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Advances in Brief

In Vivo Treatment with Antibody against IGF-1 Receptor Suppresses Growth of Human Rhabdomyosarcoma and Down-Regulates $p34^{cdc2}$

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Abstract

In a previous study, we have shown that insulin-like growth factor type 2 (IGF-2) functions as an autocrine growth factor in human rhabdomyosarcoma (RMS) cell lines. In addition, we demonstrated that the inhibition of binding of IGF-2 to the IGF-1 receptor, mediated by suramin, blocked the growth of RMS cells *in vitro*. We now report that, *in vivo*, a specific IGF-1 receptor blocking antibody (αIR-3), but not suramin, suppresses RMS tumor growth. Both progression of tumor growth in tumor-bearing animals and formation of newly established tumors were suppressed by treatment with αIR-3. Histological analysis of tumors from treated animals did not reveal necrotic lesions, implying that the treatments had no cytotoxic effect. The decrease in tumor growth was associated with a decrease of $p34^{cdc2}$, a cellular protein involved in cell cycle regulation, suggesting that treatment resulted in the arrest of cellular proliferation. We speculate, therefore, that agents which block the IGF signaling pathway may find application in treatment of RMS.

Introduction

The growth of RMS[2] cell lines is dependent upon the IGF signaling pathway (1). Similarly, abnormally high levels of IGF-2 mRNA have been shown in tissue specimens of RMS, suggesting that these tumors may use an autocrine IGF-2 growth pathway (2). The precise role of IGFs in the regulation of proliferation of these tumors, however, remains unclear. The IGFs are believed to function at the G_1-S interface during the cell cycle (3, 4). Signaling through the IGF-1 receptor increases expression of *cdc2* mRNA (5), a critically important gene implicated in the control of the eukaryotic cell cycle (6, 7). In contrast, down-regulation of the IGF-1 receptor inhibits the induction of *cdc2* mRNA (5). Furthermore, the IGF-1 receptor plays a key role in control of cellular proliferation mediated by other growth factors, including EGF and PDGF (3, 8). A functional IGF-1 receptor is required for EGF and PDGF to exert a mitogenic stimulus, while cells overexpressing the IGF-1 receptor do not need PDGF or EGF to grow (8). Previous studies have demonstrated that, *in vitro*, an antibody which blocked the IGF-1 receptor inhibited the proliferation of several cell lines, including the rhabdomyosarcoma cell line, RD (1, 9). These observations may have important implications for therapeutic strategies to control tumor growth. By blocking the IGF-1 receptor signaling pathway, the growth of tumors deriving a proliferative advantage through a perturbation of either EGF, PDGF, or IGF pathways might be inhibited. Therefore, in this study, we investigated whether the IGF-1 receptor blocking antibody may suppress growth of RMS tumors in recipient animals. We also studied the effect of suramin, a compound known to bind and inhibit several growth factors, including IGF-1 and IGF-2 (10). Finally, the level of $p34^{cdc2}$ was measured in the tumors dissected from the animals treated with αIR-3 or suramin to evaluate whether, *in vivo*, the agents blocking the IGF-1 receptor or binding the ligand may modulate the level of this protein, central to the cell cycle control mechanism.

Materials and Methods

Cells and Reagents. The human embryonal rhabdomyosarcoma cell line RD (11) was purchased from American Type Culture Collection (Rockville, MD) and maintained in RPMI 1640 (Biofluids, Inc., Rockville, MD) supplemented with 10% fetal calf serum (Biofluids). NIH nude mice were obtained from the Research Resources Units of NIH. In all experiments, male mice, matched for age and weight, were used. The monoclonal antibody against the IGF-1 receptor, αIR-3, was a gift from Drs. S. Jacobs and F. Kull (Wellcome Research Laboratories, Research Triangle Park, NC). Suramin was a gift of Dr. M. Cooper (National Cancer Institute, Bethesda, MD).

