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ORIGINAL ARTICLE

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The influence of recombinant human insulin-like growth factor-I (rhIGF-I) on cell growth and cytotoxicity of drugs in childhood rhabdomyosarcoma cell lines and xenograft models

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Abstract *Purpose*: Recombinant human insulin-like growth factor I (rhIGF-I) has been reported to ameliorate vincristine-induced neuropathy, the dose-limiting side effect of this antimitotic anticancer drug. However, rhIGF-I also might have adverse effects, as has been shown in vitro, where it stimulates growth of cancer cells and protects them from cytotoxicity of anticancer drugs. The influence of rhIGF-I on the cytotoxicity of vincristine has not yet been studied. Furthermore, studies performed have been done under serum-free conditions, which are far from physiological. *Methods*: We studied the influence of rhIGF-I on the growth of two rhabdomyosarcoma cell lines (Rh30 and Rh1) and on the antitumor effects of vincristine, cisplatin, etoposide, doxorubicin, and topotecan under serum-free and serum-containing conditions. To extend the in vitro data, we grew Rh30 cells as xenografts in mice and determined the effects of vincristine, rhIGF-I or their combination on tumor growth. *Results*: In vitro, both cell lines demonstrated a functional type I IGF receptor, as shown by the rapid activation of ribosomal p70 S6 kinase after stimulation with rhIGF-I. Under serum-free conditions, rhIGF-I stimulated growth of both cell lines. Exposure to cytotoxic drugs with and without rhIGF-I resulted in higher cell numbers in cultures exposed to rhIGF-I. However, relative to the appropriate control, fractional growth inhibition and or cell kill of the cytotoxic drugs was identical with and without rhIGF-I. Under serum-containing conditions, rhIGF-I had no effect on cell growth or drug cytotoxicity. In vivo we did not find a significant influence of rhIGF-I on HxRh30 cell growth, or on the antitumor activity of vincristine. *Conclusions*: These studies show that rhIGF-I has no adverse effects on human rhabdomyosarcoma growth or on the antitumor effect of cytotoxic drugs under serum-containing conditions in vitro or in tumor-bearing mice. Potentially, therefore, rhIGF-I may ameliorate vincristine-induced neuropathy without adversely influencing tumor growth or vincristine cytotoxicity in children.

Key words IGF-I · Vincristine · Cytotoxicity

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Introduction

Recombinant human insulin-like growth factor I (rhIGF-I) might protect against vincristine-induced peripheral neuropathy in patients, as has been shown in mice [1, 2, 3]. Peripheral neuropathy is the dose-limiting side effect of vincristine and necessitates dose reduction or cessation of therapy [4]. Thus, the potential of rhIGF-I to ameliorate this toxicity could increase the utility of this important anticancer agent.

IGF-I, or somatomedin-C, is a polypeptide hormone that is produced throughout the body, stored in the blood and regulated chiefly by growth hormone. IGF-I has endocrine, paracrine, and autocrine activity. The IGF system comprises the structurally and functionally related ligands IGF-I, IGF-II, and insulin, the IGF receptors (IGF-R), the type I, type II and insulin receptors, and the binding proteins (BPs). The IGF-Rs show cross-affinity for the ligands. BPs modulate IGF activity, either inhibiting or enhancing it [5]. The idea that

rhIGF-I might protect against vincristine-induced neuropathy came from the knowledge that endogenously produced IGF-I is important for optimal growth and development of virtually all cell types, including neurons [6]. Besides its role in physiological growth, IGF-I also plays a role in the repair of damaged neurons. For example, IGF-I production and local release is increased in denervated muscles. Uptake in damaged nerves stimulates nerve regeneration [7, 8, 9, 10, 11, 12]. Administration of rhIGF-I is being studied as treatment for different neuromuscular diseases, and damage states, including chemotherapy-induced peripheral neuropathies [10, 13, 14].

However, before exploring rhIGF-I further as a neuroprotective agent in the clinic, any adverse effect of rhIGF-I on tumor growth and on cytotoxicity of vincristine and other concomitantly administered anticancer drugs should be ruled out. Most of our knowledge about the potential adverse effects of rhIGF-I comes from preclinical studies in which the role of the endogenous IGF system in tumor growth has been studied [15, 16]. In vitro, rhIGF-I and/or rhIGF-II have been shown to stimulate proliferation of myeloid leukemic cells, Wilms' tumor, rhabdomyosarcoma, and neuroblastoma cells when added to mitogen-free medium [17, 18, 19, 20]. In vivo, rat glioblastoma cells expressing an antisense IGF-I RNA prevent tumorigenicity of syngeneic brain tumors and cause regressions of established syngeneic tumors [21]. In mice with low IGF-I levels due to diet restriction, elevation of endogenous IGF-I levels to normal by administration of exogenous rhIGF-I results in reversion of decreased bladder tumor incidence and tumor progression [22].

The IGF system has been implicated in clinical cancer as well. Overproduction of IGF-I, IGF-II and/or overexpression of IGF-IR have been demonstrated in many tumors including acute leukemias, Wilms' tumor, neuroblastoma, Ewing's sarcoma, Hodgkin's disease and central nervous system tumors [23, 24, 25, 26, 27, 28, 29]. Many of these tumors are treated with combination chemotherapy that frequently includes vincristine.

The influence of rhIGF-I on cytotoxicity of anticancer drugs has been studied in vitro, and the effects appear to be drug-specific. Under serum-free conditions of cell growth, rhIGF-I protects cancer cells against apoptosis induced by etoposide, actinomycin D, and mitomycin C, but not against apoptosis induced by adriamycin or cisplatin [30, 31, 32, 33, 34, 35, 36]. The influence of rhIGF-I on vincristine cytotoxicity has not yet been studied.

