Metastatic Behavior of the RIF-1 Murine Fibrosarcoma: Inhibited by Hypophysectomy and Partially Restored by Growth Hormone Replacement

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Background: **We recently demonstrated that hypophysectomy profoundly inhibits metastatic behavior in the MGH-OGS murine osteosarcoma model and speculated that this effect is related at least in part to ablation of the growth hormone (GH)–insulin-like growth factor I (IGF-I) axis.** ***Purpose:*** **In this study, we determined whether the administration of GH to animals rendered GH and IGF-I deficient by hypophysectomy attenuates the inhibitory effects of hypophysectomy on metastatic behavior.** ***Methods:*** **Metastatic behavior was assayed by counting visible metastases in lungs 3 weeks after tail vein injection of RIF-1 fibrosarcoma cells to control mice (n = 29), hypophysectomized mice (n = 19), and hypophysectomized mice administered 0.05 μg/g body weight recombinant human GH twice daily (n = 21).** ***Results:*** **Twenty of 21 hypophysectomized mice receiving GH, eight of 19 hypophysectomized mice not receiving GH, and 26 of 29 controls had grossly visible pulmonary metastases 3 weeks after intravenous injection of 5×10^5 cells; mean numbers ± SD of gross metastases were 38.4 ± 11.3, 6.4 ± 2.2, and 13.1 ± 2.8 in the three groups, respectively. The presence ($P<.005$, chi-square test) and number ($P = .0003$, Mann–Whitney *U* test) of metastases were significantly reduced in hypophysectomized hosts compared with control hosts and were significantly higher in hypophysectomized, GH-replaced hosts compared with hypophysectomized hosts ($P<.001$, chi-square test; $P = .011$, Mann–Whitney *U* test), while the difference in presence and extent of metastases between control and hypophysectomized, GH-replaced hosts was not statistically significant.** ***Conclusions:*** **These data support the hypothesis that the status of the host with respect to GH and/or GH-dependent factors such as IGF-I influences the metastatic behavior of certain neoplasms.** ***Implications:*** **Our results raise the possibility that compounds that reduce GH output or interfere with GH action, such as somatostatin analogues, GH antagonists, IGF antagonists, and GH-releasing hormone antagonists, may suppress metastatic behavior of certain neoplasms.**

The insulin-like growth factor (IGF) mitogenic signal transduction system includes ligands, receptors, and binding proteins. The ligands IGF-I and IGF-II have structural homology to insulin, suggesting evolutionary derivation from a parent gene (*1,2*). IGF-I is a potent mitogen for a variety of cell types (*2-6*) and is known to mediate much of the action of growth hormone (GH) on somatic growth (*7*). Although the gene encoding IGF-I is expressed in many tissues, most circulating IGF-I is produced by the liver, where transcription is regulated by a complex hypothalamic–pituitary–hepatic axis (*8,9*). GH-releasing hormone released from the hypothalamus stimulates pituitary-GH release, while somatostatin inhibits GH release. Hepatocytes synthesize and release IGF-I in response to GH. Finally, the bioavailability of IGF-I is influenced by the presence of the various IGF-binding proteins, which modulate binding interactions between IGFs and their receptors (*10*).

Data consistent with a role for IGF-I in the behavior of certain sarcomas include the demonstration of IGF-I receptors on both sarcoma cell lines (*11,12*) and primary tumors (*11-13*), the autocrine production of ligands for this receptor by a subset of primary sarcomas (*13*), the demonstration of inhibition of in vitro sarcoma proliferation by antisense oligonucleotides against the IGF-I receptor (*14*), and the demonstration that hypophysectomy, which is associated with a variety of hormonal changes including ablation of GH production and GH-dependent IGF-I gene expression, inhibits local growth rate of the MGH-OGS experimental osteosarcoma by approximately 50%, and inhibits metastatic behavior in this model by close to 100% (*15*).

