Antitumor Effects of an Adenovirus Expressing Antisense Insulin-like Growth Factor I Receptor on Human Lung Cancer Cell Lines

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ABSTRACT

Insulin-like growth factors (IGFs) are often essential for the maintenance of the malignant phenotype, and in lung cancer the IGF-I receptor (IGF-Ir) is often expressed at high levels. Stable transfection of antisense plasmids expressing the first 300 bp of the IGF-Ir reduces the tumorigenicity of a variety of tumor cell lines and has been reported to induce systemic antitumor effects on established, non-gene-modified tumors in animal model systems. We have constructed an adenovirus expressing an antisense IGF-Ir (Ad-IGF-Ir/as) in an attempt to develop these observations into a clinical therapeutic approach. A single transduction by Ad-IGF-Ir/as (at a multiplicity of infection of 10:1) decreased the IGF-Ir number by about 50% in human lung cancer cell lines NCI H460 and SCC5, as measured by an 125I-labeled IGF-I competitive binding assay. After the transduction of these human lung cancer cell lines by Ad-IGF-Ir/as, the soft agar clonogenicity was reduced by 84%. The I.p. treatment of nude mice bearing established i.p. NCI H460 cells resulted in prolonged survival compared to that of nude mice treated with a reporter virus. These results suggest that Ad-IGF-Ir/as has a therapeutic effect on established human lung cancer xenografts and may represent an effective and practical cancer gene therapy strategy.

INTRODUCTION

The continuous growth of tumors depends on the altered regulation of the cell cycle. A variety of growth factors and their receptors mediate the signal transduction pathways that modulate the cell cycle (1). Among these growth factors are the IGFs, peptides with molecular weights of about 7,500,000 that can stimulate cellular proliferation and induce cellular differentiation (2). The IGF-Ir is a heterodimer derived from the cleavage of a precursor polypeptide (3), and recently the roles of IGF-I and the IGF-Ir in cancer cells have been investigated intensively (4). In certain systems, the IGF-Ir seems to be essential for malignant transformation because fetal fibroblasts with a disruption of the IGF-Ir gene cannot be transformed by the SV40 T antigen (5).

The IGF-Ir may also be important for the maintenance of the malignant state. Trojan et al. (6, 7) demonstrated that antisense IGF-I could suppress the tumorigenicity of a rat glioblastoma and could even cause shrinkage of established tumors. Stable transfection of an antisense plasmid expressing the first 300 bp of the IGF-Ir eliminated the tumorigenicity of a variety of tumor cell lines and has been reported to induce systemic effects in established, non-gene-modified tumors (8, 9). Reduction of IGF-Ir has been shown to induce apoptosis in tumors, but it produces only growth arrest in transformed cells (1). In addition, IGF-receptor knockout mice are viable (although physically smaller than normal), indicating that relatively normal development and tissue differentiation can occur in the absence of IGF-Ir (10). These findings suggest a potential basis for tumor selectivity in therapeutic applications.

The stable introduction of an antisense plasmid or the use of antisense oligonucleotides presents a variety of obstacles in potential clinical gene therapeutic strategies. Retroviruses are hampered by the difficulty of production and their inefficiency of transduction. Adenoviruses may represent excellent vectors for the introduction of IGF-Ir antisense constructs into tumors. Adenoviruses are highly infective for the actively dividing, slowly dividing, and nondividing tumor cells that often coexist in solid tumors, and they express high levels of the transduced gene (11, 12).

The IGFs and their receptors are very important in lung development and the growth of cells in the respiratory system (13). In many lung cancer cell lines, IGF-I and IGF-Ir can mediate autocrine proliferation (14, 15). Therefore, in this study, we constructed an adenovirus expressing antisense IGF-Ir (Ad-IGF-Ir/as) and sought to determine its effectiveness in the reduction of IGF-Ir expression and in the therapy of a human lung cancer xenograft model.

MATERIALS AND METHODS

Animals, Cells, and Materials. Four-week-old female nude mice were purchased from Harlan-Sprague-Dawley. All human lung cancer cell lines (human lung adenocarcinomas A549 and SCC5, human lung small cell carcinoma NCI H460, and human small cell lung carcinoma NCI H82) were obtained from Adi F. Gazdar (University of Texas Southwestern Medical Center, Dallas, TX). All cells were passaged in RPMI 1640 with 8% fetal bovine serum. IGF-I and 125I-labeled IGF-I were purchased from Amersham.