However, the clinical relevance of these studies is questionable. The effect of exogenous rhIGF-I was studied under nonphysiological conditions of cellular growth devoid of endogenous mitogens and modulating BPs. Therefore, we studied the influence of rhIGF-I on growth of rhabdomyosarcoma cell lines (Rh30 and Rh1) and on the cytotoxicity of anticancer drugs, not only under artificial serum-free conditions but also under more physiological serum-containing conditions. We also examined the effects of rhIGF-I on tumor growth and the antitumor activity of vincristine in a rhabdomyosarcoma xenograft model in mice.

Materials and methods

In vitro studies

Cell lines and culture conditions

The alveolar rhabdomyosarcoma cell line Rh30 has been described previously [37]. A second cell line, Rh1, was established from a patient diagnosed with embryonal rhabdomyosarcoma. When cultured under serum-containing conditions cells were plated in RPMI-1640 supplemented with 2 m*M* glutamine and 10% fetal bovine serum (FBS), or where indicated, 10% human plasma-derived serum, and cultured in a humidified atmosphere containing 5% CO_2 at 37 °C. When studied under serum-free conditions, Rh30 cells were plated as described above and cultured for 1 day. Then cells were washed with serum-free RPMI-1640 and cultured in RPMI-1640 supplemented with 2 m*M* glutamine and 1 μg/ml transferrin, 20 n*M* progesterone, 30 n*M* selenium, and 100 μ*M* putrescine. Rh1 cells, were plated on fibronectin-coated plates in Dulbecco's modified Eagle's medium (DMEM) supplemented with 2 m*M* glutamine, 1 μg/ml transferrin, 20 n*M* progesterone, 30 n*M* selenium, and 100 μ*M* putrescine when cultured under serum-free conditions. rhIGF-I was generously provided by Cephalon (Westchester, Pa.). Vincristine, cisplatin, doxorubicin, and etoposide were obtained through the pharmacy at St. Jude Children's Research Hospital, and topotecan was generously provided by SmithKline Beecham (King of Prussia, Pa.).

Monolayer growth assay

For growth assays cells were plated (2.5×10^4 and 5.0×10^4 cells per well Rh30 and Rh1, respectively, when cultured with serum, or $3.0–5.0 \times 10^4$ and 10.0×10^4 cells per well, respectively, when cultured without serum) in 35-mm wells in triplicate on six-well culture dishes (Corning, N.Y.), and cultured for 1 day. The next day the medium was changed and drugs and growth factors were added. After exposure to drugs and growth factors for the required times, as described in specific experiments, cells were lysed under hypotonic conditions (10 m*M* HEPES and 1.5 m*M* $MgCl_2$) and nuclei were counted using a Coulter counter Z_m.

Ribosomal p70S6 kinase assay

To determine functional activity of the IGF-IR in both Rh30 and Rh1 cells, activation of ribosomal p70 S6 kinase ($p70^{S6K}$) was studied after stimulation by rhIGF-I. Cells (3×10^6) were seeded in 100-mm dishes and allowed to attach overnight. The cells were serum-starved for 24 h and then stimulated with rhIGF-I (50 ng/ml). Stimulation was terminated by removing the medium and washing the cells with cold phosphate-buffered saline (PBS). The cells were then lysed by gently rocking at 4 °C in 1 ml lysis buffer (50 m*M* Tris-HCl, pH 7.4, 150 m*M* NaCl, 1% NP-40, 0.5% sodium deoxycholate, 1 m*M* EGTA, 1 m*M* PMSF, 1 m*M* Na_3VO_4, and 1 m*M* NaF) containing 10 μg/ml each of aprotinin, leupeptin, and pepstatin. Lysates were centrifuged (15,000 *g*, 4 °C, 5 min) to remove nuclei. Anti-$p70^{S6K}$ polyclonal antibody (10 μl, 1 μg: Santa Cruz Biotechnology, Santa Cruz, Calif.) and 50 μl protein A/G Plus beads (Santa Cruz Biotechnology) were added to the lysates, which were then incubated overnight. The beads were washed twice with PBS and resuspended in 20 μl of $p70^{S6K}$ assay buffer (20 m*M* MOPS, pH 7.2, 25 m*M* β-glycerophosphate, 5 m*M* EGTA, 1 m*M* Na_3VO_4, 1 m*M* dithiothreitol). $p70^{S6K}$ activity was assayed using the S6 kinase assay kit (Upstate Biotechnology, Lake Placid, N.Y.), according to the manufacturer's instructions.

Assessment of apoptosis

Rh30 or Rh1 cells were plated at a density of 10,000 cells/cm^2 in serum-free DMEM/F12 medium (containing fibronectin, sodium selenite, human transferrin, and putrescine) as described above. After a 15-h incubation (37 °C, atmosphere containing 5% CO_2), cells were exposed to etoposide (20 μM) with or without rhIGF-I (10 ng/ml). After a 2-h exposure to etoposide, cells were harvested and analyzed using the ApoAlert assay, as described previously [38].

In vivo studies

Immune deprivation of mice

Female CBA/CaJ mice (Jackson Laboratories, Bar Harbor, Me.), at 4 weeks of age, were immune-deprived by thymectomy, followed 3 weeks later by whole-body irradiation (1150 cGy) using a ^{137}Cs source. Mice received 3×10^6 nucleated bone marrow cells within 6–8 h of irradiation [39].

Xenograft

The Rh30 poorly differentiated alveolar rhabdomyosarcoma xenograft (HxRh30) has been described previously [40]. It was grown from the bone marrow of a child with poorly differentiated rhabdomyosarcoma. Tumor fragments of approximately 3 mm^3 were transplanted into the subcutaneous space of both dorsal lateral flanks of mice to initiate tumor growth, 1 to 2 weeks after irradiation.