In vitro studies of MGH-OGS cells documented that these cells are growth-stimulated by IGF-I, but not by GH or other pituitary factors, which is consistent with the hypothesis that the inhibitory effects of hypophysectomy are related, at least in part, to the reduction in IGF-I gene expression and in serum levels that follow this ablative surgical procedure (*15*). In the present study, we sought to determine directly if the GH–IGF-I axis influences the metastatic behavior of an experimental sarcoma by assessing metastases in intact, hypophysectomized, and GH-replaced hosts

Materials and Methods

Control and surgically hypophysectomized C3H male mice were obtained from Charles River Laboratories (Montreal, Quebec, Canada) Hypophysectomy was performed at 6-7 weeks and hypophysectomized and control animals were both obtained at 8 weeks of age. Both hypophysectomized and control mice were fed ad libitum and climatized to a cycle of 12 hours of light and 12 hours of dark for at least 1 week prior to any experimental manipulations All protocols adhered to approved standards as determined by the Animal Care Committees of the University of Toronto and of McGill University, Montreal

Previous experiments evaluating the effect of hypophysectomy in murine sarcoma behavior used the MGH-OGS model The length of time necessary for the development of metastases using this system made it impractical for GH replacement The RIF-1 radiation-induced fibrosarcoma was therefore obtained from Dr R Hill (University of Toronto) for these purposes In vitro growth and in vivo behavior of RIF-1 cells have been described previously (*16-18*) Cell cultures of RIF-1 were grown in α-minimum essential medium and 10% fetal calf serum (GIBCO BRL, Grand Island, N Y).

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See "Notes" section following "References."

In Vitro Assays

Binding of recombinant IGF-I (rIGF-I; Amersham Corp., Arlington Heights, Ill.) and affinity labeling of IGF-I receptors were performed on plasma membrane-enriched subcellular fractions of these murine tumors prepared by differential centrifugation (*19*). Percent specific binding of cell membrane preparations to ^{125}I-labeled IGF-I was performed in the presence and absence of unlabeled IGF-I, as described previously (*11,15*). Human placenta served as a positive control tissue.

RIF-1 tumors were plated in 35-mm dishes for in vitro proliferation assays. Thymidine-incorporation experiments were performed following addition of rIGF-I (Amersham Corp.) in quadruplicate, as described previously (*15*).

Preparation of RIF-1 Cell Suspensions and Assay of Local Growth In Vivo

RIF-1 tumors were maintained in vitro and in vivo in source mice. Source tumors from four F2 generation RIF-1 sources were harvested for each RIF-1 experiment described below. An injectable cell suspension of RIF-1 cells from each harvest was prepared under sterile conditions as follows. First the source tumors were minced with scissors into a slurry and rinsed with phosphate-buffered saline (PBS) citrate. Using a cell scraper, the slurry was then passed once through a cell mesh. The sieved mixture was incubated at 37 °C with pancreatic DNA'ase and trypsin (Sigma Chemical Co., St. Louis, Mo.) and passed through a second sieve. The mixture was then centrifuged at 3000 rpm for 7 minutes at room temperature, and the resulting cell pellet was resuspended in 10% fetal calf serum. The concentration of RIF-1 cells in the cell suspension was determined manually by using a hemocytometer.

To evaluate the effect of hypophysectomy on local tumor growth, 2×10^6 RIF-1 cells prepared from a single cell suspension were injected intramuscularly into the right gastrocnemius muscle of 19 hypophysectomized and 20 control mice. Resultant tumor volumes were obtained three times weekly by measuring three orthogonal diameters ($R_1 \times R_2 \times R_3$) with 0.05-mm precision calipers and multiplying the product by $pi/6$[20]. Time for tumors to grow to 1000 mm^3 was recorded.

Experimental Metastases Assay and GH Replacement in RIF-1 Tumors

To evaluate the effect of hypophysectomy on RIF-1 metastases, hypophysectomized and control mice were randomly assigned to four experimental groups. Mice in hypophysectomized groups (n = 12 and n = 10) and control groups (n = 17 and n = 14) received either 1×10^5 or 5×10^5 RIF-1 cells prepared from a single cell suspension and injected into the lateral tail vein, as previously described by Korycka and Hill (*17*) and Ling et al. (*18*). The final inoculum volume given was 0.25 mL in a tuberculin syringe. Mice were observed for 3 weeks following this single inoculation dose and then killed; lungs were harvested at the time of death and stained in Bouin's solution for 2-3 days before metastatic colonies were counted.

