American Journal of Pathology, Vol. 135, No. 6, December 1989

Rapid Communication

Antibody to Type I Insulinlike Growth Factor Receptor Inhibits Growth of Wilms' Tumor in Culture and in Athymic Mice

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The role of the type I insulinlike growth factor (IGF) receptor in regulating growth of Wilms' tumor (WT) was evaluated by examining the effect of antibody-mediated inhibition of this receptor on tumor growth in cell cultures and as heterotransplants in athymic mice. An antibody to the human type I IGF receptor (αIR-3) inhibited ^{125}I-IGF-1 binding and prevented stimulation of thymidine incorporation by IGF-1 in vitro. Intraperitoneal administration of αIR-3 to nude mice bearing WT heterotransplants prevented tumor growth for 4 weeks and resulted in partial regression of established tumors. These data indicate the importance of IGF action in control of WT growth in vivo, and suggest potential therapeutic application using antigrowth factor receptor antibodies to block growth factor action. (Am J Pathol 189, 135:961–966)

Stimulation of tumor cell proliferation by peptide growth factors has been documented in a wide variety of neoplasms. Epidermal growth factor (EGF), transforming growth factors (TGF), platelet-derived growth factor (PDGF), fibroblast growth factor (FGF), and insulinlike growth factors (IGF) are mitogenic *in vitro* and under some conditions promote expression of phenotypic and molecular features of transformed cells including anchorage-independent growth, decreased density-dependent growth inhibition, and increased expression of some oncogenes.[1,2] Expression of growth factor mRNA and corresponding peptides have been detected *in vitro* and *in vivo*.[1,2] The initial step in the action of each of these growth factors involves binding to specific cell-surface receptors, suggesting the possibility of treating tumors by interrupting GF action at the receptor level.

Wilms' tumor is one of the most common malignant diseases in children. This kidney tumor resembles normal fetal kidney tissue histologically and histochemically and, like fetal tissue, expresses high levels of the mitogenic peptide IGF-2.[3–8] We recently demonstrated that extracts of WT bind more IGF than normal kidney tissue, and that type I IGF receptors isolated from WT display increased tyrosine kinase activity. Exposure to IGF-1 or IGF-2 *in vitro* stimulated the receptor tyrosine kinase, showing that WT cells contain receptors capable of recognizing and being activated by IGF.[9] *In vitro* propagation of WT cells requires medium supplementation with a supraphysiologic (5 μg/ml) insulin concentration. At this concentration, insulin exhibits significant interaction with the type I IGF receptor.

The present study further addresses the role of IGF in growth control of WT. We examined the ability of the anti-IGF-1 receptor antibody, αIR-3, to block IGF binding to WT extracts, and the effect of this antibody on IGF-stimulated growth of WT cultures. Finally, we evaluated the effect of αIR-3 on the growth of WT heterotransplants in athymic mice. Our data indicate that IGF action is an important component of growth control in WT *in vitro* and *in vivo* and that WT growth can be prevented by blocking IGF action at the receptor level.

Supported by NIH Grants CA37887, CA3891, DK02001, and a Medical University of South Carolina Institutional Grant.

Accepted for publication September 21, 1989.

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Materials and Methods

WT Heterotransplants and Cell Cultures

Surgical specimens of histologically documented Wilms' tumors were minced in phosphate-buffered saline into fragments approximately 1 mm in diameter. One ml of a suspension of minced tumor representing approximately 10^8 tumor cells was injected subcutaneously into the right flank of each athymic mouse. Adult male athymic mice weighing 25 to 30 g were obtained from the National Cancer Institute and housed in macroisolators. Tumor nodules were visible at 1 to 10 weeks after injection depending on the growth characteristics of each cell line. When tumors reached a diameter of approximately 2 cm, they were removed, minced, and reinjected into athymic mice as described above for the primary tumors.[4,5]

Wilms' tumor cell cultures were established from explants of athymic mouse heterotransplants as previously described.[4] Briefly, tumors were minced as for serial heterotransplantation but instead were inoculated into tissue culture flasks coated with type I collagen and adsorbed fetal calf serum (FCS) proteins. The culture medium used for growth of these cells was a 1:1 mixture of Dulbecco's Modified Eagle's Medium and Hams' F-12 supplemented with selenium (5 ng/ml), insulin (5 μg/ml), transferrin (5 μg/ml), hydrocortisone (36 ng/ml), triiodothyronine (4 pg/ml), and EGF (10 ng/ml). Cultures were split 1:3 on reaching confluence using trypsin with EDTA (0.05% and 0.02%) and were used for experiments at passages 3 to 5.

