Resistant Developing after Long-term Ganciclovir Prodrug Treatment in a Preclinical Model of NSCLC

ROLAND KURDOW, BODO SCHNIEWIND, ARND S. BOEHLE, SIEGLINDE HAYE, LARS BOENICKE, PETER DOHRMANN and HOLGER KALTHOFF

Department of General and Thoracic Surgery, Molecular Oncology Research Group, University of Schleswig-Holstein, Kiel Campus, Arnold-Heller-Strasse 7, 24105 Kiel, Germany

Abstract. Background: We recently demonstrated a 100% increase in the survival period with ganciclovir (GCV) therapy in mice bearing orthotopic HSV-TK-positive non-small cell lung cancer (NSCLC) tumors. However, long-term survival was not achieved. The aim of the present study was to evaluate tumor growth during extended GCV therapy and to monitor the herpes simplex virus thymidine kinase (HSV-TK) gene and protein in tumors at different time points. Materials and Methods: The human NSCLC cell line KNS 62 was retrovirally transduced with the HSV-TK30 gene. Cell suspensions in which 100% or 25% of the cells were TK30-positive were inoculated subcutaneously in SCID bg mice. Tumor growth was evaluated during GCV therapy and HSV-TK DNA, RNA and protein were analyzed at different time points using PCR, RT-PCR and immunoblotting. Results: HSV-TK DNA, RNA and TK30 protein were demonstrated in the tumors 21 days after subcutaneous tumor inoculation. TK-positive tumors regressed during GCV therapy and tumors in which 25% of the cells were TK-positive grew significantly more slowly than control tumors did. After 4 weeks of GCV therapy, HSV-TK DNA, RNA and TK protein were not detectable in the remaining tumors, which were therefore resistant to further GCV therapy. Conclusion: Prodrug therapy of the NSCLC cell line KNS 62, including bystander effects, is sufficient. Nevertheless, GCV-resistant tumours develop after functional loss of the TK gene. In the clinical context, further studies will need to evaluate immunological bystander effects or combinations with other drugs.

The introduction of herpes simplex virus thymidine kinase (HSV-TK) genes into tumor cells and subsequent ganciclovir (GCV) treatment can provide an effective and valuable approach to the treatment of a variety of tumor types (1-3). HSV-TK specifically phosphorylates GCV into GCV phosphate, and cellular kinases provide further phosphorylation to GCV triphosphate, which inhibits DNA polymerase and leads to apoptosis (4). In addition to this direct mechanism, activation of a T cell-mediated immune response during GCV treatment of TK-positive tumors leads to simultaneous elimination of distantly growing TK-negative tumors (5, 6).

The phenomenon of simultaneous apoptosis of neighboring TK-positive and TK-negative cells during GCV therapy is known as the "bystander effect". As transduction of the HSV-TK gene never reaches all tumor cells in vivo, the bystander effect is a prerequisite for efforts to develop clinical applications for the GCV prodrug system. Transfer of GCV triphosphate across gap junctions, in addition to having immunological vaccination effects, is generally considered to be the major mechanism for bystander effects (7, 8).

Problems that remain with regard to a clinical approach to the use of a GCV prodrug include the limited efficiency of transduction in vivo (9) and treatment resistance, which is poorly understood (10, 11). Previous research conducted by our group demonstrated a 100% increase in the survival period of mice bearing orthotopic HSV-TK-positive non-small cell lung cancer (NSCLC) tumors as a result of GCV prodrug treatment (12). However, although the HSV-TK gene was stably transduced by retroviral transfer, there was no long-term survival. The aim of the present study was to assess tumor growth and the presence of the HSV-TK gene during a prolonged period of GCV therapy. A subcutaneous tumor model was therefore selected, to allow tumor growth to be monitored precisely over an extended period. Polymerase chain reaction (PCR) and reverse transcriptase polymerase chain reaction (RT-PCR) procedures were established to detect HSV-TK DNA and RNA.

Roland Kurdow and Bodo Schniewind contributed equally to this study.

Correspondence to: Dr. R. Kurdow, Department of General and Thoracic Surgery, University of Schleswig-Holstein, Campus Kiel, Arnold-Heller Str. 7, 24105 Kiel, Germany. Tel: +49 (0)431 597 1987, Fax: +49 (0)431 597 4586, e-mail: rkurdow@surgery.uni-kiel.de

Key Words: Lung cancer, gene therapy, retroviral vector, herpes simplex virus thymidine kinase.
Materials and Methods

Retroviral vectors, transfection of cells. HSV-TK30 and enhanced green fluorescent protein (EGFP) cDNAs were inserted into retroviral vector rkatTM and used for transduction as described previously (12).

