Use of Gene Therapy in a Subcutaneous Murine Model of Lung Cancer

Manuel Rodrigo Garzón, a Íñigo Tirapu Fernández de la Cuesta, b Ainhoa Arina Iraeta, b Miguel Noel Centelles Llorente, b and Javier Zulueta Francés a

a Servicio de Neumología, Clínica Universitaria de Navarra, Centro de Investigación Médica Aplicada (CIMA), Universidad de Navarra, Pamplona, Navarra, Spain.

b Departamento de Medicina Interna, Clínica Universitaria de Navarra, Centro de Investigación Médica Aplicada (CIMA), Universidad de Navarra, Pamplona, Navarra, Spain.

OBJECTIVE: To assess the effectiveness of in vivo gene therapy to treat subcutaneous tumors generated from murine lung cancer cells.

MATERIAL AND METHODS: C57BL/6 mice received subcutaneous injections of 5 \times 10^6 cells from the murine Lewis lung cancer cell line. By 10 days, subcutaneous tumors of approximately 5 mm diameter were formed. At that point, treatment was provided by intratumoral injection of a replication-defective recombinant adenovirus carrying the gene for thymidine kinase (AdCMV-Tk) or interleukin (IL) 12 (AdCMV-IL12), or by injection of syngeneic dendritic cells previously transduced with adenovirus containing the IL-12 gene (DC-IL12). Control groups were treated with saline or adenovirus containing the gene for β-galactosidase (AdCMV-LacZ), which functions as a reporter gene and does not have a therapeutic effect. The number of animals in each group ranged from 14 to 25 in experiments using adenovirus and from 10 to 12 in experiments using dendritic cells. Tumor size was followed for 3 weeks in the case of treatment with adenovirus and 4 weeks for treatment with dendritic cells.

RESULTS: A significant reduction in subcutaneous tumor growth was observed in the groups treated with AdCMV-Tk, AdCMV-IL12, and DC-IL12 compared to control groups treated with saline or AdCMV-LacZ. The difference was statistically significant from day 7 of treatment in the AdCMV-Tk group, from day 9 in the AdCMV-IL12 group, and from day 10 in the DC-IL12 group, and in all cases it was maintained until the end of the follow-up period.

CONCLUSIONS: Gene therapy with AdCMV-Tk, AdCMV-IL12, or DC-IL12 is effective in our model of subcutaneous tumors arising from cells of the Lewis lung cancer cell line. The treatment leads to a significant reduction in tumor growth compared with control groups.

Key words: Gene therapy. Lung cancer. Adenovirus. Thymidine kinase. Interleukin 12. Dendritic cells.

Aplicación de tratamiento oncogénico a un modelo subcutáneo de cáncer de pulmón murino

OBJETIVO: Demostrar la utilidad del tratamiento génico (TG) in vivo en los tumores subcutáneos de cáncer de pulmón murino.

MATERIAL Y MÉTODOS: Se inyectaron a ratones C57BL/6 por vía subcutánea 5 \times 10^6 células de la línea de cáncer de pulmón murino de Lewis, A los 10 días se formaron tumores subcutáneos de unos 5 mm de diámetro. En ese momento se trataron mediante inyección intratumoral con un adenovirus recombinante defectivo portador del gen de la timidincinasa (AdCMV-Tk), o del gen de la interleucina 12 (AdCMV-IL12), o con células dendríticas (CD) singénicas transducidas con el gen de la interleucina 12 (CD-IL12).

Como grupos control se incluyeron tumores tratados con suero salino o con un adenovirus con el gen de la β-galactosidasa (AdCMV-LacZ), que es un gen indicador sin efecto terapéutico. El número de animales por grupo osciló entre 14 y 25 con adenovirus y entre 10 y 12 con CD. A continuación se realizó un seguimiento del tamaño tumoral desde el primer día de tratamiento hasta la tercera (adenovirus) o cuarta (CD) semanas para comparar su evolución.