Preparation of αIR-3

αIR-3-producing myeloma cells were the gift of Dr. Steven Jacobs, Wellcome Laboratories, Research Triangle Park, NC. αIR-3 ascites was produced in BALB/c $\times$ SJLF1 mice pretreated with pristane as previously described.[10,11] The IgG_1 was purified on affigel protein A (Biorad, Richmond, CA).

Receptor Purification from WT Heterotransplants

Tissue was finely ground in a mortar containing liquid nitrogen and then homogenized at 4 C in buffer containing 50 mM N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (HEPES), 1.5% Triton X-100, 4 mM ethylenediamine tetraacetic acid disodium salt, 1 trypsin-inhibitory unit/ml aprotinin, 1 mg/ml benzamidine, 1 mg/ml benzoyl arginine methyl ester, 0.03 mg/ml leupeptin, and 2 mM phenylmethylsulfonyl-fluoride, pH 7.4. After centrifugation (10,000 *g*, 30 minutes), the supernate was centrifuged a second time (150,000 *g*, 90 minutes, 4C). This supernate was applied to a wheat germ agglutinin-agarose (WGA) column, washed with 50 mM HEPES/0.1% Triton X-100, and eluted with HEPES/Triton containing 0.3 M N-acetyl-glucosamine.

Hormone Binding to Soluble Receptors

Aliquots of WGA eluate were incubated (4 C, 16 hours) with A14-^{125}I-insulin or ^{125}I-IGF-1 (the gift of Dr. Richard deMarchi, Lilly Research Laboratories, Indianapolis, IN) at approximately 10^{-11} M (200 to 300 μCi/μg). Unlabeled recombinant human IGF-1 or porcine insulin (gifts of Dr. deMarchi and Dr. Ronald Chance, Lilly Research Laboratories), MOPC-21 IgG (Sigma, St. Louis, MO) or αIR-3 IgG were added in varying concentrations. Receptors and bound hormones were precipitated by addition of polyethylene glycol (12.5%) and bovine gamma globulin (0.1 mg/ml), and the associated radioactivity was counted.

Quantitation of Thymidine Incorporation

Cells were plated onto collagen/FCS-coated 96-well trays. At confluence, the medium was replaced with basal medium (usual growth medium without insulin, EGF, or transferrin). Two days later, cells were refed with basal medium and the indicated concentrations of IGF-1, insulin, αIR-3 or MOPC-21 control IgG. Eight hours later, the cells received a 12-hour pulse of methyl-^{3}H-thymidine (0.5 μCi/well, New England Nuclear, Boston, MA). The cells were detached with trypsin and EDTA, and the DNA-associated radioactivity was immobilized on glass fiber filters, using a semiautomatic cell harvester (Skatron Inc., Sterling, VA). Tritium was quantitated by liquid scintillation counting and the mean values of triplicate wells was expressed as percentage of control (basal medium only) values.

Administration of αIR-3 to Athymic Mice

Athymic mice were inoculated subcutaneously with approximately 10^8 WT cells as described above for serial heterotransplantations. After 1 week, tumor nodules were visible in approximately two thirds of the mice. At that time, intraperitoneal injections of αIR-3 or MOPC-21 were begun, with each mouse receiving 0.5 mg of IgG three times weekly. The diameters of tumor nodules were recorded every 2 or 3 days. This experiment was performed three times. One set of seven mice was inoculated with a histologically favorable WT line in its eighth nude mouse passage. A second set of ten mice received a histologi-