To evaluate the effect of hypophysectomy and GH replacement on RIF-1 metastases, hypophysectomized mice were allocated into replacement and placebo groups. A dose of 0.05 μg human GH (HuGH) per gram of body weight given twice daily has been shown to normalize skeletal growth rates in hypophysectomized mice (*21-24*). The adequacy of hypophysectomy and GH replacement was assessed by comparing weekly mouse weights in groups. Treatment mice (hypophysectomy + HuGH; n = 21) received 0.05 μg of HuGH per gram of body weight twice daily by subcutaneous administration; hypophysectomized control mice (hypophysectomy + normal saline; n = 19) received equivalent doses of normal saline subcutaneously. Normal control mice (control + normal saline; n = 29) also received normal saline placebo. Seven days after initiation of daily injections, mice received 5×10^5 RIF-1 cells by lateral tail vein injection. Lungs were harvested 3 weeks following the tumor inoculum. This experiment was performed in duplicate, using two independent sets of RIF-1 sources to prepare single cell suspensions for tail vein injections.

Statistical Analyses

Unpaired Student's *t* tests were used to analyze the average time for tumors in control and hypophysectomized groups to grow to 1000 mm^3. Chi-square statistics were used to compare presence or absence of gross metastases between experimental groups; nonparametric Kruskal–Wallis and Mann–Whitney *U* tests were used to compare the number of metastases in RIF-1 experimental groups, where appropriate. Analysis of variance (ANOVA) was used to evaluate intergroup differences in mouse weights in replacement experiments.

Results

In Vitro Assays

Preliminary in vitro studies were performed on the murine tumors to quantitate levels of cell membrane IGF-I receptors and to determine the effect of IGF-I and GH on in vitro cell proliferation. The results of affinity-binding studies using ^{125}I-labeled IGF-I are shown in Fig. 1. Compared with our known positive controls for the IGF-I receptor (human placenta; 19.9% specific binding of ^{125}I-labeled IGF-I), RIF-1 tumors showed 9.0% specific binding. RIF-1 tumor proliferation in vitro was stimulated to 140% ± 8% of control in the presence of 1×10^{-9} *M* rHuIGF-I (P<.05), as determined by thymidine incorporation and cell number assays. Recombinant human GH (rHuGH) had no mitogenic activity for RIF cells in these assays.

Effect of Hypophysectomy on RIF-1 Local Growth

The local growth of the RIF-1 tumors was significantly slower when implanted in hypophysectomized hosts. The time

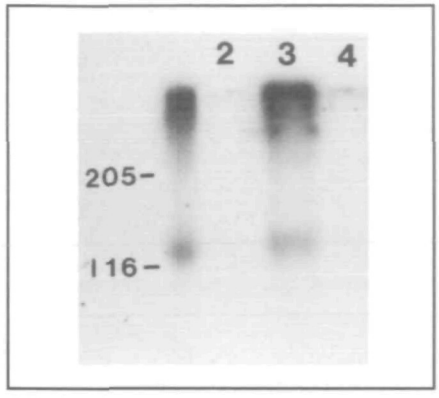

Fig. 1. Affinity labeling of IGF-I receptors of RIF-1 sarcoma. Affinity labeling experiments using ^{125}I-labeled IGF-I were performed on plasma membrane-enriched subcellular fractions of RIF-1 tumors (lanes 1 and 2) and human placenta (lanes 3 and 4) prepared as described previously (*11*). Lanes 1 and 3 were incubated in the absence of excess unlabeled IGF-I, and lanes 2 and 4 were incubated in the presence of excess unlabeled IGF-I.

(means ± SD) required for tumors to reach 1000 mm^3 averaged 23 ± 4 days in hypophysectomized mice compared with only 15 ± 2 days in control mice (P<.001; unpaired *t* test).

Effect of Hypophysectomy on Metastases in RIF-1 Tumors

The effect of hypophysectomy on the frequency distribution of RIF-1 metastases following injection of 1×10^5 or 5×10^5 cells demonstrated significant differences between groups (P<.0014). There was no difference in the presence (P>.05) and number (P>.05) of metastases observed between hypophysectomized and control mice 3 weeks following injections with 1×10^5 cells. However, following an inoculation dose of 5×10^5 RIF-1 cells, a significant difference was observed in number of metastases (P = .001) between hypophysectomized and control groups (Fig. 2).

Effect of HuGH Replacement

Analysis of mouse weights by ANOVA demonstrated that mice randomly assigned to hypophysectomized treatment (hypophysectomy + HuGH) and control (hypophysectomy + normal saline) showed no significant variation in size prior to commencement of the experiment (P>.05); however, both groups of hypophysectomized mice were significantly smaller than their intact control counterparts (P = .0001). Hypophy-

sectomized mice receiving twice-daily injections of HuGH (hypophysectomy + HuGH) gained significantly more weight than hypophysectomized mice receiving placebo (hypophysectomy + normal saline) ($P<.0001$). The amount of weight gained by the hypophysectomized replacement group during the course of the experiment was equivalent to that of the control group.

