Inhibition of Proliferation in Human MNNG/HOS Osteosarcoma and SK-ES-1 Ewing Sarcoma Cell Lines in Vitro and in Vivo by Antagonists of Growth Hormone-Releasing Hormone

Effects on Insulin-Like Growth Factor II

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BACKGROUND. Antagonists of growth hormone-releasing hormone (GH-RH) can inhibit the proliferation of various tumors either indirectly through the suppression of the pituitary growth hormone/hepatic insulin-like growth factor I (*IGF-I*) axis and the lowering of serum *IGF-I* concentration or directly by reducing the levels of *IGF-I* and *IGF-II* and their mRNA expression in tumors and blocking the effect of autocrine GH-RH. In this study, the authors investigated the effects of the GH-RH antagonist JV-1-38 on MNNG/HOS human osteosarcoma and SK-ES-1 human Ewing sarcoma cell lines.

METHODS. Male nude mice bearing subcutaneous xenografts of MNNG/HOS or SK-ES-1 tumors were treated subcutaneously with JV-1-38 at a dose of 20 μg twice daily for 4 weeks. The concentrations of *IGF-I* and *IGF-II* in serum and in tumor tissue were measured by radioimmunoassay. Tumor and liver levels of mRNA for *IGF-I* and *IGF-II* were determined by reverse transcriptase-polymerase chain reaction analysis. The effects of JV-1-38, *IGF-I*, and *IGF-II* on cell proliferation in vitro were evaluated.

RESULTS. GH-RH antagonist significantly ($P < 0.05$) inhibited the tumor volume and tumor weight of MNNG/HOS and SK-ES-1 tumors by $> 50\%$ after 4 weeks and increased tumor doubling time. JV-1-38 lowered the serum *IGF-I* level, decreased the expression of mRNA for *IGF-I* in the liver, and significantly ($P < 0.05$–0.01) reduced the concentration of *IGF-II* and mRNA levels for *IGF-II* in both sarcomas. The concentration of *IGF-I* was lowered only in SK-ES-1 tumors. In vitro, the proliferation of SK-ES-1 and MNNG/HOS cells was inhibited by JV-1-38 and by antisera to *IGF-I* and *IGF-II*.

CONCLUSIONS. The inhibition of MNNG/HOS osteosarcoma and SK-ES-1 Ewing sarcoma by GH-RH antagonists was linked to a suppression of *IGF-II* production in tumors. However, in SK-ES-1 tumors, the effects on *IGF-I* also may be involved.
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KEYWORDS: osteosarcoma, Ewing sarcoma, insulin-like growth factor I, insulin-like growth factor II, growth hormone-releasing hormone antagonists.

Osteosarcoma represents the most common primary bone malignancy in children and young adults.[1,2] Surgery and high-dose chemotherapy with methotrexate, doxorubicin, cisplatin, and ifosfamide[1,2] are effective mainly in patients with localized disease and can improve overall survival.[3,4] However, despite aggressive treat-

ment, more than one-third of patients develop recurrent high-grade osteosarcomas, with pulmonary metastases the most common site of recurrence.[5] Ewing sarcoma is the second most common malignant tumor of bone affecting children and adolescents and is treated with chemotherapy, surgery, and radiation.[6] Unfortunately, many patients with localized disease and most patients with metastatic disease ultimately die of this malignancy. Consequently, it is necessary to explore other therapeutic approaches for patients with osteosarcomas and Ewing sarcomas.

Numerous observations indicate that growth hormone (GH) and insulin-like growth factors (IGFs), which stimulate growth and metabolism of the normal bone, also are involved in the proliferation of human and murine osteogenic sarcomas.[7–10] Thus, a new therapeutic strategy for this malignancy may be based on the suppression of GH release from the pituitary and a subsequent reduction in serum and tissue levels of *IGF-I*.[8,9,11] However, somatostatin analogs, which can effectively reduce serum GH in acromegalics, caused only a modest decrease in *IGF-I* concentration in patients with neoplastic diseases.[12] This inadequate inhibition of serum *IGF-I* may be explained by compensatory mechanisms, such as an increased secretion of GH-releasing hormone (GH-RH), which may attenuate the inhibitory effect of somatostatin analogues during the long-term treatment of patients with intact hypothalamopituitary systems.[11,12]

The inhibition of GH-RH/GH/*IGF-I* endocrine axis also can be achieved with antagonistic analogs of GH-RH.[13–15] In view of the involvement of *IGF-I* in the progression of several neoplasms, potent GH-RH antagonists that were intended for therapeutic application were developed in our laboratory.[13–15] In the initial oncologic investigation of these antagonists in carcinoma models, we assessed their effects on the GH/*IGF-I* axis in vivo.[15–17] We showed that treatment with the GH-RH antagonist MZ-4-71 significantly inhibited the growth of SK-ES-1 bone tumors derived from a Ewing sarcoma and from MNNG/HOS osteosarcomas in nude mice, and MZ-4-71 suppressed the serum levels of GH and *IGF-I* as well as the concentration of *IGF-I* in liver and tumor tissues.[16] Subsequently, we demonstrated that GH-RH antagonists can act directly on tumor cells and inhibit their proliferation in vitro.[15,18] In the course of subsequent investigations on various carcinoma cell lines, it became evident that these direct effects of GH-RH antagonists, which do not require the suppression of the GH/*IGF-I* axis, play a major role in the tumor inhibition observed during in vivo therapy.[15,17,19–24] Studies with human prostatic, pancreatic, colorectal, breast, and ovarian carcinomas and glioblastomas showed that the antiproliferative effect of GH-RH antagonists was accompanied by a decrease in the synthesis of tumoral autocrine *IGF-II* in vitro and in vivo.[15,17,18,20–25] This effect is independent of the actions of GH-RH antagonists on the pituitary, because the biosynthesis of *IGF-II* is not controlled by serum levels of GH.[15] *IGF-II*, acting on the same receptors as *IGF-I*, is a potent mitogen for various malignancies.[15,17,26–28]

Scheven et al.[10] and Fournier et al.[29] demonstrated that not only *IGF-I* but also *IGF-II* can stimulate the growth of SaOS-2 and OHS-4 human osteogenic sarcoma cells. Immunoactive *IGF-II* and mRNA for *IGF-II* were found in U-2 OS human osteosarcoma cells.[30] mRNA for *IGF-I* and *IGF-II* and receptors for *IGF-I* and *IGF-II* were demonstrated in surgical specimens obtained from patients with osteosarcomas.[31] Thus, therapeutic agents that suppress the synthesis of tumoral *IGF-II* may be useful for the treatment of patients with malignancies, including patients with osteosarcomas that require *IGF-II* as an autocrine growth factor.

