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Preclinical report

No evidence of tumor growth stimulation in human tumors *in vitro* following treatment with recombinant human growth hormone

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In a recent study we demonstrated that recombinant human growth hormone (r-hGH; Saizen®) delayed tumor-induced cachexia in human tumor xenografts *in vivo*. Such a therapeutic effect could have a great impact in the supportive care of advanced cancer patients. Before large clinical studies are initiated possible growth stimulation should be excluded. This question was investigated *in vitro* in 20 human tumor models, which had been established in serial passage in nude mice. The effect of continuous exposure of r-hGH was investigated at dose levels ranging from 0.3 ng/ml up to 0.1 μg/ml in colorectal (*n*=2), gastric (*n*=1), non-small cell lung (*n*=4), small cell lung (*n*=1), mammary (*n*=3), ovarian (*n*=2), prostate (*n*=2) and renal cancers (*n*=2), and melanoma (*n*=3) using a modified Hamburger and Salmon clonogenic assay. The results show that there was neither tumor growth inhibition nor any evidence for tumor growth stimulation in any of the tumors studies. Therefore this preclinical study in 20 human tumor models indicated no direct risk for tumor growth enhancement. [© 2000 Lippincott Williams & Wilkins.]

Key words: Clonogenic assay, human tumor xenografts, *in vitro*, recombinant human growth hormone.

Introduction

Cachexia is commonly observed in patients with advanced cancers. The survival time of patients experiencing cachexia is shortened and the response to chemotherapy is reduced significantly.[1] Cachexia is the result of alterations in carbohydrate, lipid and protein metabolism. It is a multifactorial process of which the precise mechanism of action is not yet well understood. Several cytokines, including tumor necrosis factor-α, interleukin (IL)-1, IL-6 and interferon (IFN)-γ, and other factors such as catecholamines and a sulfated glycoprotein, increasing prostaglandin E_2 levels, are probably involved in the etiology of cachexia.[2,3] Reduction of cachexia by increasing body weight has been achieved by agents stimulating food intake or inhibiting the catabolic effects of certain tumors on the host.[4]

In a recent study we demonstrated that recombinant human growth hormone (r-hGH; Saizen®) delayed tumor-induced cachexia in nude mice bearing human tumor xenografts.[5] In clinical studies r-hGH has been shown to stimulate muscle protein synthesis, improve nitrogen balance and promote wound healing in a variery of catabolic states.[6–8] In addition, r-hGH acts as an immunoregulatory cytokine.[9,10] These properties suggest that r-hGH might be beneficial in preventing cancer-induced cachexia. Therapeutic use of r-hGH in the supportive care of advanced cancer patients is controversial due to the assumption that it could stimulate tumor growth. For instance, r-hGH inhibited tumor growth and metastasis is some experimental rat tumor models,[11,12] whereas in a rat sarcoma tumor growth inhibition following hypophysectomy was partially restored by growth hormone[13] and in another rat tumor model an increase in the proportion of tumor aneuploid tumor cells was found.[14] Clinical data showed that the occurrence of second malignancies or recurrence rate did not increase in children with brain tumors or lymphoblastic leukemia receiving long-term r-hGH substitution.[15] In order to study the direct effect of r-hGH on the growth of a wide spectrum of solid tumors, this question was investigated in 20 human tumor xenografts *in vitro*.

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Table 1. *In vitro* effect of recombinant human growth hormone in human tumor xenografts

Tumor designation and passage no.	Tumor histology	Test/control (%) at drug concentration (ng/ml)					
		0.3	1	3	10	30	100
Colon							
CXF 609/8	colorectal		113−		101−		109−
CXF 1103/8	colorectal		114−		96−		104−
Gastric							
GXF 251/13	gastric		102−		104−		110−
Lung							
LXFA 289/10	adeno		87−		108−		64−
LXFA 526/7	adeno		124−		96−		121−
LXFE 211/19	epidermoid	112−	116−	84−	88−	85−	94−
LXFL 529/6	large cell		99−		108−		94−
LXFS 650/11	small cell		72−		105−		72−
Mammary							
MAXF MCF7/11	adeno ductal		104−		93−		99−
MAXF 449/14	adeno poorly differentiated	93−	98−	90−	93−	73−	76−
MAXF 1322/7	adeno poorly differentiated		83−		67−		78−
Melanoma							
MEXF 514/9	melanotic		83−		82−		75−
MEXF 535/13	amelanotic	77−	69−	69−	55−	79−	84−
MEXF 1341/16	amelanotic	108−	92−	87−	106−	111−	105−
Ovarian							
OVXF 899/16	adeno moderately differentiated	101−	98−	96−	91−	90−	88−
OVXF 1023/9	adeno poorly differentiated		80−		90−		59−
Prostate							
PRXF PC3M/7	adeno	90−	99−	95−	88−	84−	77−
PRXF DU145/6	adeno		114−		107−		99−
Renal							
RXF 423/15	adeno clear cells		70−		98−		36+
RXF 1332/14	adeno clear cells		97−		99−		93−
Stimulation T/C > 130%		0/6	0/20	0/6	0/20	0/6	0/20
Inhibition T/C < 50%		0/6	0/20	0/6	0/20	0/6	1/20

Key: −, T/C ⩾ 50% of control; +, T/C < 50% of control.