Cell lines and culture conditions. The human NSCLC cell line KNS 62 and its suppliers have been described previously (12). Non-derivatized engineered cells were routinely maintained in RPMI 1640 supplemented with 10% fetal calf serum (FCS), 2 mM glutamine and 1 mM sodium pyruvate (Life Technologies, Inc., Eggenstein, Germany). Retrovirally transduced cells expressing EGFP or HSV-TK received neomycin (G418, 700 mg/mL) in addition. All cells were kept in a water-saturated atmosphere containing 5% CO2 at 37°C. Cells to be implanted were washed in phosphate-buffered saline (PBS; Life Technologies, Inc.), resuspended in a serum-free culture medium, counted in a hemocytometer and equilibrated at a density of 2 x 10^6 cells/200 μL for subcutaneous injections.

In vitro cytotoxicity assay. For the assay, 1 x 10^4 rkatTM EGFP/ne or rkatTM TK30/neo KNS 62 cells were seeded into each well of a 96-well plate. Mixtures of rkatTM EGFP/neo and rkatTM TK30/neo cells at ratios of 90:10, 75:25 and 50:50 were seeded at the same density. Twenty-four hours later, various concentrations of ganciclovir (Cymevene, Syntex Pharmaceuticals, Aachen, Germany) were added at volumes of 200 μL to each well. The medium and GCV were changed after 3 days. After 5 days, cytotoxicity was measured using the EZ4U cytotoxicity kit (Bio-Rad Laboratories, Munich, Germany) according to the manufacturer's instructions. After addition of the chromogenic substrate, the plates were scanned in an enzyme-linked immunosorbent assay reader (Anthos, Salzburg, Austria) at a wavelength of 450 nm. The values were determined as a percentage of the value for control wells without GCV treatment (n=6 for each GCV concentration). LD50 was calculated by regression analysis.

Immunoblot analysis. Preparation of protein extracts was carried out as described previously (13). For immunoblotting, 28 μg of total cellular protein (measured with BCA Protein Assay Reagent, Pierce Chemical Co., Illinois, USA, according to the manufacturer's instructions) was separated by 12% or 15% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to a polyvinylidene fluoride membrane (Immobilon-P; Millipore, Eschborn, Germany). Membranes were blocked with PBS containing 5% non-fat dry milk overnight at 4°C, washed several times with PBS containing 0.2% Tween 20 (PBS/Tween) and then incubated with the primary antibody (polyclonal rabbit anti-TK, kindly provided by Prof. William Summers, Yale University, New Haven, Connecticut, USA) at a concentration of 1:200. After washing with PBS/Tween, blots were incubated with appropriate peroxidase-conjugated mule anti-rabbit secondary antibody 1:5000 and developed with the ECL chemiluminescent detection kit (Amersham, Brunwick, Germany) following the manufacturer's protocol.

Detection of HSV-TK mRNA and cDNA by PCR. Total RNA or DNA was isolated using the DNA/RNeasy mini-kit (QIAGEN, Basle, Switzerland). The analyzed HSV-TK gene was intron-free, therefore the product length in RT-PCR and PCR are equal. Isolated total cellular RNA was digested by DNase prior to RT-PCR analysis to exclude DNA contamination in the specimen. For cDNA synthesis of extracted RNA (2 μg RNA per sample to be analyzed), Taqman Reverse Transcription Reagents (including random hexamers) were used according to the ABI Prism Detection System (Perkin Elmer Applied Biosystems, Foster City, California, USA). Amplification of HSV-TK cDNA was carried out using 5'-GGCGCGGTGTGTAATGACAAGC-3' as the forward primer and 5'-GGTATGGGACCTGGGGTGGTG-3' as the reverse primer. In these conditions, a 692-base sequence of the HSV-TK cDNA was amplified. The PCR conditions were as follows: 92°C for 2 min, 66°C for 1 min and 72°C for 1.5 min, 30 cycles; and 72°C for 10 min for the final extension.