In the current study, we investigated the mechanism involved in the inhibition of human experimental bone tumors by a new potent GH-RH antagonist, JV-1-38. We assessed the effect of this GH-RH antagonist on the growth of MNNG/HOS osteosarcoma cells[32] and SK-ES-1 Ewing sarcoma cells[33] in vivo, serum *IGF-I* and liver mRNA for *IGF-I*, and the concentration of *IGF-I* and *IGF-II* and their mRNA levels in tumors. We also evaluated the effects of JV-1-38, *IGF-I*, and *IGF-II* on the proliferation of these bone tumors in vitro.

MATERIALS AND METHODS

Peptides

The GH-RH antagonist JV-1-38 [PhAc-Tyr^{1}, D-Arg^{2,28}, Phe(4-Cl)^{6}, Har^{9}, Tyr(Me)^{10}, Abu^{15}, Nle^{27}, Har^{29}]hGH-RH(1–29)NH_2 was synthesized in our laboratory by solid-phase methods, as described previously.[14] For daily injections, the peptide was dissolved in 0.1% dimethyl sulfoxide (DMSO) in sterile, aqueous, 10% propylene glycol (vehicle solution).

Animals

Male athymic nude mice, age approximately 6 weeks on arrival, were purchased from the Frederick Cancer Research Facility of the National Cancer Institute (Frederick, MD) and were housed in laminar air-flow cabinets under pathogen free conditions on a 12 hours light-12 hours dark schedule. All animal studies were in accordance with institutional guidelines for the care and use of experimental animals.

Cell Lines

MNNG/HOS human osteosarcoma[32] and SK-ES-1 Ewing sarcoma[33] cell lines were purchased from the American Type Culture Collection (Manassas, VA). MNNG/HOS cells were cultured as a monolayer in minimal essential medium supplemented with 10% fetal bovine serum, antibiotics, antimycotics (100 U/mL penicillin G sodium, 100 μg/mL streptomycin, and 0.25 μg/mL amphotericin B). All culture media components were purchased from Gibco (Grand Island, NY). The SK-ES-1 cell line was grown as a monolayer in McCoy 5A medium supplemented with 15% fetal bovine serum, antibiotics, antimycotics, as specified above. Cultures were incubated in air containing 5% CO_2 at 37 °C. Tumor cells growing exponentially were harvested by a brief incubation with 0.25% trypsin-ethylenediamine tetraacetic acid (EDTA) solution (Gibco).

In Vivo Studies

Experiment 1

Xenografts of human osteosarcoma MNNG/HOS were initiated by subcutaneous injections of 1×10^7 cells into the right flanks of four mice. After 4 weeks, tumors were dissected and mechanically minced, and 3 mm^3 tumor tissue fragments were transplanted subcutaneously with a trocar needle into 18 nude mice. One week after the transplantation, when tumors had grown to approximately 50 mm^3, mice were divided randomly into two groups and received treatment with either GH-RH antagonist JV-1-38 (20 μg subcutaneously twice daily) or vehicle solution (control group). Tumor volume (length $\times$ width $\times$ height $\times$ 0.5236) and body weight were measured once per week. Tumor volume doubling time was evaluated between the start and the end of the study. After 4 weeks of treatment, the mice were killed, and tumors as well as various organs were removed, dissected, and weighed. Specimens of tumor tissue were snap frozen and stored at -70 °C for further analyses.

Experiment 2

Experiment 2, using nude mice bearing SK-ES-1 sarcomas, was designed and conducted as outlined above for Experiment 1. The treatment with either GH-RH antagonist JV-1-38 (20 μg subcutaneously twice daily) or vehicle solution lasted for 4 weeks.

In Vitro Studies

The effect of various agents on the rate of cell proliferation in serum free medium was evaluated by the 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) assay, as described previously.[16] Briefly, SK-ES-1 and MNNG/HOS cells were seeded into 96-well microplates, cultured for 18 hours, then washed once with Dulbecco modified Eagle medium/F12 1:1 medium with 15 mM N-2-hydroxyethyl-piperaz-ine-N′-2-ethane sulphonate. This was replaced by N_2E medium containing the test compounds dissolved in 0.1% DMSO (all media components were from Gibco or Sigma [St. Louis, MO]; for a description of the media, see Pinsky et al.[16]). Test compounds included *IGF-I* (5 ng/mL, 10 ng/mL, and 25 ng/mL; Gibco), *IGF-II* (5 ng/mL, 10 ng/mL, and 25 ng/mL; Bachem, Torrance, CA), antisera to *IGF-I* and *IGF-II* at dilutions of 1:200 and 1:50, as well as JV-1-38 (3 μM) alone and in combination with *IGF-I* (25 ng/mL) or *IGF-II* (5 ng/mL), each in octuplicate wells. The rabbit antibody (UB2-495) against human *IGF-I*, which was used in the proliferation experiments, was produced by Drs. L Underwood and J. J. Van Wyk (both at the University of North Carolina at Chapel Hill) and was supplied by the National Hormone and Pituitary Program (NHPP). This antibody shows 0.5% cross reaction with *IGF-II*. Human *IGF-II* specific rabbit antiserum, with less than 0.1% cross reactivity for *IGF-I*, was obtained from GroPep (Adelaide, Australia). Controls received only DMSO in N_2E medium. After 72–96 hours of culture, when the cells approached the end of the exponential phase of growth, MTT (Sigma) was added, and in vitro cell growth was measured as described elsewhere.[16] Results were calculated as $\%T/C$, where T is the optical density of treated cultures, and C is the optical density of control cultures.

The effects of JV-1-38 (3×10^{-6} M) on the expression of mRNA for *IGF-I* and *IGF-II* and on the amounts of *IGF-I* and *IGF-II* secreted by the cells into the serum free medium also were measured. The culture media (see above) of SK-ES-1 and MNNG/HOS cells grown in culture flasks (triplicate flasks with 10 million cells in each) were replaced either by 10 mL N_2E medium containing 3 μM JV-1-38 or by 10 mL N_2E medium alone for controls, using the procedure described above. For the measurement of *IGF-I* and *IGF-II* in the media of SK-ES-1 and MNNG/HOS cells, the samples were taken at the time when the serum free medium was placed on the cells as well as 24 hours and 48 hours later. After 48 hours, the cells were harvested, total RNA was extracted from control cells and treated cells, and the expression of mRNA for *IGF-I* and *IGF-II* was measured as described below.