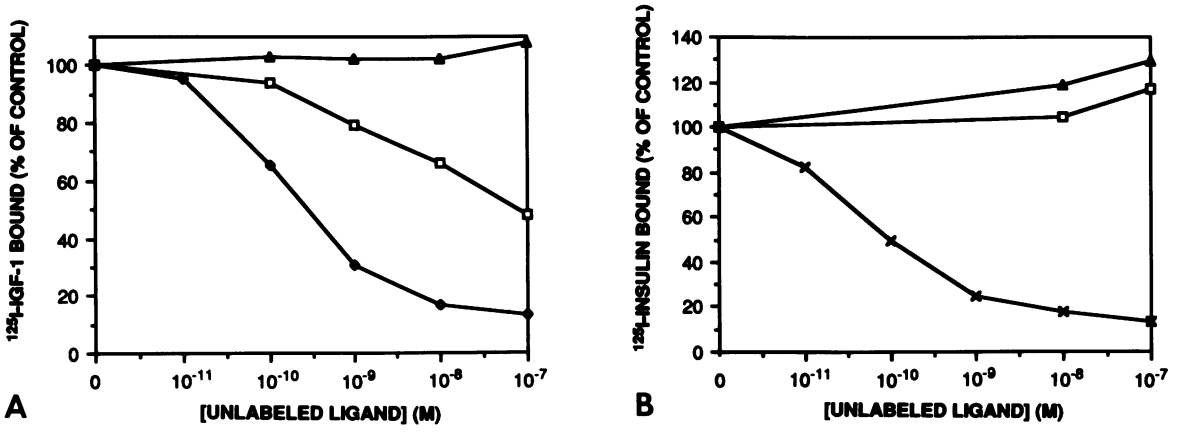

Figure 1. *Hormone binding to WT extracts.* **A:** *Competition of ^{125}I-IGF-1 binding by unlabeled IGF-1 (diamonds), αIR-3 (squares), or MOPC-21 (triangles).* **B:** *Competition of ^{125}I insulin-binding by unlabeled insulin (x), αIR-3 (squares), or MOPC-21 (triangles). Points represent the means of duplicate or triplicate samples, and are representative of two experiments.*

cally unfavorable (anaplastic) WT line in its 29th passage. Both of these experiments were terminated after three weeks of antibody treatment. In the third experiment, ten mice received a histologically favorable WT line in its 20th nude mouse passage. For this third set, mice were not sacrificed after 3 weeks of treatment, but instead one half of the mice in each group were reassigned to receive the other antibody. At that time, mice were ranked by tumor size with even- and odd-numbered mice reassigned to the two new subgroups, thereby avoiding any bias in this process. Two mice were sacrificed after 2 weeks of treatment to observe tumor histology during regression. The remaining mice were sacrificed 1 week later. At the conclusion of each of the three experiments, the mice were sacrificed by cervical dislocation, body weights, and weights of tumors and most organs were recorded. Portions of tumor were fixed in 10% buffered formalin, embedded in paraffin, sectioned at 4 μm, and stained with hematoxylin and eosin. Sections were examined microscopically, noting the extent of stromal and epithelial differentiation and the presence or absence of tumor infiltration by inflammatory cells.

Results

Radioligand-binding studies demonstrated that WT extracts contained IGF receptors and insulin receptors (Figure 1). One half maximal inhibition of ^{125}I-IGF-1 and ^{125}I-insulin binding was observed with unlabeled hormone concentrations of 3×10^{-10} M and 10^{-10} M, respectively. Binding of IGF-1 but not insulin was substantially inhibited by 5×10^{-7} M αIR-3. A control antibody (MOPC-21) of the same subclass (IgG_1) had no effect on the binding of either hormone.

Exposure of four different WT cell lines to IGF-1 or insulin resulted in stimulation of thymidine incorporation into DNA by a factor of 2 (representative dose-response curve shown in Figure 2). Although the maximal levels of stimulation produced by the two hormones were similar (225% ± 10.3% and 235% ± 6.2% of control for IGF-1 and insulin, respectively), one half maximal stimulation by IGF-1 and insulin was achieved at $1.4 \pm 0.04 \times 10^{-9}$ M and $5.5 \pm 1.4 \times 10^{-8}$ M, suggesting that insulin action was mediated mainly by an IGF receptor. The addition of 5×10^{-7} M αIR-3 to these assays essentially blocked the action of 10^{-8} M IGF-1 and inhibited the effect of 10^{-7} M IGF-1, ($P < 0.05$), supporting the concept that IGF-1 was acting *via* the type I IGF receptor (Table 1).