Implantation of cells and GCV treatment of the tumors in vivo. In groups of five animals, female SCID bg mice (Harlan Winkelmann, Borchen, Germany) received subcutaneous xenotransplantation into the left flank of 2 x 10^6 cells of KNS 62, transduced with rkatTM TK30.neo, rkatTM EGFP/neoe, or a mixture containing 75% TK-positive and 25% EGFP-transduced cells. At a subcutaneous tumor size of about 100-150 mm^3, intraperitoneal GCV therapy with 50 mg/kg body weight per day, split into two injections, was started. For evaluation of subcutaneous tumor growth, animals were treated with GCV therapy for 4 weeks. After 4 weeks, GCV treatment was stopped and the tumors were examined for PCR and RT-PCR analysis of HSV-TK. In case of 100% rkatTM TK30.neo-transduced tumors, two animals were sacrificed for PCR and RT-PCR analysis. After 10 days, GCV therapy was started again for the rest of the animals in this group. To evaluate HSV-TK expression at the start of GCV therapy, HSV-TK-positive tumors were inoculated into two more animals and were examined 21 days after inoculation for PCR and RT-PCR analysis. Animals with a tumor volume of 1000 mm^3 or more, or with occurrence of any clinical symptoms, were sacrificed.

Clinical and postmortem evaluation. The animals were monitored daily for signs of decreased physical activity and visible tumor growth. The animals' weight and subcutaneous tumor growth were measured twice a week; tumor volume was calculated using the formula for a prolate ellipsoid (length x width^2 x 0.52) (14).
Statistics. Analysis of variance (ANOVA) and Scheffe’s method, as well as regression analysis of cytotoxicity assays, were carried out using the Statistical Package for the Social Sciences (SPSS) program (SPSS Inc., Chicago, USA).

Results

In vitro evaluation of GCV sensitivity and bystander effects. As reported in data published previously (12), HSV-TK-transduced KNS 62 cells displayed a dose-dependent reduction in cell survival during GCV therapy. With regard to co-cultures with 50% TK30-transduced cells, the effect of GCV therapy was similar to that in 100% TK30-positive KNS 62 cells. In co-cultures of only 10% TK30-positive cells, a significant effect of GCV therapy was still observed for KNS 62 cells (Figure 1).

Since the growth rate of wild-type, EGFP-transduced and HSV-TK-transduced cells of KNS 62 were shown to be similar in a 3H-thymidine incorporation assay in vitro (data not shown), EGFP-transduced cells were used as negative controls afterwards.

Subcutaneous tumor growth. KNS 62 TK30 subcutaneous tumors regressed during GCV therapy from 157 mm³ (67.0 SD) to 22.5 mm³ (31.7 SD) after 25 days of therapy and were significantly smaller than EGFP controls from the seventh day of therapy onwards (Figure 2). After a GCV-free interval of 10 days, subcutaneous tumors grew up to 295 mm³ (173.4 SD) and did not respond to further GCV therapy. Bystander KNS 62 tumors regressed from 152 mm³ (131 SD) to 114 mm³ (80 SD) after 7 days of GCV therapy. They grew more slowly than EGFP controls, with a statistically significant difference, from the seventh day of therapy onwards, but became significantly larger than TK-positive tumors after 20 days of GCV therapy (Figure 2).

PCR and RT-PCR analysis. HSV-TK gene expression was detected at the DNA and RNA level in vitro after gene transfer. Twenty-one days after inoculation of TK-positive
cells into SCID mice, TK<sup>30</sup> RNA and DNA were still detectable in tumors. After 4 weeks of GCV therapy, neither TK<sup>30</sup> RNA nor DNA were detected in TK<sup>30</sup>-positive tumors or bystander tumors (Figure 3).

**Immunoblot analysis.** HSV-TK protein was detected by polyclonal antibodies in cell lysates after transduction. Twenty-one days after inoculation of subcutaneous tumors, TK protein was detectable, whereas after 4 weeks of GCV therapy, the tumors no longer contained any detectable levels of HSV-TK protein (Figure 3).

**Discussion**

The efficacy of treatment involving the transfer of thymidine kinase as a suicide or chemosensitivity gene and subsequent ganciclovir therapy has been proven for various malignant tumors in vitro and in a variety of different tumor models (1,2). Despite this, clinical studies have failed to demonstrate any noticeable treatment response (9,15). On the one hand, this could be a consequence of low or absent transduction efficiency, while on the other hand treatment resistance has also been reported with a variety of human tumor cell lines, including gastrointestinal cancer entities and NSCLC cell lines (10, 11).