Radioimmunoassays for GH, *IGF-I*, and *IGF-II*

Trunk blood was collected from nude mice at the end of the study, centrifuged at $\times$2000 *g* for 30 minutes at 4 °C, and serum was frozen for hormone studies. Serum GH levels were determined with a double-antibody radioimmunoassay (RIA) using materials pro-

vided by Dr. A. F. Parlow (Pituitary Hormones and Antisera Center, Torrance, CA): mouse GH reference preparation AFP10783B, mouse GH antigen AFP10783B, and antirat GH-RIA-5/AFP411S.

IGF-I and *IGF-II* were measured in the cytosol fraction of tumors as well as in serum and cell culture media. Tumor tissue was homogenized on ice at a ratio of 1 part of tissue to 5 parts of buffer consisting of 0.05 M Tris-HCl, 0.005 M EDTA, 0.005 M $MgCl_2$, and 0.25 M sucrose also containing protease inhibitors. To obtain the cytosol fraction, the homogenate was centrifuged at $\times$1300 *g* for 10 minutes, the pellet was discarded, and the supernatant was centrifuged at $\times$45,000 *g* for 45 minutes at 4 °C. *IGF-I* and *IGF-II* from serum and cytosol fractions were extracted using a modified acid-ethanol cryoprecipitation method that was described previously.[34,35] The method eliminates most of the IGF binding proteins, which can interfere with the RIA. In the cell culture media, *IGF-I* and *IGF-II* were measured directly without an extraction. Protein determination was performed on the cytosol using the Bio-Rad protein assay kit (Bio-Rad, Hercules, CA). *IGF-I* concentration was measured by RIA using *IGF-I* (DSL Inc., Webster, TX) as a standard in the range of 2–2000 pg per tube. *IGF-I* was iodinated by using the chloramine-T method. Goat anti-*IGF-I* antibody specific for *IGF-I* (DSL Inc.) was used at a final dilution of 1:20,833. *IGF-II* concentration was measured by RIA using human recombinant *IGF-II* standard (Bachem) in the range of 2–500 pg per tube. *IGF-II* was iodinated by using the lactoperoxidase method and was purified by reverse-phase high-performance liquid chromatography on a Vydac C18 column. Antirat *IGF-II* (10 μg/mL) monoclonal antibody (Amano International Enzyme, Troy, VA) was used at a final dilution of 1:14,285. This antibody cross reacts 100% with human *IGF-II* and rat *IGF-II* and 10% with human *IGF-I*.[36]

RNA Extraction and Reverse Transcriptase-Polymerase Chain Reaction Analysis

Total RNA was extracted from cultured MNNG/HOS and SK-ES-1 human sarcoma cells, from tumors grown in nude mice, and from mouse livers using the Micro RNA Isolation Kit (Stratagene, La Jolla, CA) and was quantified spectrophotometrically at 260 nm. Reverse transcriptase-polymerase chain reaction (RT-PCR) analysis was performed using the Gene Amp RNA Core Kit (Perkin-Elmer, Foster City, CA). Total RNA (2 μg) was reverse transcribed at 42 °C for 1 hour using 3.5 μM random hexamers and 2.5 units Moloney murine leukemia virus reverse transcriptase in 20 μL reaction containing 5 mM $MgCl_2$, 1 $\times$ PCR buffer, 1 mM of each dNTP, and 1 unit of RNase inhibitor. The RT reaction was terminated by heating at 99 °C for 5 minutes. The PCR for *IGF-I* and *IGF-II* was performed as follows: 2.5 μL of cDNA were amplified in 12.5 μL mixture containing 1 $\times$ PCR buffer, 2 mM $MgCl_2$, 0.3 mM of each dNTP, 2.5 units of Ampli Taq DNA polymerase, and 0.5 μM of the respective oligonucleotide primer pair. The primers used were 5′-ACA TCT CCC ATC TCT CTG GAT TTC CTT TTG C-3′ (sense) and 5′-CCC TCT ACT TGC GTT CTT CAA ATG TAC TTC C-3′ (antisense) for mouse and human *IGF-I* (not species specific) and, 5′-AGT CGA TGC TGG TGC TTC TCA CCT TCT TGG C-3′ (sense) and 5′-TGC GGC AGT TTT GCT CAC TTC CGA TTG CTG G-3′ (antisense) for human *IGF-II*. PCR for *IGF-I* consisted of initial denaturation at 97 °C for 100 seconds; 5 cycles at 97 °C for 30 seconds, 60 °C for 30 seconds, and 72 °C for 30 seconds; followed by 33 cycles (liver), 28 cycles (SK-ES-1 tumors), 25 cycles (SK-ES-1 cells), or 35 cycles (MNNG/HOS tumors and cells) at 95 °C for 35 seconds, 60 °C for 30 seconds, and 72 °C for 30 seconds with a final extension at 72 °C for 7 minutes. The PCR profile for *IGF-II* consisted of 1 cycle of denaturation at 95 °C for 3 minutes; 26 cycles (MNNG/HOS tumors), 22 cycles (MNNG/HOS cells), 30 cycles (SK-ES-1 tumors), or 29 cycles (SK-ES-1 cells) at 95 °C for 1 minute, 60 °C for 1 minute, and 72 °C for 1 minute; followed by 1 cycle at 72 °C for 7 minutes. The sequences of primers and PCR conditions for mouse and human β-actin (internal controls) and human *IGF-I* receptor were described previously.[19,23] For each PCR, the number of cycles was set at the exponential phase. PCR was performed in a Gene Amp PCR System 2400 (Perkin-Elmer). PCR products were separated by electrophoresis on 1.8% agarose gels and stained with ethidium bromide. The intensity of bands was measured with a scanning densitometer (model GS-700; Bio-Rad) coupled with Bio-Rad PC analysis software. The relative levels of PCR products were normalized compared with mRNA for β-actin.

Statistical Methods

The SigmaStat software package was used for statistical analysis of the data. Data are presented as mean $\pm$ standard error. The differences between groups were assessed with the two-tailed Student *t* tests, with $P < 0.05$ considered significant. The results of cell proliferation assays were subjected to a one-way analysis of variance followed by a Tukey test.

