Inhibition of Metastatic Behavior of Murine Osteosarcoma by Hypophysectomy

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Background: We recently reported that human osteogenic sarcoma cells are mitogenically responsive in tissue culture to insulin-like growth factor 1 (IGF-I), a mitogen important in the regulation of cellular proliferation of many tissues, including bone. Purpose: The present study was designed to determine whether these in vitro observations could be extended to an in vivo experimental system and whether reduction of IGF-I levels by hypophysectomy could inhibit the aggressive metastatic behavior of osteosarcoma. Methods: We used standard competitive binding and affinity-labeling techniques to characterize the IGF-I-binding sites of MGH-OGS, a model of human osteosarcoma. Radioimmunooassay of serum, preprocessed to remove IGF-binding proteins, was used to quantitate IGF-I levels. In vitro proliferative response of MGH-OGS cells to IGF-I and other pituitary-dependent factors was determined by thymidine-incorporation experiments. In vivo growth of the neoplasm in 12 hypophysectomized C3H mice and in 14 control C3H mice was determined by serial measurements of implanted tumors and by gross and microscopic examination of the lungs for metastases. Results: MGH-OGS exhibited specific binding sites for 1.39 pmol IGF-I per milligram MGH-OGS cellular membrane protein, a concentration similar to that which we previously reported for human osteosarcoma. In tissue culture, MGH-OGS exhibited mitogenic response to IGF-I (P < .01) but not to other pituitary-dependent factors. Hypophysectomy reduced levels of circulating IGF-I to 15% of control, significantly inhibited local growth of MGH-OGS tumors (increased time for growth to 1 cm³ from 49 to 84 days, P < .001), and profoundly inhibited metastatic behavior (decrease in mean number of metastases per host from 16 to less than one; P < .001). Conclusions: This study is the first to document the profound inhibitory effect of hypophysectomy on the metastatic behavior of an experimental sarcoma. We conclude that the metastatic behavior exhibited by MGH-OGS osteosarcoma is dependent on pituitary factors, and we suggest that the inhibitory effects of hypophysectomy are related, at least in part, to the reduction of IGF-I levels.

Insulin-like growth factor I (IGF-I) is a peptide mitogen known to have a key role in the regulation of cellular proliferation of many tissues, including bone (1,2). Elaborate physiological control systems exist to regulate circulating and tissue levels of this growth factor (1). The bulk of circulating IGF-I is synthesized in the liver, where transcription of the IGF-I gene is positively regulated by growth hormone (3). Pituitary secretion of growth hormone is regulated by a complex neuroendocrine control system involving the stimulatory hypothalamic peptide growth hormone-releasing hormone and the inhibitory peptide somatostatin (4). IGF-I is also expressed in extrahepatic tissues, where it may act in a paracrine rather than in an endocrine fashion, but the regulation of IGF-I gene expression in extrahepatic tissues is incompletely characterized (5). The bioactivity of IGF-I is further regulated by several specific high-affinity binding proteins present in both tissues and...
serum (1). At target cells, the first step in IGF-1-stimulated cellular proliferation involves the binding of IGF-1 to specific cell-surface receptor molecules of the tyrosine kinase class known as type 1 IGF receptors (6).

Because IGF-1 has been shown to be a key mitogen for normal osteoprogenitor cells (2), we recently studied its effect on osteogenic sarcoma. Our results demonstrated that human osteogenic sarcoma tissue is IGF-I receptor positive and that IGF-1 is a potent mitogen for osteogenic sarcoma cells in vitro (7). These observations suggested the possibility of novel endocrine therapies.

Despite recent advances in adjuvant chemotherapy and in locoregional management of osteogenic sarcoma, the aggressive metastatic behavior of this neoplasm remains an important clinical challenge. We therefore conducted experiments to determine if our in vitro observations could be extended to an in vivo experimental system and if hormonal manipulation could inhibit the metastatic behavior of osteosarcoma.

