Stimulation of Tumor Growth by Recombinant Human Insulin-like Growth Factor-I (IGF-I) Is Dependent on the Dose and the Level of IGF-I Receptor Expression

Andrew A. Butler, Vicky A. Blakesley, Maria Tsokos, Vasilikki Pouliki, Teresa L. Wood, and Derek LeRoith

ABSTRACT

The insulin-like growth factors (IGF-I and II) regulate metabolism, mitogenesis, differentiation, and apoptosis. The therapeutic uses of IGF-I have been discussed extensively; however, excessive activity of the IGF ligands and IGF-I receptor has been suggested as a factor in tumorigenesis. The inhibition of apoptosis by IGF-I is believed to be particularly important for the stimulation of tumor growth. This study examined whether systemic recombinant human IGF-I (rhIGF-I) therapy affects the growth of fibrosarcomas derived from fibroblasts expressing the IGF-I receptor at high or naturally occurring densities (1.9 x 10^4 compared with 1.6 x 10^4 IGF-I receptors/cell) in athymic nude mice. Treatment with 4 or 10 mg/kg rhIGF-I resulted in a marked reduction in the tumor latency and stimulated the growth of fibrosarcomas that overexpressed the IGF-I receptor. The latency and growth of fibrosarcomas expressing parental levels of the IGF-I receptor were not affected by rhIGF-I therapy. Analysis of mitosis by histone H3 mRNA in situ hybridization and of apoptosis by terminal deoxynucleotidyl transferase-mediated nick end labeling assay indicated that rhIGF-I-stimulated tumor growth was associated with a marked increase in mitogenesis; however, there was no evidence for any significant effect on apoptosis. These data imply that: (a) systemic rhIGF-I can stimulate the growth of tumors directly by stimulating mitosis; and (b) a reasonable level of IGF-I receptor expression is required for stimulation of tumor growth by systemic rhIGF-I.

INTRODUCTION

The mitogenic actions of IGF-I and IGF-II are believed to be mediated primarily by the activation of the type I or IGF-I receptor (1). The IGF-I receptor is closely related to the insulin receptor, consisting of two aß heterodimers, and shares many of the signal transduction pathways associated with insulin receptor activation (2, 3). The IGF-I receptor is essential for fetal development, with extreme growth retardation and postnatal lethality associated with the deletion of the IGF-I receptor (4). The IGF-I receptor has also been implicated in the regulation of chemotaxis, programmed cell death (apoptosis), and differentiation (5).

The anti-apoptotic and mitogenic actions of the IGF-I receptor are presently believed to be an important factor in tumorigenesis (2, 6, 7). The regulation of the synthesis of IGF ligands and IGF receptors may be a contributing factor to the pro-apoptotic and antiproliferative functions of tumor suppressors. For example, the Wilms' tumor suppressor WT1 inhibits the transcriptional activity of both the IGF-I receptor promoter (8, 9) and the IGF-II promoter (10). Suppression of IGF-I receptor synthesis by WT1 is associated with a reduction in IGF-I mediated biological effects in vitro (8). The tumor suppressor p53 similarly inhibits transcription of IGF-I receptor mRNA (11) and IGF-II mRNA (12). Viral oncoproteins may also act through the IGF system, with the hepatitis viral oncogene HBx increasing the IGF-I receptor promoter activity and protein levels (13).

Further evidence for the essential role of the IGF-I receptor in tumorigenesis has come from studies in which the use of antisense- or antibody-based therapies to impede the synthesis or activation of the IGF-I receptor results in the inhibition of tumor growth and, in some cases, complete regression (14–19). Overexpression of the IGF-I receptor has been reported to confer a transformed phenotype, correlating with: (a) an increase in mitotic activity in vitro; (b) the acquisition of the ability for growth in serum-free conditions supplemented solely with IGF-I; and (c) an increased ability for anchorage-independent growth (20). Overexpression of the IGF-I receptor has been shown to increase the tumorigenic activity of several cell lines in nude mice (21, 22). The IGFs in the circulation can also affect tumor growth. Chronic elevation of IGF-II, which is associated with the expression of a liver-specific transgene, leads to an increase in the incidence of a variety of tumors (23). Hypophysectomy or dietary restriction, both of which reduce the amount of IGF-I in the circulation, can inhibit tumorigenesis (24, 25). Treatment of nutritionally restricted rats with growth hormone or IGF-I increases the proliferation of a mononuclear leukemia cell line in diffusion chambers implanted in dietary-restricted rats (26).

