Insulinlike Growth Factor I: A Potent Mitogen for Human Osteogenic Sarcoma

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Insulinlike growth factor I (IGF-I) is among the peptide mitogens that play key roles in the regulation of normal skeletal growth. To investigate the possibility that certain skeletal neoplasms retain a sensitivity to mitogenic stimulation by IGF-I, we studied the effects of this growth factor on human osteosarcoma. Competitive-binding assays and affinity-labeling experiments on membranes prepared from MG-63 immortalized human osteosarcoma cells and primary human osteogenic sarcoma cells demonstrate the presence of specific IGF-I receptors. Furthermore, we show that IGF-I is a potent stimulator of proliferation of MG-63 cells in vitro and is active at concentrations as low as 10^{-10} *M*. A blocking antibody against the IGF-I receptor (α-IR3) significantly reduces IGF-I-stimulated proliferation in a dose-dependent manner. These results are consistent with the hypothesis that at least a subset of human osteogenic sarcomas are responsive to IGF-I and indicate that it may be possible to exploit this responsiveness therapeutically.

Insulinlike growth factor I (IGF-I), also known as somatomedin C, is a 76-amino acid mitogen known to stimulate the proliferation of many tissues (*1*). The binding of IGF-I to specific cell-surface receptor molecules is the first step in the mitogenic signal transduction pathway leading to IGF-I-stimulated cellular proliferation. Like other mitogen receptors, the IGF-I receptor consists of an extracellular ligand-binding domain, a transmembrane region, and an intracellular tyrosine-specific protein kinase domain (*2*). In vivo, serum IGF-I is dependent on growth hormone levels. Although the liver is a major site of growth hormone-dependent IGF-I synthesis, recent work indicates that a variety of normal and neoplastic tissues, including sarcomas, may also produce IGF-I (*3-5*). Thus IGF-I may act as a regulator of cellular proliferation via autocrine or paracrine as well as endocrine mechanisms.

In view of evidence that IGF-I is a mitogen for normal osteoprogenitor cells (*6,7*), we undertook the present studies to determine the degree to which osteogenic sarcoma might retain a dependence on this mitogen for optimum proliferation.

Materials and Methods

Cells, growth factors, and antibodies. MG-63 immortalized human osteosarcoma cells (*8*), recombinant human IGF-I, insulin, and growth hormone were obtained from the American Type Culture Collection (Rockville, MD), Amersham Corp. (Arlington Heights, IL), Eli Lilly & Co. (Indianapolis, IN), and Sigma Chemical Co. (St. Louis, MO), respectively. The α-IR3 blocking antibody against the IGF-I receptor (*9*) was provided by S. Jacobs.

Tissue culture and membrane preparation. MG-63 cells were routinely cultured in RPMI-1640 medium supplemented with 10% fetal calf serum. A plasma membrane-enriched subcellular fraction was prepared from primary tumor tissue (unfixed and flash-frozen) and 10^9 cultured MG-63 cells as previously described (*10*). For growth curves, cells were plated in quadruplicate in 2.5-cm^2 dishes in medium with 10% fetal calf serum, which was changed after 24 hours to serum-free media with various concentrations of IGF-I. After 6 days, the cells were trypsinized and counted with a hemacytometer.

Binding studies. Aliquots of the plasma membrane-enriched subcellular fractions were incubated at 4 °C for 20 hours with labeled IGF-I and varying concentrations of unlabeled growth factors as previously described (*10*).

Thymidine incorporation. We plated 3×10^4 cells in 2.5-cm^2 wells with medium containing serum. After 24 hours, the medium was changed to serum-free medium, and the cells were cultured for an additional 24 hours. Incubation was performed with various concentrations of IGF-I under serum-free conditions. After 22 hours, 1 μCi of tritiated thymidine/mL was added to each well. Two hours later, cells were washed in phosphate-buffered saline and precipitated in 10% trichloroacetic acid (TCA). TCA-insoluble material was collected by centrifugation and washed by repeated centrifugation in TCA. The final TCA-insoluble pellet was dissolved in NaOH and neutralized with HCl. We measured radioactivity using a liquid scintillation counter, and the numbers of cells were determined in replicate cultures. Rates of thymidine incorporation were calculated as disintegrations per minute per cell over 2 hours, and values were expressed as percentages of control values. Experiments were performed in quadruplicate.

