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Consequences of Chemoresistance for the Herpes Simplex Virus Thymidine Kinase/Ganciclovir-induced Bystander Effect in a Human Small Cell Lung Cancer Cell Line Model

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Abstract. *This paper focuses on the influence of chemoresistance on the herpes simplex virus (HSV-tk)/ganciclovir (GCV)-induced bystander effect (BE), as studied in a human small cell lung cancer (SCLC) cell line (GLC_4) and its sublines with in vitro acquired resistance to adriamycin (GLC_4/ADR), mitoxantrone (GLC_4/MITO) and cisplatin (GLC_4/CDDP). Chemoresistance for adriamycin, mitoxantrone and cisplatin significantly changed GCV sensitivity. A significant BE was found in all GLC_4 cell lines. Compared to the parental GLC_4 cell line, the BE was significantly higher only for the GLC_4/ADR cell line. No expression of the nucleoside transporters MRP4 and MRP5 was detected. In all cell lines expression of connexin 43 was found, but modulation of gap junctional intercellular communication (GJIC) by 18-α-glycyrrhetinic acid did not significantly change the BE in any of the GLC_4 cell lines. In conclusion, chemoresistance can influence the HSV-tk/GCV-induced BE, which seems not to be related to differences in MRP4/MRP5 expression or to differences in GJIC.*

Chemotherapy of patients with advanced cancer often leads to tumor remissions but only rarely results in cure. The existence or development of chemoresistance is a major obstacle in reaching this goal. To overcome this problem, new strategies are being explored in various stages of basic and clinical research.

One of these new anti-tumor approaches is suicide gene therapy. The goal of this approach is to transfect tumor cells with a foreign gene, which encodes an enzyme that is capable of transforming a nontoxic prodrug into cytotoxic metabolites. Transfecting tumor cells with the gene encoding for herpes simplex virus thymidine kinase (HSV-tk) has appeared to be a successful strategy for sensitizing tumor cells to the nucleoside analogue ganciclovir (GCV). Only tumor cells expressing the HSV-tk enzyme are capable of phosphorylating GCV to its toxic metabolites (1). After DNA incorporation of the GCV monophosphate in the nascent strand, cells die due to DNA strand breaks, chromosomal aberrations, chain termination and inhibition of DNA polymerase (2).

Besides the direct toxic effect of GCV on HSV-tk-positive cells, an indirect toxic effect induces tumor cell kill of HSV-tk-negative bystander cells. This is called the bystander effect (BE) (3). GCV metabolites can be transported from HSV-tk-positive to HSV-tk-negative cells *via* gap junctional intercellular communication (GJIC). Differences in expression of gap junction proteins, the so-called connexins (Cx), and differences in functionality are important determinants in the occurrence of the BE (4,5).

The multidrug resistance-related proteins MRP4 and MRP5 are recently discovered efflux pumps, capable of transporting nucleoside analogues (6,7). It was demonstrated that overexpression of MRP4 can lead to a decrease of the HSV-tk/GCV-induced BE (8).

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Key Words: Herpes simplex virus thymidine kinase, ganciclovir, bystander effect, gap junctional intercellular communication, multidrug resistance.

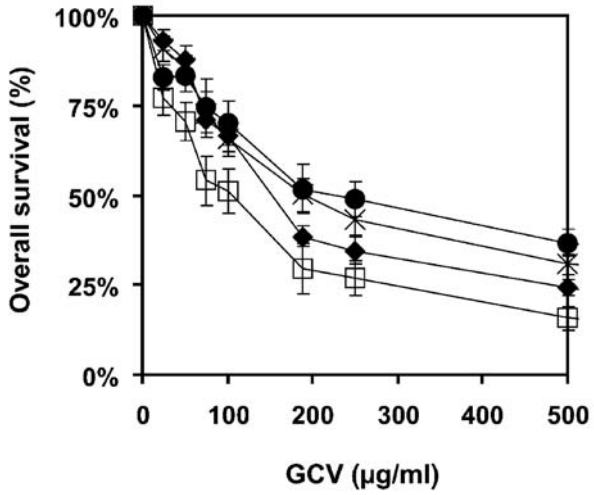

Figure 1. *Influence of chemoresistance on GCV sensitivity. Survival of GLC_4 (◆), GLC_4/ADR (x), GLC_4/MITO (●) and GLC_4/CDDP (Z), after continuous incubation with GCV, measured by MTA (n≥4). Error bars represent standard error of the mean.*