RESULTADOS: Se objetivó una disminución significativa del crecimiento de los tumores subcutáneos en los grupos tratados con AdCMV-Tk, AdCMV-IL12 y CD-IL12 comparados con los grupos control tratados con suero salino y AdCMV-LacZ. En el grupo AdCMV-Tk esta diferencia fue estadísticamente significativa desde el día 7, en AdCMV-IL12 desde el día 9 y en CD-IL12 desde el día 10 y se mantuvo hasta el final del seguimiento.

CONCLUSIONES: El TG con AdCMV-Tk, AdCMV-IL12 o CD-IL12 es efectivo en nuestro modelo de tumores subcutáneos de células de carcinoma pulmonar de Lewis, ya que es capaz de disminuir su tasa de crecimiento de forma significativa respecto a los grupos de control.


Introduction

Gene therapy, which involves the use of genetic material for therapeutic purposes, is a new form of treatment that has appeared in the last decade as a result of advances in microbiology, virology, organic

Chemistry, molecular biology, biochemistry, cell biology, genetics, and genetic engineering. It differs from classical pharmacology in its use of genes and vectors in place of other active molecules. To date, its use has been studied most extensively in oncology, particularly in relation to bronchogenic carcinoma.

The vectors used can be classified as viral and nonviral and a large number of different genes can be used for therapeutic purposes. In this study, we used a viral vector, namely a first-generation, serotype 5, replication-defective, recombinant adenovirus. The virus is unable to replicate by itself since the part of the genome necessary for replication is replaced with the recombinant gene of interest. The genes used were thymidine kinase (Tk) and interleukin 12 (IL-12).

Gene therapy for cancer utilizes various approaches, and they can be combined and used alongside classical pharmacology in its use of genes and vectors in place of other active molecules. To date, its use has been studied most extensively in oncology, particularly in relation to bronchogenic carcinoma.

In this study, we analyzed the usefulness of gene therapy using suicide-gene and immunotherapy strategies in a subcutaneous model of murine lung cancer.

Material and Methods

Animals and Cell Lines

The female, 6-week-old, C57BL/6 mice (Harlan, Barcelona, Spain) used in the study were cared for according to current guidelines in our hospital. Three human lung cancer cell lines (A549, adenocarcinoma; HTB58, squamous cell carcinoma; and H441, bronchioloalveolar adenocarcinoma) and 1 murine lung cancer cell line (Lewis lung carcinoma, LLC) were used for in vitro experiments. LLC, which is syngeneic with C57BL/6 mice, was used for in vivo experiments. All cell lines were obtained from the American Type Culture Collection. LLC and A549 cell lines were maintained in Dulbecco’s modified Eagle’s medium (DMEM) and HTB58 and H441 were cultured in Roswell Park Memorial Institute (RPMI) medium. Both media were supplemented with 10% fetal bovine serum (FBS), 100 U/mL penicillin, and 100 mg/mL streptomycin. Cultures were incubated at 37°C in an atmosphere containing 5% CO₂.

Adenovirus Construction

Adenoviruses were constructed containing the genes for Tk, IL-12, and Escherichia coli β-galactosidase. β-galactosidase is a reporter gene used as a control and has no therapeutic effect. All genes were placed under the control of the ubiquitous cytomegalovirus (CMV) promoter. The viruses were constructed according to a previously published protocol in the laboratories of the Gene Therapy Unit, Department of Internal Medicine, Clinica Universitaria de Navarra, Spain.

Synergistic Dendritic Cells

Bone marrow was taken from the femur and tibia of syngeneic mice according to a previously published protocol. Cells were infected in vitro with the adenovirus AdCMV-IL12 at a multiplicity of infection (MOI) of 3000.