Affinity labeling. Plasma membrane-enriched subcellular fractions were prepared as previously described (*10*) from human placenta, cultured cells, or primary tumor tissue and incubated for 24 hours at 4 °C with radiolabeled IGF-I (20,000 cpm) in the presence or absence of excess unlabeled IGF-I. Electrophoresis was performed on a 7.5% polyacrylamide gradient gel after cross-linking with disuccinimidyl suberate, solubilizing with sodium dodecyl sulfate, and reduction in 100 m*M* 1,4-dithiothreitol (*11*).

Results

The results of binding studies on MG-63 osteosarcoma cells and primary osteogenic sarcoma cells are shown in figures 1 and 2, respectively. In each figure, panel A illustrates compe-

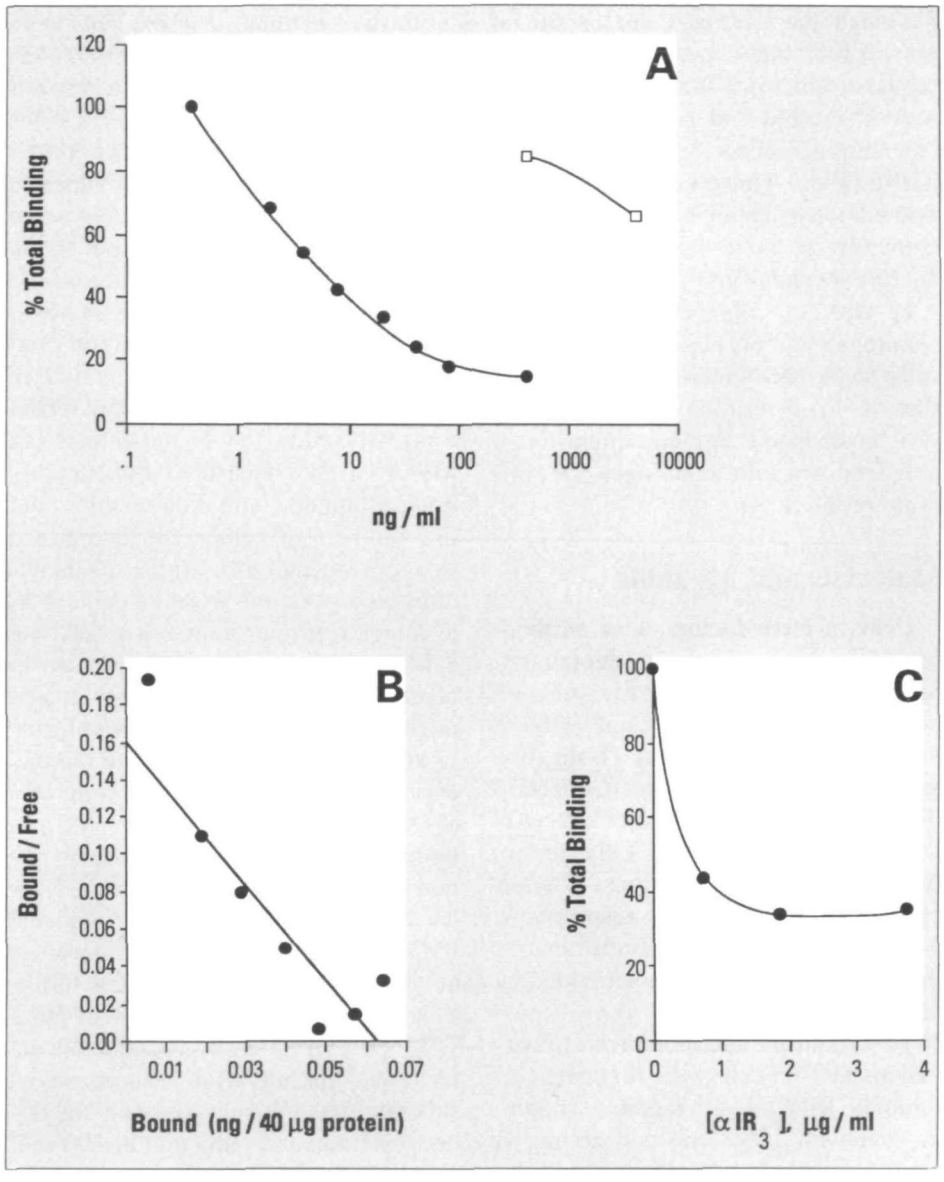

Figure 1. Binding of IGF-I to membranes prepared from human MG-63 osteosarcoma cells. A: Competition of binding of radiolabeled IGF-I with unlabeled IGF-I (●) and insulin (□). B: Scatchard plot of binding data. C: Displacement of bound IGF-I by increases in concentration of α-IR3 antibody, a blocking antibody against the IGF-I receptor.

tition of radiolabeled IGF-I with unlabeled IGF-I and insulin for IGF-I binding sites; panel B is a Scatchard plot of the binding data; and panel C illustrates displacement of bound radiolabeled IGF-I from binding sites by increases in the concentration of α-IR3 antibody (*9*), a blocking antibody against the IGF-I receptor.

Under our experimental conditions, we observed 16.4% and 16.0% specific binding of IGF-I to membranes prepared from MG-63 cells and primary osteogenic sarcoma cells, respectively, while human placenta, a tissue known to have a particularly high concentration of IGF-I receptors (*12*), showed 25% specific binding. Scatchard analysis revealed equilibrium affinity constants (K_d) of 2.9 and 2.5 n*M* for MG-63 cells and primary osteogenic sarcoma cells, respectively. The calculated binding capacity for IGF-I per milligram of protein is 0.17 pmol for primary osteogenic sarcoma cells and 0.21 pmol for MG-63 cells.