To investigate whether the systemic administration of IGF-I influences tumor growth directly, the effect of rhIGF-I on the growth of tumors arising from murine fibroblast (NIH3T3) lines expressing different numbers of the IGF-I receptor was examined in athymic nude mice. In three separate experiments, rhIGF-I therapy over a 12-week period stimulated the development and growth of fibrosarcomas overexpressing the IGF-I receptor but had no effect on a line expressing parental levels of the receptor. Thus, the sensitivity of the tumor cells to circulating IGF-I exhibits a dependency on the IGF-I receptor number.

MATERIALS AND METHODS

MATERIALS. Cell culture media and reagents were purchased from Biofluids, Inc (Rockville, MD), Advanced Biotechnologies (Columbia, MD), and Life Technologies, Inc. (Grand Island, NY). Polyclonal antibodies to the IGF-I receptor (3B7) were purchased from Santa Cruz Biotechnologies, Inc (Santa Cruz, CA). Cell culture reagents were purchased from Biofluids, Inc. and Life Technologies, Inc.

CELL LINES. The fibroblast lines used have been described previously (21). Frozen stocks of each line were prepared within two passages after Scatchard analysis, with fresh stocks used for each study. Cells were cultured in DMEM supplemented with 10% fetal bovine serum, 2 mm glutamine, 100 units/ml penicillin, and 100 mcg/ml streptomycin.

ANIMAL STUDIES. Male NIH Athymic nude mice were purchased from the Research Resource Units of the NIH, and were maintained in accordance with the NIH Guide for the Care and Use of Laboratory Animals. To examine the effects of chronic treatment with rhIGF-I on tumor growth, 15–25 mice were inoculated with 5 x 10^6 cells suspended in 100–130 μl of PBS (pH 7.4), injected s.c. into the left flank. The vehicle [100 mM NaCl, 50 mM sodium acetate, 9 mg/ml benzyl alcohol, and 2 mg/ml Tween 20 (pH 5.4)] or rhIGF-I p53 similarly inhibits transcription of IGF-I receptor mRNA (11) and IGF-II mRNA (12). Viral oncoproteins may also act through the IGF system, with the hepatitis viral oncogene HBx increasing the IGF-I receptor promoter activity and protein levels (13).
Systemic IGF-I Treatment Reduces the Latency Period and Stimulates Postpalpable Growth of Fibrosarcomas Overexpressing the IGF-I Receptor in Vivo. The determination of the IGF-I receptor number by Scatchard analysis showed that the NWTc43 and pNeo1 lines expressed in the order of 1.9 X 10^3 and 1.6 X 10^4 IGF-I receptors per cell, respectively (data not shown). The specificity of the NWTc43-derived fibrosarcomas for overexpression of the human IGF-I receptor was confirmed by immunostaining (data not shown). The time taken for the tumors to attain an arbitrary size (10% of maximum tumor volume of 2700 mm^3) was used to examine the effects of rhIGF-I therapy on the time taken for the appearance of tumors except for experiment 3, in which most fibrosarcomas derived from pNeo1 cells did not achieve this volume and in which a volume of 100 mm^3 was used. The “maximum” tumor volume was established by calculating the mean volume of tumors in animals killed when the tumor burden was considered unethical, which for this study was a length of 20 mm.

Palpable tumors were observed 70 to 80 days after the s.c. injection of either NWTc43 or pNeo1 cells into the posterior flank of athymic nude mice (Table 1). The effects of rhIGF-I therapy on the growth of NWTc43- and pNeo1-derived fibrosarcomas was examined in three separate experiments, with each experiment beginning with five animals per treatment group. The latency period of NWTc43-derived fibrosarcomas was significantly reduced in mice treated with 4 or 10 mg/kg rhIGF-I in experiments 1 and 2 (Table 1). The growth of fibrosarcomas after the appearance of a palpable tumor was also stimulated in mice treated with 4 or 10 mg/kg rhIGF-I (Fig. 1). In experiment 3, only the 1 mg/kg dose stimulated postpalpable tumor growth (Fig. 1). Analysis of the combined data indicated that the effects of the 4 and 10 mg/kg doses on latency and postpalpable growth were highly significant (P < 0.01). Treatment with rhIGF-I did not significantly stimulate the development of fibrosarcomas derived from pNeo1 cells (Table 1; Fig. 1).