In Vitro Transduction of Cell Lines With AdCMV-LacZ

Cells from each cell line were seeded at a density of 2×10⁴ cells in 2 mL of either DMEM or RPMI containing 2% FBS. After 24 hours, the medium was removed and the cells infected with AdCMV-LacZ in a volume of 500 µL per well of un-supplemented medium. After 4 hours, 1.5 mL of DMEM or RPMI containing 2% FBS was added to the wells and the cells were incubated for a further 48 hours. Infection was performed at an MOI of 1, 10, 100, 1000, and 10 000. A control well was prepared containing cells incubated in DMEM without adenovirus infection. The medium was removed after 2 days and the cells fixed with glutaraldehyde. Following fixation, 500 µL of 5-bromo-4-chloro-3-indolyl β-D-galactoside (X-gal) was added to each well and the cells were incubated for 4 hours at 37°C. X-gal stains cells blue if they contain β-galactosidase protein in their cytoplasm. Thus, cells that are successfully transduced with AdCMV-LacZ and contain the protein encoded by the transgene will appear blue. The percentage transfection can be assessed by optical microscopy to count the total number of cells and the number of cells stained blue. This experiment was performed twice and the number of transfected cells counted in 5 fields per well.

In Vitro Transduction of LLC Cells With AdCMV-Tk

On day 1, 1.5×10⁴ cells per well were seeded in 2 6-well plates, each well containing 2 mL DMEM supplemented with 2% FBS. On day 2, the cells were infected with AdCMV-Tk by replacing the medium with 500 µL of AdCMV-LacZ at an MOI of 1. E3 LLC cells were infected with AdCMV-LacZ at an MOI of 10000 and AdCMV-Tk at an MOI of 1. The cultures were incubated for 4 hours at 37°C. After 24 hours, the medium was replaced with fresh medium containing 2% FBS. On day 4, the medium was replaced with fresh medium containing 2% FBS. On day 5, the medium was replaced with fresh medium containing 2% FBS. On day 6, the medium was replaced with fresh medium containing 2% FBS. On day 7, the number of live cells was counted in each well. The percentage transfection was calculated by optical microscopy to count the total number of cells and the number of cells stained blue. This experiment was repeated twice to confirm the results.

In Vivo Transduction of Subcutaneous LLC Tumors With AdCMV-LacZ

Subcutaneous injections of 5×10⁶ LLC cells were administered in the flanks of 3 C57BL/6 mice. AdCMV-LacZ was injected into the tumors when they reached a diameter of 5 mm. A dose of 1×10⁸ plaque forming units (pfu) was used in a volume of 40 µL physiologic saline. Animals were sacrificed 2 days later and the subcutaneous tumors extracted. The tumors were embedded in optimal cutting temperature (OCT) compound (Tissue-Tek Sakura, Tokyo, Japan) and frozen immediately in isopentane immersed in liquid nitrogen. The samples were stored at −80°C. Tissue sections were obtained with a cryostat. For staining, the sections were first fixed in glutaraldehyde for 10 minutes and then washed...
twice with a phosphate-buffered solution before incubating overnight in X-gal solution. The following day, the sections were washed with a phosphate buffer and viewed under a Nikon Eclipse E 800 optical microscope. Cells that were transduced with the adenovirus were stained blue.

Subcutaneous Model and Treatment

Subcutaneous tumors were generated as described in the previous section. When the tumors reached 5 mm in diameter, they were injected with either adenovirus or dendritic cells. Animals were anesthetized for tumor induction and treatment by intraperitoneal injection of thiazine hydrochloride and ketamine. Animals were divided into 4 groups for the adenovirus experiment: 2 control groups, one treated with physiologic saline (n=25 for Tk; n=16 for IL-12) and the other with AdCMV-LacZ (n=23 for Tk; n=14 for IL-12), and 2 therapeutic groups, one treated with AdCMV-Tk (n=23) and the other treated with AdCMV-IL12 (n=17). Treatments were performed in 40 µL of physiologic saline. The adenovirus doses used were 1×10^9 pfu in the Tk group, 5×10^8 pfu in the IL-12 group, and 1×10^9 or 5×10^8 pfu in the LacZ group. The animals in the Tk group received GCV treatment via daily intraperitoneal injection (50 mg/kg/day in a volume of 200 mL physiologic saline) for 14 days, starting 2 days after adenovirus injection. Two groups were formed for experiments involving dendritic cells: a control group (n=10), treated with physiologic saline, and a treatment group (n=12), which received 5×10^5 DC-IL12 injected into the tumor in 75 µL of physiologic saline on day 1 and day 3. Tumor growth was then followed and compared between groups by taking measurements 3 times per week starting on the day of treatment. Follow-up was undertaken for 21 days in the experiment involving adenovirus and 28 days in the dendritic cell experiment.