The mechanisms of resistance are not fully understood, but appear to be variable. Possible reasons for the inadequate effect of GCV on TK-positive tumors with GCV may be changes in the susceptibility of cells to GCV, exit of tumor cells from the cell cycle, inactivation or loss of the HSV-TK gene, or structural changes in herpes simplex virus mRNA. Golumbek and colleagues describe recurrent melanoma cells after 4 weeks of therapy, which were fully sensitive to further GCV treatment (16). Graessmann et al. showed that a single methylation of the HSV-TK gene within its coding region was responsible for a complete loss of function (17). Deletion or loss of the HSV-TK gene as a cause of GCV resistance was documented by Yang et al. and Moolten et al. (3, 10). Chou et al. (18) and Wolff et al. (19) report GCV resistance in patients after infection by a cytomegalovirus encoding a mutant UL97 gene, inhibiting the phosphorylation of GCV into its active metabolite. Zhang et al. found ongoing expression of HSV-TK mRNA in GCV-resistant NSCLC cell populations, but this was significantly lower than it was directly after transduction of tumor cells (11). The size of mRNA was reduced and GCV susceptibility was significantly decreased in recultivated tumor cells after 2 weeks of GCV therapy in nude mice. Yang et al. found that deletions of parts of the whole TK gene were responsible for GCV resistance. They found that 1-5% of cells were resistant to GCV therapy after transduction and antibiotic selection in vitro. This implies the appearance of resistant cell clones during GCV therapy if resistant clones are not killed by a sufficient bystander effect. Yang et al. therefore consider that bystander effects play a central role in the appearance of GCV-resistant tumors (10).

We have demonstrated that the KNS 62 cell line has an adequate bystander effect. This is in accordance with its higher connexin-43 expression (12) and leads to significant GCV susceptibility in vitro, even if only 10% of cells are TK<sup>30</sup>-positive (Figure 1). The subcutaneous model in the present study used tumors in which 25% of the cells were TK-positive (which has been documented for KNS bystander tumors during GCV therapy in comparison with EGFP vector controls. Nevertheless, even completely transduced KNS tumors recur after initial regression and grow exponentially during GCV therapy. Recurrence of tumors during therapy is in accordance with our previously published results in orthotopic NSCLC tumors, which led to a survival benefit of 100% in TK-positive tumors during GCV therapy, but did not provide long-term survival (12).

To explore the mechanism underlying tumor recurrence further, HSV-TK DNA and RNA were measured using PCR and RT-PCR at different time points. In addition, HSV-TK protein was detected by immunoblot analysis using polyclonal antibodies. After transduction, there was a strong HSV-TK DNA and RNA signal in cell lysates and HSV-TK protein was detected. Twenty-one days after tumor inoculation in SCID mice, just before the start of GCV therapy, there was still a strong DNA and RNA signal and HSV-TK protein was still detectable. During GCV therapy, HSV-TK tumors regressed incompletely. After 4 weeks of GCV therapy, two animals were sacrificed. In the small residual tumors, neither DNA nor RNA encoding HSV-TK or the TK-protein were detected. After an additional 10 days, GCV therapy was started again in the remaining animals. The tumors grew exponentially and did not respond to further GCV therapy.

We conclude that a loss of functional TK<sup>30</sup> gene in the tumor clones led to GCV-resistant tumor clones. This is an interesting finding, since stable retroviral transduction and subsequent neomycin selection, together with an adequate bystander effect (which has been documented for KNS 62 cells), ought to provide perfect conditions for complete tumor regression. In general, possible reasons for the loss of a functional gene might include single methylation (17), partial deletion (10), or loss of the whole gene. Intrinsic resistance to GCV can be excluded in this case.

These results are difficult to transfer to a clinical context, as immunodeficient SCID mice were used in this study. As an immunological bystander effect itself can result in complete tumor regression (20), it might be possible for complete tumor regression to take place with cell line KNS 62 in an immunocompetent tumor model.

A combination of prodrug therapy with other chemotherapeutic agents might be an option for further
studies, although current studies on this have not so far been successful (21, 22). In addition, current studies combining immunomodulation with GCV prodrug therapy have shown promising results and may be the subject of further investigation (23).

In summary, the present study is one of the few investigations that have been conducted into the limitations of GCV prodrug therapy after long-term therapy with GCV. An NSCLC cell line, with good susceptibility against GCV and a sufficient bystander effect after HSV-TK transduction, was studied in a subcutaneous murine xenotransplantation model. During GCV therapy, an initially adequate GCV effect with nearly complete tumor regression was detectable. Nevertheless, in residual tumors left after 4 weeks of GCV therapy, functional HSV-TK DNA, RNA and HSV-TK protein were not detectable. Tumor growth is resistant to further GCV therapy at this time point. Gene inactivation by methylation, deletion, or loss of the gene is therefore assumed to be the reason for functional loss of the TK gene, rather than intrinsic resistance against GCV.

The documented limitations of GCV prodrug therapy might be overcome in a clinical setting by an immunological bystander effect or a combination with chemotherapeutic agents and this should be evaluated in further studies.

References


Received October 9, 2003
Revised December 12, 2003
Accepted January 12, 2004