Materials and Methods

Hormones and Growth Factors

Recombinant human IGF-1 was obtained from Amersham Corp. (Arlington Heights, Ill.). Fetal calf serum was purchased from GIBCO BRL (Grand Island, N.Y.). Mibolerone was a gift from Dr. L. Pinsky, and human prolactin was donated by Dr. Paul Kelly. Other hormones were obtained from Sigma Chemical Co. (St. Louis, Mo.). Collagenase was purchased from Worthington Biochemical Corp. (Freehold, N.J.).

IGF-I Binding and Affinity Labeling

Procedures for studies of IGF-I binding and affinity labeling of IGF-I receptors were carried out on plasma membrane-enriched subcellular fractions of MGH-OGS tumors prepared by differential centrifugation, as previously described (7,8). These procedures were used to generate specific binding data, competitive binding data, and Scatchard plots. Human MG-63 osteosarcoma cells and human placenta were processed in the same way and served as control tissues. Recombinant human IGF-1 was used in these experiments.

In Vitro MGH-OGS Cell Proliferation

For the in vitro work, we did not obtain a large number of independent primary MGH-OGS tumors as sources of cells. Our reason for not doing so was that the MGH-OGS system was well described in our previously published studies (9,10) as being clonal, and biological variability was demonstrated to be negligible between specimens carried in different hosts.

MGH-OGS cells were obtained for in vitro studies by surgically removing one tumor from each of two hosts, removing surrounding normal tissues, mincing the pooled neoplastic tissue with scissors in nutrient mixture F-12 Ham medium (Sigma Chemical Co.), and digesting the tissue in 0.09% collagenase. Cells were then plated in 10% fetal calf serum, and debris was removed by changing the medium after 2 days. Subsequently, cells were replated in 35-mm plates for experiments to measure proliferation by assaying the incorporation of radiolabeled thymidine, as previously described (7,11,12). We assayed proliferative response to IGF-I, triiodothyronine, estradiol, the dihydrotestosterone analogue mibolerone, cortisol, prolactin, and growth hormone. Thymidine incorporation experiments were conducted in quadruplicate.

Mice

Appropriate approval for animal care was obtained from the Animal Care Committees of the University of Toronto and McGill University. Intact and hypophysectomized 8-week-old male C3H mice were obtained from Charles River Breeding Laboratories (Wilmington, Mass.) and kept in cages under standard conditions.

Radioimmunoassay for IGF-I

Radioimmunoassay of serum preprocessed to remove IGF-binding proteins was used to quantitate IGF-I levels. Radioimmunoassay was conducted as described and validated for rat serum by Breier et al. (13), using an antibody (14) provided by the National Institute of Diabetes and Digestive and Kidney Diseases (National Institutes of Health, Bethesda, Md.). Serum specimens (50 μL) were diluted 1:5 in 87.5% absolute ethanol and 12.5% 2 M hydrochloric acid. They were then processed to remove serum IGF-I-binding proteins by cryoprecipitation prior to the radioimmunoassay procedure, which was initiated by the incubation of samples with rabbit anti-IGF-I polyclonal antibody (14) at a 1:18000 final dilution. After 24 hours, radiolabeled IGF-I (20000 counts per minute) was added to each sample, and a further 24-hour incubation was carried out before the addition of 100 μL of normal rabbit serum (Sigma Chemical Co.) at a 1:100 dilution and 100 μL of sheep antibody to rabbit immunoglobulin (Sigma Chemical Co.) at a 1:18 dilution. After a final 24-hour incubation, samples were centrifuged at 2200g for 30 minutes, and radioactivity of the pellets was measured with a gamma counter. All incubations were performed at 4 °C. IGF-I levels were read from a standard curve, obtained by assaying samples containing known amounts of human recombinant IGF-I. Samples were assayed in duplicate, and the interassay variation was less than 15%.

Artifacts due to IGF-binding proteins present in serum were minimized by acid-ethanol cryoprecipitation and 1:200 dilution of serum prior to assay. The methodology used has been validated for rat serum (13).