As the α-IR3 antibody is specific for IGF-I receptors, the data suggest that IGF-I binds to these receptors, rather than to receptors for insulin or IGF-II. The similarity of binding data from the primary osteogenic sarcoma cells and the cultured MG-63 osteosarcoma cells indicates that in vitro proliferation of the latter cells may, in certain respects, reflect the in vivo behavior of the former cells. In similar studies with radiolabeled growth hormone, we did not observe significant binding.

Chemical cross-linking of ^{125}I-labeled IGF-I to binding sites in membranes derived from MG-63 cells and primary osteogenic sarcoma cells and analysis by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (fig. 3) showed labeled proteins at the 130-kilodalton band in a position similar to that seen in control membranes from human placenta. Labeling was totally abolished by the presence of excess unlabeled IGF-I, a finding consistent with the presence of specific IGF-I receptors. Some labeling was observed at the 260-kilodalton band. As this band is also abolished by excess cold IGF-I, it likely represents a receptor dimer.

Figure 4A shows the stimulatory effect of IGF-I on the in vitro proliferation of MG-63 osteosarcoma cells, as measured by the number of cells. The number of cells at day 6 in the media containing IGF-I was significantly greater than in control media ($P < .01$). Figure 4B shows dose-response data for the same cell line incubated at various IGF-I concentrations, as determined by thymidine incorporation. Figure 4C illustrates the inhibition of IGF-I-stimulated proliferation of MG-63 cells by the α-IR3 antibody. These data provide further support for the hypothesis that IGF-I stimulates proliferation of MG-63 cells by binding with IGF-I receptors.

Discussion

Our data suggest that at least a subset of human osteogenic sarcomas are IGF-I receptor positive and can be stimulated to proliferate by IGF-I. It is not possible to directly extrapolate our in vitro dose-response data to in vivo or clinical situations, partly because the nature and concentration of IGF-I binding proteins, which influence the bioactivity of somatomedins, are not identical in vitro and in vivo (*13,14*). However, the fact that IGF-I was ac-

