An adenoviral vector expressing functional heterogeneous proteins herpes simplex viral thymidine kinase and human interleukin-2 has enhanced in vivo antitumor activity against medullary thyroid carcinoma

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Abstract

To explore a more efficient multi-gene antitumor treatment, we developed an adenoviral vector expressing both herpes simplex virus thymidine kinase (HSVtk) and human interleukin-2 (hIL-2) (AdCMVTKhIL2). Production of hIL-2 is expected to augment antitumor T cell and natural killer cell activity. Two separate cassettes expressing HSVtk and hIL-2, each under the control of the human cytomegalovirus (CMV) immediate early gene promoter, were inserted into the early 1 region of adenovirus type 5. This vector showed similar direct cytotoxicity towards infected rat medullary thyroid carcinoma (rMTC) cells as did the single gene vector, AdCMVtk. rMTC cells infected with the virus in vitro showed high sensitivity to ganciclovir.

After infection with AdCMVTKhIL2 at 100 m.o.i. for 1 h, more than 20,000 U hIL-2 were produced during 24 h by 1×10^6 rMTC cells on day 2 and day 3. hIL-2 was also detected in the supernatants of primary cultures from tumors treated in vivo by the AdCMVTKhIL2 vector.

Infected cells lost their tumorigenicity when transplanted subcutaneously into syngeneic rats, whereas all control animals developed tumors.

More than 63% of tumors (19 out of 30 treated tumors) were destroyed when AdCMVTKhIL2 was injected intratumorally, compared with 38% when tumors were treated with AdCMVIL2, and 12% when treated with AdCMVtk, indicating an antitumor effect superior to that of each single vector given alone at the same dosage.

These results indicate that the AdCMVTKhIL2 vector efficiently produces both HSVtk and hIL-2, and provides an enhanced antitumor activity.

Introduction

One strategy in cancer therapy is to introduce into tumor cells suicide genes that can kill the cells after administration of the appropriate prodrug. Herpes simplex virus thymidine kinase (HSVtk) is one of the best studied, and most widely used, suicide genes. HSVtk, a non-mammalian enzyme, can phosphorylate ganciclovir (GCV) into GCV-monophosphate (GCV-MP), whereas normal mammalian cellular thymidine kinase (TK) does not. GCV-MP is then further phosphorylated by cellular TK to toxic GCV-triphosphate (GCV-TP). GCV-TP is a potent inhibitor of viral DNA polymerase, and selectively inhibits mammalian δ-polymerase (Smee et al. 1985, St Clair et al. 1987, Ilsley et al. 1995). The suicide-gene-containing cells are killed by the gene/prodrug system. In addition, cells neighboring the suicide-gene-containing cells are also killed because of the bystander effect (Freeman et al. 1993, Yang et al. 1998).

Exciting results were obtained using the HSVtk/GCV system in animal studies (Hwang et al. 1995, Kianmanesh et al. 1998).
revealed an inability of this system to inhibit tumor growth (Marcel & Grausz 1997). However, some studies have
reasoned that certain cells lack the bystander effect (Touraine et al. 1998, Zhang & DeGroot 2000a). One
second reason is that not all tumors are appropriate for suicide-gene therapy, and limit the
application of such cancer therapy in humans at this time.

Another strategy for cancer treatment is to introduce genes that alter the local immunological microenvironment and
activate immune effector cells. A variety of molecules have been used for this purpose, including co-stimulator
histocompatibility complex (MHC) molecules (Hoglund et al. 1993, Lee et al. 1995), cytokine genes (Tepper et al.
colony-stimulating factor and granulocyte-monocyte colony-stimulating factor (Vieweg et al. 1994, Colombo &
Rodolfo 1995, Xing et al. 1996, Lee et al. 1997, Yano et al. 1997). Among these molecules, interleukin-2 (IL-2) has
been extensively studied and constantly showed antitumor activity.