Tumorigenicity Assays and Treatment with αIR-3 or Suramin. RD cells were injected s.c. into the lower dorsal region of recipient animals. Each mouse received 0.1 ml of a single cell suspension containing 1×10^7 viable cells, as determined by trypan blue exclusion. Ten days following injection, the size of the tumors was measured, and mice bearing tumors of approximately the same size were used for further study. Those mice were then divided into groups, each containing five animals and treated with αIR-3 (50 μg/injection) or suramin (50 μg/injection). The injections of αIR-3 or suramin were done s.c. in proximity to the tumors, twice weekly. Tumor growth was monitored by measuring twice weekly their volume, calculated as $V = a \times b^2/2$ (a, length; b, width), according to the method of the National Cancer Institute (12). In the control group, the injection of RD cells was followed by injection of a class-matched mouse IgG (MOPC-21). All mice were sacrificed at the same time, and autopsies were performed to examine internal organs for gross pathological alterations. Subsequently, histological sections were prepared and analyzed microscopically. These experiments were repeated two times, each time using five animals per group.

To monitor the capacity of RD cells to initiate tumor formation in the presence of αIR-3, RD cells were preincubated with αIR-3 (10 ng/ml) for 3 h and then injected (1×10^7 cells/mouse) s.c. into four groups of mice, each containing four mice. Following the injection of tumor cells, each group was treated with either 5, 50, or 500 μg of αIR-3, twice weekly for 50 days. A group receiving class-matched mouse IgG was used as control. Tumors were detected by palpation and measured twice weekly. All mice were sacrificed 50 days after injection of tumor cells.

Western Blot Analysis. The level of $p34^{cdc2}$ was measured in cellular extracts isolated from tumors dissected at autopsy from the animals receiving either αIR-3 or suramin, as described above. Samples containing equal amounts of protein were fractionated on 10% sodium dodecyl sulfate-polyacrylamide gels and transferred onto nitrocellulose filters (Schleicher & Schuell, Inc., Keene, NH), then incubated for 4 h with anti-$p34^{cdc2}$ (Oncogene Science, Uniondale, NY). After three washes, the filter was incubated with ^{125}I-labeled donkey anti-rabbit IgG (Amersham Corp., Arlington Heights, IL) for 2 h. Finally, the filter was washed, dried, and exposed to XAR-5 X-ray film (Kodak). Densitometric quantification of $p34^{cdc2}$ was performed by scanning the autoradiogram in an Bio-Rad model 620 video densitometer (Bio-Rad, Richmond, CA).

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[2] The abbreviations used are: RMS, rhabdomyosarcoma; IGF, insulin-like growth factor; EGF, epidermal growth factor; PDGF, platelet-derived growth factor.

Results

The Effect of αIR-3 or Suramin on Progression of Rhabdomyosarcoma Growth in Nude Mice Receiving Injections of RD Cells. In order to evaluate the effect of αIR-3 or suramin on progression of rhabdomyosarcoma growth, tumor-bearing animals were treated with either αIR-3 (50 μg/injection) or suramin (50 μg per injection). The treatment was initiated 10 days after injection of RD cells, when tumors had reached a size of approximately 70 mm^3, and continued twice weekly. When compared to controls, the average size of tumors in animals treated with αIR-3 was >80% smaller. Specifically, in a representative experiment (Fig. 1), the average increase in tumor size, as measured at autopsy, was 264 mm^3 in αIR-3-treated animals compared to 1381 mm^3 in control animals. As determined by a two-tailed *t* test, αIR-3 produced a significant decrease in tumor size, when compared to the control ($P < 0.005$), while suppression of tumor size in suramin-treated animals was not statistically significant ($P < 0.6$). As expected, the tumor mass measured at autopsy was also significantly lower in αIR-3-treated animals than in controls (Fig. 1*b*). These findings were consistent with the results obtained in two other independent experiments. Nevertheless, if the established tumors were larger then 70 mm^3 at the beginning of treatment, the degree of suppression of tumor growth was smaller (data not shown). Histological examination of tumor specimens did not reveal an appreciable change in the number of rhabdomyoblastic cells in the tumors from animals receiving different treatments (data not shown). Similarly, no difference in the amount of necrosis was detected, suggesting that the treatments did not cause a cytotoxic effect.