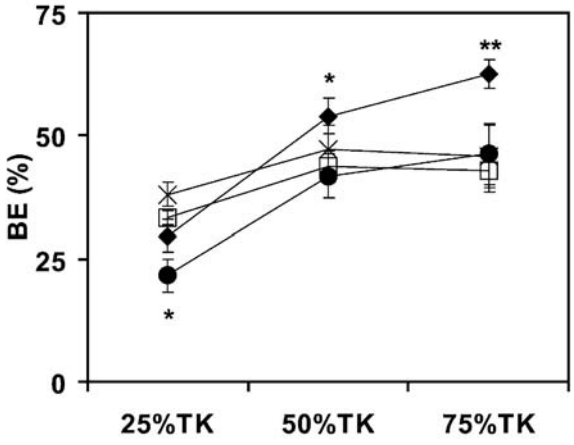

Figure 2. *The HSV-tk/GCV-induced BE in a panel of GLC_4 cell lines. Different mixtures of C6-rTK-18 cells and GLC_4 (□), GLC_4/ADR (◆), GLC_4/MITO (x) and GLC_4/CDDP (●) were plated and incubated for three days in the presence or absence of GCV (25 µg/ml). Survival was measured by MTA (n>3). Error bars represent the standard error of the mean. Significant differences of the BE compared to GLC_4 are indicated with *p<0.05 ** p<0.005.*

Besides this, induction of an immune response, phagocytosis of apoptotic bodies and intercellular transportation of other cytotoxic substances have been mentioned as contributing factors to the BE.

In contrast to the substantial anti-tumor cell responses observed in *in vitro* and animal experiments, the anti-tumor effect obtained in clinical trials is less convincing (9). One of the differences between animal experiments and clinical trials is the fact that patients have already been treated with various therapeutic modalities, *e.g.* chemotherapy. It is well-known that chemotherapy often leads to resistance of the tumor cells to the chemotherapeutic agent, due to changes in tumor cell characteristics, like drug efflux pumps, DNA repair *etc*. If acquired drug resistance influences HSV-k/GCV gene therapy, treatment and anti-tumor effects might be improved by preselection of patients on the basis of their treatment history.

Our study focused on the influence of acquired drug resistance on the HSV-tk/GCV-induced BE. To address this issue, GCV sensitivity, HSV-tk/GCV-induced BE and expression of MRP4, MRP5 and Cx43 were determined in human small cell lung cancer (SCLC) cell lines which differ in chemoresistance. Additionally, we determined if the HSV-tk/GCV-induced BE could be modulated by inhibition of GJIC with the long-term GJIC blocker, 18-α-glycyrrhetinic acid (AGA).

Materials and Methods

Chemicals. Paraformaldehyde, saponin, 3-(4,5-dimethylthiazol-2-yl) 2,5-diphenyltetrazolium bromide (MTT), AGA, 3-amino-9-ethyl carbazole (AEC), sodium vandate, Tween 20, dithiothreitol (DTT), Triton X-100, glycerol, sodium dodecyl sulphate (SDS), protease inhibitors and ß-mercaptoethanol were purchased from Sigma (St. Louis, MO, USA). Haematoxylin and dimethyl sulfoxide (DMSO) were purchased from Merck (Darmstadt, Germany). Skim milk, ECL Plus™ chemoluminescense detection system and nitrocellulose membranes were obtained from Amersham Biosciences (Orsay, France). GCV was obtained from Roche Diagnostics (Basel, Switzerland). Dulbecco's minimum essential medium (DMEM), Roswell Park Memorial Institute (RPMI) 1640 medium, nutrient mixture F-12 (Ham), bovine serum albumin (BSA), trypsin (2.5% (w/v)) and geneticine were purchased from Invitrogen (Merelbeke, Belgium). Fetal calf serum (FCS) was obtained from PAA laboratories (Brunschwig, Amsterdam, The Netherlands).

