</td>
<td>0.049</td>
</tr>
<tr>
<td>Mean ± SE</td>
<td>298.5 ± 25.7</td>
</tr>
</tbody>
</table>

% of control

| % of control | 114.0 | 74.9 | 35.5 |

A, not investigated; * P ≤ 0.05 versus control; ***, P < 0.001 versus control.

**Fig. 2.** A, analysis of the expression of mRNA for IGF-I and human β-actin in control (Lanes 1–7) and JV-1-38-treated (Lanes 8–15) H838 human NSCLC tumors grown in nude mice. Products of the expected sizes of 514 bp (IGF-I) and 459 bp (human β-actin) were found in H838 tumors. Lane M, 100-bp DNA molecular weight marker. B, expression of mRNA for IGF-IR in H838 human NSCLC tumors grown in nude mice. Products of the expected size of 447 bp were found in control tumors (Lanes 1–7). Lane M, 100-bp DNA molecular weight marker.

**Table 3**

<table>
<thead>
<tr>
<th>Time (h)</th>
<th>GH-RH (ng/ml medium)</th>
<th>IGF-I (ng/ml medium)</th>
</tr>
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<tbody>
<tr>
<td>0</td>
<td>ND*</td>
<td>1.74</td>
</tr>
<tr>
<td>24</td>
<td>0.20</td>
<td>4.36</td>
</tr>
<tr>
<td>48</td>
<td>0.45</td>
<td>10.67</td>
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* ND, not detectable.

**DISCUSSION**

Although some progress has been made in the past 10 years in the management of patients with NSCLC, the 5-year relative survival rates have not improved substantially (39). The outlook for patients with NSCLC remains poor (40) and the need for the development of novel therapeutic modalities persists. This study demonstrates that GH-RH antagonist JV-1–38 significantly inhibits the growth of H838 human NSCLC tumors xenografts in nude mice. After 4 weeks of treatment, the tumor volume and the tumor weight of the animals given JV-1–38 were significantly reduced by >50% as compared with those of the controls, and the tumor-doubling time was also significantly extended.

GH-RH antagonists were initially developed for treatment of cancers dependent on hepatic endocrine IGF-I, stimulated by pituitary GH (17, 20). However, more recent evidence points toward a major role of local GH-RH and its tumoral receptors in the pathogenesis of various cancers, indicating that most of the antitumor effects of GH-RH antagonists could be exerted through a direct action on the cancer cells (9, 19, 27–29, 31, 33–35, 47). It has been established that locally produced GH-RH is present in appreciable quantities both in the normal lung (41) and in NSCLC and other lung tumor specimens (41–44). However, the role of GH-RH in NSCLC has not been investigated thus far. We detected mRNA for GH-RH in H838 tumors grown in nude mice. The quantitation of GH-RH secreted by the cells in vitro revealed that its concentration in the culture medium increased between 24 and 48 h of culture. In cell cultures in vitro, GH-RH did not affect the growth of H838 cell line, but both GH-RH antagonist
tumors (Lanes 1 and 2). Lane M, 100-bp DNA molecular weight marker; Lane N, negative control. B, RT-PCR analysis of the expression of mRNA for SVs of GH-RH receptors in H838 human NSCLC tumors grown in nude mice. A product of the expected size of 322 bp corresponding to GH-RH was found in mRNA extracts pooled from three to four H838 control NSCLC tumors grown in nude mice. A product of the expected size of 720 bp corresponding to SV1 was found in mRNA extracts pooled from three to four H838 control tumors (Lanes 1 and 2). Lane N, negative control; Lane M, 100-bp DNA molecular weight marker.

Fig. 3. A, RT-PCR analysis of the expression of mRNA for GH-RH in H838 human NSCLC tumors grown in nude mice. A product of the expected size of 322 bp corresponding to GH-RH was found in mRNA extracts pooled from three to four H838 control tumors (Lanes 1 and 2). Lane M, 100-bp DNA molecular weight marker; Lane N, negative control. B, RT-PCR analysis of the expression of mRNA for SVs of GH-RH receptors in H838 human NSCLC tumors grown in nude mice. A product of the expected size of 720 bp corresponding to SV1 was found in mRNA extracts pooled from three to four H838 control tumors (Lanes 1 and 2). Lane N, negative control; Lane M, 100-bp DNA molecular weight marker.