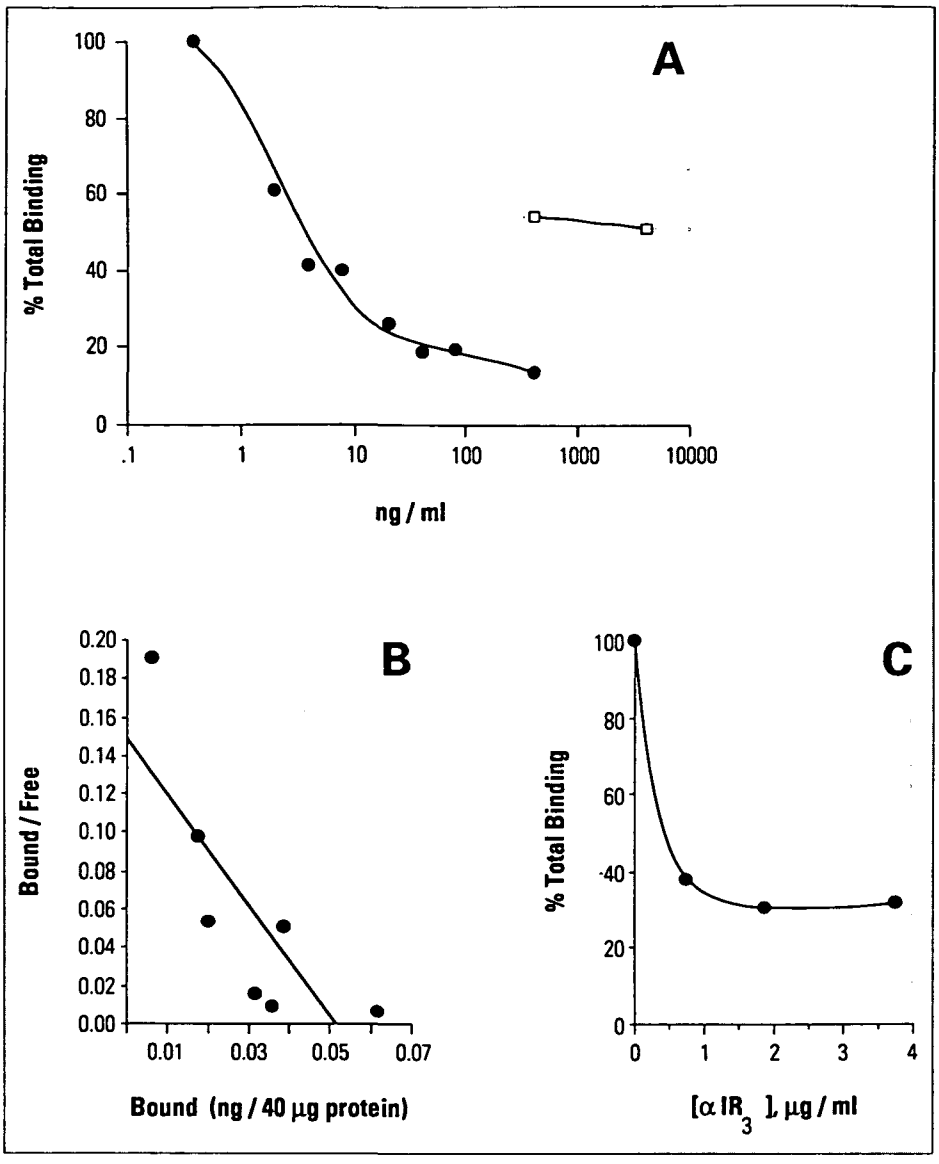

Figure 2. Binding of IGF-I to membranes prepared from human primary osteogenic sarcoma cells. A: Competition of binding of radiolabeled IGF-I with unlabeled IGF-I (●) and insulin (□). B: Scatchard plot of binding data. C: Displacement of bound IGF-I by increasing concentrations of α-IR3 antibody, a blocking antibody against the IGF-I receptor.

tive under our experimental conditions at nanomolar concentrations indicates that normal serum IGF-I levels may be sufficient to stimulate the proliferation of osteogenic sarcomas in patients.

The degree of stimulation of proliferation we observed (approximately sevenfold) is high compared with that observed in other IGF-I-responsive systems (*1,11*). Our data do not suggest that this responsiveness is related to an unusually high density of IGF-I receptors, although a previous study showed an association between hypersensitivity to epidermal growth factor and a high density of receptors for this growth factor in certain cell lines (*15*). We have considered the possibility that a derangement in the IGF-I receptor gene of MG-63 cells might result in abnormally high IGF-I-dependent tyrosine kinase activity, but studies to date have not shown such aberrations, at least in terms of phosphorylation of an artificial polytyrosine substrate (data not shown).