Antibodies. Monoclonal mouse anti-human MRP4 antibody was kindly provided by Prof. Gary D. Kruh (Fox Chase Cancer Center, Philadelphia, PA, USA). Prof. Dr. R.J. Scheper (Free University Medical Center, Amsterdam, The Netherlands) kindly supplied the monoclonal rat anti-human MRP5 antibody (M_5I-1). Polyclonal rabbit anti-human/anti-rat Cx43 antibody was purchased from Zymed (San Francisco, CA, USA). Horseradish peroxidase conjugated swine anti-rabbit antibody was obtained from Amersham Bioscience. Rabbit anti-rat biotinylated IgG rabbit anti-mouse biotinylated IgG, and horseradish peroxidase conjugated streptavidin were purchased from Dako (Glostrup, Denmark).

Cell lines. As described previously (10), HSV-tk-positive C6 rat glioma cells (C6-rTK-18) were acquired by transfection of C6 cells (American Type Culture Collection) with supernatant of PA317-tk packaging cells containing replication incompetent retroviruses carrying the HSV-1-tk gene and the NeoR gene. Subcloning was performed by single cell sorting through flow cytometry. Subclones were tested for HSV-tk expression and GCV sensitivity. The stability of TK expression was checked and followed for two

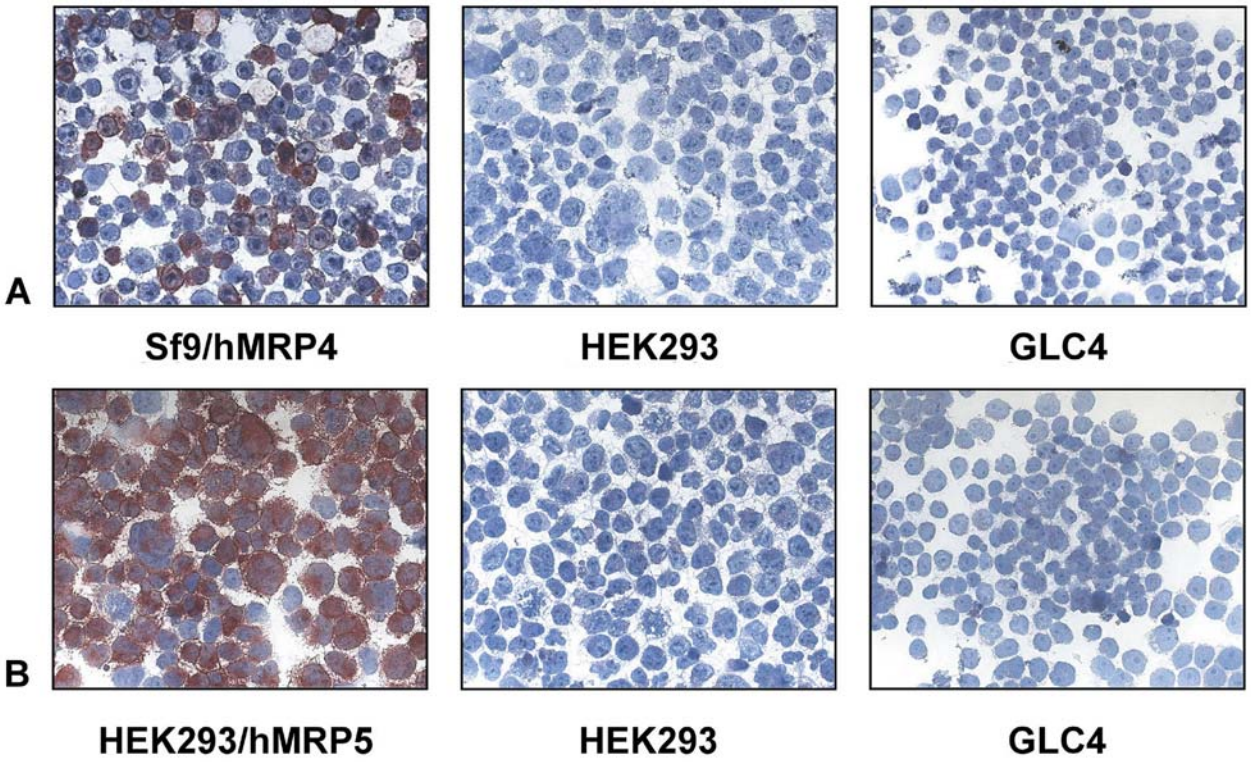

Figure 3. *MRP4 (A) and MRP5 (B) expression in a panel of GLC_4 cell lines. Only a representative example of GLC_4 is shown.*

months (Van Dillen *et al.*, manuscript submitted). Cells were cultured as monolayers in DMEM supplemented with 5% FCS and 1.0 mg/ml geneticine and grown in a humidified atmosphere with 5% CO_2 at 37˚C.

The human SCLC line GLC_4 (11) was derived from a pleural effusion in our laboratory and kept in culture in RPMI 1640 medium supplemented with 10% heat-inactivated FCS at 37˚C in a humidified atmosphere with 5% CO_2. GLC_4/ADR (11), GLC_4/MITO (12) and GLC_4/CDDP (13) obtained resistance by stepwise increasing