RESULTS

The Effect of GH-RH Antagonist JV-1-38 on the Growth of Malignant Bone Tumors in Nude Mice

The antiproliferative effect of JV-1-38 became significant within 7 days of therapy in MNNG/HOS osteo-

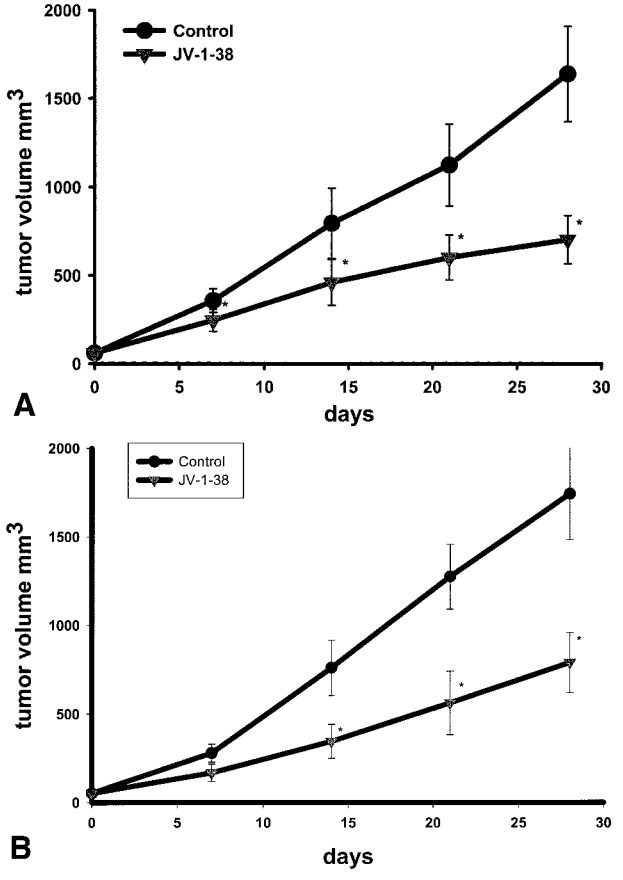

FIGURE 1. The effect of growth hormone-releasing hormone (GH-RH) antagonist JV-1-38 on the growth of MNNG/HOS human osteosarcoma (A) and SK-ES-1 human Ewing sarcoma (B) in nude mice. Treatment with JV-1-38 at a dose of 20 μg twice daily was started when the tumors measured approximately 50 mm^3 and lasted for 4 weeks. Vertical bars represent the standard error. An asterisk indicates $P < 0.05$ compared with the control.

sarcomas (Fig. 1A) and within 14 days in the SK-ES-1 model (Fig. 1B). The final tumor volume of MNNG/HOS osteosarcomas in the group treated with JV-1-38 for 28 days decreased significantly ($P < 0.05$) to 702 $\pm$ 136 mm^3 compared with controls, which measured 1638 $\pm$ 270 mm^3, corresponding to a 52.2% reduction (Table 1, Fig. 1A). In mice with SK-ES-1 sarcomas, the final tumor volume in the group that received JV-1-38 for 28 days was 789 $\pm$ 169 mm^3 compared with 1744 $\pm$ 237 mm^3 for the control group; thus, the GH-RH antagonist caused 54.1% inhibition ($P < 0.05$) (Table 1, Fig. 1B). The doubling time of MNNG/HOS tumors was extended significantly ($P < 0.01$) to 9.81 $\pm$ 1.98 days in mice treated with JV-1-38 compared with 6.07 $\pm$ 0.34 days for the untreated control mice (Table 1). The tumor doubling time for SK-ES-1 sarcomas, likewise, was increased significantly by treatment ($P < 0.01$) to 7.81 $\pm$ 0.52 days compared with the control value of 5.70 $\pm$ 0.21 days (Table 1). At the end of both experiments, tumor weights in the animals that received the antagonist were significantly lower compared with the animals in the control groups, with 50.6% and 50.1% inhibition in the MNNG/HOS and SK-ES-1 tumors, respectively ($P < 0.05$ for both) (Table 1). There were no significant changes in the body weights or in the weights of liver and kidneys in treated mice compared with control mice, indicating that the antagonist was devoid of any systemic toxicity (Table 1).

The Effect of JV-I-38 on Serum Levels of GH, *IGF-I*, and *IGF-II* and on Tumor Concentrations of *IGF-I* and *IGF-II*

After administration of JV-1-38 for 28 days, there were no statistically significant differences between the serum GH levels of treated groups and control groups in either experiment (data not shown). The concentrations of *IGF-I* and *IGF-II* in serum and tumor samples are presented in Table 2. Therapy with JV-1-38 reduced serum levels of *IGF-I* by 35% ($P < 0.05$) in animals with MNNG/HOS osteosarcomas and by 26% ($P < 0.05$) in animals with SK-ES-1 tumors. Serum levels of *IGF-II* were not affected by the treatment with JV-1-38 (Table 2). The presence of immunoreactive *IGF-I* and *IGF-II* also was detected in samples of both tumors. The tissue concentration of *IGF-I* was higher in SK-ES-1 tumors compared with MNNG/HOS tumors. Therapy with JV-1-38 significantly decreased *IGF-I* levels in SK-ES-1 tumors ($P < 0.01$) by 31% but did not affect *IGF-I* levels in MNNG/HOS tumors. The tissue concentration of *IGF-II* was similar in both types of tumors. Chronic administration of JV-1-38 suppressed the levels of *IGF-II* by 23% ($P < 0.05$) in MNNG/HOS tumors and by 33% ($P < 0.05$) in the SK-ES-1 model (Table 2).

The Effects of JV-1-38 on the Expression of mRNA for *IGF-I* in the Liver and the Expression of mRNA for *IGF-I* and *IGF-II* in Tumors

To evaluate the suppression of the pituitary GH/hepatic *IGF-I* axis, we investigated the changes in expression of mRNA for *IGF-I* in the livers of mice bearing MNNG/HOS or SK-ES-1 tumors that were treated with JV-1-38. Figure 2A,B illustrates the RT-PCR analysis of mRNA for *IGF-I* and β-actin in mice with MNNG/HOS osteosarcomas. Densitometric analysis showed that the GH-RH antagonist reduced expression of the *IGF-I* gene in liver tissue to 63% $\pm$ 3.9% ($P < 0.05$) in mice xenografted with the MNNG/HOS osteosarcoma cell line and to 68% $\pm$ 6.4% ($P < 0.05$) in mice with SK-ES-1 Ewing sarcomas compared with control animals, with an accepted expression level of 100% (Fig. 2C). Expression of the *IGF-I* gene was found in SK-ES-1

TABLE 1
The Effect of Growth Hormone-Releasing Hormone Antagonist JV-1-38 on the Growth of Subcutaneously Transplanted MNNG/HOS and SK-ES-1 Human Malignant Bone Tumors in Nude Mice[a]

	Tumor volume (mm^3)				
Treatment group	Initial	Final (% inhibition)	Tumor volume doubling time (days)	Tumor weight (g) (% inhibition)	Body weight (g)
MNNG/HOS control	59.8 ± 6.38	1638 ± 270	6.07 ± 0.34	2065 ± 290	28.83 ± 0.93
JV-1-38	59.4 ± 6.36	702 ± 136 (52.2)[b]	9.81 ± 1.98[c]	1119 ± 241 (50.6)[b]	27.92 ± 0.78
SK-ES-1 control	51.9 ± 10.11	1744 ± 237	5.70 ± 0.21	2138 ± 266	30.28 ± 0.66
JV-1-38	51.8 ± 5.12	789 ± 169 (54.1)[b]	7.81 ± 0.52[c]	1066 ± 239 (50.1)[b]	29.94 ± 1.27

[a] Values are expressed as mean ± standard error.
[b] $P < 0.05$ versus control.
[c] $P < 0.01$ versus control.