Changes in Tumor Morphology Associated with IGF-I Receptor Overexpression and IGF-I Treatment. Staining of paraffin sections with H&E revealed significant differences in the morphology of tumors derived from pNeo1 and NWTc43 cells (Fig. 2, A-D). The tumors derived from pNeo1 cells were well-differentiated, mature fibrosarcomas with collagen. pNeo1 fibrosarcomas contained a few well-formed vessels with evidence of invasion into the surrounding host tissue, notably adipose and muscle. Fibrosarcomas derived from NWTc43 cells exhibited atypia and pleiomorphism, were poorly differentiated, and lacked the extensive extracellular matrix observed in pNeo-derived fibrosarcomas.

Necrosis was observed in the center of large fibrosarcomas derived from NWTc43 cells but was rarely observed in fibrosar-
rhIGF-I, IGF-I RECEPTOR EXPRESSION, AND TUMOR GROWTH

Table 1 Time (days) taken for tumors to achieve a volume 10% of the maximum allowable tumor burden (2700 mm$^3$)

Athymic nude mice were inoculated with 1–5 × 10$^6$ tumor cells in the right posterior flank and the following day began a treatment regimen of a daily s.c. injection in the opposite flank with the vehicle or with rhIGF-I at the doses indicated. Tumor measurements were recorded biweekly and were used to calculate the tumor volume (length × width$^2$ × 0.52). The data were analyzed for statistical significance by one-way ANOVA followed by post hoc comparisons using the Newman-Keuls test.

<table>
<thead>
<tr>
<th>IGF-I dose (mg/kg/day)</th>
<th>0.0</th>
<th>0.25</th>
<th>1.0</th>
<th>4.0</th>
<th>ND</th>
</tr>
</thead>
<tbody>
<tr>
<td>Experiment 1</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>pNeo1</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>78.4 ± 4.2</td>
<td>ND$^b$</td>
<td>75.6 ± 2.7</td>
<td>71.4 ± 4.7</td>
<td>ND</td>
</tr>
<tr>
<td>2</td>
<td>81.2 ± 4.1</td>
<td>ND</td>
<td>83.5 ± 1.0</td>
<td>83.4 ± 0.9</td>
<td>ND</td>
</tr>
<tr>
<td>3$^a$</td>
<td>80.4 ± 2.8</td>
<td>83.5 ± 2.3</td>
<td>83.9 ± 2.0</td>
<td>73.0 ± 7.0</td>
<td>80.9 ± 2.4</td>
</tr>
<tr>
<td>Mean</td>
<td>79.8 ± 2.8</td>
<td>83.5 ± 2.3</td>
<td>80.5 ± 2.2</td>
<td>77.4 ± 3.0</td>
<td>80.9 ± 2.4</td>
</tr>
<tr>
<td>% control</td>
<td>100</td>
<td>102</td>
<td>101</td>
<td>97</td>
<td>100</td>
</tr>
<tr>
<td>Experiment 2</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>pNeo1</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>67.4 ± 5.4</td>
<td>64.9 ± 7.6</td>
<td>63.9 ± 4.9</td>
<td>58.0 ± 5.5</td>
<td>46.3 ± 3.6</td>
</tr>
<tr>
<td>2</td>
<td>81.2 ± 1.5</td>
<td>79.0 ± 2.8</td>
<td>70.1 ± 7.7</td>
<td>49.1 ± 4.4$^d$</td>
<td>47.2 ± 4.0$^d$</td>
</tr>
<tr>
<td>3</td>
<td>75.7 ± 6.4</td>
<td>84.1 ± 2.1</td>
<td>66.0 ± 7.7</td>
<td>70.1 ± 6.2</td>
<td>71.5 ± 1.9</td>
</tr>
<tr>
<td>Mean</td>
<td>74.1 ± 3.0</td>
<td>76.0 ± 3.4</td>
<td>66.7 ± 3.7</td>
<td>59.1 ± 3.8$^e$</td>
<td>52.5 ± 3.6$^d$</td>
</tr>
<tr>
<td>% control</td>
<td>100</td>
<td>101</td>
<td>89</td>
<td>79</td>
<td>70</td>
</tr>
</tbody>
</table>

$^a$ In experiment 3 with cell line pNeo1, volume used was 100 mm$^3$.

$^b$ The data are mean ± SEM.

$^c$ ND, dose not included in this experiment.

$^d$ P < 0.01 compared with 0.0, 0.25, and 1.0 mg/kg.

$^e$ P < 0.01 compared with 0.0 and 0.25 mg/kg.