Local and Metastatic Behavior of the Murine MGH-OGS Osteosarcoma Model

The murine MGH-OGS osteosarcoma was carried by serial transplantation and implanted in the lateral gastrocnemius muscle of 12 hypophysectomized mice and 14 control mice, as previously described (9,10,15). Tumor volumes were determined three times per week by measuring three orthogonal diameters with calipers and multiplying the product by π/6 (15). Mice were killed when their primary tumors grew to 1 cm 3, and the lungs were assayed for metastasis at this time. Metastatic involvement of the lungs was estimated by inspection at the time of autopsy—by weighing the metastatic lesions and by microscopic examination of serial sections of the lungs (9,10,15).
Results

IGF-I-Binding Studies and Affinity Labeling of IGF-I Receptors

Figs. 1 and 2 give the results of experiments conducted to determine if the experimental MGH-OGS tumor bears IGF-I receptors. Fig. 1 shows competitive binding curves for the binding of IGF-I to membranes prepared from MGH-OGS tumors, human MG-63 osteosarcoma cells, and human placenta. Scatchard analysis (Fig. 1, inset) yielded estimates of specific high-affinity (K_d = 0.6 nM) binding sites for 1.39 pmol exogenous IGF-I per milligram MGH-OGS cellular membrane protein.

Under our assay conditions, specific binding of 20% of added IGF-I was observed with membranes prepared from MGH-OGS tumors, compared with 28% seen with human placenta, a tissue known to have a particularly high concentration of IGF-I receptors (data not shown). These results suggest that MGH-OGS tumors have a density of IGF-I receptors comparable to that which we previously described for human osteosarcoma (7).

Affinity labeling with IGF-I showed the presence of labeled proteins in the 130-kd region, consistent with the presence of type I IGF-I receptors (Fig. 2).

In Vitro Studies of MGH-OGS Cell Proliferation

Results of experiments conducted in vitro with disaggregated MGH-OGS cells in primary culture were also consistent with the presence of type I IGF-I receptors. Thymidine incorporation studies (Fig. 3) showed the rate of proliferation in the presence of medium supplemented with 5 x 10^{-9} M IGF-I to be significantly higher (P<.01, Mann-Whitney U test) than control values obtained when proliferation of MGH-OGS cells was assayed in the absence of exogenous IGF-I. In contrast, there was no statistically significant difference between control values and values obtained when proliferation of MGH-OGS cells was assayed in the presence of other pituitary-dependent hormones at concentrations known to be mitogenic for cells that respond to these factors. The concentrations used were as follows: 5 x 10^{-8} M triiodothyronine, 10^{-8} M estradiol, 6 x 10^{-7} mibolerone (dihydrotestosterone analogue), 5 x 10^{-7} M cortisol, 100 ng/mL human prolactin, and 100 ng/mL recombinant human growth hormone.

Radioimmunoassay for IGF-I

To assess the adequacy of hypophysectomy as an IGF-I-lowering intervention, we obtained serum samples from the hypophysectomized and the control mice when they were killed. Radioimmunoassay estimates of serum IGF-I were 195 ± 8 ng/mL (SE) for the control group and 29 ± 6 ng/mL for the hypophysectomized group (Table 1).

Although we have been able to demonstrate, by the use of ligand blots, some residual binding proteins following the acid-ethanol-cryoprecipitation procedure (Pollak M, Polychronakos C: unpublished observations), the results presented here are sufficient to demonstrate the adequacy of hypophysectomies performed on the mice used in our experiments.

| Table 1. Effect of hypophysectomy on host serum IGF-I levels and metastatic behavior of MGH-OGS sarcoma |
|----------------------------------------------------------|---------------------------------|
| Control (n = 14)                                           | Hypophysectomized (n = 12)     |
| Serum IGF-I, ng/mL. mean ± SE (range) | 195 ± 8 (189-202)               | 29 ± 6 (22-36)             |
| No. of mice with metastases (%)                          | 12 (83.7)                      | 1 (8.3)                    |
| No. of metastases per host. mean ± SE                   | 16 ± 6                         | <1*                        |