TABLE 2
Concentrations of Insulin-Like Growth Factor I and II in Serum and Tumor Tissue of Nude Mice Bearing MNNG/HOS and SK-ES-1 Human Sarcomas and Treated with Growth Hormone-Releasing Hormone Antagonist JV-1-38[a]

	Serum concentration (ng/mL)		Tissue concentration (pg/100 μg protein)	
Treatment group	*IGF-I*	*IGF-II*	*IGF-I*	*IGF-II*
MNNG/HOS control	129.6 ± 7.05	39.92 ± 2.88	258.75 ± 26.7	266.0 ± 30.41
JV-1-38	84.48 ± 7.62[b]	44.96 ± 6.53	270.5 ± 15.41	204.26 ± 17.65[b]
SK-ES-1 control	143.33 ± 10.12	19.64 ± 2.02	385.27 ± 37.7	239.17 ± 29.19
JV-1-38	107.21 ± 3.38[b]	18.62 ± 1.85	266.37 ± 21.47[c]	160.07 ± 16.1[b]

IGF: insulin-like growth factor.
[a] Values are expressed as the mean ± standard error.
[b] $P < 0.05$ versus control.
[c] $P < 0.01$ versus control.

tumors (Fig.3A), but the levels of mRNA for *IGF-I* did not change after therapy with JV-1-38 (data not shown). In contrast to SK-ES-1, we could not detect *IGF-I* mRNA in MNNG/HOS tumors under the conditions used (up to 40 cycles of PCR) (Fig. 3A). *IGF-II* gene expression was found in both models of human sarcoma (Fig. 3B). The normalized level of mRNA for *IGF-II* in MNNG/HOS tumors was about five times greater compared with the level in SK-ES-1 tumors. Therapy with JV-1-38 lowered *IGF-II* mRNA by 24% ($P < 0.01$) in MNNG/HOS sarcomas and by 20% ($P < 0.05$) in SK-ES-1 tumors (Fig. 3E) compared with control levels. Both sarcomas expressed similar levels of mRNA for *IGF-I* receptor (Fig. 3C).

Secretion of *IGF-I*, *IGF-II*, and Their mRNA Levels in Vitro

The MNNG/HOS cell line released 0.47–0.52 ng/mL *IGF-I* and 0.38–0.48 ng/mL *IGF-II* into serum free media after 24 hours and 48 hours of culture, respectively, whereas the SK-ES-1 cell line secreted 0.58–1.3 ng/mL *IGF-I* and 0.82–1.25 ng/mL *IGF-II* in the same culture periods. We also evaluated the effect of JV-1-38 (3 μM) on the secretion of *IGF-I* and *IGF-II* in the MNNG/HOS and SK-ES-1 cell lines after 24 hours and 48 hours of treatment but found no significant differences compared with untreated cells (data not shown). The expression of mRNA for *IGF-I* and *IGF-II* also was evaluated in the cell lines by RT-PCR analysis. In accordance with the tumor results, we found mRNA for *IGF-II* in MNNG/HOS cells; however, *IGF-I* mRNA could not be detected using our method with up to 40 cycles of PCR. SK-ES-1 cells expressed mRNA for both *IGF-I* and *IGF-II*, like the tumor samples. After a 48-hour exposure of either cell line to 3 μM JV-I-38, the levels of the mRNA for *IGF-I* or *IGF-II* were not affected significantly compared with controls (data not shown).

In Vitro Proliferation Studies

Exogenous *IGF-I* at a concentration of 5–25 ng/mL did not influence the proliferation rate of MNNG/HOS cells (data not shown). *IGF-II* slightly stimulated cell growth, but only at the 5 ng/mL level and not at the 10 ng/mL and 25 ng/mL levels (data not shown). Al-

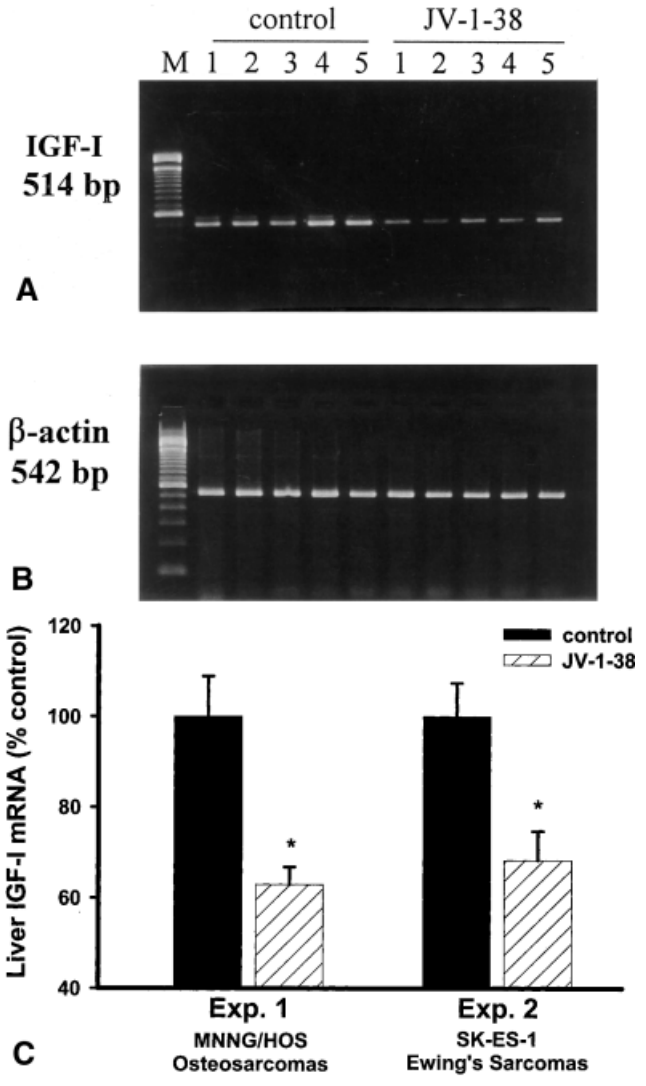

FIGURE 2. Suppression of mRNA for insulin-like growth factor I *IGF-I* in the liver of nude mice bearing MNNG/HOS human osteosarcoma by growth hormone-releasing hormone (GH-RH) antagonist JV-1-38. Polymerase chain reaction (PCR) products of the expected size of 514 base pairs (bp) for *IGF-I* (A) and 542 bp for β-actin (B) were separated on 2% agarose gels. PCR products from five controls and from five mice that were treated with JV-1-38 are shown. (C) Densitometric analysis of mRNA for IGF-1 in livers of nude mice bearing MNNG/HOS osteosarcomas (Exp. 1) and SK-ES-1 Ewing sarcomas (Exp. 2). The values were standardized according to mouse β-actin mRNA levels and expressed as a percentage of the control. Vertical bars indicate the standard error. An asterisk indicates $P < 0.05$ compared with the control.