IL-2, originally called T cell growth factor, is produced by CD4+ T cells and to a lesser extent by CD8+ T cells
( Abbas et al. 1994). It stimulates proliferation of cytotoxic T cells (Moller 1980, Kasaian & Biron 1989), and helper T
cells (Mosmann & Coffman 1987), causes activation of natural killer cells, and enhances their cytolytic function as
lymphokine-activated killer cells (Grimm et al. 1982, Trinchieri 1989). IL-2 also stimulates synthesis of other
T-cell-derived cytokines and lymphotoxin ( Abbas et al. 1994). Although systemic administration of IL-2 gave
moderate benefits in the treatment of certain tumors in animal models and human trials (Rosenberg 1988), therapeutically
effective concentrations of IL-2 are always accompanied by undesirable side effects (Siegel & Puri 1991, Lentsch et al.
1999). Transfection of IL-2 cDNA into tumor cells greatly reduced toxic effects, because sufficient amounts of IL-2
are released at the tumor site but without leading to high systemic concentrations (Fearon et al. 1990, Gansbacher et
al. 1990, Sarna et al. 1990). In these studies, IL-2-secreting tumor cells lost their tumorigenicity, and induced an efficient
immune response after implantation into syngeneic animals, causing them to reject a subsequent challenge with parental
While efficacious in producing IL-2 secreting cells, this method did not demonstrate efficiency against established
tumors, and routine clinical application has been limited by the inability to culture and transfet many types of common
tumors.

Development and application of viral vectors including adenovirus have made cancer gene therapy feasible. Adenoviruses
infect a wide range of host cells, can be produced at very high titers, can express transgenes without integration into the DNA of infected cells, and are therefore suitable for direct in vivo gene transfer. Numerous reports show the effectiveness of adenoviral vectors in transducing antitumor genes for cancer gene therapy (Haddada et al. 1993, Cordier et al. 1995, Bramson et al. 1996, Huang et al. 1996, Gilligan et al. 1998).

Various researchers have used two or more vectors to treat tumors (Chen et al. 1996, O’Malley et al. 1996, 1997,
Coll et al. 1997, Uckert et al. 1998), including the combination of a suicide gene and a cytokine gene (Coll et
al. 1997, O’Malley et al. 1997). Some of these studies showed a benefit from the combined therapy, but with some
limitations. For example, to keep the total amount of the virus in the treatment to a practical level, a much smaller
amount of each viral vector must be used, and this limits the effectiveness of the therapy. More recently, vectors
expressing two heterogeneous proteins have been constructed and initial animal studies indicated a beneficial effect of this
approach (Putzer et al. 1997, Emtage et al. 1999, Palu et al. 1999). In this study, we describe the construction of a new
adenoviral vector expressing both HSVtk and hIL-2 genes, analyze the functional expression of these two genes in rat
medullary thyroid carcinoma (rMTC) cells, and evaluate in vivo antitumor activity in an rMTC animal model.

Materials and methods

Plasmid construction and adenovirus rescue

Plasmids pCA13, pCA14 and pAE1sp1B, which contain the left end of the adenovirus type 5 (Ad5) genome (0–16.1 mu),
were obtained from Microbox Biosystem Inc. (Ontario, Canada). pJM17, which contains the entire Ad5 sequence
Figure 1 Structure of (A) AdCMVtk and (B) AdCMVTKhIL2. The expression cassette for HSVtk or HSVtkhIL2 replaces E1 of the Ad5 genome. Both inserted genes are under control of the human CMV IE promoter and are terminated by the polyadenylation signal (PA) of simian virus 40. The Ad vector was obtained by homologous recombination via co-transfection of Ad5 integrated in its genome (Graham & van der Eb 1973, 1977), was purchased from Microbox Biosystems Inc., and maintained in complete MEM supplemented with 10% fetal bovine serum, 100 U/ml penicillin and 100 µg/ml streptomycin. 293 cells, a transformed human cell line that has the E1 region of the Ad5 integrated in its genome (Graham & van der Eb 1973, Graham et al. 1977), were purchased from Microbox Biosystems Inc., and maintained in complete MEM (containing 10% fetal bovine serum, 100 U/ml penicillin and 100 µg/ml streptomycin). CTLL-2 cells were kindly provided by Dr Edward Cohen (University of Illinois at Chicago, IL, USA) and maintained in complete RPMI 1640 medium supplemented with 50 mmol/l 2-mecaptoethanol and 20 U/ml recombinant hIL-2.