Treatment of Wilms' tumor-bearing athymic mice by intraperitoneal injection of αIR-3 was initiated seven days after tumor transplantation. In each of three experiments using three different WT lines (two with favorable histology, one with unfavorable histology), statistically significant ($P < 0.029$, $P < 0.016$, $P < 0.008$, Wilcoxn–Mann–Whitney test) differences between tumor diameters in αIR-3 and control MOPC-21 groups were evident within 7 to 12 days of treatment (Figure 3). After 3 weeks of treat-

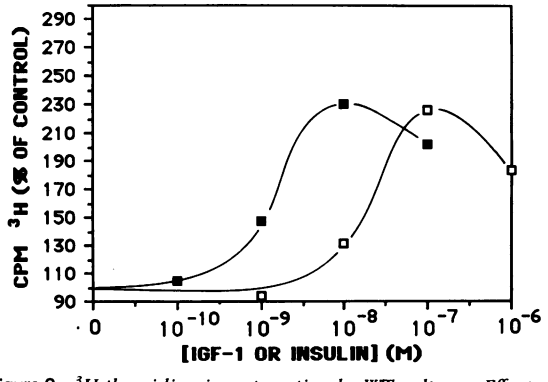

Figure 2. *^{3}H-thymidine incorporation by WT cultures. Effect of IGF-1 (filled squares) or insulin (open squares). Points shown are the mean values of triplicate samples and are representative of 2 to 3 experiments for each of four WT cell lines.*

Table 1. *Effect of αIR-3 on IGF-1-Stimulated ^{3}H-Thymidine Incorporation of WT Cultures*

	10^{-8} M IGF-1		10^{-7} M IGF-1	
WT type	MOPC-21	αIR-3	MOPC-21	αIR-3
F	123.0 ± 7.4	90.6 ± 4.6*	234.5 ± 18.7	127.3 ± 3.2*
F	230.0 ± 23.7	102.8 ± 8.8*	202.1 ± 2.1	160.3 ± 4.2*
F	152.9 ± 8.5	123.0 ± 33.3	202.9 ± 41.0	169.0 ± 26.6
U	159.9 ± 9.3	89.8 ± 6.5*	169.9 ± 5.1	161.3 ± 7.6
Mean ± SEM	166.5 ± 22.6	101.6 ± 7.7*	202.4 ± 13.2	154.5 ± 9.3*

Values (means of triplicates ± SEM) shown represent CPM ^{3}H incorporated into DNA, expressed as percentage of control (cells fed with basal medium).
F = favorable histology, U = unfavorable (anaplastic) histology.
* αIR-3 < MOPC-21; $P < 0.05$.

ment, tumor diameters were 521.7% ± 102.1% and 112.6% ± 23.6% of pretreatment values for control and antireceptor antibody groups, respectively (mean ± SEM of the three experiments). At this time, one half of the mice in each group from the third experiment were reassigned to receive the other antibody. The mice initially in the MOPC-21 group that were switched to αIR-3 showed a 31% reduction in tumor diameter between day 2 and day 10 of αIR-3 treatment, while tumors in the MOPC-21 group continued to grow, increasing to 144% of their initial diameter during the same period (Figure 4A). The effect of αIR-3 was temporary and by day 42 after tumor transplantation, tumors began to grow in all mice, even in those continuously treated with αIR-3 (Figure 4B). The mice initially treated with αIR-3 and subsequently changed to MOPC-21 (Figure 4B) showed a prominent increase in tumor size, beginning 4 to 6 days after this change. The increase in tumor diameter from days 28 to 49 was 0.58 cm (range, 0.45 to 0.75) for mice remaining on αIR-3 *versus* 1.03 cm (range, 0.9 to 1.15) for mice switched from αIR-3 to MOPC-21.