Construction of Ad-IGF-Ir/as. The cDNA of the IGF-Ir was made by reverse transcription-PCR of mRNA from NCI H82 (a human small cell lung cancer cell line). The forward primer used was AGCTG AATTC ATCCC TATCT C. The reverse primer used was AGCTG AATTC AAGAG G, and the reverse primer used was AGCTG AATTC GGGGA AGAGG TCTCC GAGGC T. The resulting cDNA fragment contained 321 bp of the IGF-Ir cDNA open reading frame, including the ATG initiation codon. Both ends of the cDNA were engineered to contain EcoRI restriction sites that were used to clone the fragment in an antisense direction into the polylinker site of the pAC shuttle plasmid (a gift of Dr. Robert Gerard, University of Texas Southwestern Medical Center). pAC contains the CMV immediate early enhancer and promoter and the SV40 polyadenylation. The entire insert was sequenced to verify structure, and the resulting pAC-CMV-IGF-Ir/as and vector plasmid plm17 (also a gift of Dr. Gerard) were cotransfected into 293 cells by standard calcium phosphate coprecipitation methods. Ad-IGF-Ir/as was generated by homologous recombination (16). The resulting adenovirus was confirmed by sequencing of PCR products and plaque purified three times. A recombinant adenovirus expressing the luciferase gene under the control of CMV promoter was used as a control virus (Ad-luc).

Cell Growth Assay. Tumor cells were transduced with 10 m.o.i. of Ad-IGF-Ir/as or Ad-luc for 1 h, and 3 x 10^4 cells were plated in 6-well microtiter plates. These were maintained in complete medium for 48 h and then were switched to serum-free medium + 0.1% BSA (fraction V) with or without 10 ng/ml of IGF-I. Cell numbers were counted by hemocytometer after 7 days.

Soft Agar Clonogenicity. Anchorage-independent growth was assessed by soft agar clonogenicity assays. Briefly, tumor cells were transduced with 20 m.o.i. of Ad-IGF-Ir/as and then were detached and plated in 0.2% agarose with 1% underlay (5 x 10^5 cells/plate). After 1 week, medium with 20 m.o.i.
The medium overlay was changed after 1 week. Colonies greater than 125 /j.m of Ad-IGF-Ir/as (Ad-luc as control adenovirus) was added over the soft agar.

Consisted of animals injected with PBS and Ad-luc on the same schedule. Mice bound activity. Radioactivity was counted by gamma counter. The IGF-Ir of lysis buffer (0.1% SDS, 0.01 N NaOH, and 0.1% Triton) to measure cell concentration of 50 pM I25I-labeled IGF-I for 2 h at 4°C. After incubation, cells were washed twice with RPMI + 0.1% BSA. Cells were then lysed in 0.5 ml RPMI + 0.1% BSA containing varying concentrations of IGF-I and a constant concentration of 50 pm 125I-labeled IGF-I for 2 h at 4°C. After incubation, cells were washed twice with RPMI + 0.1% BSA. Cells were then lysed in 0.5 ml of lysis buffer (0.1% SDS, 0.01 N NaOH, and 0.1% Triton) to measure cell bound activity. Radioactivity was counted by gamma counter. The IGF-Ir number was calculated by standard Scatchard analysis (18, 19).

Therapeutic Studies. To assess the effect of Ad-IGF-Ir/as on established tumors, 3.0 X 10^3 NCI H460 cells were injected i.p. into 4-week-old nude mice irradiated with 300 rad. Three days later, 50 m.o.i. (based on the injected tumor cell number) of Ad-IGF-Ir/as was injected i.p. for 5 consecutive days. Controls consisted of animals injected with PBS and Ad-luc on the same schedule. Mice were euthanized when they developed preterminal symptoms, and the time to this point was assessed as survival.