Statistical Analysis

Nonparametric methods were used for comparison of tumor size. The Wilcoxon test was used for comparisons between 2 groups and the Friedman test for comparisons between more than 2 groups. In all cases, P values less than or equal to .05 were considered statistically significant. Analysis was performed with the Instat program (GraphPad Software Inc, San Diego, USA).

Results

In Vitro Transduction of Cell Lines With AdCMV-LacZ

Prior to performing experiments in animals, the in vitro transduction efficiency of AdCMV-LacZ was assessed in various cell lines. The results are shown in Table 1. The left hand column shows the MOI used and the remaining columns the percentage of transduced cells in each cell line. The transduced cells are stained blue with X-gal solution (Figure 1). All 4 cell lines were transduced by AdCMV-LacZ but the efficiency varied. The most permissive cell line was A549 and the most resistant, LLC, in which the first blue-stained cells were observed at an MOI of 1000 and an MOI of 10 000 was required to achieve 42% transduction.
In Vitro Transduction of LLC Cells With AdCMV-Tk

The efficacy of the Tk-GCV system was tested in vitro with the LLC line prior to its use in vivo. Table 2 shows the results obtained using a fixed MOI of 100 and a fixed dose of GCV of 100 mmol/L. With a fixed MOI of 100, cell death was observed at all doses of GCV tested. Cell death increased with increasing concentration of the drug. With a fixed dose of 100 mmol/L GCV, cell death occurred at all MOIs tested, including the lowest MOI of 1. Cell death increased with increasing MOI. The results obtained with an MOI of 100 and 100 mmol/L GCV were similar in both experiments: 3.8% and 3.4% live cells compared with the control.

In Vivo Transduction of Subcutaneous LLC Tumors With AdCMV-LacZ

Tissue sections of subcutaneous tumors transduced with AdCMV-LacZ were stained with X-gal (Figure 2). The blue-stained cells transduced with the adenovirus were visible along the trajectory of the needle in the tumor.

Treatment of Subcutaneous LLC Tumors With AdCMV-Tk

Tumor size was followed in 3 groups, treated with physiologic saline, AdCMV-LacZ, and AdCMV-Tk. The results are shown in Figure 3. The y axis shows the area of the tumors (mm$^2$) and the x axis shows the time elapsed. Growth was slower in tumors treated with AdCMV-Tk and GCV. Thus, the area of the tumors was smaller in that group compared with those treated with physiologic saline and AdCMV-LacZ. The differences began 2 days after initiation of treatment and were statistically significant from 7 days until the end of the study ($P < .05$). No significant differences were observed between the groups treated with physiologic saline and AdCMV-LacZ.

Treatment of Subcutaneous LLC Tumors With AdCMV-IL12

Tumor size was followed in 3 groups, treated with physiologic saline, AdCMV-LacZ, and AdCMV-IL12.
The results are shown in Figure 4. The y axis shows the area of the tumors (mm²) and the x axis shows the time elapsed in days. Delayed tumor growth was observed in the group treated with AdCMV-IL12 compared with the other 2 groups. The differences became apparent on day 4 and were statistically significant from day 9 (P<.01). No significant differences were observed between the mice treated with physiologic saline and those treated with AdCMV-LacZ.