The effect of exogenous HuGH replacement in hypophysectomized mice on the metastatic potential of the RIF-1 tumor is illustrated in Fig. 3. Almost all of the control and HuGH replacement animals demonstrated visible metastases (26 of 29 controls and 20 of 21 replacement mice) compared with fewer than 50% of hypophysectomized animals (eight of 19; $P<.001$). The mean number ± SD of gross metastases observed in the three groups also differed significantly (38.4 ± 11.3, 13.1 ± 2.8, and 6.4 ± 2.2, respectively; $P = .0006$). In paired comparisons of groups, a significant difference in presence ($P<.005$) and number ($P = .0003$) of metastases was observed between control mice treated with normal saline and hypophysectomized mice treated with normal saline; hypophysectomized mice treated with HuGH and hypophysectomized mice treated with normal saline also demonstrated significant differences in presence ($P<.001$) and number ($P = .011$) of metastases. Using nonparametric analysis, the difference between the number of metastases in hypophysectomized mice treated with HuGH and control mice treated with normal saline did not reach statistical significance ($P = .08$). Similar results were observed when this experiment was repeated.

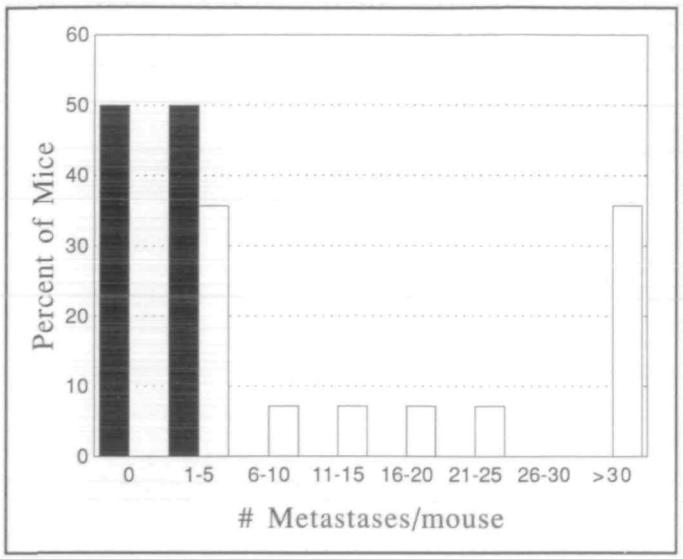

Fig. 2. Metastatic growth of RIF-1 in hypophysectomized (solid bars) and control (open bars) mice. Assay of RIF-1 metastases demonstrated that presence and number of metastases were significantly different between hypophysectomized and control mice inoculated with 5×10^5 cells ($P<.001$ by Mann–Whitney U test; $P>.05$ by chi-square test).

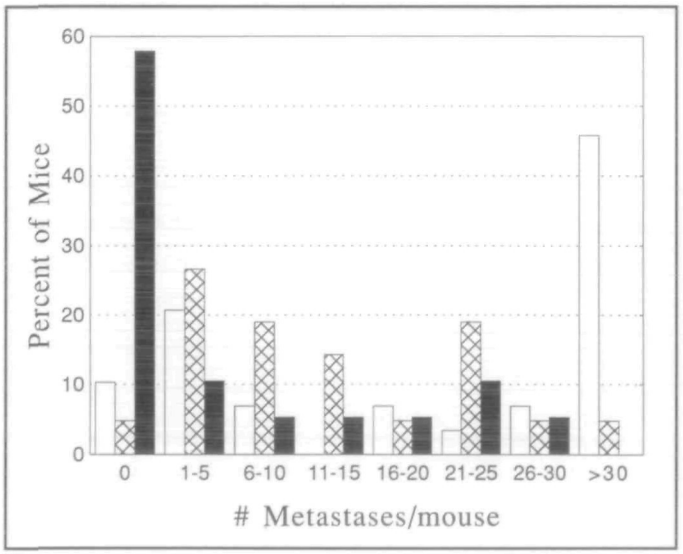

Fig. 3. Effect of GH replacement on RIF-1 metastases. Control mice treated with normal saline (open bars) and hypophysectomized mice treated with normal saline (solid bars) differed in presence ($P<.005$) and number ($P = .0003$) of metastases. Similarly, hypophysectomized mice treated with HuGH (cross-hatched bars) and hypophysectomized mice treated with normal saline differed in presence ($P<.05$) and number ($P = .0021$) of metastases. Hypophysectomized mice treated with HuGH and control mice treated with normal saline demonstrated no difference in metastases.