Mice were sacrificed at the times indicated in Figures 3 and 4. Tumors and organs were weighed and examined histologically. Whereas tumor weights differed significantly ($P < 0.01$) between the antireceptor groups and control groups (reflecting the tumor diameter values shown in Figure 3), tumor-free body weight did not vary. This is to be expected because αIR-3 does not react with IGF receptors from mouse tissues and therefore should not inhibit growth of the host animal. Histologic features of all tumors were similar, with predominance of the blastemal component and rare foci of stromal and epithelial

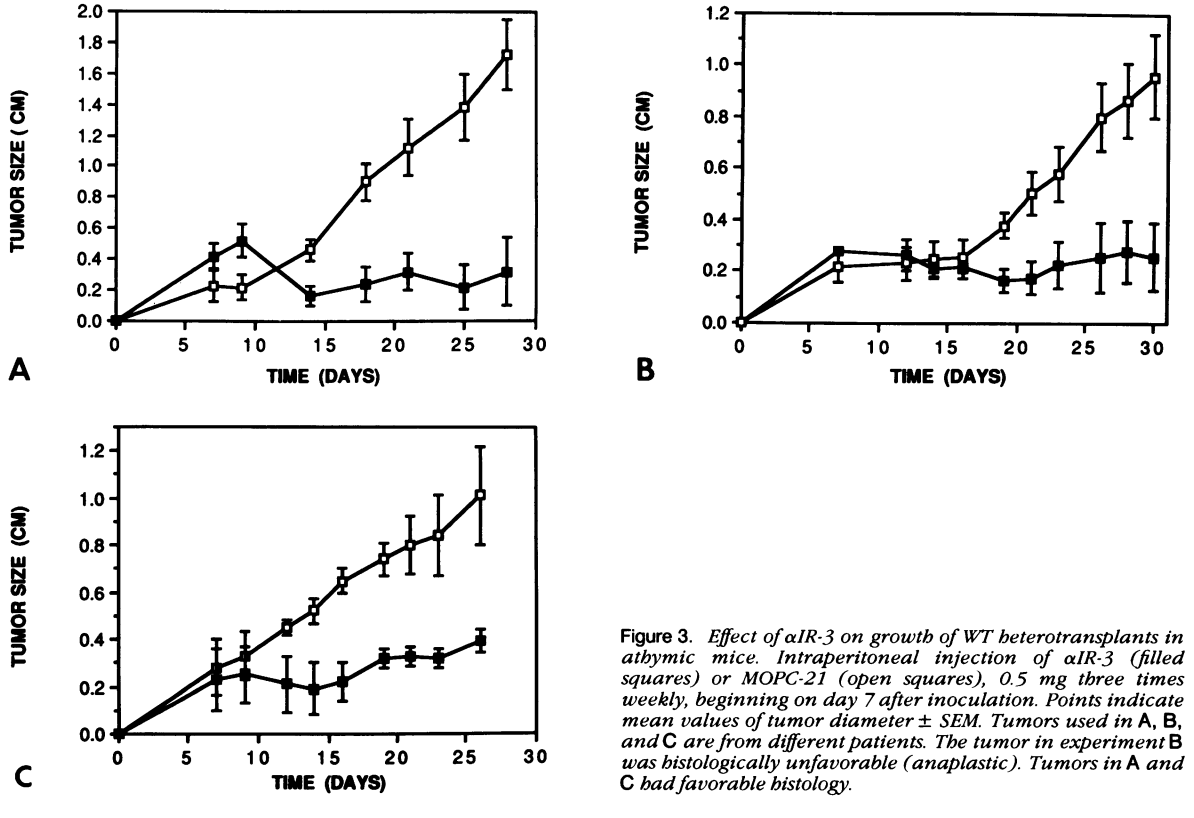

Figure 3. *Effect of αIR-3 on growth of WT heterotransplants in athymic mice. Intraperitoneal injection of αIR-3 (filled squares) or MOPC-21 (open squares), 0.5 mg three times weekly, beginning on day 7 after inoculation. Points indicate mean values of tumor diameter ± SEM. Tumors used in* A, B, *and* C *are from different patients. The tumor in experiment* B *was histologically unfavorable (anaplastic). Tumors in* A *and* C *had favorable histology.*