RESULTS

Ad-IGF-Ir/as Reduces the Number of IGF-Irs by 125I-labeled IGF-I Competitive Binding. We were able to confirm the high level expression of the IGF-Ir RNA in our human lung cancer cell lines by RT-PCR using IGF-Ir specific primers (data not shown). We then sought to determine whether transduction with the Ad-IGF-Ir/as virus could induce a measurable reduction in the number of surface receptors, as measured by a competitive binding assay. In the NCI H460 cells, the observed B_max for control, Ad-luc-, and Ad-IGF-Ir/as-transduced cells was 5.10 pm, 6.10 pm, and 3.10 pm, respectively (for 1.0 X 10^5 cells). The receptor numbers calculated by Scatchard analysis were 1.53 X 10^4/cell in the control group, 1.86 X 10^4/cell in Ad-luc-transduced group, and 9.3 X 10^3/cell in the Ad-IGF-Ir/as-transduced group. In SCC5 cells, B_max was 7.63 pm in the control group, 8.23 pm in Ad-luc-transduced group, and 3.75 pm in Ad-IGF-Ir/as-transduced group (for 7 X 10^4 cells; Fig. 1). The calculated receptor numbers were 3.27 X 10^4/cell in the control group, 3.52 X 10^4/cell in the Ad-luc-treated cells, and 1.60 X 10^4/cell in the Ad-IGF-Ir/as-treated cells. Therefore, a single transduction by Ad-IGF-Ir/as decreased the receptor number by over 50% compared to the control virus-transduced cells. It should be stressed that these cells were not selected in vitro for transduction but were tested after a single bulk transduction, more closely approximating potential clinical therapeutic situations than previous studies.

In Vitro Growth Characteristics of Ad-IGF-Ir/as-transduced Human Lung Cancer Cell Lines. The ability of Ad-IGF-Ir/as to block IGF-mediated growth stimulation was tested as described in “Materials and Methods.” In the Ad-luc-transduced group, the addition of IGF-I resulted in an enhancement ratio of 1.44 ± 0.18, and in the Ad-IGF-Ir/as-transduced group, only a 1.15 ± 0.08 ratio was observed (mean ± SE of triplicates). These data suggest that Ad-IGF-Ir/as-transduction can blunt the mitogenic effect of IGF-I.

Soft Agar Clonogenicity. Previous studies have suggested that the inhibition of the IGF-Ir has a more profound effect on tumorigenicity than on in vitro growth on plastic. Therefore, we sought to test the ability of human lung cancer cells to form colonies in soft agar (Table 1). Three weeks after plating, 448 and 410 colonies were found in control and Ad-luc-transduced groups, respectively. The Ad-IGF-Ir/as group showed only 68 colonies (an 84% decrease compared to the Ad-luc group). This finding suggests that Ad-IGF-Ir/as is capable of suppressing the tumorigenicity of NCI H460 cells.

Treatment with Ad-IGF-Ir/as Prolongs the Survival of Nude Mice with Established Human Lung Cancer Xenografts. To test the therapeutic potential of Ad-IGF-Ir/as, nude mice bearing established i.p. H460 cells were treated 3 days after tumor inoculation as described in “Materials and Methods.” Ad-IGF-Ir/as treatment resulted in a statistically significant prolongation of survival of these mice (median survival of 35 days in the control group, 33 days in Ad-luc-transduced group, and 40 days in Ad-IGF-Ir/as-treated group, as shown in Fig. 2). Thus, there was a 1-week increase in median survival for animals treated with the antisense virus. No survival benefit was observed with a once per week for 3 weeks dosing schedule (data not shown), suggesting that perhaps higher infective doses would achieve greater therapeutic effect.

DISCUSSION

IGF-I seems to be required for the optimal growth of most normal cells. Other growth factors vary widely in their necessity in different cell types (20). The IGF peptides, binding proteins, and receptors have very important roles in normal growth and development, including

<table>
<thead>
<tr>
<th>Group of NCI H460</th>
<th>Colony number</th>
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<tbody>
<tr>
<td>Control</td>
<td>448</td>
</tr>
<tr>
<td>Ad-luciferase-transduced</td>
<td>410</td>
</tr>
<tr>
<td>Ad-IGF-Ir/as-transduced</td>
<td>68</td>
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* Each number represents the mean of triplicate plates.
that of the lung. IGF-mediated growth responsiveness is also found in many cancer cells. Studies in the past few years have shown that IGFs are important mitogens in many malignancies and may enhance in vivo tumor formation, growth, and metastasis (21–23). SV40 T antigen, for example, increases the expression of IGF-I, enhancing the malignant transformation of BALB/c 3T3 cells. Conversely, BALB/c 3T3 cells lacking the IGF-Ir are resistant to malignant transformation by the SV40 T antigen (6).