Overall, inhibition of apoptosis was apparently not a major factor in the stimulation of the growth of fibrosarcomas derived from NWTc43 cells by rhIGF-I treatment, inasmuch as only a few isolated cells exhibiting staining typical of apoptosis were observed in fibrosarcomas from mice treated with both the vehicle and 10 mg/kg rhIGF-I.

Fig. 2. Micrograph of H&E-stained sections showing the altered morphology of fibrosarcomas derived from pNeo1 cells (A and C) compared with those derived from NWTc43 cells (B and D). Fibrosarcomas derived from pNeo1 cells seemed less cellular (compare panels A and B; ×2.5) and contained more collagen (compare C and D; ×20) than NWTc43 cells. The cells in NWTc43-derived tumors also exhibit larger, more irregular nuclei. Well-formed vessels are apparent in both kinds of tumors (C and D).

A

B

C

D
Doses of rhIGF-I, the differences in histone H3 mRNA signaling was examined by apoptotic cells (C; X10). Analysis of DNA Synthesis by Histone H3 mRNA Expression. Histone H3 mRNA is specifically expressed in cells entering S phase and is recognized as a reliable marker of mitosis (29). An irregular pattern of histone H3 mRNA was observed in all tumors; close examination revealed H3 mRNA signaling from scattered individual cells. In NWTc43-derived fibrosarcomas from mice treated with high doses of rhIGF-I, the differences in histone H3 mRNA signaling was more extreme than in the appropriate controls, with H3 mRNA-positive cells occurring at high densities in some areas whereas other areas of healthy cells were devoid of H3 mRNA signaling. This effect was not observed in fibrosarcomas derived from pNeol cells, which exhibited the same pattern of H3 mRNA staining in both the vehicle and the 4-mg/kg rhIGF-I treatment groups.

**DISCUSSION**

This study has examined the effect of systemic IGF-I administration on tumorigenesis. The model of tumorigenesis that was used—fibrosarcoma development in the athymic nude mouse—has been used by this and other laboratories in previous studies (21, 22, 30, 31) to examine the tumorigenic properties of the IGF-I receptor. The results of the previous experiments indicate that overexpression of the IGF-I receptor results in the early appearance of large tumors relative to the appropriate control fibroblast lines. The present study indicates that the systemic administration of rhIGF-I can further stimulate the appearance and growth rate of tumors derived from cell lines over-expressing the IGF-I receptor. The present study complements the results of a recent study that demonstrated that IGF-I enhanced the tumor growth in p53-deficient mice (32).

Tumorigenesis is a multistage, complicated process that involves the combined effects of a multitude of genetic and environmental factors. In the model used for these experiments, previous studies suggest that the majority of suspended cells that are injected die within 24 h, followed by the establishment of a pretumorous mass embedded in a supportive stroma (33). The period of latency before the appearance of a palpable tumor involves a balance between the rates of mitosis and cell death caused by necrosis, apoptosis, or a combination of both (34). Because diffusion is insufficient to maintain tumor growth beyond a radius of 150–200 μm, the development of new blood supplies (angiogenesis) represents a critical factor for progression from the latent period to the exponential growth phase—a transition known as the "angiogenic switch" (34). Angiogenesis is believed to be regulated by the balance between angiogenic inhibitors (e.g., thrombospondin-1, the M₁₆,000 fragment of prolactin, IFN-α and -β, angiostatin, endostatin) and inducers (e.g., acidic fibroblast growth factor, basic fibroblast growth factor, and vascular endothelial growth factor). The mechanisms that increase the ratio of angiogenic promotores to inhibitors, thus leading to angiogenesis, are poorly understood. However, a combination of genetic and environmental factors are believed to be involved (34). Tumor growth after the angiogenic switch represent the combined effects of angiogenesis and growth factors, of which IGF-II seems to be particularly important (10, 12, 35–38).

A previous study indicated that the IGF-I receptor number is a factor in determining the duration of the latency period, possibly because of an inhibition of apoptosis in the postimplantation stage (39). By reducing the IGF-I receptor number by two-thirds in C6 glioblastoma cells using antisense oligonucleotides to the IGF-I receptor, the appearance of palpable tumors was retarded sixfold in the hind leg of nude mice. However, in the present study, it was observed that, despite a 10-fold difference in the IGF-I receptor number, there was only a minor difference in the time taken for palpable tumors to appear in three separate experiments. The C6 glioblastoma cell line used in the study of Resnicoff et al. (39) produced palpable tumors within 4 days, compared with 80 days in the present study. It is possible that the difference observed in the effect of the IGF-I receptor number on tumor latency reflects the very short period between inoculation and the appearance of a palpable tumor in the C6 line compared with the fibroblast line used in the present study. The data also suggest that, unlike the C6 line, the injected fibroblasts may enter
a relatively long latent period. The subsequent appearance and exponential growth of solid fibrosarcomas may therefore be dependent on environmental and/or genetic stimuli akin to that which has been proposed to occur in de novo tumorigenesis (34). Indeed, that Balb/3T3 cells cultured on plastic in vitro, or on glass beads transplanted s.c. in vivo, undergo spontaneous neoplastic transformation has been known for some time (40, 41).