Fig. 5. A, effect of GH-RH antagonist JV-1–38, an antiserum to GH-RH or IGF-I on the proliferation of H838 NSCLC cell line. Relative cell number in treated, and control plates was determined by crystal violet staining and expressed as percent T/C values, where T = absorbance of treated cultures and C = absorbance of control cultures. Measured absorbance is proportionate to cell number. B, [3H]Thymidine incorporation into H838 NSCLC cells. Confluent cell cultures were incubated with 0.1–10 μM GH-RH antagonist JV-1–38, SV-95 antiserum 1:2500 and 1:1000, or 5–25 ng/ml IGF-I. After 20 h of incubation, the cells were pulsed with 0.25 μCi/well [3H]thymidine for 3–4 h. Columns, mean value of six replicates of a representative experiment. Bars, SE. *, P < 0.05; **, P < 0.01; ***, P < 0.001.

Fig. 4. Representative example of saturation of [125I]JV-1–42 binding to H838 NSCLC tumor membrane fraction. The total (○), nonspecific (■), and specific (○) binding is plotted as a function of radiolabeled peptide concentration. Inset, representative Scatchard plot derived from specific [125I]JV-1–42 binding to the membrane fraction isolated from H838 human NSCLC. Each point represents the mean of triplicate determinations.

The participation of SV1 in cell proliferation signaling has been shown by transfection in NIH-3T3 mouse fibroblast cells (47). In that study, transfection of 3T3 cells with SV1 produced specific high-affinity binding sites for radiolabeled GH-RH antagonist and conferred a greatly increased sensitivity to GH-RH analogues in the cell-proliferation assays. Thus, the expression of SV1 augmented the mitogenic responses of 3T3 cells to GH-RH, reagents JI-38 and the antimitogenic responses to GH-RH antagonist JV-1–38 compared with control cells not expressing SV1 (47). In addition, using an endometrial carcinoma cell line that normally expresses SV1 receptors, our group demonstrated that ablation of SV1 by an antisense RNA-based approach reduced the rate of cell proliferation in the absence of exogenous GH-RH and decreased the sensitivity of the cells to exogenous GH-RH (48).

In our investigation, using [125I]labeled GH-RH antagonist JV-1–42 as a new radioligand, we were able to detect tumor binding sites for GH-RH with a high affinity and specificity in H838 NSCLC tumor.

JV-1–38 and an antibody to GH-RH inhibited the proliferation based on crystal violet assay. Similar effects were observed using a quantitative approach such as [3H]Thymidine incorporation assay. The lack of stimulation by exogenous GH-RH in vitro might reflect the fact that H838 cells may already produce GH-RH at levels that can cause maximal stimulation of this cell line in vitro. The inhibition of cell proliferation observed in the presence of a GH-RH antiserum supports this hypothesis. The suppression of growth produced by JV-1–38 in vitro is likely due to the ability of this antagonist to block the actions of autocrine GH-RH on the tumoral GH-RH receptors.

Attempts to detect the presence of pituitary-type GH-RH receptors in cancer cells and tumors were unsuccessful (14, 26, 33, 45, 46). However, recently we were able to identify splice variants of GH-RH receptors on tumors that might mediate the direct effects of GH-RH and its antagonists (33, 34). These tumoral GH-RH receptors are distinct from the pituitary-type GH-RH receptors or receptors for other peptides of the secretin/glucagon family. The isolation and sequencing of cDNAs corresponding to the tumoral GH-RH receptor mRNAs revealed that they are splice variants of the pituitary GH-RH receptors (33). All of the SVs of the GH-RH receptor have a retained intronic sequence at their 5′ end but lack the first three exons (33, 34). We assume that the lack of the first three exons observed in SV1 could result in the formation of a tumoral GH-RH receptor protein in which most of the large NH2-terminal extracellular domain, characteristic of the pituitary receptor, is truncated (33, 34). Thus, SV1 could encode a functional receptor protein with binding affinity for GH-RH and its analogues different from that of the pituitary GH-RH receptor (34). The participation of SV1 in cell proliferation signaling has been shown by transfection in NIH-3T3 mouse fibroblast cells (47). In that study, transfection of 3T3 cells with SV1 produced specific high-affinity binding sites for radiolabeled GH-RH antagonist and conferred a greatly increased sensitivity to GH-RH analogues in the cell-proliferation assays. Thus, the expression of SV1 augmented the mitogenic responses of 3T3 cells to GH-RH and the antimitogenic responses to GH-RH antagonist JV-1–38 compared with control cells not expressing SV1 (47). In addition, using an endometrial carcinoma cell line that normally expresses SV1 receptors, our group demonstrated that ablation of SV1 by an antisense RNA-based approach reduced the rate of cell proliferation in the absence of exogenous GH-RH and decreased the sensitivity of the cells to exogenous GH-RH (48).

In our investigation, using [125I]labeled GH-RH antagonist JV-1–42 as a new radioligand, we were able to detect tumor binding sites for GH-RH with a high affinity and specificity in H838 NSCLC tumor.
tissue. Moreover, RT-PCR analysis revealed the expression of SV₁ of GH-RH receptors in the tumors. Because SV₁ may participate in the regulation of cell proliferation and mediate the actions of GH-RH and its antagonists (47, 48), the direct inhibitory effect of GH-RH antagonist JV-1 on the growth of H838 tumors could have been exerted through these receptors. SV₁ is also expressed in other normal and malignant human extrapituitary cells and tissues (23, 27–29, 31, 33, 34, 46).