though the effect of *IGF-II* at 5 ng/mL was modest (9% stimulation), it was significant statistically ($P = 0.01$). Rabbit antibodies specific for *IGF-I* or *IGF-II* strongly inhibited the proliferation of MNNG/HOS cells at dilutions of 1:200 (by 30% and 18%, respectively) and 1:50 (by 34% and 37%, respectively) (Fig. 4A). Normal rabbit serum was without effect at the same dilutions (data not shown). GH-RH antagonist JV-1-38 (3 μM) suppressed the growth of MNNG/HOS cells in vitro even more powerfully (41%), and this effect could not be nullified by *IGF-I* (25 ng/mL) or *IGF-II* (5 ng/mL) (Fig. 4B).

The growth of SK-ES-1 cells was stimulated significantly by *IGF-I* at concentrations of 10 ng/mL and 25 ng/mL (22% and 23%, respectively; $P < 0.001$), but *IGF-II* had no effect (data not shown). The antisera against *IGF-I* and *IGF-II* both strongly decreased the proliferation rate of SK-ES-1 cells by 37–48% ($P < 0.001$) (Fig. 4C) in contrast to normal rabbit serum, which slightly stimulated the proliferation (data not shown). Again, GH-RH antagonist JV-1-38 (3 μM) had an even greater antiproliferative effect on the SK-ES-1 cell line (54% inhibition) compared with the antisera to IGFs, and this inhibitory effect could not be offset by the addition of *IGF-I* (25 ng/mL) or *IGF-II* (5 ng/mL) (Fig. 4D).

DISCUSSION

New therapeutic modalities are needed to improve the treatment of patients with osteogenic sarcomas and other malignant bone tumors. The MNNG/HOS and SK-ES-1 cell lines represent different models of human sarcoma. MNNG/HOS is a chemically transformed cell line that was derived from an osteogenic sarcoma in a Caucasian female age 13 years.[32] The SK-ES-1 Ewing sarcoma line was established using a primary biopsy from a male age 18 years.[33] Both of these sarcomas are tumorigenic in nude mice and, thus, can be used for in vivo investigations. In the current study, we evaluated the effects of GH-RH antagonist JV-1-38 in the two models of primary bone neoplasms.

Our findings demonstrate that the new GH-RH antagonist, JV-1-38, has distinct antineoplastic activity and can inhibit the growth of MNNG/HOS and SK-ES-1 sarcomas xenografted into athymic nude mice significantly. The decrease in plasma *IGF-I* levels of nude mice treated with JV-1-38 indicates that the GH-RH antagonist can act in part through an inhibition of the effects of hypothalamic GH-RH on the pituitary. The resulting suppression in the release of GH from the pituitary gland leads to the reduction in the synthesis of *IGF-I* in the liver and other organs.[15,37] A decrease in the expression of mRNA for *IGF-I* in the livers of nude mice supports the view that the antiproliferative effect of GH-RH antagonists may be exerted in part through this indirect endocrine mechanism. Our inability to demonstrate an inhibition of serum GH in our experiments may be related to the fact that the suppression of pituitary GH release after administration of GH-RH antagonists in vivo lasts for

< 1 hour.[14,15] In addition, serum levels of GH show pulsatile fluctuations, and circulating *IGF-I* is correlated better with integrated 24-hour GH concentrations than with a single sampling of GH levels.[38] However, MNNG/HOS osteosarcomas are not stimulated in vitro by *IGF-I*, no mRNA for *IGF-I* could be demonstrated in the cells, and no changes in *IGF-I* content after JV-1-38 treatment were found in tumors. Thus, the indirect endocrine mechanism may not be important in MNNG/HOS osteosarcomas and can account only in part for the growth suppression of SK-ES-1-Ewing sarcomas, considering the reduction in *IGF-II* and its mRNA levels in both types of tumors produced by GH-RH antagonists in vivo and an inhibition of proliferation of these sarcomas in vitro in the presence of GH-RH antagonist JV-1-38 or the specific antisera to *IGF-II*.

Nevertheless, various results indicate that at least a subset of human osteogenic sarcomas must be responsive to *IGF-I*.[8–12,16,17] The mitogenic effects of *IGF-I* have been reported in some osteosarcomas.[8,9] Thus, compared with our findings in MNNG/HOS osteosarcomas, Pollak et al.[8,9] showed that *IGF-I* was a potent stimulator of proliferation in human MG-63 osteosarcomas and MGH-OGS murine osteosarcomas. In the current study and in previous work,[16] we also found that exogenous *IGF-I* can stimulate, and an antibody to *IGF-I* inhibits, the proliferation of SK-ES-1 Ewing sarcomas in vitro.

RT-PCR analysis revealed that SK-ES-1 tumors and cells expressed the gene for *IGF-I*; however, in MNNG/HOS tumors and cells, no measurable levels of mRNA for *IGF-I* could be found under the conditions of our experiments. However, *IGF-I* protein was detected in the media of both MNNG/HOS cells and SK-ES-1 cells cultured in vitro. Measurement of the *IGF-I* levels in tumors grown in nude mice by RIA showed that both sarcomas contained *IGF-I* protein, with higher concentrations found in SK-ES-1 tumors compared with MNNG/HOS tumors, in accordance with previous observations.[16] The differences seen in the expression levels of *IGF-I* and *IGF-II* in the MNNG/HOS and SK-ES-1 cell lines could be attributed to