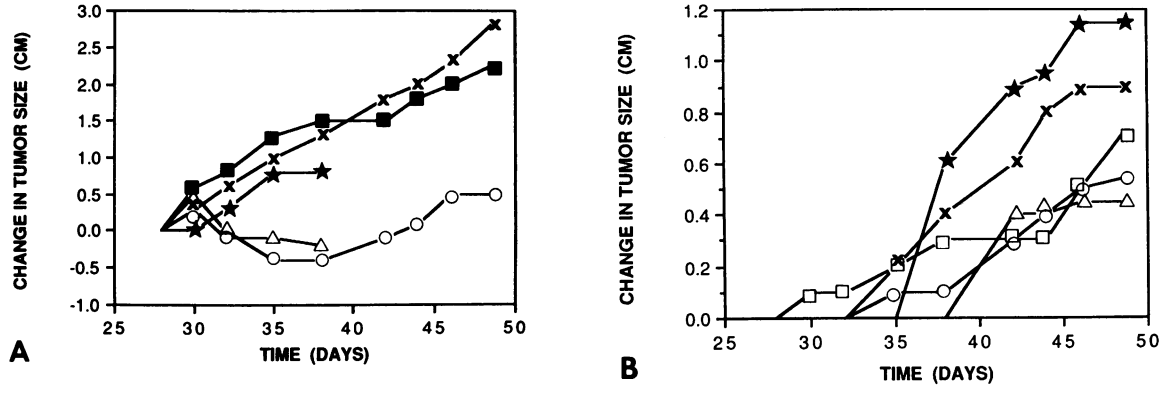

Figure 4. **A:** *Tumor diameters of mice treated initially with MOPC-21 and switched to αIR-3 (open circles and triangles) or continued on MOPC-21 (X, stars, and solid squares).* **B:** *Tumor diameters of mice treated initially with αIR-3 and switched to MOPC-21 (X and stars) or continued on αIR-3 (open squares, circles, and triangles). In both* **A** *and* **B**, *tumor diameters of all mice were normalized to 0 cm on day 28 to facilitate comparison of growth during the second phase of the experiment. (Growth of tumors in these mice before postinjection day 28 is shown in Figure 3A). In graph* **B**, *baseline values have been omitted to avoid overlapping symbols.*

differentiation. There was no host inflammatory response to the tumors noted in either control or αIR-3 groups.

Discussion

Although the IGFs have been shown to be mitogenic for a large number of transformed cell types *in vitro,* their role in stimulating the growth of tumors *in vivo* has not been defined. Wilms' tumors previously were shown to contain increased levels of IGF-2 mRNA and protein.[6-8] We recently reported that membrane extracts prepared from WT contain more type I IGF receptors than those of normal kidney, that the receptors from WT have increased basal tyrosine kinase activity, and that autophosphorylation of the type I receptor was stimulated *in vitro* by IGF-1 or IGF-2.[9] These observations suggested to us that the IGFs may play a role in the control of proliferation and differentiation of WT *in vivo* and formed the basis for the experiments reported here.

Our first goal for the present study was to document the presence of IGF receptors in WT heterotransplants from athymic mice. Radioligand assays detected binding activity for IGF-1 and insulin. The presence of distinct receptors for insulin and IGF-1 is supported by relative affinity profiles of the two hormones and by the observation that antibody to the type I IGF receptor inhibited binding of ^{125}I-IGF-1 but not ^{125}I-insulin.

The presence of IGF receptors in a tissue does not necessarily indicate that the IGFs are mitogenic for that tissue. For this reason, we examined the effect of IGF-1 and insulin on ^{3}H-thymidine incorporation into DNA by WT cell lines. In all four WT cell lines tested, both hormones stimulated thymidine incorporation approximately twofold above basal levels. However, the relatively high concentration of insulin required for one half maximal stimulation (5.5×10^{-8} M *vs.* 1.4×10^{-9} M IGF-1) suggests that the mitogenic effect of insulin on these cells is mediated by the type I IGF receptor. Inhibition of IGF-1-stimulated thymidine incorporation by αIR-3 confirmed that this effect is mediated mainly by the type I IGF receptor. Although some exceptions have been reported,[11-13] the mitogenic actions of both IGFs and insulin are usually mediated by the type I IGF receptor.[10,11,13-16] These data suggest that IGFs are important in regulation of WT growth. However, stimulation of thymidine incorporation does not necessarily prove that a growth factor induces cells to progress completely through the cell cycle *in vivo* or even *in vitro.*