*The single animal with metastasis had two lesions.
Local and Metastatic Behavior of the Murine MGH-OGS Osteosarcoma Model

Fig. 4 shows data demonstrating the effect of prior hypophysectomy on the local growth of 1-mm³ MGH-OGS tumors transplanted into the lateral gastrocnemius muscle of mice. We obtained data on local tumor growth in 14 control mice and in 12 hypophysectomized mice by measuring the time required for each of the 26 tumors to reach 1 cm³. In summary, the slower growth in the hypophysectomized group was demonstrated by a longer time required for the tumor to grow to 1 cm³ (84 days versus 49 days for controls; P<.001, Student's t test).

Lungs were examined macroscopically at the time of death. The photographs shown in Fig. 5 and the data given in Table 1 illustrate the profound effect of hypophysectomy, which virtually abolished the metastatic behavior of MGH-OGS tumors. In the control group, examined when primary tumors were 1 cm³, 12 (85.7%) of 14 mice were positive for metastatic disease, while only one (8.3%) of 12 of the hypophysectomized mice had evidence of metastasis at the time the primary tumors were 1 cm³. The difference between these groups, with respect to incidence of...
metastasis, had high statistical significance ($P<.001$). In the control group, the mean number of metastatic lesions per animal was 16, while in the hypophysectomized group, the only animal with metastasis had two lesions. The mean total weight of the metastatic lesions was 25.0 mg/animal in the control group versus 0.1 mg/animal in the hypophysectomized group. Serial sectioning of randomly selected lungs from both groups revealed that, in the control group, micrometastatic lesions were frequently detectable in the lungs that also had macroscopic lesions, while micrometastases were not seen in the lungs of hypophysectomized mice. The degree of inhibition of metastasis that we were able to achieve with hypophysectomy in this study was considerably greater than that which we achieved with adjuvant or neoadjuvant chemotherapy in previous work (9).

### Discussion

The murine MGH-OGS osteosarcoma has been previously shown to share many characteristics with human osteogenic sarcoma, e.g., histological appearance, local invasive behavior, chemosensitivity, and metastatic pattern, with a predilection for the lung as a metastatic site (9,10,15). Thus, it is an excellent model of the human disease.

In previous studies, we observed the responsiveness of human osteogenic sarcoma cells to IGF-I. One of our goals in this study was to determine whether the murine MGH-OGS osteosarcoma model could be used to extend these in vitro observations to an in vivo experimental system. To make this determination, we initially characterized MGH-OGS cells with respect to IGF-I receptors and response to IGF-I. Our results suggest that the experimental MGH-OGS tumor is IGF-I receptor positive and that, like human osteogenic sarcoma cells, MGH-OGS cells are mitogenically responsive to IGF-I.

Pituitary ablation, as expected, resulted in a profound reduction in levels of circulating IGF-I in the C3H mice used in the experimental MGH-OGS system. Our data show that hypophysectomy markedly inhibited local proliferation of MGH-OGS tumors. These results are consistent with those of a previous report (16) demonstrating that hypophysectomy reduced local growth of a transplantable rat chondrosarcoma. Our present study, however, is the first to document the profound effect of hypophysectomy on the metastatic behavior of an experimental sarcoma.

We speculate that the IGF-I-lowering effect of hypophysectomy is responsible for the inhibitory effects of this ablative surgery on local growth and on metastatic behavior. This hypothesis is supported by our observation that in vitro proliferation of MGH-OGS cells is stimulated by IGF-I but not by the other pituitary-dependent hormones tested. Further experiments to determine the effect of replacing growth hormone and/or IGF-I on the metastatic behavior of the MGH-OGS neoplasm in the hypophysectomized host will be required to conclusively establish if IGF-I reduction is the particular consequence of pituitary ablation that is responsible for the inhibition seen.

The degree of inhibition of the metastatic process is remarkable, especially since our experimental design involved assaying for metastatic lesions when hypophysectomized and control animals had primary lesions of similar size. Because the primary tumors grew more slowly in the hypophysectomy group (Fig.4), these animals were in fact at risk for developing metastasis over a longer period of time than were the controls.