Both IGF-I and IGF-II have been shown to influence factors affecting all stages of tumor development, stimulating the synthesis of angiogenic factors and ultimately angiogenesis (42–44), stimulating
mitosis (21), and inhibiting apoptosis (14, 39, 45, 46). IGF-I has been shown to be an important factor in the induction of ischemia-associated retinal neovascularization (43). In two of the three experiments performed in the present study, treatment with rhIGF-I at the highest doses reduced the latency period by 20 to 30%, suggesting that the progression from the latent to the rapidly growing tumor was stimulated. A possible explanation for this is that increasing systemic IGF-I levels increases the ratio of angiogenic inducers to inhibitors, leading to the stimulation of angiogenesis.

The IGFs can stimulate tumor growth by either stimulating mitosis or inhibiting apoptosis, or through a combination of both mechanisms (45). The amount of histone H3 mRNA, an indicator of cells in S phase, was higher in NWTc43 fibrosarcomas from mice treated with the high doses of rhIGF-I compared with vehicle-treated controls. The increased number of cells in S phase suggests that IGF-I was exerting a mitogenic effect on tumor cells. In pancreatic B-cell carcinomas induced by the expression of the SV40 T antigen, IGF-II primarily stimulates tumor growth by inhibiting apoptosis (45). However, in the present study, there were few apoptotic cells, which indicated that programmed cell death was not a major factor influencing tumor growth. Cell death was largely confined to areas of necrosis in the central regions of the larger tumors. Because no evidence of apoptosis on the periphery of the necrotic regions was evident, the necrotic death was probably due to toxicity associated with anaerobic condition prevalent in the centers of rapidly growing tumors.

Large rapidly growing tumors often contain areas of necrosis in which cell division has outstripped the capacity of the vasculature to supply nutrients and oxygen. This was also evident in the present study, especially in tumors in which growth had been stimulated by rhIGF-I therapy. In general, the H3 mRNA in situ data indicated that rhIGF-I therapy increased the amount of DNA synthesis in NWTc43-derived fibrosarcomas. However, the regions of high-density H3 mRNA signaling tended to be localized with areas of apparently healthy cells exhibiting no sign of cell division. It was also evident in rapidly growing NWTc43-derived fibrosarcomas from mice treated with rhIGF-I that the cells adjacent to necrotic regions, although seeming healthy, were not undergoing mitosis. Possible explanations for the regions of quiescent cells around areas of necrosis are the presence of toxins released from necrotic cells inhibiting cell division or a reduction in blood supply resulting in a diminished capacity for proliferation. The latter may also explain the patches of quiescent cells in areas that exhibit no signs of necrosis.

IGF-I has been proposed as a therapeutic agent in the amelioration of wasting syndromes associated with illness, injury, degenerative diseases, and aging (47). The data from the present study are encouraging in that IGF-I treatment affected significantly only the growth of tumors overexpressing the IGF-I receptor. However, the levels of IGF-I receptor have been shown to be increased in a number of tumor cell lines (31), and mutations in p53, a tumor suppressor that inhibits IGF-I receptor synthesis, is one of the most frequently observed occurrences in human cancers (48). Furthermore, work from the laboratory of R. Baserga (Jefferson University, Philadelphia, PA) has shown that the transforming capability of a cell is determined by the level of expression of the IGF-I receptor (20). In light of the data from the present study and from the growing body of evidence that implicates systemic IGF-I as a stimulator of tumor growth, the use of IGF-I as a therapeutic agent should be viewed with the appropriate precautions against potential effects on tumorigenesis.

ACKNOWLEDGMENTS

We thank Genentech, Inc., for providing the rhIGF-I for this study and Ricardo Dreyfus for the expert photomicrography.


Stimulation of Tumor Growth by Recombinant Human Insulin-like Growth Factor-I (IGF-I) Is Dependent on the Dose and the Level of IGF-I Receptor Expression

Andrew A. Butler, Vicky A. Blakesley, Maria Tsokos, et al.