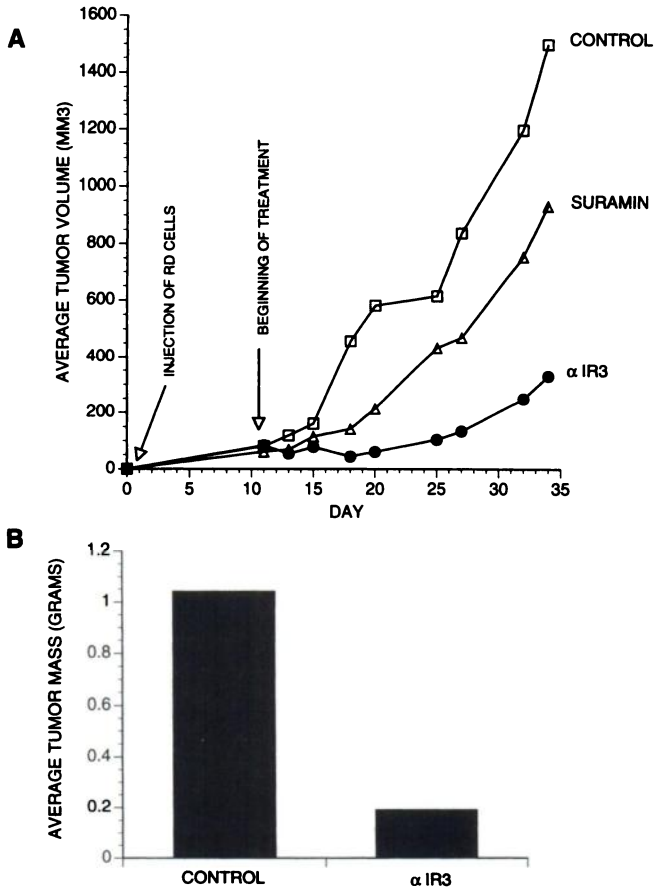

Fig. 1. The effect of αIR-3 or suramin in tumor-bearing animals on progression of tumor growth. *A*, data from a representative experiment show the average tumor volume for each group, containing five animals, measured at the indicated time points. The measurements obtained at autopsy had shown a statistically significant decrease in tumor size ($P < 0.005$), produced by αIR-3, but not suramin ($P < 0.6$). *Arrows*, the time of the first injection of αIR-3 (50 μg/injection) or suramin (50 μg/injection) and the initial size of tumors (70 mm^3). *B*, average weights of tumors dissected at autopsy 35 days after injection of tumor cells. Injections s.c. of αIR-3 or suramin were given twice weekly in proximity to the tumors. SD was <15% at all points. Comparable results were obtained in two other independent experiments.