While it is possible that the inhibition of metastasis we observed is a direct result of the inhibition of IGF-I-dependent proliferation of neoplastic cells, effects of IGF-I other than stimulation of neoplastic cell proliferation may be involved. Work in other experimental systems (17) has shown that IGF-I can function as a motility factor, which might enhance metastatic potential independent of stimulation of proliferation. Furthermore, we cannot exclude the interesting possibility that hypophysectomy inhibits the metastatic process by mechanisms that do not involve neoplastic cells directly, e.g., inhibition of aspects of neovascularization that require IGF-I or other pituitary-dependent factors. To investigate this possibility further, studies of the effects of hypophysectomy on the metastasis of other IGF-I receptor-positive tumors as well as IGF-I receptor-negative tumors should be undertaken.

In our experimental system, gross differences in anterior pituitary function profoundly affect metastatic behavior. Because there is considerable variability in anterior pituitary function among individuals, our data justify studies to determine if serum IGF-I level or other variables related to pituitary function are correlated with prognosis and/or risk of metastasis in human malignancies.

Surgical hypophysectomy has, in the past, been undertaken as a palliative measure in advanced metastatic breast cancer (18). Hypophysectomy has never been evaluated early in the evolution of malignancy in a surgical adjuvant or neoadjuvant setting. It is, however, at this early stage that inhibition of metastatic proliferation might be detected by an appropriately designed clinical trial of pituitary ablation using surgical, pharmacological, or radiotherapeutic modalities in the treatment of human sarcomas or other neoplasms. The morbidity associated with such treatment, given with appropriate replacement of thyroid and adrenocortical hormones, would not be greater than that associated with current adjuvant therapies.

Somatostatin analogues have been observed to reduce local proliferation of experimental sarcomas (19-21). The mechanism of action has not been well characterized, and the effect of these compounds on the metastatic process has not been studied. The data presented here suggest that the IGF-I-lowering effect of somatostatin analogues may, at least in part, mediate their inhibitory effect on the local growth of sarcomas and provide justification for studies of the effects of these drugs on metastatic behavior.

Clinical use of somatostatin analogues, in an attempt to achieve a selective (growth hormone-specific) "medical hypophysectomy," has resulted in only a modest decrease in IGF-I levels (22). This finding is probably due to compensatory homeostatic mechanisms, such as increased growth hormone-releasing hormone secretion, which attenuate the inhibitory effect of somatostatin analogues on growth hormone secretion. We have proposed that more complete pharmacological ablation of the pituitary-IGF-I axis may be possible by using somatostatin analogues in conjunction with growth hormone-releasing hormone antagonists (23). Other interesting approaches to the inhibition of IGF-I-dependent neoplastic cell proliferation involve the pharmacological use of...
recombinant human IGF-I-binding proteins (Pollak M, Maack C: manuscript submitted for publication) or suramin (11). These strategies may also have relevance to IGF-I receptor-positive neoplasms other than sarcomas, e.g., carcinomas of the breast and colon (8, 12).

Clinical syndromes associated with low levels of circulating IGF-I in childhood provide strong evidence that IGF-I is crucial in the regulation of cellular proliferation during preadult stages of development (24). In contrast, low levels of circulating IGF-I appear to be well tolerated in adults (25). Our data suggest that certain neoplasms exhibit considerable dependence on circulating IGF-1. Such malignancies would be expected to be IGF-I receptor positive but lacking in autocrine or paracrine sources of IGF-I stimulation. If further research demonstrates that such neoplasms exist in humans, development of therapeutic strategies to decrease circulating IGF-I levels will be warranted because such treatment might reduce neoplastic proliferation without causing serious toxic effects.

References

(20) REDDING TW, SCHALLY AV: Inhibition of growth of the transplantable rat chondrosarcoma by analogs of hypothalamic hormones. Proc Natl Acad Sci USA 80:1078–1082, 1983