Tumor growth and inhibition studies

Tumor-bearing mice were randomized into four groups of five to seven mice prior to initiating therapy. When tumors were approximately 0.20–1 cm in diameter treatment was started according to the following protocols. The vincristine-treated group received 1 mg/kg vincristine every 7 days for 5 weeks intravenously (i.v.); the rhIGF-I treated group received 1 mg/kg rhIGF-I on Monday, Wednesday, and Friday for 5 weeks subcutaneously (s.c.); the vincristine plus rhIGF-I-treated group received 1 mg/kg vincristine every 7 days for 5 weeks i.v. and 1 mg/kg rhIGF-I on Monday, Wednesday, and Friday for 5 weeks s.c.; the control group received no treatment. Vincristine was dissolved in normal saline to a concentration of 0.05 mg/ml; rhIGF-I was dissolved in PBS to a concentration of 200 μg/ml.

Tumor volumes were calculated from weekly measurements of biperpendicular tumor diameters using the formula $[(\pi/6) \times d^3]$, where 'd' is the mean diameter assuming tumors to be spherical. For measurement of biperpendicular tumor diameters digital Vernier calipers interfaced with a Macintosh computer were used [39].

In growth studies animals were killed when both tumors in one animal reached 400% of the baseline volume or after 12 weeks. In growth inhibition studies animals were killed after 12 weeks. The number of weeks until tumors grew to 400% (tumor failure time) or regressed to 25% (tumor regression time) was used as a measure of tumor growth and inhibition rate. Since tumors were implanted in both lateral flanks, the tumor failure and regression times from each mouse are clustered observations. To account for the clustering effect due to the mouse without explicitly specifying the correlation structure, the time to failure or regression was defined as the minimum of the failure times of the bilateral tumors.

Statistical methods

The exact log-rank test was used to compare tumor failure times and tumor regression times among treatment groups. No adjustments were made for multiple comparisons.

Results

In vitro studies

IGF-IR function in Rh30 and Rh1 cells

Previously, the presence of a functional IGF-IR has been demonstrated on Rh30 cells: the addition of the neutralizing antibody α-IR3 to serum-free medium inhibits the growth of these cells [19, 41, 42]. In contrast, the growth of Rh1 cells is not significantly inhibited by the addition α-IR3 [42]. To further examine whether both cells had an intact IGF-I signaling pathway, we first determined whether rhIGF-I would stimulate induction of ribosomal p70^{S6K} activity. This pathway links mitogen stimulation to protein translation and cell cycle progression [43]. rhIGF-I (50 ng/ml) significantly stimulated p70^{S6K} activity in both Rh30 and Rh1 cells when added for 30 min to cells which had been cultured for 24 h under serum-free conditions, as shown in Fig. 1. Thus, Rh1 cells also clearly have a functional IGF-IR-mediated signaling pathway.

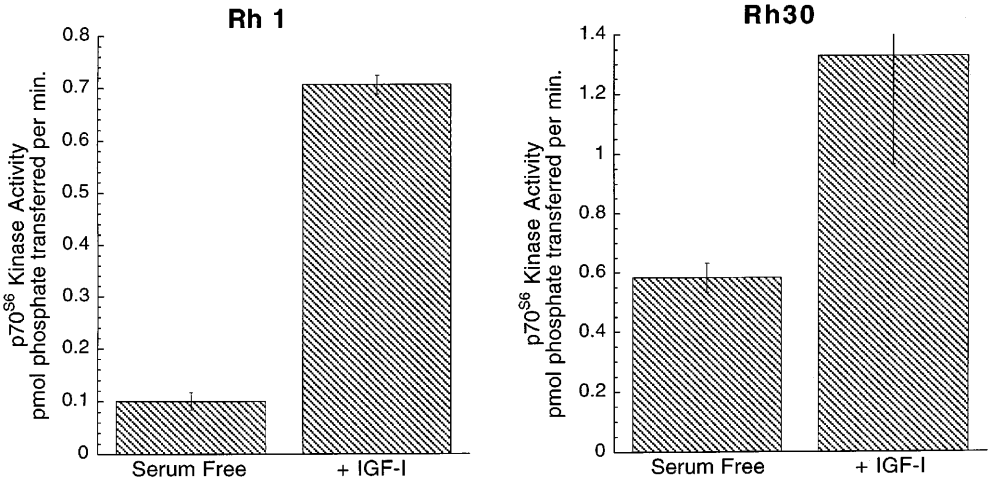

Fig. 1 Activation of ribosomal p70 S6 kinase by exogenous IGF-I in rhabdomyosarcoma cell lines. Rh30 and Rh1 cells were serum-starved overnight, then cells were either stimulated by addition of rhIGF-I (50 ng/ml), or not stimulated. Cells were harvested 30 min after the addition of rhIGF-I, lysed, and immunoprecipitated as described in Materials and methods. rhIGF-I stimulated p70^{S6} kinase activity by 225% and 700% in Rh30 and Rh1 cells, respectively (mean ± SD, $n = 3$)

Influence of rhIGF-I on Rh30 and Rh1 growth

Serum-free conditions. rhIGF-I had a stimulatory effect on Rh30 and Rh1 growth when cells were cultured under serum-free conditions. In the presence of increasing concentrations of rhIGF-I (0 to 1000 ng/ml) in the culture medium, the number of nuclei after 3 days was positively influenced by rhIGF-I in a concentration-dependent way (Fig. 2). The growth of Rh30 cells more than doubled in the presence of 100 ng/ml rhIGF-I. The effect of rhIGF-I on the growth of Rh1 cells was less pronounced with a 50% increase in growth after 3 days at a concentration of 100 ng/ml rhIGF-I. However, even at the highest rhIGF-I concentrations growth of both cell lines was less than growth achieved in medium containing 10% FBS.

Serum-containing conditions. In contrast, the addition of rhIGF-I (0–1000 ng/ml) did not have an influence on the growth of Rh30 or Rh1 cells when the cells were cultured with 10% FBS for 3 days (Fig. 3, only Rh30 data shown). Similarly, when Rh30 cells were cultured in the presence of 10% human plasma-derived serum, rhIGF-I did not have any influence on growth (data not shown).