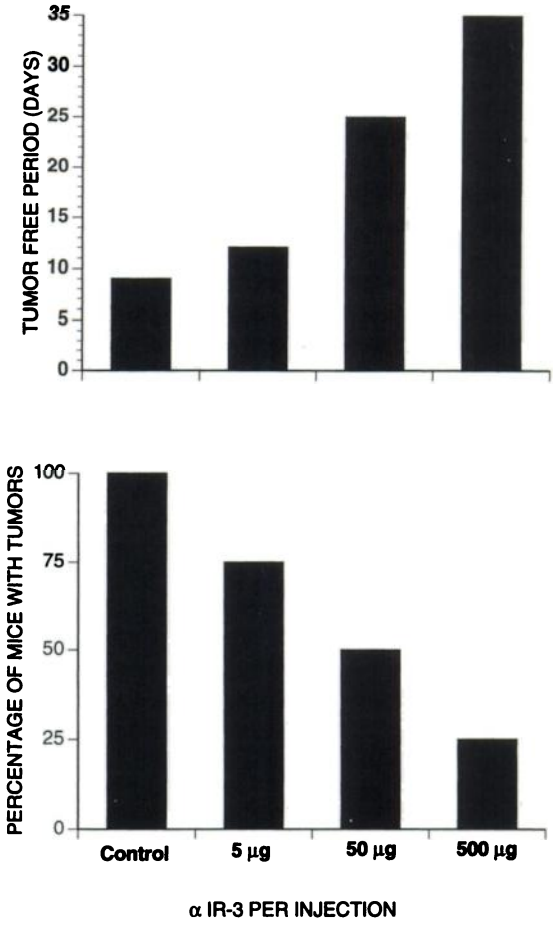

Fig. 2. Treatment of nude mice with αIR-3 following the injection of RD cells pretreated with αIR-3 prolongs the tumor-free period and decreases the incidence of tumor formation. The tumor-free period, shown on the *upper panel*, is the time between the injection of tumor cells and the appearance of the first tumor in a group of mice. The percentage of tumor-bearing animals, as assessed 50 days after the injection of tumor cells, is shown in the *lower panel*. RD cells were incubated with αIR-3 (10 ng/ml) for 3 h at 37°C and then injected (1×10^7 cells/animal) s.c. into groups of four nude mice. Following the injection of RD cells, each group was treated twice weekly with 5, 50, or 500 μg of αIR-3 per mouse, injected s.c. into the same area where the cells were injected. In control mice, the s.c. injection of RD cells was followed by injections of class-matched mouse IgG (50 μg). Comparable results were obtained when this experiment was repeated.

The Effect of αIR-3 on Formation of Rhabdomyosarcoma in Nude Mice Receiving Injections of RD Cells Pretreated with αIR-3. RD cells were incubated with 10 ng/ml αIR-3 for 3 h and then injected s.c. (1×10^7 cells/animal) into recipient animals to examine whether an antibody blocking the IGF-1 receptor affects the initial stage of tumor formation. Following injection of tumor cells, the mice were treated twice weekly with different doses of αIR-3. We observed that the treatment with αIR-3 produced a dose-dependent delay of tumor formation; a representative experiment is illustrated in Fig. 2. In the group of mice treated with 50 μg of αIR-3 per injection, a measurable tumor first appeared 25 days after injection of tumor cells, while in the group of mice treated with 500 μg of αIR-3 per injection, a measurable tumor mass appeared only after 35 days. (Fig. 2, *upper panel*). In contrast, a 9-day tumor-free latency period was observed in the control animals receiving class matched mouse IgG. The delay in

tumor formation in αIR-3-treated animals was associated with a decreased incidence of tumor formation. As determined at autopsy, 50 days after injection of tumor cells, 25% of mice had tumors in the group treated with 500 μg of αIR-3 (Fig.2, *lower panel*), while 75% of mice had tumors in the group treated with 5 μg of αIR-3. All of the control animals had tumors. In αIR-3-treated animals, when compared to the controls, tumor size decreased in a dose-dependent manner (data not shown). This experiment demonstrated that αIR-3, when injected simultaneously with tumor cells, had a dose-dependent effect on tumor-forming latency period, as well as the incidence and the size of tumors in recipient animals.

The Effect of αIR-3 on p34^{cdc2}. Based on the observation that the growth of tumors *in vivo* was significantly decreased after treatment with αIR-3, we studied the level of expression of p34^{cdc2}, a cellular kinase that plays a central role in cell cycle regulation. The tumors dissected from αIR-3-treated mice had a marked decrease in levels of p34^{cdc2}, compared to control-treated mice, as determined by Western blot analysis (Fig. 3). This finding suggests that treatment with a blocking antibody against the IGF-1 receptor interferes with the pathway that regulates the synthesis or stability of p34^{cdc2}. The decreased level of p34^{cdc2} associated with the decreased size of tumors implies that treatment with αIR-3 might have induced cell cycle arrest. In contrast, suramin, which did not significantly alter tumor growth *in vivo*, had no effect on p34^{cdc2} levels.