**Direct cytotoxicity of AdCMVTKhIL2 on infected cells**

rMTC cells were infected by AdCMVTKhIL2 or AdCMVtk using different ratios of viral p.f.u. to cells (multiplicity of infection between 0.1 and 10 p.f.u./cell).
infection, m.o.i.) for 1 h in 500 µl infection solution (DMEM supplemented with 2% fetal bovine serum), and then incubated for 3 days in 96-well plates. On the last day of incubation, 20 µl [3H]thymidine solution (0.5 µCi) were added into each well. Cells were harvested and incorporation of [3H]thymidine into transduced cells was determined by scintillation counter.

**Analysis of functional hIL-2 expression**

hIL-2 bioactivity was measured by a standard bioassay protocol using an IL-2-dependent murine T cell line, CTLL-2, as described previously (Davis et al. 1994). The transduced rMTC cells were cultured and supernatant was recovered for hIL-2 assay every 24 h. Different numbers of rMTC cells were used in the cultures, depending on the period of incubation (from 2×10⁵ cells/well to 1×10⁶ cell/well in 6-well plate). CTLL-2 cells (5×10⁴) were incubated with an hIL-2-containing sample in a volume of 180 µl for 24 h at 37 °C in 96-well microtiter plates. [3H]Thymidine (0.5 µCi/well in 20 µl) was added, and the incorporation of [3H]thymidine into DNA was determined after overnight incubation (Davis et al. 1994).

**GCV sensitivity assay**

AdCMVTKhIL2- or AdCMVtk-infected or wild-type rMTC cells were incubated in 96-well plates using 5×10⁴ cells in 180 µl complete medium for 24 h. A series of concentrations of GCV was added into the culture in sextuple (20 µl solution) and incubation was continued for another 24 h. [3H]Thymidine (0.5 µCi in 20 µl) was then added to each well and samples were incubated for another 24 h. Cells were harvested after detaching them from the plates by three freeze-thaw cycles. Incorporation of [3H]thymidine was determined in a scintillation counter.

**Tumorigenicity of AdCMVTKhIL2-transduced rMTC cells**

rMTC cells were infected with 50 m.o.i. AdCMVTKhIL2 for 1 h in 500 µl infection solution. Infected cells were then washed with serum-free medium. A total of 1×10⁷ infected cells in 100 µl serum-free medium were injected subcutaneously into one flank of WAG/Rij rats. Animals transplanted subcutaneously with rMTC cells infected by AdCMVLacZ or AdCMVtk virus served as the controls. Injected animals were inspected every 2 days for development of tumors.

**Development of rMTC tumors in WAG/Rij rat**

rMTC cells were harvested in the exponential growth phase and washed three times in serum-free medium, then 1×10⁶ cells were injected subcutaneously into the right flank of each rat. Tumors usually developed in 2 weeks.

**In vivo antitumor efficiency study**

Adenoviral vectors were directly injected into the tumors using 2×10⁹ p.f.u. in 100 µl solution. Forty-eight hours after the viral treatment, GCV was administered i.p. at 100 µg/kg body weight for 7 consecutive days. Tumor size was measured every 2 days. The subcutaneous tumor volumes were determined from the formula \(v = a^2b/2\), where \(a\) is the shortest diameter, and \(b\) is the longest diameter of the tumor.

**Challenge of rats cured of tumor by reinjection of tumor cells**

Rats that were tumor-free after treatment with AdCMVTKhIL2 were challenged with wild type rMTC cells injected in the opposite flank. The same cell number was used as in the first injection.

**Statistical calculations**

Student’s \(t\)- and \(\chi^2\)-tests were used to analyze the data. \(P>0.05\) was considered significant.