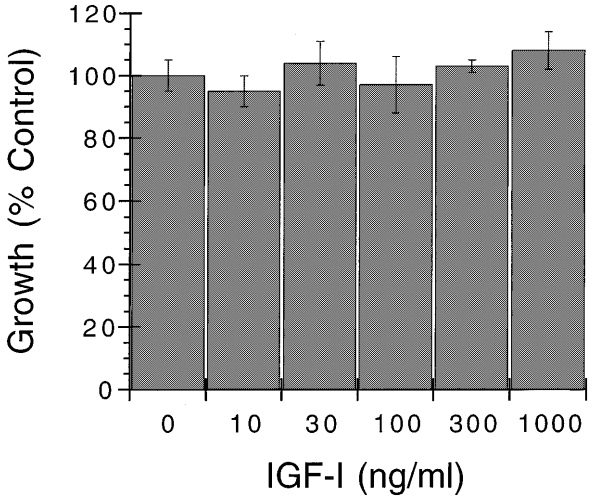

Fig. 3 Influence of rhIGF-I on growth of Rh30 cells when cultured under serum-containing conditions. Cells were cultured in RPMI-1640 medium containing 10% FBS together with IGF-I from 10 to 1000 ng/ml. Cells were counted after 3 days. Each result is the mean ± SD ($n = 3$) and shows growth relative to that in serum-containing medium without added IGF-I

Influence of rhIGF-I on vincristine cytotoxicity

Serum-free conditions. In the first set of experiments, cells were grown under serum-free conditions with or without rhIGF-I for 3 days, and cell numbers determined relative to control cultures (without vincristine). Consistent with the data presented in Fig. 2, growth of control cultures with rhIGF-I (no vincristine) compared to control cultures without rhIGF-I (no vincristine), showed a 1.5–2-fold increase. This differential was maintained between vincristine-treated cells in cultures with or without rhIGF-I over the range of vincristine concentrations used, as shown in Fig. 4. Thus, the cell number was always greater for the same drug concentration in the presence of rhIGF-I compared to the appropriate culture without rhIGF-I (Fig. 4A). In order to differentiate the influence of rhIGF-I on cell growth from the influence of rhIGF-I on the cytotoxicity of vincristine, cell numbers for treated cultures were normalized to the appropriate control (no vincristine, with or without rhIGF-I). When growth rates were normalized there was no difference in fractional growth inhibition and or cell kill as shown in Fig. 4B.

In the second set of experiments, we eliminated the influence of rhIGF-I on growth by exposing Rh30 cells for an equal number of cell doublings rather than equal exposure time to vincristine with or without rhIGF-I (100 ng/ml). Cells were harvested at 48 h (with rhIGF-I)

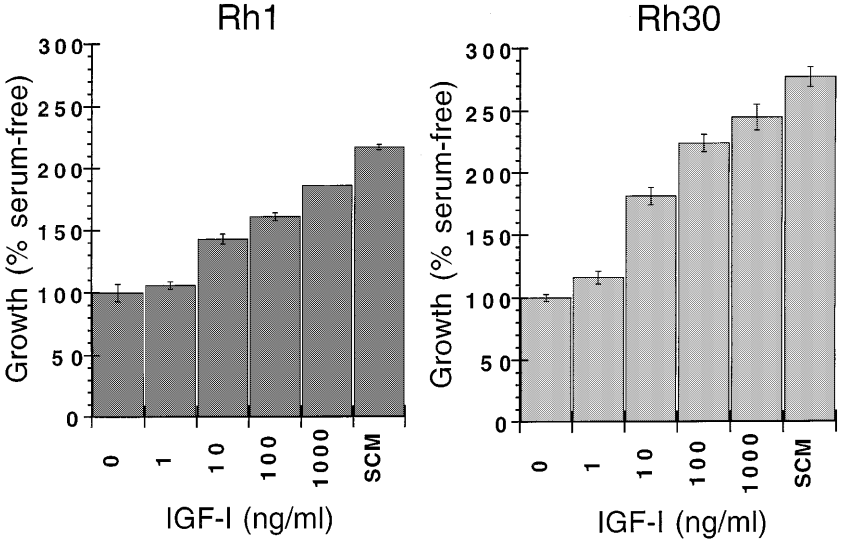

Fig. 2 Influence of rhIGF-I on the growth of Rh1 and Rh30 cells when cultured under serum-free conditions for 3 days. The results are expressed as the percentage of growth of cells cultured without rhIGF-I, and compared to growth in serum-containing medium (SCM) without additional rhIGF-I. In this experiment all Rh1 cells (including SCM) were plated on fibronectin-coated plates and cultured for 1 day under serum-free conditions prior to the addition of IGF-I or serum