Treatment of Subcutaneous LLC Tumors With DC-IL12

Two groups were used, one receiving injections of physiologic saline or syngeneic dendritic cells transduced with the gene for interleukin 12 (DC-IL12). Tumor area is plotted against time. Tumor growth was significantly reduced in the group treated with DC-IL12. P<.05, from this point onwards.

Discussion

In the last 60 years, lung cancer has moved from being a relatively uncommon disease to become the most common cancer in the world (12.3% of all malignant tumors), with an estimated 1.2 million new cases in the year 2000, 52% of which occurred in developed countries. Given the low survival rate of lung cancer patients treated conventionally, new treatments must be sought. Gene therapy has emerged in recent years as an alternative to more traditional forms of treatment in bronchogenic carcinoma, such as surgery, chemotherapy, or radiotherapy. The aim of this study was to assess the efficacy of gene therapy in a subcutaneous model of murine lung cancer.

The first step was to assess the efficacy of the system in vitro. To this end, cultured LLC cells were infected with AdCMV-LacZ. LLC cells were later used for in vivo experiments. In addition, 3 human lung cancer cell lines were analyzed. All of the cell lines could be transduced using adenovirus-based gene therapy techniques, but the efficiency varied according to the cell line used (Table 1 and Figure 1). Adenovirus enter cells via endocytosis mediated by the coxsackievirus and adenovirus receptor (CAR) and integrins αβ3 and αβ5 in the cell membrane. CAR binds the fibers projecting from the adenovirus capsid and the integrins in the cell membrane bind viral capsid proteins and internalize the adenovirus in the cell. The transduction efficiency depends on the number of membrane receptors for adenovirus that the cells have, leading the efficiency to vary according to the cell line used. Cells with a high number of adenovirus receptors will display a high transduction efficiency. The results obtained in this study are similar to those published with other lung cancer cell lines. The LLC cell line required an MOI of 1000 before the first stained cells were visible and a high MOI (10,000) to achieve 42% transduction. These infection ratios are high but are consistent with those published previously for the LLC cell line, which was reported to require an MOI of 20,500 to achieve a transduction efficiency of 100%.

The next step was to assess the efficacy of the Tk-GCV system in vitro. This system employs the suicide gene strategy mentioned in the Introduction. The strategy involves introduction of the gene for Tk into tumor cells in order for them to convert GCV, which is nontoxic, into GCV triphosphate, which is highly cytotoxic. In addition to being toxic for the transduced cell, the effect extends to surrounding cells by diffusion of GCV triphosphate through cell junctions. This is known as the bystander effect and it amplifies the efficacy of the system. LLC cells were infected in culture with AdCMV-Tk using different MOIs followed by a range of concentrations of GCV. The aim was to determine whether introduction of the gene for Tk into cultured LLC cells was effective and led to cell death following treatment with GCV. The results showed that this system worked (Table 2). Even at a low MOI (1, 10, or 100), when transgene expression was not observed with AdCMV-LacZ, cell death nevertheless occurred with the Tk-GCV system. This finding is explained by the low sensitivity of the LacZ reporter gene used to assess adenovirus transduction and the consequent tendency to underestimate transduction levels. Cells can contain β-galactosidase protein in their cytoplasm but not be stained blue with X-gal because the amount of protein is small and the visualization threshold is unable to distinguish it. Consequently, although blue-stained cells were not observed with

---

**Figure 5.** Subcutaneous Lewis lung carcinoma tumors treated with physiologic saline or syngeneic dendritic cells transduced with the gene for interleukin 12 (DC-IL12). Tumor area is plotted against time. Tumor growth was significantly reduced in the group treated with DC-IL12. P<.05, from this point onwards.
AdCMV-LacZ infected at MOIs of 100, 10, or 1, cell death occurred with AdCMV-Tk and GCV, the action of which would also be enhanced by the bystander effect.2,12