**Results**

**Direct cytotoxicity of AdCMVTKhIL2 on infected rMTC cells**

The direct cytotoxicity of AdCMVTKhIL2 on infected rMTC cells was evaluated by in vitro cell proliferation assay as described in Materials and methods. The toxicities of AdCMVTKhIL2 and AdCMVtk for infected rMTC cells were similar (Fig. 2). A clear inhibitory effect was observed when the cells were infected at more than 100 m.o.i. of either virus.

**AdCMVTKhIL2-directed expression of functional hIL-2 in infected rMTC cells**

The expression of hIL-2 in infected rMTC cells was determined using an hIL-2-dependent CTLL-2 cell proliferation assay (Fig. 3). The infected cells produced large amounts of hIL-2 in the first few days (Fig. 4), and production then decreased, probably because of loss of virus, as cells replicate and some die. On day 2 and 3, more than 60 000 IU hIL-2 were produced by 1×10⁶ cells each 24 h as calculated using this assay.
Figure 2  Cytotoxicity of vectors towards rMTC cells infected by AdCMVTkhlIL2 or AdCMVtk at different m.o.i. for 1 h, and then incubated for 3 days in 96-well plates. On the last day of incubation, [3H]thymidine solution was added to each well. Cytotoxicity was evaluated by measuring the incorporation of [3H]thymidine by infected cells.

Figure 3  AdCMVTkhlIL2-directed expression of functional hIL-2 in transduced rMTC cells (1×10^6) infected in vitro at 100 m.o.i. for 1 h in 500 µl solution. After three washes with serum-free medium, the infected cells were incubated in 6-well plates. Supernatants were harvested after 24 h incubation. The activity of hIL-2 in the supernatants was tested by CTLL-2 cell proliferation assay. Recombinant (r)IL-2 served as positive control: '1x' rIL-2 equals 80 U/ml. The medium served as negative control.
Figure 4 Dynamic expression of hIL-2 in rMTC cells infected with AdCMVTKhIL2 at 100 m.o.i. for 1 h, and then incubated in 6-well plates at 2×10^5 cells/4 ml complete medium. Supernatants were saved and replaced every 24 h for 6 consecutive days. The activity of hIL-2 was detected in supernatants by CTLL-2 cell proliferation assay.

Figure 5 GCV sensitivity of AdCMVTKhIL2-infected rMTC cells incubated and treated with GCV at different concentrations as described in Materials and methods. Proliferation fraction (PF) equals 100 multiplied by the value of the c.p.m. in cells treated with GCV divided by the c.p.m. in cells not treated with GCV. The GCV sensitivity of transduced rMTC cells was calculated according to the extent of proliferation inhibition in different conditions. The key in the figure indicates the various m.o.i. used in the infection.
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Analysis of expression of HSVtk by GCV sensitivity assay using AdCMVTKhIL2 infected cells

To determine the expression of HSVtk gene in infected cells, rMTC cells were transduced with AdCMVTKhIL2 at various m.o.i. When rMTC cells were infected at 2 m.o.i., cell proliferation was inhibited by more than 34% at a GCV concentration of 1 mmol/l, compared with 5% inhibition in uninfected GCV control cells. When cells were infected at 20 m.o.i., cell proliferation was inhibited more than 76%. At a GCV concentration of 100 mmol/l, using 2 m.o.i., cell proliferation was inhibited by about 60%, compared with 30% inhibition in uninfected control cells, and inhibition was 92% at 20 m.o.i. These results demonstrate the expression of HSVtk and high sensitivity of infected rMTC cells to the HSVtk/GCV system in vitro (Fig. 5).

Expression of hIL-2 after intratumoral administration of AdCMVTKhIL2

In order to verify the expression of hIL-2 in infected tumor cells in vivo, tumors were treated by injection of 2×10⁸ p.f.u. of either AdCMVTKhIL2 or AdCMVLacZ virus in 100 µl serum-free medium. The treated tumors were then excised 2 days after the injection. A single cell suspension was prepared, primary cultures were made at 2×10⁶ cells/µl, and cells were cultured overnight in complete RPMI-1640 media. Supernatants were then saved and equal aliquots of the supernatants were used to examine the presence of hIL-2 using the IL-2-dependent CTLL-2 cell proliferation assay. No proliferation of the CTLL-2 cells was observed using supernatants from uninfected tumors or tumors treated with AdCMVLacZ, whereas supernatants from AdCMVTKhIL2-treated tumors induced a very good proliferation (Fig. 6), indicating the production of functional hIL-2 by the AdCMVTKhIL2 treated tumor cells.