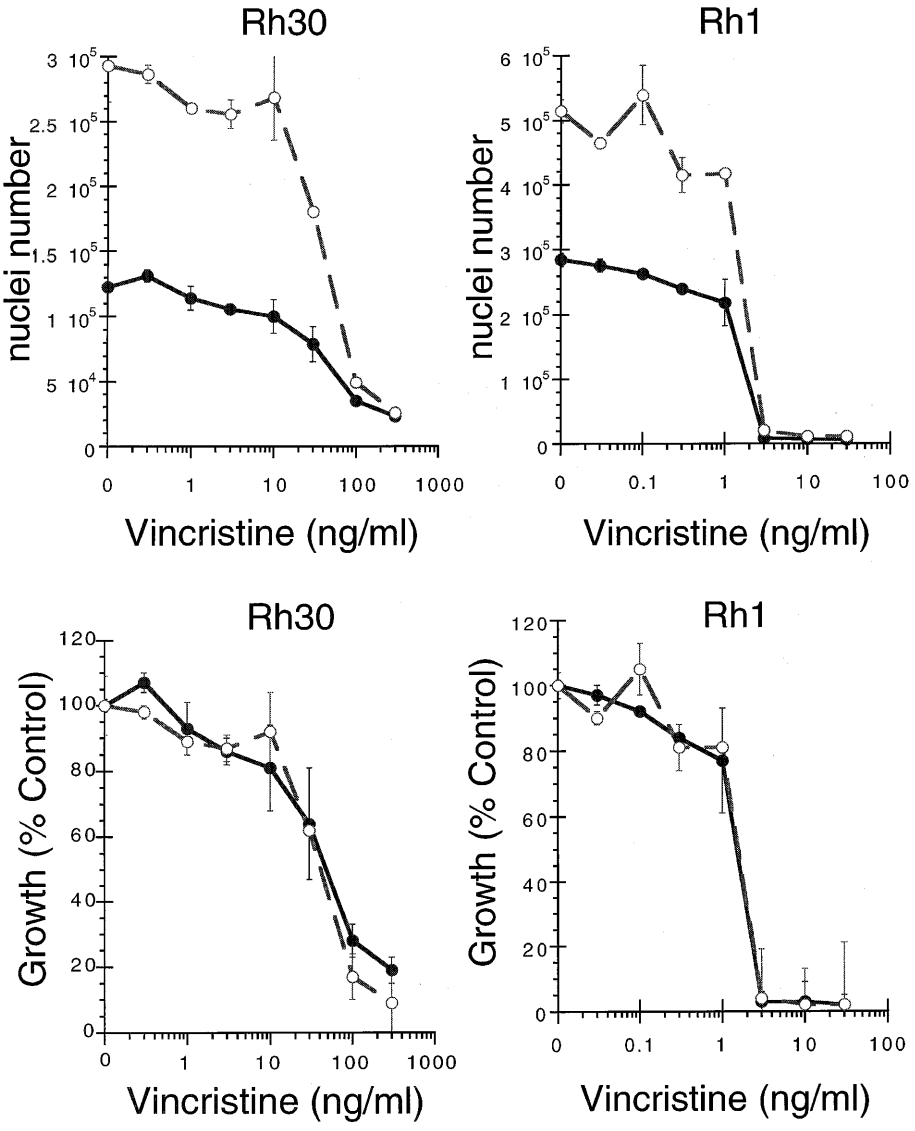

Fig. 4 Influence of rhIGF-I on vincristine cytotoxicity in Rh30 and Rh1 cells when cultured under serum-free conditions for 3 days (*top panels* absolute number of nuclei; *lower panels* data expressed as percentage of the growth of the appropriate control; *solid lines* without rhIGF-I, *broken lines* + 100 ng/ml rhIGF-I). Each point represents the mean ± SD ($n = 3$)

and at 73 h (without rhIGF-I) representing 1.3 cell doublings for each condition. Nuclei counts for controls (no vincristine, with or without rhIGF-I) were similar. For both conditions vincristine reduced cell counts equally. These results indicate that, in Rh30 and Rh1 cells, the effect of rhIGF-I is through the promotion of growth (proliferation or survival) of cells under serum-free culture conditions, rather than through the protection of cells from the antimitotic action of vincristine.

In the experiments described above, cells were exposed to drugs and rhIGF-I simultaneously. We next determined whether pre- and postincubation with rhIGF-I protected cells from vincristine. Rh30 cells were preincubated (18 h) with rhIGF-I (100 ng/ml), then exposed to vincristine (1000 ng/ml) for 1 h, and postincubated with rhIGF-I (100 ng/ml, 0–48 h). Cells were counted 12 to 48 h after vincristine exposure, and cell numbers were compared between different groups that had received similar concentrations of vincristine. Pre- and postincubation with rhIGF-I did not influence the degree of vincristine cytotoxicity nor did rhIGF-I delay cytotoxicity under serum-free culture conditions when data were normalized for differences in cell growth (data not shown).

Serum-containing conditions. When cells were maintained under conditions with serum (10% FBS) the addition of rhIGF-I (100 ng/ml) did not have any influence on growth rates of control cultures (without vincristine) for either Rh30 or Rh1 cells. Further, under these conditions, the addition of rhIGF-I (100 ng/ml) did not alter vincristine cytotoxicity in either cell line, when cultured for 3 days. Similarly, rhIGF-I did not have any influence on vincristine cytotoxicity in Rh30 when cells were cultured with 10% human plasma.

Influence of rhIGF-I on cytotoxicity of other anticancer drugs

We examined the potential protective effect of rhIGF-I on the antitumor effect induced by cisplatin, doxoru-

bicin, etoposide, or topotecan, cytotoxic drugs having mechanisms of action different from vincristine. Rh30 and Rh1 cells were exposed to increasing concentrations of these other cytotoxic drugs under serum-free conditions, in the absence and in the presence of 100 ng/ml rhIGF-I. After 3 days drug exposure, cells were harvested and the nuclei counted. rhIGF-I did not influence fractional growth inhibition of Rh30 or in Rh1 cells and/or cell kill for any drug if cell numbers were normalized to the appropriate control (with or without rhIGF-I, no drug). The results for Rh30 cells are shown in Fig. 5.

To determine whether rhIGF-I is able to protect against drug-induced apoptosis, we studied the influence of rhIGF-I on apoptosis of Rh1 cells which undergo typical morphological apoptosis when treated with etoposide. To quantitate apoptosis, control and treated cells (etoposide 20 μM, 24 h, with or without IGF-I 100 ng/ml) were stained with annexin V-FITC and propidium iodide (Table 1). Etoposide significantly increased the proportion of annexin V-positive cells. rhIGF-I reduced the proportion of annexin V-positive cells, but increased the proportion of cells that stained positive for both annexin V and propidium iodide. Thus, rhIGF-I did not protect Rh1 cells from apoptosis. Similar results were obtained for Rh30 cells (data not shown).