It is possible, therefore, that the MG-63 cells studied were rendered unusually responsive to IGF-I by alterations in steps in the mitogenic signal transduction pathway that follow receptor binding. As osteogenic sarcomas have been observed to have a homozygous deletion at the retinoblastoma locus (*16*), the responsiveness to IGF-I exhibited by MG-63 cells may be related to a derangement of negative growth controls, as discussed by Weinberg (*17*). It has been reported that platelet-derived growth factor is not a mitogen for MG-63 cells (*18*), despite the fact that these cells have receptors for this growth factor (*19*). The responsiveness of MG-63 cells to IGF-I stimulation is therefore specific rather than a reflection of general hypersensitivity to all mitogenic signals.

Our results obtained with human tissues are consistent with previous data from an in vivo rat chondrosarcoma model (*20*). In this system, the effect of IGF-I was not studied directly, but tumor growth was greatly accelerated in the presence of growth hormone-dependent serum factors.

The responsiveness of osteogenic sarcoma to IGF-I may have clinical relevance, given that the peak incidence of osteogenic sarcoma occurs in adolescence, coincident with the pubertal peak in IGF-I levels. In view of our data, it is reasonable to speculate that lowering systemic IGF-I levels might reduce the rate of proliferation of osteogenic sarcomas in patients.

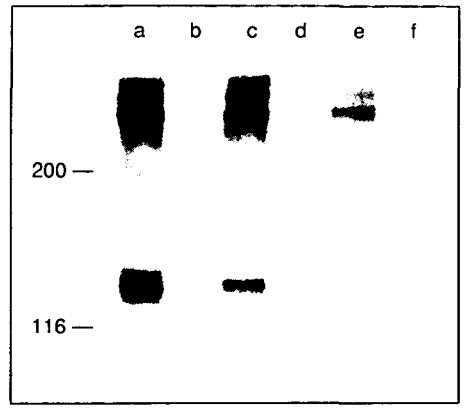

Figure 3. Affinity labeling of IGF-I receptors of human osteogenic sarcoma cells. Membranes were incubated with labeled IGF-I in presence (lanes b, d, and f) or absence (lanes a, c, and e) of excess unlabeled IGF-I. Cross-linking was carried out, and electrophoresis was performed on 7.5% polyacrylamide gel in presence of 2% sodium dodecyl sulfate. Lanes a and b: human placenta. Lanes c and d: MG-63 osteosarcoma cells. Lanes e and f: primary human osteogenic sarcoma cells.