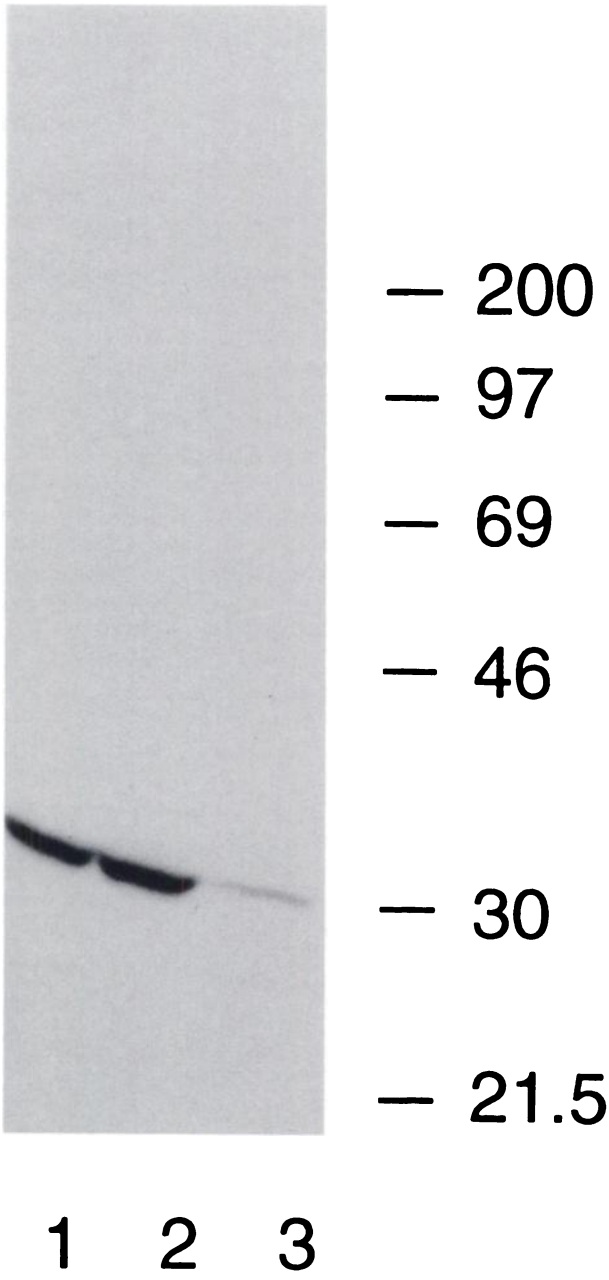

Fig. 3. Levels of p34^{cdc2} are decreased in tumors dissected from αIR-3- treated nude mice. Tumor-bearing nude mice were treated with αIR-3 (50 μg/injection) or suramin (50 μg/injection) twice weekly over a period of 35 days. The tumors were dissected at autopsy, and cellular lysates were prepared as described in "Materials and Methods." Western blot analysis of cellular lysates containing equal amounts of proteins is shown. *Lane 1*, tumors from control animals. *Lane 2*, tumors from suramin-treated animals. *Lane 3*, tumors from αIR-3-treated animals.