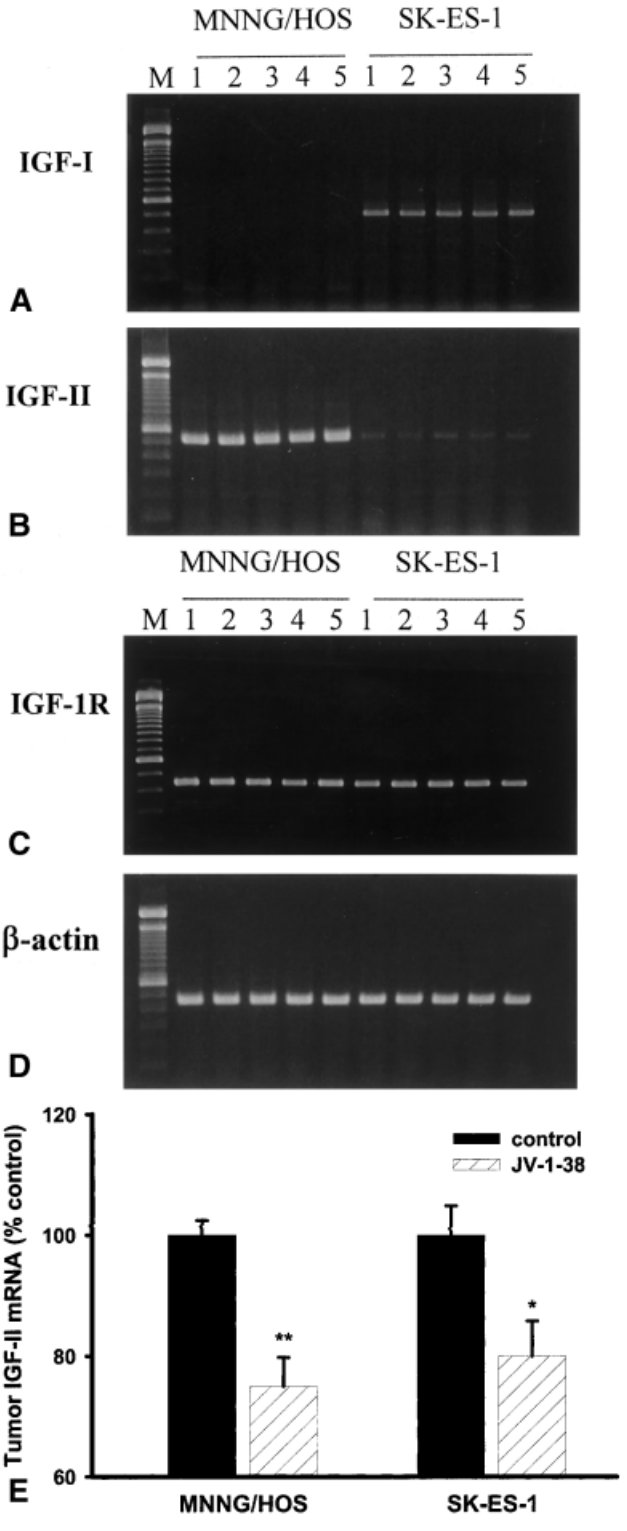

FIGURE 3. The expression of mRNA for insulin-like growth factor I (*IGF-I*) (A), *IGF-II* (B), *IGF-I* receptor (*IGF-IR*) (C), and β-actin (internal control) (D) in MNNG/HOS and SK-ES-1 human sarcomas. Polymerase chain reaction (PCR) products of the expected sizes (514 base pairs [bp] for *IGF-I*, 538 bp for *IGF-II*, 447 bp for IGF-1R, and 459 bp for β-actin) were separated by electrophoresis on 1.8% agarose gels. No mRNA for *IGF-I* was detected in MNNG/HOS tumors under the conditions used (up to 40 cycles of PCR). (E) The effect of JV-1-38 on the expression of *IGF-II* in MNNG/HOS and SK-ES-1 human bone tumors xenografted into nude mice. The optical densities of bands representing *IGF-II* (538 bp) were normalized according to human β-actin mRNA levels and expressed as a percentage of the control. A single asterisk indicates $P < 0.05$ compared with the control, and double asterisks indicate $P < 0.01$ compared with the control. ND: not detected.

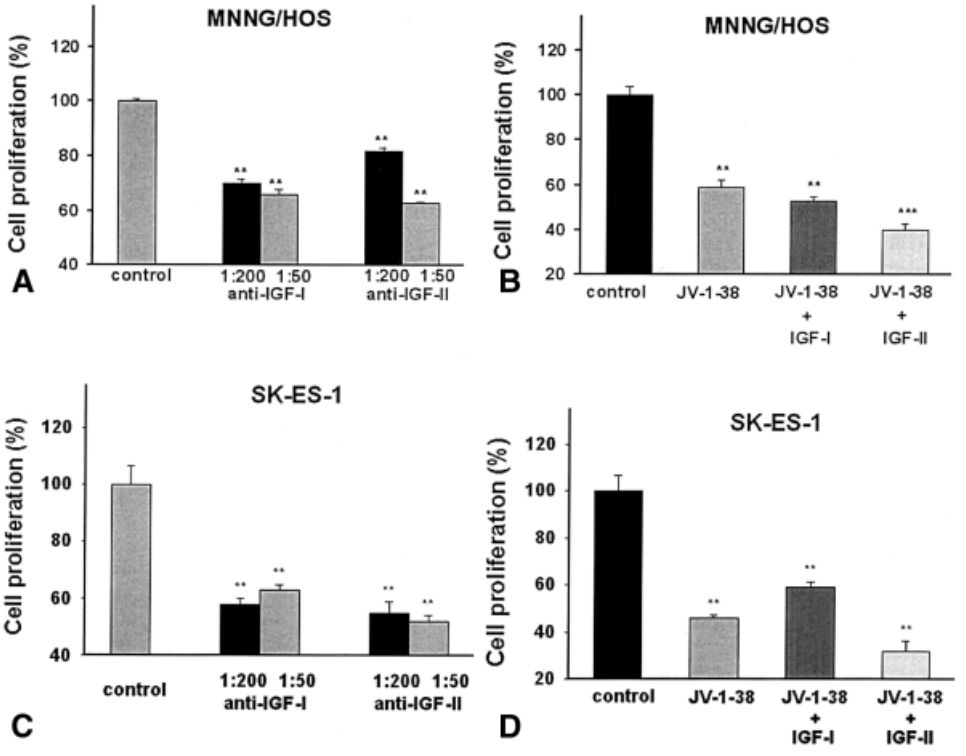

FIGURE 4. The effects of anti-insulin-like growth factor I (anti-*IGF-I*) and anti-IGF II antibodies (A,C) and of the growth hormone-releasing hormone (GH-RH) antagonist JV-1-38 (3 μM) alone or in combination with *IGF-I* (25 ng/mL) or *IGF-II* (5 ng/mL (B,D) on the proliferation of MNNG/HOS human osteosarcoma cells (A,B) or SK-ES-1 human Ewing sarcoma cells (C,D), in vitro. Vertical bars represent the standard error. Double asterisks indicate $P < 0.001$ compared with the control, and triple asterisks indicate $P < 0.001$ compared with the control and $P < 0.05$ compared with JV-1-38 alone.

differences in tumor type. The concentration of *IGF-I* protein in cytosol fractions of SK-ES-1 tumors was decreased significantly by treatment with JV-1-38, although the levels of *IGF-I* in MNNG/HOS tumors were not reduced after treatment with the GH-RH antagonist. Thus, the role of endocrine and autocrine *IGF-I* in MNNG/HOS osteosarcomas remains to be clarified. Our results support the view that *IGF-I* is an endocrine and autocrine growth factor for SK-ES-1 sarcomas, and the reduction of serum and tumor levels of *IGF-I* after treatment with GH-RH antagonists may be one of the mechanisms responsible for tumor inhibition.