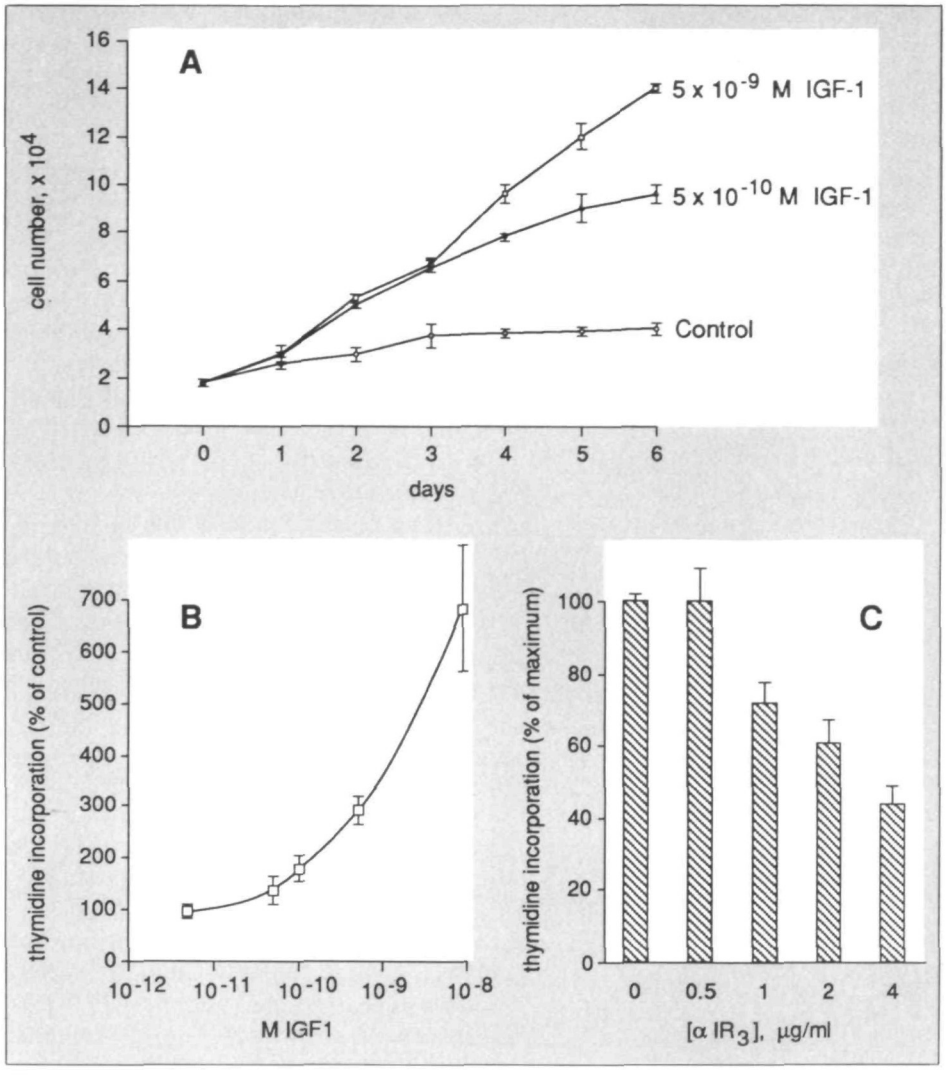

Figure 4. Effect of IGF-I on proliferation of human MG-63 osteosarcoma cells. A: Effect of IGF-I on proliferation over 6 days, as measured by numbers of cells. B: Dose-response curve showing thymidine incorporation. C: Antiproliferative effect of IGF-I receptor blockade by α-IR3 antibody on human MG-63 osteosarcoma cells, as determined by thymidine incorporation. Cells were allowed to proliferate in serum-free media with IGF-I (10^{-9} *M*), with varying amounts of receptor-blocking antibody.

Apart from hypophysectomy, there are a variety of nonsurgical measures to lower IGF-I levels or block IGF-I action. These measures include treatment with long-acting somatostatin analogs and/or antagonists to growth hormone-releasing factor (*21,22*) and development of competitive antagonists to the IGF-I receptor.

Lowering of IGF-I levels cannot be expected to slow the proliferation of sarcomas that are IGF-I receptor negative or those that produce significant quantities of IGF-I in an uncontrolled (growth hormone-independent) autocrine fashion. Further studies are required to determine what proportion of sarcomas have IGF-I receptors but no autocrine mechanism. Sarcomas with these characteristics might respond to treatments that lower IGF-I levels.

The therapeutic potential of IGF-I-lowering strategies is supported by a significant decrease in the in vivo proliferation of experimental sarcomas achieved in two studies by treatment with somatostatin (*23,24*). As the sarcoma used in these experiments was somatostatin receptor negative, a direct antiproliferative effect of somatostatin is ruled out, and in view of our findings, an IGF-I-lowering mechanism of action is likely. Measures that reduce IGF-I stimulation of tumor proliferation are not tumoricidal. Nevertheless, given the precedent provided by the efficacy of hormonal treatments of steroid-dependent prostate and breast cancers, the potential role of IGF-I-lowering manipulations as well-tolerated and effective palliative treatment deserves further study.

A separate implication of our results is the possibility that the efficacy of cytotoxic chemotherapy of osteogenic sarcoma might be enhanced by the use of IGF-I in mitogenic stimulation of target cells in a manner analogous to the use of estrogens prior to chemotherapy for breast cancer (*25*). This synchronization strategy has been attempted clinically only with mitogenic steroids. As peptide growth factors are often more potent mitogens than steroids, they may be more effective in recruiting malignant cells into S phase prior to cytotoxic treatment.

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