GH-RH belongs to the family of structurally related peptide hormones that also includes secretin, glucagon, vasoactive intestinal peptide and pituitary adenylate cyclase-activating polypeptide. Vasoactive intestinal peptide and pituitary adenylate cyclase-activating polypeptide as well as their receptors are present in NSCLC and may be involved in the autocrine regulation of this malignancy (49–52). GH-RH appears to fulfill some of the parameters for an autocrine growth factor in H838 human NSCLC cell line, because it is apparently expressed and secreted by the tumor and a tumoral GH-RH receptor isofrom is also present on tumors. GH-RH antagonists can inhibit H838 tumor growth in vitro presumably by an action on GH-RH receptors nullifying the effect of locally produced GH-RH.

Various studies demonstrate the involvement of IGF-I, IGF-II, and IGF-IR in proliferation of NSCLC (21, 22, 52, 53). Thus, Quinn et al. (22) found that IGF-I and IGF-II mRNAs were coexpressed with GH-RH in the majority of human lung cancer cell lines. In our study, we have also found that H838 tumors express the mRNA for IGF-IR, which is known to mediate the mitogenic effects of IGF-I (53). Zia et al. (21) reported that monoclonal antibody to IGF-I receptor inhibited growth of NSCLC in vitro and in vivo. Recently, a statistically significant positive association was reported between plasma IGF-I levels and the risk of lung cancer (54). Immunoactive IGF-I is detectable in primary lung tumor tissue including non-SCLC (squamous cell and adenocarcinomas) at higher levels than in normal lung (53). Immunoactive IGF-I is secreted into the media of both small cell lung cancer (SCLC) and non small cell lung cancer (NSCLC) cells grown in culture (53). In our oncological study in vivo, JV-1–38 was administered at a low dose that had only a weak effect on the pituitary GH-hepatic IGF-I axis, as seen from the lack of a significant reduction of serum IGF-I level. Nevertheless, significant decreases in the tumoral concentration of IGF-I and its mRNA expression were observed after treatment with the GH-RH antagonist. Thus, the inhibition of synthesis of IGF-I in H838 tumor tissue could be a consequence of the direct effect of the GH-RH antagonist on the H838 cells in vitro, mediated by the tumoral GH-RH receptors, although the same effect could not be reproduced in vitro during a shorter, 48-h treatment period. This phenomenon would be similar to that observed in IGF-II-producing prostatic, pancreatic, colorectal and ovarian cancers, glioblastomas and sarcomas in vivo, where the treatment with GH-RH antagonists caused an inhibition of the synthesis of tumoral IGF-II (10–13, 16, 18, 45, 46). The ability of the GH-RH antagonist to reduce the concentration of IGF-I in H838 tumor is likely to provide some contribution to the antitumor effect, because IGF-I is a growth factor for this NSCLC. In our experiments in vitro, exogenous IGF-I significantly stimulated the proliferation of H838 cells. However, the inhibition of tumoral IGF-I synthesis could be of relatively minor importance, because in vitro the GH-RH antagonist was able to decrease the proliferation of H838 cells without exerting a parallel inhibitory effect on the synthesis of autocrine IGF-I. Thus, the anti-proliferative effect of blocking of GH-RH receptors in vitro may not be mechanistically linked to an inhibition of IGF-I signaling through the suppression of IGF-I synthesis/secretion. In addition, the decreases in tumoral IGF-I protein concentrations appear to be smaller than those in the mRNA levels, although no firm conclusions could be drawn, because only four control tumors and two treated tumors were examined for protein content, because of a shortage of tumor tissue samples.

In conclusion, our work extends previous findings suggesting an autocrine role for GH-RH in various cancers and demonstrates that GH-RH antagonists inhibit the growth of H838 human NSCLC tumors in nude mice, probably by interfering with the stimulatory effects of tumoral (autocrine) GH-RH. Inhibitory effects on the autocrine and endocrine IGF-I system could be also involved in the antitumor mechanisms of GH-RH antagonists, but to a lesser extent. Our findings indicate the merit of further investigations with GH-RH antagonists in the management of NSCLC. Our studies suggest that future therapies, based on GH-RH antagonists, might be beneficial for at least a subset of patients with non small cell lung carcinomas that express GH-RH and IGF-I receptors and are dependent on autocrine stimulation by GH-RH and/or IGF-I.

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INHIBITION OF LUNG CARCINOMAS BY GH-RH ANTAGONIST

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