In vivo studies

We examined whether IGF-I influenced the growth or antitumor activity of vincristine in vivo, using Rh30 xenografts. Previously, we have shown that Rh30 xenografts are very sensitive to vincristine [40]. The results are shown in Fig. 6. In this experiment there were

Table 1 Determination of apoptosis in Rh1 cells by ApoAlert assay (A^-, A^+ annexin V negative, positive; P^-, P^+ propidium iodide negative, positive)

	A^-/P^-	A^-/P^+	A^+/P^-	A^+/P^+
Control	82.12	0.36	14.36	3.16
Etoposide	17.53	0.32	73.63	8.52
Etoposide + IGF-I	19.91	8.00	25.62	46.7

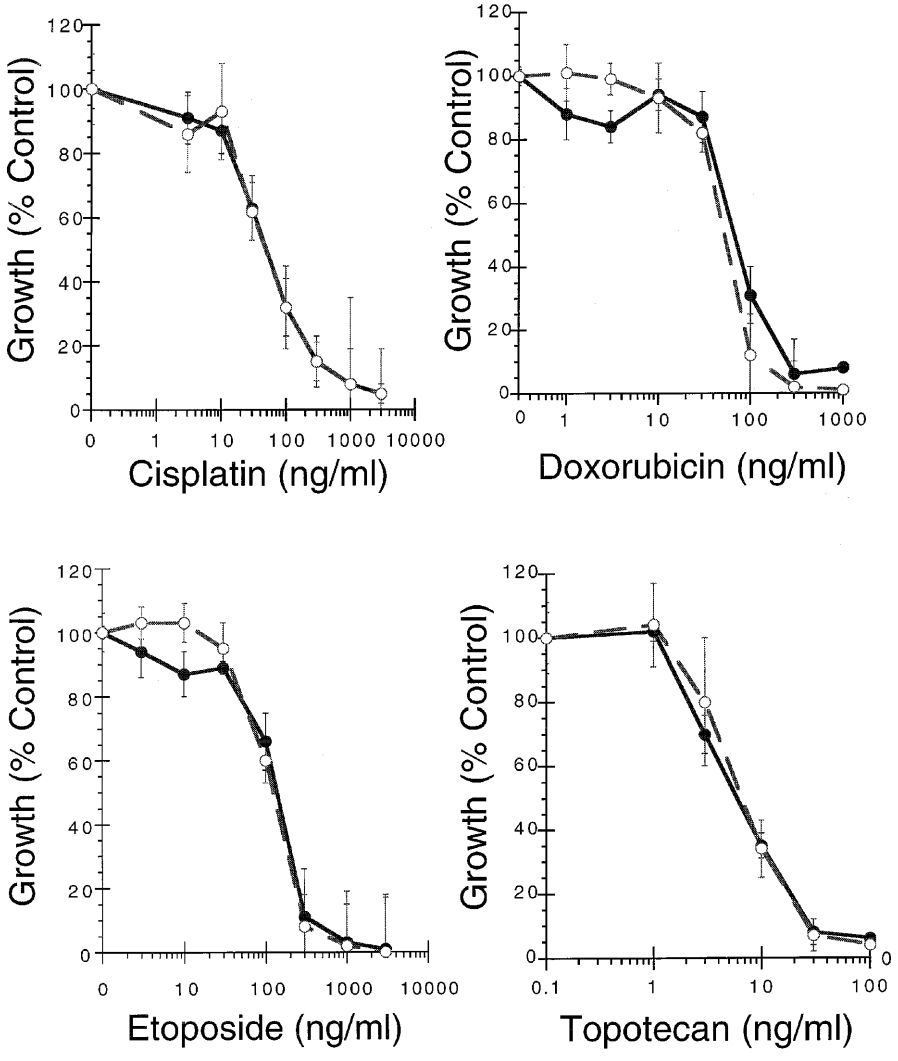

Fig. 5 Influence of rhIGF-I on cisplatin, doxorubicin, etoposide, and topotecan cytotoxicity in Rh30 cells when cultured under serum-free conditions for 3 days. The results are expressed as percentage of the growth of the appropriate control (mean ± SD, $n = 3$) (*solid lines* without rhIGF-I, *broken lines* + 100 ng/ml rhIGF-I)