Discussion

Previous in vitro studies (*11-13*) have identified human osteosarcoma and soft-tissue sarcoma cell lines that exhibit IGF-I receptors on their cell membranes and that also respond mitogenically to IGF-I in culture. We were therefore interested in identifying murine tumor models from which in vivo experiments might be extrapolated to the human clinical disease. The murine MGH-OGS osteosarcoma (*20*), which parallels the human disease, has been shown to be IGF-I responsive in vitro; in vivo, both the local and metastatic growth of this tumor were inhibited by hypophysectomy (*15*). We postulated that these observations in the MGH-OGS osteosarcoma tumor are due to sequelae of GH reduction. We have therefore tested the effect of GH replacement on the metastatic behavior of an experimental sarcoma in which metastatic behavior is inhibited by hypophysectomy. The RIF-1, rather than the MGH-OGS experimental system, was selected for these experiments because the long latency period of the former model made GH replacement impractical.

Evaluation of the effect of hypophysectomy on RIF-1 metastases demonstrated a difference in the presence of and the number of metastases between groups of hypophysectomized and control mice inoculated with doses of 5×10^5 cells but not with 1×10^5 cells. Other studies (*17*) have shown that tumor load is important in the development of metastases in this model, and adequate tumor inoculum appears necessary to demonstrate the effect of hypophysectomy.

While the results of the experiments reported here are consistent with the hypothesis that certain tumors are dependent on the GH–IGF-I axis, the precise molecular mechanisms underlying our observations remain to be determined. IGF-I bioactivity in the microenvironment of neoplastic cells is a function not only of IGF-I concentration but also of the concentration of specific IGF-binding proteins that modulate responsiveness to IGFs. In addition to the changes in serum IGF-I level that are induced by

hypophysectomy with or without GH replacement, these interventions affect the concentrations of serum IGF-binding proteins and may also affect local expression of IGFs and IGF-binding proteins in neoplastic tissue and/or target organs for metastasis.

The profound in vivo effect of hypophysectomy on both local and metastatic growth in RIF-1 tumors and the ability to decrease the effect of hypophysectomy on metastasis by GH replacement has not been reported previously. These observations suggest a novel approach to the management of human tumors that may be dependent on the GH–IGF-I axis in vivo for optimum cell proliferation and/or metastatic behavior. A variety of approaches for pharmacologically ablating the GH–IGF-I axis have been proposed (*25*). Somatostatin analogues have been observed to reduce IGF-I levels both in experimental systems and in clinical trials, but the degree of suppression falls short of that achieved by hypophysectomy (*25*). Recent findings (*26*) suggest that there may be synergy between tamoxifen and somatostatin analogues in reducing IGF-I gene expression. Other approaches include GH antagonists (*27*), GH-releasing hormone antagonists (*28*), and pharmacologic interference with binding of IGF-I to its receptor by means of peptide IGF-I receptor antagonists (*29*), antibodies (*30*), or agents such as suramin, which have been observed to inhibit sarcoma proliferation in vitro (*31*) and in vivo (*32*). Another novel therapeutic approach would involve the use of GH or IGF-I as synchronization-recruitment agents prior to the administration of cytotoxic drugs (*33,34*).

It remains to be determined if stimulation of metastatic behavior of neoplasms by GH and/or by GH-dependent factors is a rare or a common phenomenon. While data obtained with both the MGH-OGS (*15*) and the RIF-1 systems are consistent with a stimulatory role of GH-dependent factors, no significant effect of hypophysectomy on local growth was seen in the murine SCC-7 squamous cell carcinoma model that has low IGF-I receptor expression (data not shown). In addition to preclinical investigation of the activity of the therapeutic approaches listed above, it will be necessary to investigate expression of genes for the IGF-I receptor, as well as the IGF-binding proteins, which may allow prediction of in vivo responsiveness to GH-dependent factors in both experimental tumors and clinical tumor specimens. To gain insight into the mechanisms by which changes in the hormonal milieu affect metastatic behavior, it also will be of interest to identify genes whose expression in neoplastic tissue is modulated by hypophysectomy and GH replacement.

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