The efficiency of AdCMV-LacZ transduction was assessed for in vivo experiments by injection into subcutaneous LLC tumors. Following staining, blue cells were observed inside the tumor, mainly located along the trajectory of the needle (Figure 2). The efficiency in vivo depends on the dose of adenovirus used, the promoter controlling the transgene, and the injection technique. It is better to perform a single injection in order for the adenovirus to diffuse as widely as possible throughout the tumor, aided by the pressure produced during injection. The integrity of the tumor also affects the efficacy of the procedure, since if it is poor the adenovirus can escape and infect peritumoral tissue.23

Having confirmed that the adenovirus was able to transduce subcutaneous tumors in vivo, the therapeutic adenoviruses were studied. Physiologic saline, AdCMV-LacZ, and AdCMV-Tk were injected into the tumors. Tumor cells transduced with AdCMV-Tk produce Tk and will transform GCV into GCV triphosphate, which is toxic to dividing cells as a result of its inhibition of DNA polymerase. Its effect is enhanced by the bystander phenomenon, which also functions in vivo.8,24 During follow-up, a reduction in the growth of the tumors was observed in the group treated with AdCMV-Tk; this effect was apparent from 2 days after initiation of treatment. This coincided with the first day of treatment with intraperitoneal GCV. The differences increased and were statistically significant from day 7 until the end of the study (Figure 3). No toxicity due to administration of either the adenovirus or GCV alone was observed at any time. Thus, treatment with AdCMV-Tk and GCV causes a significant reduction in the growth of tumors in this model compared with those treated with saline or AdCMV-LacZ.

In another group of animals the tumors were treated with physiologic saline, AdCMV-LacZ, or AdCMV-IL12. The aim was to enhance the immune response of the host to the tumor in order to eliminate it. IL-12 was used because it is able to activate both types of immune response, namely innate and acquired immunity. In addition, antitumor effects have been shown in animal models: it impedes the growth of new tumors and causes regression of those that are already established.25,26 Delayed tumor growth was observed 4 days after injection of the adenovirus in the group treated with AdCMV-IL12 compared with those treated with either saline or AdCMV-LacZ. The differences were statistically significant from day 9 onwards and remained so until the end of the follow-up period (Figure 4). No toxic side effects of treatment were observed. AdCMV-IL12 was used at a dose of 5 × 10⁹ pfu. Lower doses of 1 × 10⁹ or 2.5 × 10⁹ did not display differences from the control groups treated with physiologic saline or AdCMV-LacZ, while higher doses (1 × 10⁹ pfu) did not lead to greater benefits than 5 × 10⁹ pfu (data not shown). The differences were apparent from 4 days of treatment, 2 days later than with AdCMV-Tk. This delay is due to the fact that the effects of Tk following GCV administration are immediate, whereas IL-12 induces a cascade of secondary cytokines that are ultimately responsible for its biologic effects and therefore delay its action.26 In conclusion, a significant delay in the rate of growth of tumors in this model is seen with treatment using AdCMV-IL12.

Finally, syngeneic dendritic cells transduced with adenovirus in order that they secrete IL-12 were also injected. Animals treated with physiologic saline were used as controls. The dendritic cells in the cavity of the tumor capture antigens and present them to the immune system to initiate an antitumor response.10,12,15,29 Differences between the groups were observed at 3 days of treatment, with lower growth in the group that received DC-IL12. The differences were statistically significant from day 10 onwards and remained so until the end of the follow-up period (Figure 5). Thus, treatment with DC-IL12 caused a significant reduction in the growth of the subcutaneous tumors.

It can be concluded that the 3 treatments (AdCMV-Tk plus GCV, AdCMV-IL12, and DC-IL12) were effective in our in vivo model. Those treatments were able to reduce the growth of subcutaneous tumors compared with control groups (physiologic saline and AdCMV-LacZ). Consequently, it can be envisaged that in the future gene therapy will be employed as a new treatment for lung cancer alongside conventional treatments, although further studies and clinical trials will be necessary.30

REFERENCES