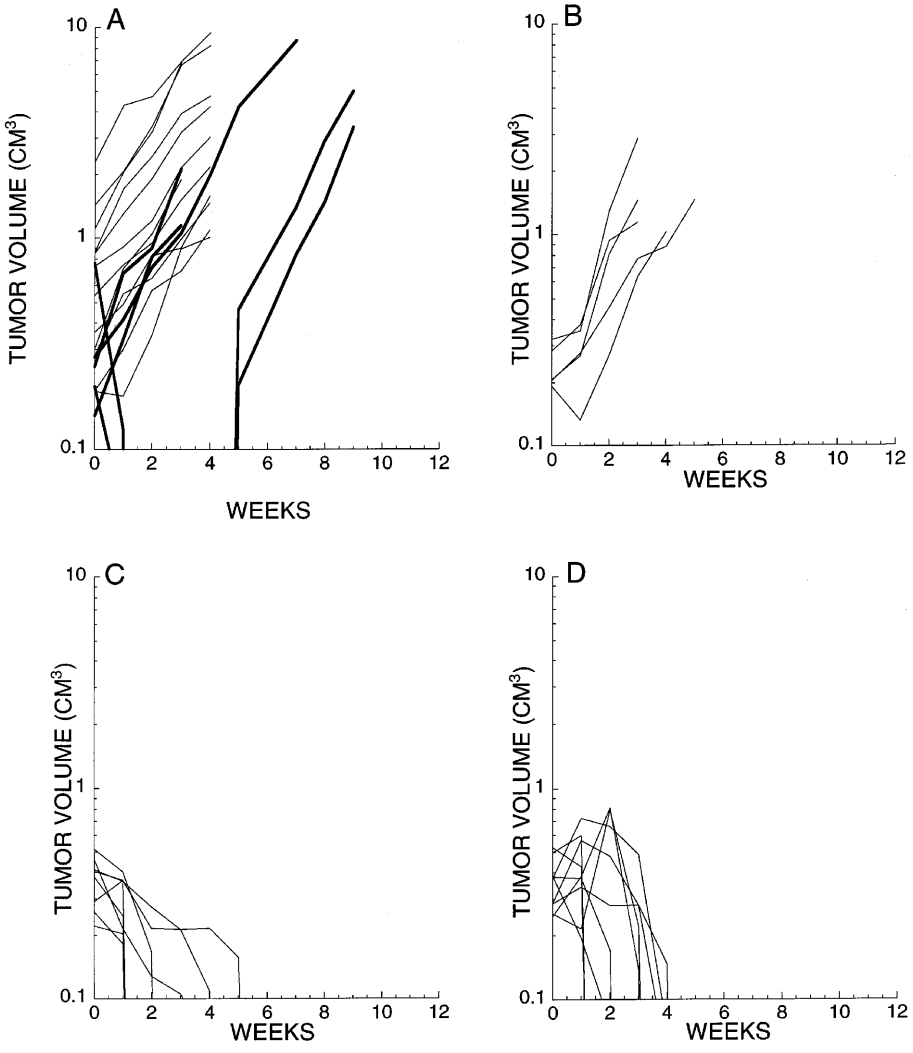

Fig. 6A–D Antitumor activity of vincristine or vincristine plus IGF-I combination treatment in mice bearing Rh30 xenografts. **A** Controls; **B** IGF-1 1 mg/kg s.c. Monday, Wednesday, Friday for 5 consecutive weeks; **C** vincristine 1 mg/kg i.v. weekly for 5 weeks; **D** vincristine plus IGF-I. Each curve represents the growth of an individual tumor. Mice were observed for 12 weeks after initiating treatment, or until tumors reached four times the volume at the start of therapy

only three control mice (group A, no treatment), and three mice that received IGF-I alone (group B). In group A spontaneous regression, then regrowth of both tumors in one mouse occurred. Data from this experiment (shown as bold tumor growth curves) was combined with those of a control group from the next experiment using Rh30 tumors on the same passage. As shown, growth in the controls from both experiments, as well as tumor growth in IGF-I-treated mice was very similar ($P = 0.27$). Vincristine administered at 1 mg/kg every 7 days for 5 consecutive weeks caused complete regressions with no regrowth of any tumor during the period of observation (12 weeks), irrespective of IGF-I treatment. There was no significant difference between responses of tumors in mice treated with vincristine alone, or in combination with IGF-I ($P = 1$).

Discussion

We studied the influence of rhIGF-I on human alveolar and embryonal rhabdomyosarcoma growth and on cytotoxicity of vincristine and several other anticancer drugs in cell culture and xenograft models. When serum-starved cells were exposed to rhIGF-I, there was a rapid induction of ribosomal $p70^{S6K}$ activity, indicating a functional IGF-IR-mediated pathway in both cell lines. Growth of Rh30 and Rh1 cells was stimulated 1.5–2-fold by rhIGF-I under serum-free conditions, but there was no influence of rhIGF-I on growth when cells were cultured under serum-containing conditions. Of note was that, when data were normalized for differences in cell growth, rhIGF-I did not influence the cytotoxicity of the antimitotic agent vincristine, nor the cytotoxicity of agents that mediate different forms of DNA damage (cisplatin, doxorubicin, etoposide and topotecan) in either cell line when cultured under serum-free conditions. Nor did we find an influence on cytotoxicity of vincristine in Rh30 and Rh1 cells, when cultured under serum-containing conditions.

In our study, 100 ng/ml rhIGF-I increased growth of Rh30 and Rh1 cells to about 200% and 150% of the growth under serum-free conditions, which is, respectively, about 81% and 74% of that in medium

containing 10% FBS. The less pronounced influence of rhIGF-I on the growth of Rh1 cells compared to that of Rh30 cells is in accordance with previous findings that treatment with antibody against the IGF-IR (α-IR3) has much more influence on growth of Rh30 cells than of Rh1 cells [42]. Thus, although rhIGF-I more potently stimulated induction of $p70^{S6K}$ activation in Rh1 cells than in Rh30 cells, the influence of this hormone on the growth rate of Rh1 cells was somewhat less.

The failure of exogenous rhIGF-I to augment the growth of rhabdomyosarcoma cells in the presence of serum might be explained by the presence of IGF BPs, by the presence of endogenous IGF-I and other growth factors in serum or by a combination of both. The most abundant BP present in serum is BP 3 (IGFBP3), which has a very high affinity for IGF-I. Binding of IGF-I to IGFBP3 attenuates IGF-I activity [44]. In vivo IGFBP3 complexes in the bloodstream allow transport and distribution of IGF-I throughout the body [45]. The presence of endogenous growth factors in serum might saturate IGF-IRs on cells, thereby preventing an additional effect of rhIGF-I on growth. Because the concentrations of IGFBPs and endogenous growth factors in FBS have not been characterized, and might be different from those in human serum, we studied both. The human serum used was plasma-derived, which means that the growth factors released from thrombocytes were lacking, so that growth factor concentrations present in the physiological situation were mimicked more closely. Also in the presence of this human plasma-derived serum, rhIGF-I did not stimulate growth of Rh30 cells.

We did not find an influence of rhIGF-I on drug cytotoxicity for any of the drugs we studied under serum-free conditions when data were normalized for differences in cell growth. Both Harrington et al. and Sell et al. [33, 34] have found a protective effect of rhIGF-I on the cytotoxicity of etoposide. They studied specifically the influence of rhIGF-I on etoposide-induced apoptosis. Harrington et al. studied clones of a rat fibroblast cell line expressing c-myc (ER). In these clones c-myc is expressed under the control of an estrogen-responsive promoter. Apoptosis was induced only in the presence of β-estradiol in the culture medium. Exposure to etoposide for 1 day followed by the addition of β-estradiol in the absence or presence of 100 ng/ml rhIGF-I (under serum-free conditions) produced a delay in the initiation of apoptosis by rhIGF-I of 5 h and suppression of apoptosis of approximately 20% after 20 h. The influence of rhIGF-I after a longer time periods were not studied.