Tumorigenicity of rMTC cells infected in vitro with AdCMVTKhIL2 in Wag/Rij rats

To evaluate the effect of hIL-2 secretion by AdCMVTKhIL2-infected cells on tumorigenicity, parental rMTC cells, or rMTC cells transduced with AdCMVLacZ, AdCMVtk or AdCMVTKhIL2 were injected subcutaneously into the flanks of groups of Wag/Rij rats. Parental rMTC cells induced tumor development in 100% of injected rats. All rats injected with AdCMVLacZ-infected rMTC cells or with AdCMVtk-infected cells and treated with phosphate-buffered saline (PBS), developed tumors. No rats injected with AdCMVtk-infected cells and treated with GCV, or with AdCMVTKhIL2-infected cells, developed tumors (Table 1). In animals injected with AdCMVtk-infected cells and treated with GCV, the expression of HSVtk presumably phosphorylated GCV, destroying the tumor cells. In the AdCMVTKhIL2-infected and PBS-treated group, the secretion of hIL-2 in the tumor cells is presumed to activate host antitumor immune responses, and thus induce inhibition of the tumor development (Moller 1980, Grimm et al. 1982, Mosmann & Coffman 1987, Kasaian & Biron 1989, Trinchieri 1989).
Table 1 Tumorigenicity of infected rMTC cells.

<table>
<thead>
<tr>
<th>Infection</th>
<th>Treatment</th>
<th>Tumor-free rats</th>
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<tbody>
<tr>
<td>Untreated</td>
<td>GCV</td>
<td>0/5</td>
</tr>
<tr>
<td>AdCMVLacZ</td>
<td>GCV</td>
<td>0/5</td>
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<tr>
<td>AdCMVtk</td>
<td>PBS</td>
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<tr>
<td>AdCMVtk</td>
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<tr>
<td>AdCMVTKhIL2</td>
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<tr>
<td>AdCMVTKhIL2</td>
<td>GCV</td>
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rMTC cells were infected in vitro with different adenoviral vectors as indicated in the Table at 10 m.o.i. for 1 h in 500 µl infection solution, then infected cells were injected subcutaneously into one flank of WAG/Rij rats after washing. Two days after the injection, animals were treated with either GCV at 100 mg/kg per day or PBS for 5 consecutive days. Animals were inspected every 2 days for 2 months for the development of tumors.

Superior in vivo antitumor activity with the bivalent vector

To evaluate the antitumor effect of AdCMVTKhIL2 in a rMTC animal model, the different adenoviral vectors were used at the same dosage to treat tumors. Tumors of different sizes were used in different experiments. Tumor growth was significantly inhibited after treatment with AdCMVTKhIL2 compared with the effect of a control vector. Table 2 summarizes the treatment efficiency with different vectors. AdCMVTKhIL2 destroyed 63% of tumors after treatment, whereas, at the same dosage, AdCMVIL2 destroyed 38% and AdCMVtk destroyed 11.8% of tumors, reflecting the benefit of the combination of HSVtk and IL-2 expression (Table 2).

Development of long-term antitumor immunity after treatment with AdCMVTKhIL2

To determine whether animals developed antitumoral immunity after AdCMVTKhIL2 treatment, rMTC cells were re-injected subcutaneously into the opposite flanks of animals cured of tumors. Only four of 19 rats developed tumors after the injection of parental rMTC cells (78.9% tumor free, Table 3, indicating that most animals were highly immunized against the tumor cells, whereas all five control animals developed tumors after injection of rMTC cells. These results demonstrate that a systemic and long-term antitumor immunity was established in most rats after direct intratumor delivery of AdCMVTKhIL2 virus.

Table 3 Long-term antitumor immunity in cured rats.