Material and methods

Human tumors

Twenty human solid tumors established in serial passages in athymic nude mice were used as tumor material in the study (Table 1). The xenografts comprised the following tumor types: colorectal, gastric, small cell and non-small cell lung, mammary, ovarian, prostate and renal cancer, and melanoma. The histology of these mouse-grown tumors reflects the histology of the original tumors. The human origin of the tumors was confirmed by isoenzymatic and immunohistochemical methods.[16] A high correlation of drug response in nude mice versus the response in the patients has been demonstrated.[17–19] The xenografts have not been characterized for the presence of growth hormone or IGF-I receptors.

Clonogenic assay

The *in vitro* activity of r-hGH was studied using a modification[20] of the two-layer soft agar culture system introduced by Hamburger and Salmon.[21] The target cell population in this assay system is the stem cell, responsible for unlimited growth of a tumor

Preparation of a single-cell suspension

Solid human tumor xenografts were mechanically disaggregated, and subsequently incubated with an enzyme cocktail consisting of collagenase 40 U/ml, DNAse 375 U/ml and hyaluronidase 96 U/ml in RPMI 1640 at 37°C for 30 min. The cells were washed twice, and passed through sieves of 200 and 50 μm mesh size to remove any remaining clumps. The percentage of

viable cells was determined in a hemocytometer using Trypan blue exclusion.

Culture methods

The bottom layer consisted of 0.2 ml Iscove's medium with 20% fetal calf serum and 0.7% agar which were plated in 24-well plates. Between 20 000 and 200 000 viable cells (the density was adjusted to the known growth rate) were added in a volume of 0.2 ml of the same culture medium and 0.4% agar over the base layer. r-hGH was given in 0.2 ml medium (drug overlay). The control group received the vehicle only.

Cultures were incubated at 37°C and 7% CO_2 in a humidified atmosphere for 6–16 days and monitored closely for colony growth using an inverted microscope. Within this period *in vitro* tumor growth led to the formation of colonies (diameter greater than 50 μm). At the time of maximum colony formation counts were performed with an automatic image analysis system (Bausch and Lomb Omnicon FAS IV). Twenty-four hours prior to evaluation, viable colonies were stained with tetrazolium chloride.[22]

Drug treatment in the clonogenic assay

r-hGH was applied 1 day after cell seeding in the culture medium by continuous exposure until the end of the experiment. The incubation time ranged from 5 to 15 days, depending on the proliferation rate of each tumor. Three to six dose levels were studied in triplicate. In the first experiments tumors were treated at six dose levels of r-hGH ranging from 0.3 to 100 ng/ml. In later experiments three dose levels were used (1, 10 and 100 ng/ml). In each assay six vehicle-treated cultures were plated for determination of control growth. 5-Fluorouracil at a toxic dose of 1000 μg/ml (continuous exposure) was used as positive reference compound.

Evaluation

Drug effects were expressed in terms of the percentage of survival, obtained by comparison of the mean number of colonies in the treated plates with the mean colony count of the untreated controls according to the formula: $T/C\% = (\text{colony count}_{\text{treated group}}/\text{colony count}_{\text{control group}}) \times 100\%$.

Tumor growth stimulation was considered relevant if colony formation was increased to more than 30% of the control group value (T/C $>130\%$). For tumor growth inhibition a colony survival of less than 50% of the control group value (T/C $<50\%$) was required for a weak effect and T/C $<30\%$ for a clinically relevant effect.

An assay was considered fully evaluable if the following quality control criteria were fulfilled:[17,20] mean number of colonies in the control group dishes $\geqslant 20$ with a colony diameter of $\geqslant 50$ μm; initial plate counts on day 0 or 2 were $\leqslant 30\%$ of the final control group count; coefficient of variation in the control group $\leqslant 50\%$ and the positive reference compound 5-fluorouracil must effect a colony survival of $\leqslant 30\%$ of the control.

r-hGH

Serono (Geneva, Switzerland) supplied r-hGH (Saizen®). The clinical formulation was used in the study. r-hGH was further diluted with RPMI 1640. The controls received the vehicle only.

Results

The effects of r-hGH on tumor growth were investigated *in vitro* in 20 human tumor xenografts, comprising eight different tumor types. Growth hormone was applied in dose levels ranging from 0.3 ng/ml to 0.1 μg/ml under continuous drug exposure. The results are summarized in Table 1. The mean colony number ranged from 32 to 180 per plate. Applying the criteria of tumor growth (T/C $>130\%$) and tumor growth inhibition (T/C $<50\%$), r-hGH did not show any evidence for tumor stimulation nor inhibition *in vitro*. Only the renal cancer RXF 423 showed a slight reduction of the colony number to 36% of the control at the highest dose of 0.1 μg/ml.