The roles of IGF-I and the IGF-Ir have been underscored by a number of studies indicating the antitumor efficacy of transfection by antisense constructs. The expression of antisense message to IGF-Ir renders C6 rat glioblastoma cells nontumorigenic and can cause the regression of established tumors (7, 8). The transfection of antisense plasmids to IGF-Ir demonstrates a reduction in tumorigenicity of a variety of human cancer cell lines, which is at least sometimes accompanied by the induction of apoptosis (1, 9, 24). Surprisingly, antisense IGF-Ir can cause antitumor effects on preestablished, non–gene-modified tumors by unknown (but probably immune-mediated) mechanisms (24).

The importance of IGFs and the IGF-Ir in human cancers is also becoming increasingly clear, especially in breast cancer, rhabdomyosarcoma (25), and osteosarcoma (26). Most lung cancer cell lines express IGF-I and IGF-Ir that mediate autocrine proliferation (14, 22). In our study, strong expression of IGF-Ir mRNAs were found in all lung cancer cell lines screened by RT-PCR. Our data are the first to show that the malignant features of human lung cancer can also be effectively reduced in vitro and in vivo by even modest reductions in the level of expression of the IGF-Ir. A decrease in IGF-Ir expression through the introduction of antisense constructs may act via the induction of apoptosis in lung cancer cells as well.

Previous therapeutic strategies designed to interfere with IGF-I-mediated signal transduction have used antisense plasmid transfection or synthetic antisense oligonucleotides. We are the first to report the antitumor effects of IGF-Ir inhibition in human lung cancer cells, and the design and therapeutic effectiveness of an adenoaviral vector expressing antisense IGF-Ir, a potentially more clinically relevant approach. In our study, a single transduction of Ad-IGF-Ir/as into human lung cancer cell lines decreased the IGF-Ir number by 50%. This is comparable to the 70% decrease in serum-free medium (from 44% to 14%) is also of a similar magnitude to that observed in other systems (24). A 50% decrease in the receptor number after a single transduction without in vitro selection indicates that this delivery system is actually very efficient, and it is limited by the inherent problems with antisense approaches. The potential for repeated transduction in a clinical setting may improve effectiveness.

In spite of the relatively modest effects on the receptor number and proliferation on plastic, treatment with Ad-IGF-Ir/as induced a dramatic suppression of colony formation in soft agar, consistent with a selective effect on the transformed phenotype. We therefore tested the therapeutic potential of this virus in an i.p. tumor model of the human lung cancer cell line NCI-H460. In this i.p. model, we were able to observe a statistically significant increased survival. However, compared with the treatment effect of antisense plasmid stably transfected syngeneic rat glioblastoma cells on non–gene-modified tumor cells (24), this increase in survival is not striking. The systemic antitumor effects observed for antisense IGF-Ir plasmids in a syngeneic animal model system are probably the result of an immunity induction in addition to the reduction of IGF-Ir, which would not play a role in this human xenograft model. In addition, if more effective inhibition of receptor expression could be achieved (perhaps by the use of dominant negative constructs), the magnitude of the effect may be improved.

Two important findings were demonstrated in our study: (a) we demonstrated the in vitro and in vivo antitumor efficacy of antisense IGF-Ir in human lung cancer cells, which broadens the potential clinical indications of antisense IGF-Ir therapy in human cancers; and (b) we demonstrated that the delivery of antisense IGF-Ir via recombinant adenoaviruses was effective both in vitro and in vivo. In contrast to antisense plasmid transfection, adenoavirus vectors are a very efficient and practical method to deliver therapeutic genes to cancer cells. The fundamentally nontumor-selective nature of adenoaviruses transduction is not a concern for this approach because in contrast to its dramatic effects on tumor cells, antisense IGF-Ir seems to only marginally reduce the growth rate of normal cells, and the expression of adenoviruses is transient, lasting only 1–2 weeks. Thus, Ad-IGF-Ir/as extends the preclinical data on the inhibition of IGF-Ir to a potentially practical clinical therapy.

REFERENCES

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