<table>
<thead>
<tr>
<th>No. of tumor-free rats after challenge</th>
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</thead>
<tbody>
<tr>
<td>Naive Wag/Rij</td>
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<tr>
<td>Cured Wag/Rij</td>
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Animals cured from their tumor after treatment with AdCMVTKhIL2 were re-injected with 1×10^6 wild-type rMTC cells per rat, 2 months after AdCMVTKhIL2 treatment. Control animals were Naive Wag/Rij rats.*** P<0.001 compared with naive controls.

Discussion

Our previous studies showed that adenovirus expressing murine IL-2 can be used to modify rMTC cells in vitro, and abrogated tumorigenicity in vivo (Zhang et al. 1998a). Direct treatment in vivo induced more than 40% of tumors to regress, and the remaining tumors had a low growth rate (Zhang et al. 1998a, 1999). The benefit of combination gene therapy with mixtures of different adenoviral vectors expressing HSVtk/GCV or cytokine genes has been reported. The combination of HSVtk/GCV with either IL-2 or IL-12 has been reported to have a better therapeutic efficacy than either one alone (Chen et al. 1996, O’Malley et al. 1996, 1997, Uckert et al. 1998).

HSVtk/GCV treatment can kill infected tumor cells, and the killed cells may then release specific tumor antigens. Cytokines secreted from transduced cells producing IL-2 or IL-12 will activate and enhance the function of both non-specific and specific immune cells. The activated immune cells will be more efficiently primed by taking up the released tumor antigens. These factors will synergize and more efficiently destroy the tumor mass. On the basis of this hypothesis, we constructed an adenoviral vector expressing HSVtk and hIL-2 (AdCMVTKhIL2), in which two
separately expressing cassettes are inserted into the Ad5 E1 region. This differs from other reported vectors, in which two cDNA pieces were inserted in the same region, using an encephalomyocarditis virus internal ribosome entry site sequence to initiate expression of the second cDNA. We compared the expression of HSVtk and IL-2, using AdCMVTKhIL2, with expression from AdCMVtk and AdCMVIL2, and did not find a significant difference. Another benefit is that we can insert multiple expression cassettes in suitable regions of Ad5 within the range of the insertion capacity. A combination of three or more different antitumor genes may be powerful enough to destroy the treated tumors.

Our in vivo study confirmed that the co-expression of HSVtk and IL-2 in one vector is superior to the effect of either one alone, or the mixture of two separate vectors. When we applied this new vector to treat rMTC tumors, 63% of tumors were destroyed. Only 38% of treated tumors were destroyed when AdCMVIL2 vector was used alone, and 11.8% of tumors were destroyed when AdCMVtk was used alone at the same dosage and in similar size tumors (Table 2).

We did not observe benefit from injecting a mixture of the two adenoviral vectors AdCMVIL2 and AdCMVtk (data not shown). Because of the low transduction efficiency, two viruses may not always infect the same cell in the treated tumor, and this may reduce the benefit of the combination.

We believe that a combined gene strategy is the best way to obtain high therapeutic efficiency. New vectors expressing both IL-12 subunits and the HSVtk gene, and other combinations, are therefore now under consideration.

Acknowledgements

We are grateful to Dr Bernard Roizman for the use of his laboratory, the Marjorie B Kovler Viral Oncology Laboratories at The University of Chicago for producing and preparing adenoviral vectors, Dr Cyprian Gardine for his careful reading of the manuscript and important suggestions, Miss Myrna Zimberg for her excellent secretarial assistance, and to all our fellow colleagues in our laboratory for their advice and useful discussion during this project.

This project was supported by the David Wiener Fund, the Elsa U Pardee Foundation, and a Center of Excellence Award from Knoll Pharmaceutical Corporation.

References


Moscovici S & Attramadal H 1993 The activation of IL2 transcription in L3T4+ and Lyt2+ lymphocytes during virus infection in vivo. Journal of Immunology 142 1287–1292.


Sen SK, Lambe CU & Furman PA 1987 Inhibition by ganciclovir of cell growth and DNA synthesis of cells