IGF-II is homologous structurally to *IGF-I*, and most of its mitogenic/metabolic effects are mediated through the *IGF-I* receptor.[26] The expression of *IGF-II* is widespread during fetal life and is restricted much more after birth.[39] However, bone is among the few adult tissues that express *IGF-II* and in which *IGF-II* seems to play a physiologic role.[39] *IGF-II* is synthesized locally and is used as an autocrine growth factor by a variety of malignancies, including osteosarcomas, although it is independent of GH.[15,17,26–30] The expression of mRNA for *IGF-II* and *IGF-I* receptors was found in the majority of human osteosarcoma specimens investigated.[31,40]

In our study, we showed the presence of immunoreactive *IGF-II* protein and mRNA for *IGF-II* in MNNG/HOS and SK-ES-1 tumors and cells. Treatment with the GH-RH antagonist in vivo suppressed the synthesis of autocrine *IGF-II* in both tumors, as shown by the quantitation of *IGF-II* protein by RIA and the estimation of gene expression for *IGF-II* by RT-PCR analysis; however, as expected, the treatment with JV-1-38 had no influence on the serum *IGF-II* levels in nude mice. It is likely that the reduction of *IGF-II* levels in the tumor xenografts produced by GH-RH antagonists contributed importantly to the inhibition of tumor growth, because *IGF-II* and the *IGF-I* receptors appeared to form an autocrine stimulatory loop in both sarcomas. Although the exposure to exogenous *IGF-II* in vitro only slightly stimulated the proliferation of MNNG/HOS cells at one dose and did not affect the growth of SK-ES-1 cells, a specific antibody to *IGF-II* significantly inhibited the proliferation of both sarcomas. A lack of proliferative response or only weak stimulatory effects by exogenous growth factors are quite common in carcinoma cell lines that produce such growth factors in an autocrine manner and in which the corresponding autocrine loops already are stimulated maximally or submaximally.

GH-RH specific receptors on tumors that may mediate the effects of GH-RH and its antagonists were identified recently.[17,41,42] Using ^{125}I-labeled GH-RH antagonist JV-1-42 as a special ligand, we demonstrated the presence of specific, high-affinity binding sites for GH-RH and its antagonists on CAKI-1 renal, MiaPaCa-2 pancreatic, LNCaP and PC-3 prostatic, and OV-1063 ovarian carcinomas.[17,23,41,42] The isolation

and sequencing of cDNAs corresponding to the tumoral GH-RH receptor mRNAs revealed that they are splice variants (SV) of the pituitary GH-RH receptors.[42] RT-PCR analyses showed the expression of receptor SV in several carcinoma cell lines, including LNCaP, PC-3, and MDA-PCa-2B prostatic carcinoma cells; MiaPaCa-2 pancreatic carcinoma cells; CAKI-1 renal carcinoma cells; H-69 small cell lung carcinoma and MDA-MB-468 breast carcinoma cells; and OV-1063 ovarian carcinoma cells.[17,23,41,42] The direct inhibitory effect of GH-RH antagonists appears to be mediated by the tumoral GH-RH receptors, by mechanisms dependent or independent of *IGF-I* and *IGF-II*.[17,43] Thus, GH-RH antagonists inhibit the production of *IGF-I* and *IGF-II* and the expression of *IGF-II* mRNA in many human carcinoma cell lines in vitro and in vivo, including prostatic, renal, pancreatic, and colon carcinomas; glioblastomas; ovarian carcinomas; and non-small cell lung carcinomas.[15,17,18,20–25] Because *IGF-II* is a potent mitogen for many tumors, a suppression of its production would inhibit tumor growth. However, we also found recently that, in various tumor types, the extent of tumor inhibition produced by GH-RH antagonists was not always correlated with the magnitude of reduction in tumoral IGF-II, indicating that mechanisms independent of *IGF-II* also participate and play an important role in the antineoplastic mechanism.[17,43,44] In some tumor models, such as the H-69 small cell lung carcinoma, GH-RH antagonists inhibit proliferation without actually reducing the synthesis of autocrine *IGF-II*.[43] Similarly, the inhibition of growth of MDA-MB-435 breast carcinoma was not linked to any effects on *IGF-I* or *IGF-II*.[44] Those investigations indicate that GH-RH synthesized by various tumor cells can serve as an autocrine growth factor and that GH-RH antagonists inhibit tumor growth, nullifying these mitogenic actions of GH-RH.[17,43] Our latest studies revealed that MNNG/HOS and SK-ES-1 sarcomas also express SV1 of GH-RH receptors and produce GH-RH peptide, which may be involved in the pathophysiology of these tumors.[45] A direct inhibitory effect in vitro of GH-RH antagonist JV-1-38 on the two cell lines of human malignant bone tumors is in accord with these observations. In the current study, we did not evaluate the relative contribution of various mechanisms of action of GH-RH antagonists to inhibition of sarcomas. This topic will be the subject of our future investigations.

In conclusion, both the suppression of hepatic and/or tumoral *IGF-I* and a decrease in the synthesis of tumoral autocrine/paracrine *IGF-II* appear to contribute to the inhibitory effects of GH-RH antagonists on the growth of SK-ES-1 Ewing sarcoma cells. However, in MNNG/HOS osteosarcoma cells, only the latter mechanism could be demonstrated. Because GH-RH antagonists inhibit *IGF-II* dependent tumors like MNNG/HOS, they should be superior to GH antagonists, because the synthesis of *IGF-II* is not controlled by GH. GH-RH antagonists also would be preferred to somatostatin analogs for the suppression of bone tumors, because some human sarcomas, such as SK-ES-1, do not express somatostatin receptors.[15,17] Our findings support the merit of further investigations aimed at the development of new therapies for patients with bone tumors based on GH-RH antagonists.

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