Discussion

Growth hormone is a potent anabolic agent that can reverse many nutritional and metabolic abnormalities associated with severe catabolic states. Clinical studies show that growth hormone improves nitrogen balance in burns and postoperative patients, increases protein levels, promotes lipid mobilization and protein synthesis, and accelerates wound healing.[8] Growth hormone has become available for clinical and basic investigations through recombinant DNA technology. It is potentially useful as an adjunct to nutritional support of the cachectic patient.

The role of growth hormone in cancer cachexia and its potential effect on primary and metastatic tumor growth is still controversial. Cancer cachexia is a complex syndrome, which is associated with extensive abnormalities in protein, lipid, carbohydrate and energy metabolism. Growth hormone might act as a

potential tumor growth factor by providing nutrient substrates or directly stimulating growth hormone receptors on tumor cells. An association between increased tumor growth and elevated growth hormone levels and somatomedin activity in patients with osteosarcoma was reported,[23] and also stimulatory effects of growth hormone on rat bladder carcinogenesis but no increase in tumor volume.[24] Growth hormone played some role in the promotion, but not the acceleration, of myelodysplastic syndrome in a child treated with r-hGH after treatment for neuroblastoma.[25] In contrast, other preclinical and clinical reports show that r-hGH administration does not lead to tumor proliferation, increased risk on secondary tumors[26–28] or carcinogenesis.[29] In addition, the presence of a functional receptor for human growth hormone on acute leukemias is not sufficient to induce proliferation.[30]

In the present study the effect of r-hGH in the dose range of 0.3–100 ng/ml was investigated on 20 human tumor xenografts of different histology *in vitro* using a clonogenic assay. The results showed that there was neither tumor growth stimulation nor inhibition. This means that r-hGH had no direct effect on the growth of human tumor cells *in vitro*. Serum concentrations of growth hormone in human controls and patients with chronic renal failure were 1.3 ± 1.0 and 1.5 ± 1.4 ng/ml, respectively,[31] and in advanced lung cancer patients with a weight loss of less than 10% 0.7 ng/ml and in patients with a weight loss of greater than 10% 1.6 ng/ml.[32] In addition, pharmacokinetic studies showed that a serum level of 3–4 ng/ml was reached in growth hormone deficient adults receiving 3 IU/m^2/day r-hGH by s.c. injection,[33] 8–9 ng/ml when 4 IU/m^2 was given[34] and infusion of 310 μg/m^2. r-hGH in controls and in patients with chronic renal failure resulted in a steady-state plasma growth hormone concentration of 45–50 ng/ml.[31] These data show that the dose levels investigated in our study correspond to physiologic serum concentrations, and concentrations obtained in a clinical setting.

Earlier we showed that r-hGH had also no effect on tumor growth stimulation and tumor growth inhibition in nude mice bearing human tumor xenografts.[5] These xenografts, two non-small cell lung cancers (LXFE 397 and LXFE 211) and a renal cancer (RXF 423), induced cachexia in nude mice. r-hGH administration showed a dose-dependent delay of the body weight loss and in one of the models, LXFE 397, an increase in survival time of the mice. Two of the tumors, RXF 423 and LXFE 211, were also used in the present study *in vitro*. These *in vitro* and *in vivo* results show that r-hGH had no direct or indirect effects on tumor growth stimulation. This data are in agreement with the *in vitro* and *in vivo* results of r-hGH on the rat pituitary tumor 7315b[35] and MIA PaCa-2 pancreatic carcinoma tumor.[36] In both studies r-hGH had no effect on tumor growth *in vivo* and *in vitro*, although r-hGH increased the serum IGF-1 level, which stimulated *in vitro* tumor growth. Other *in vivo* studies using mouse and rat tumor models also do not show any evidence that r-hGH administration causes tumor growth.[37–39].

In a clinical study,[40] in which r-hGH was given to 12 patients with advanced breast cancer 2 days prior to chemotherapy, the median serum IGF-1 level increased from 63.5 to 112 ng/ml, while the median tumor IGF-1 level decreased from 4.64 to 1.8 ng/g. The mean tumor labeling index increased however from 1.3 to 2.6%. If this kinetic recruitment is associated with a tumor volume increase *in vivo* could not been assessed since all patients were treated with chemotherapy. In this respect a parallel might be drawn with the results reported by Ng *et al.*[14] in which a rat tumor model showed also an increase in labeling index but which was not accompanied by tumor volume increase. Thus, the majority of the available data indicate that r-hGH administration does not stimulate tumor growth.

Conclusions

r-hGH showed neither direct tumor stimulation nor any evidence of tumor inhibition in a total of 20 human tumor xenografts tested *in vitro*. This was observed in eight different tumor types including cancers of the gastrointestinal system, lung, breast and skin. This preclinical study does not indicate a direct risk for tumor enhancement in cancer patients.

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