To learn more about the role of the IGFs in stimulating the *in vivo* growth of WT, we examined the effect of passive immunotherapy with αIR-3 on the growth of WT heterotransplants. Intraperitoneal injection of 0.5 mg of αIR-3 three times weekly prevented tumor growth for at least 3 weeks and, in some mice, resulted in complete regression of all palpable tumor for a period of 4 to 6 weeks. All mice receiving the control antibody, MOPC-21, showed progression of their tumors. Inhibition of WT growth by αIR-3 was observed in three experiments using three different tumor lines. After 3 weeks of treatment, mice treated with antireceptor antibody had tumors with diameters 28.1% ± 4.73% of those of mice in the control group. We ended two experiments after 3 weeks of antibody treatment. The third experiment followed a double-crossover protocol in which mice were treated for 3 weeks as in the other experiments, then one half of the mice in each group were reassigned to receive other antibody. Mice remaining in the control (MOPC-21) group showed continuing tumor enlargement, while mice switched to αIR-3 had 31% reduction of tumor diameter over a period of eight days, followed by a gradual return to their tumors' original sizes. Mice switched from αIR-3 to MOPC-21 had greater tumor enlargement during the second phase of the study than mice remaining on αIR-3 treatment, although after 4 to 5 weeks of continuous αIR-3 therapy tumor growth accelerated in treated mice. The data clearly indicate that IGFs play an important role in stimulating the growth of WT heterotransplants in athymic mice and suggest that

there is a similar role for these agents in stimulating tumor growth in humans.

Histologic examination of heterotransplanted tumors showed that they consisted predominantly of blastema with a minor component of stromal differentiation and rare foci of tubular differentiation. Treatment with αIR-3 did not alter these morphologic features. There was no infiltration of tumor by lymphocytes or other inflammatory cells. Thus the mechanism of tumor regression and suppression by αIR-3 is probably due to its blockade of IGF receptors and not related to induction of an immune response against the tumor cells.

The source of the IGF responsible for stimulating WT growth has not been identified. In humans, WT have been shown to express IGF-2 mRNA and protein, which presumably contribute to tumor growth. We have not yet determined if the heterotransplants also produce IGF-2. If IGF-2 production by WT is under hormonal regulation, it is possible that murine hormones (*ie,* growth hormone) may be unable to stimulate its production in the human tumor tissue. Endogenous murine IGFs, including IGF-1, may contribute to stimulating tumor growth. Whatever the source (autocrine production by tumor cells or endocrine production by murine tissues) and nature (IGF-1 or IGF-2) of the IGF that is stimulating growth of these heterotransplants, the effect appears to be mediated predominantly by the type I IGF receptor because tumor growth is effectively inhibited by an antibody specific to this receptor.

In the present study, we found that by 42 days after WT transplantation tumors began to grow in all mice, even those continuously treated with αIR-3. There are a number of possible explanations for this. Immunotherapy with αIR-3 may result in the selection of clones whose growth is IGF independent. Alternatively, the therapy may select for clones that express increased numbers of type I IGF receptors or receptors with altered immunoreactivity. Studies are underway to evaluate these possibilities.

These data not only demonstrate the importance of IGF action in promoting proliferation of WT cells but also suggest the possible use of anti-GF receptor immunotherapy for neoplastic diseases. Obviously the athymic mouse heterotransplant model represents a highly favorable (but unrealistic) situation for evaluation of any immunotherapy. In the present study, our mouse antibody reacted with the human-derived tumor but not the IGF receptors of normal mouse tissues. The success of this approach in clinical oncology will depend on developing dosage schedules that might exploit differences in growth factor control of proliferation of the patients' normal and neoplastic cells.

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Acknowledgment

The authors thank Carol Hill for editorial assistance.