Discussion

This study demonstrates that αIR-3 suppresses the growth of human rhabdomyosarcoma in recipient animals. As a model of rhabdomyosarcoma, we used RD cells s.c. injected into nude mice. These results are consistent with previous studies that demonstrated that αIR-3 inhibited the proliferation of RD cells *in vitro* (1). Similarly, αIR-3 treatment inhibits the growth of breast cancer and melanoma cells in athymic mice (13, 14). αIR-3 interferes with the binding of IGF-2 to the type 1 IGF receptor, which is thought to be one of the pivotal factors that regulates proliferation of rhabdomyosarcoma cells (1, 3). In our study, the most effective suppression of tumor growth was obtained after injection of αIR-3 in close proximity to the tumors. An i.v. or i.p. injection of αIR-3 produced lower levels of growth suppression (data not shown), indicating that the route of administration and, presumably, the intratumoral concentration of these agents affected the efficacy of the treatment. A dose-dependent inhibition of breast cancer cell growth has also been observed in nude mice treated with αIR-3 (13). We also observed that the extent of suppression of tumor growth depended on the initial size of tumors. At the given dose of αIR-3, the inhibition of growth was >80% when the initial size of tumors was 70 mm^3, while a lower level of growth suppression was observed when the initial size of tumors was 120 mm^3. Interestingly, if the RD cells were treated with αIR-3 prior to their injection into the animals, tumor formation was remarkably delayed, and the number of tumors per group was decreased. We had shown previously that incubation of cells with a comparable concentration with αIR-3 decreased the binding of IGFs to its receptor (1). These results suggest that blocking the IGF-1 receptor may interfere at multiple steps in the tumor growth process. αIR-3 could presumably inhibit *in vivo* tumor cell proliferation, as it does *in vitro*. In addition, αIR-3 may affect an early stage in the sequence of events that lead to tumor formation, perhaps survival of cells in the host environment and/or migration and adhesion to the extracellular matrix. It was previously described that preincubation with laminin fragments that block the laminin receptor, which mediates attachment to basement membrane, decreased dramatically the number of secondary tumors (15, 16).

Histological analysis showed no appreciable morphological differences between tumors from treated and control animals. Similarly, no differences in the number or extent of necrotic lesions was observed, suggesting that the decreased size of tumors in treated animals was not due to cytotoxic effects or massive cellular death. Moreover, no clear pattern of apoptosis was detected when DNA from treated tumors was analyzed (data not shown). However, in view of the fact that apoptotic cells have a limited life time, a more detailed study would be necessary to completely exclude that apoptosis did not occur during the treatment with αIR-3. Nevertheless, based on our observations, we speculated that αIR-3 induced cell cycle arrest; therefore, we evaluated the amount of p34^{cdc2}, a protein which plays a key role in cell cycle control (6, 17). It was reported that, in normal fibroblasts,

micro-injection of antibody to $p34^{cdc2}$ led to G_2 arrest (18). In addition, antisense cdc2 oligonucleotides reduced the entry of T lymphocytes into the S phase and DNA synthesis (19). In contrast, the amount of $p34^{cdc2}$ increased in T lymphocytes 24 h after stimulation with phytohemagglutinin, which corresponded to late G or early S phase. Moreover, in fibroblasts overexpressing IGF-1 receptor, the induction of cdc2 mRNA was mediated by IGF-1, while antisense oligonucleotides to the IGF-1 receptor inhibited the IGF-1-mediated induction of cdc2 mRNA (5). In our experiments, the level of $p34^{cdc2}$ was decreased in tumors dissected from αIR-3-treated animals. The decreased level of $p34^{cdc2}$ correlated with a strong suppression of tumor growth. It is possible that the αIR-3-induced decrease in $p34^{cdc2}$ expression may be secondary to a more direct effect on other cell cycle regulatory proteins such as cyclins. In contrast, treatment with suramin did not suppress $p34^{cdc2}$ and did not significantly inhibit tumor growth. Our observation that a decreased level of $p34^{cdc2}$ was associated with a decreased tumor size suggests that αIR-3 treatment may lead to the arrest of tumor cell proliferation. Other investigators, using synthetic peptide analogues of IGF-1 that block the IGF-1 receptor, observed, *in vitro*, a dramatic inhibition of the growth of several cell types, including prostate carcinoma cells (20). Similarly, decreased levels of IGF-1 receptor (21) or the endogenous IGF-1 (22, 23), produced by transfection of the respective antisense RNAs or complementary DNA, lead to suppression of tumorigenicity of glioblastoma cells.

In view of the critical role of IGF-1 receptor signal transduction in regulation of cellular proliferation, we speculate that blocking IGF-1 receptors, under controlled conditions, might prove useful in developing novel clinical strategies. Although the mechanism that causes the suppression of tumor growth in animals treated with antibody against the IGF-1 receptor remains to be elucidated, this study shows that inhibition of rhabdomyosarcoma growth may be successfully achieved *in vivo* by targeting one critical molecule that regulates a cellular proliferation pathway.

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