Sell et al. studied a rat fibroblast cell line with overexpression of the IGF-IR. After exposure for 48 h to etoposide in the absence and presence of 50 ng/ml rhIGF-I (under serum-free conditions) there was a significantly higher percentage of viable cells in the presence of rhIGF-I, measured by trypan blue exclusion. Furthermore, the apoptotic peak found by flow cytometry in cells exposed for 6 h to etoposide almost completely disappeared under the influence of rhIGF-I. In the parental cell line in which the IGF-IR was not overexpressed the influence of rhIGF-I on viability was much less pronounced.

Unlike these investigators, we could not study apoptosis by flow cytometric analysis/TUNEL assay because Rh30 cells treated with cytotoxic agents do not show the classical DNA laddering. However, both cell lines show classical apoptosis determined by annexin V reactivity, nuclear shrinkage, and caspase 3 activation following etoposide treatment. Etoposide increased the proportion of cells that were annexin V-positive, but rhIGF-I did not protect against etoposide-induced annexin V positivity. RhIGF-I appeared to increase the proportion of cells stained by propidium iodide, indicating loss of membrane integrity. This suggests that in the cell lines we studied there is no protection against etoposide-induced cell death. This is supported by the fact that in our study the difference in cell number between the cells exposed to drug under the influence of rhIGF-I could be explained completely by differences in cell growth under the influence of rhIGF-I.

Possible explanations for this difference in the influence of rhIGF-I on the antitumor effect of etoposide might be found in the different cell lines used. The number of IGF-I receptors per Rh30 cell (~32,000) [41] is much lower than in the P6 cell line (5×10^5) used by Sell et al. [34]. Support for the explanation that the IGF-R (number) is important for the difference in the influence of rhIGF-I found by these investigators and our results is found in several studies. Stable transfection with the IGF-IR, in neuroblastoma (SHEP) cells, which express only low levels of IGF-IR, but not transfection with IGF-II, results in protection against etoposide-induced apoptosis, indicating the quantitative importance of IGF-IR (number) to protect cells against apoptosis [30]. Furthermore, Resnicoff et al. have shown a correlation between IGF-IR number and protection against apoptosis [46, 47].

Besides the number of receptors, mutations of the wild-type receptor might play a role in the difference in the influence of rhIGF-I on cytotoxicity of drugs in different cell lines. Different domains of the IGF-IR are required for proliferation and for protection against apoptosis [48]. In contrast, the findings of Geier et al. are not supportive of the idea that the difference in cell lines and IGF-IR might be the explanation for the difference in influence of rhIGF-I. They studied the influence of rhIGF-I on actinomycin D and doxorubicin both in the same human breast cancer cells. rhIGF-I was found to protect against actinomycin D cytotoxicity, but not against doxorubicin cytotoxicity [31, 32]. Other factors which might explain the difference in our findings and the results of other investigators are the apoptotic pathways involved, or the status of the different genes involved in apoptotic pathways, which could be different in the different cell lines. Under serum-containing conditions, rhIGF-I did not influence the antitumor effect of vincristine in Rh30 and Rh1 cells.

Our specific interest was to determine whether rhIGF-I stimulates rhabdomyosarcoma growth and protects rhabdomyosarcoma cells against cytotoxicity of vincristine, because rhIGF-I might be a protective agent against vincristine-induced neuropathy. Thus, potentially, rhIGF-I could be used to abrogate vincristine-induced neuropathy, allowing for increased dose intensity, or for more prolonged administration schedules than are currently tolerated. Our results show that rhIGF-I promotes growth of two rhabdomyosarcoma cell lines, but does not protect cells from vincristine and other drug cytotoxicity as found under serum-free conditions. Clearly, serum-free culture conditions are artificial in the extreme, but it is probable that protective effects of rhIGF-I would be observed under these conditions. Serum-containing conditions with IGFBPs, and endogenous growth factors present in the medium more accurately mimic the physiological situation of systemic administration of exogenous rhIGF-I than serum-free conditions do. Under serum-containing conditions rhIGF-I did not affect tumor growth or the antitumor effect of cytotoxic drugs. Obviously, in any in vitro situation, the dynamics of the in vivo situation, for example allowing release of rhIGF-I from BPs, are lacking. Also the balance between inhibiting and enhancing BPs in tissue might be of influence in the in vivo situation. Thus, we speculate that the influence of exogenously administered rhIGF-I in vivo might be intermediate.

To test this speculation directly, we examined the influence of rhIGF-I on rhabdomyosarcoma growth and the antitumor activity of vincristine, in HxRh30- bearing mice. We could not find a significant influence of rhIGF-I on rhabdomyosarcoma growth. Because there was only spontaneous regression in the control group but not in the rhIGF-I treated group, this difference might develop under the influence of rhIGF-I. However, when we checked growth in the control group from the next experiment (same passage of HxRh30) (Fig. 6, non-bold growth curves) it appeared to be very similar to the growth both in controls and in the rhIGF-I-treated group of this experiment. Our results are consistent with the results of Dong et al., who studied the potential beneficial anabolic effects of rhIGF-I for cachectic cancer patients in tumor-bearing rats [49]. While a beneficial effect on body weight was found, no effect on tumor growth was seen after 7 days of continuous infusion.

We also could not find a significant influence of rhIGF-I on the antitumor effect of vincristine in vivo. The results of our study suggest that administration of rhIGF-I may not adversely affect rhabdomyosarcoma growth or antagonize the antitumor activity of vincristine in vivo. Of course, the numbers of mice in these experiments were too small to prove that there is no influence. However, these results encourage further studies of the effect of rhIGF-I in ameliorating vincristine-induced neuropathy.

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