concentrations of adriamycin, mitoxantrone, or cisplatin, respectively. Compared to the parental GLC_4 cell line, the resistance factors for adriamycin, mitoxantrone and cisplatin in the GLC_4/ADR, GLC_4/MITO and GLC_4/CDDP were, respectively, 350, 60 and 15. GLC_4/ADR and GLC_4/MITO were cultured continuously in the presence of the corresponding drug. Before the cells were used in the experiments, they were cultured without the drug for 18-21 days. GLC_4/CDDP was incubated with cisplatin once every three months. All experiments were performed with exponentially growing cells.

Cytotoxicity. To determine GCV sensitivity, the microculture tetrazolium assay (MTA) was used. The linear relationship of cell number to MTT formazan crystal formation and the exponential growth of cells in the wells were checked. In a total volume of 200 µl, 2,250 cells for C6-rTK-18, 2,000 cells for GLC_4 and 4,000 cells for GLC_4/ADR, GLC_4/MITO and GLC_4/CDDP were incubated. Treatment consisted of continuous incubation with various concentrations of GCV.

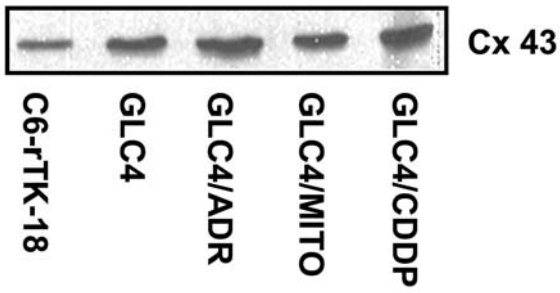

Figure 4. *Western blot analysis of Cx43 expression levels in C6-rTK-18 cells and in a panel of GLC_4 cell lines.*

After a total incubation period of 96 h, 20 µl of MTT (5 mg/ml) was added. After 3 h and 45 min, the plates were centrifuged and the supernatant was aspirated. After dissolving the formazan crystals in DMSO, the plates were read immediately at 520 nm using a microtiter well spectrometer (Benchmark microplate reader, Biorad, Hercules, CA, USA). The light absorption of untreated cells served as a control (survival 100%). After background correction, cell survival was defined as the light absorption of treated cells compared to the light absorption of untreated cells. The resistance factors were calculated by dividing the IC_{50} (GCV concentration that

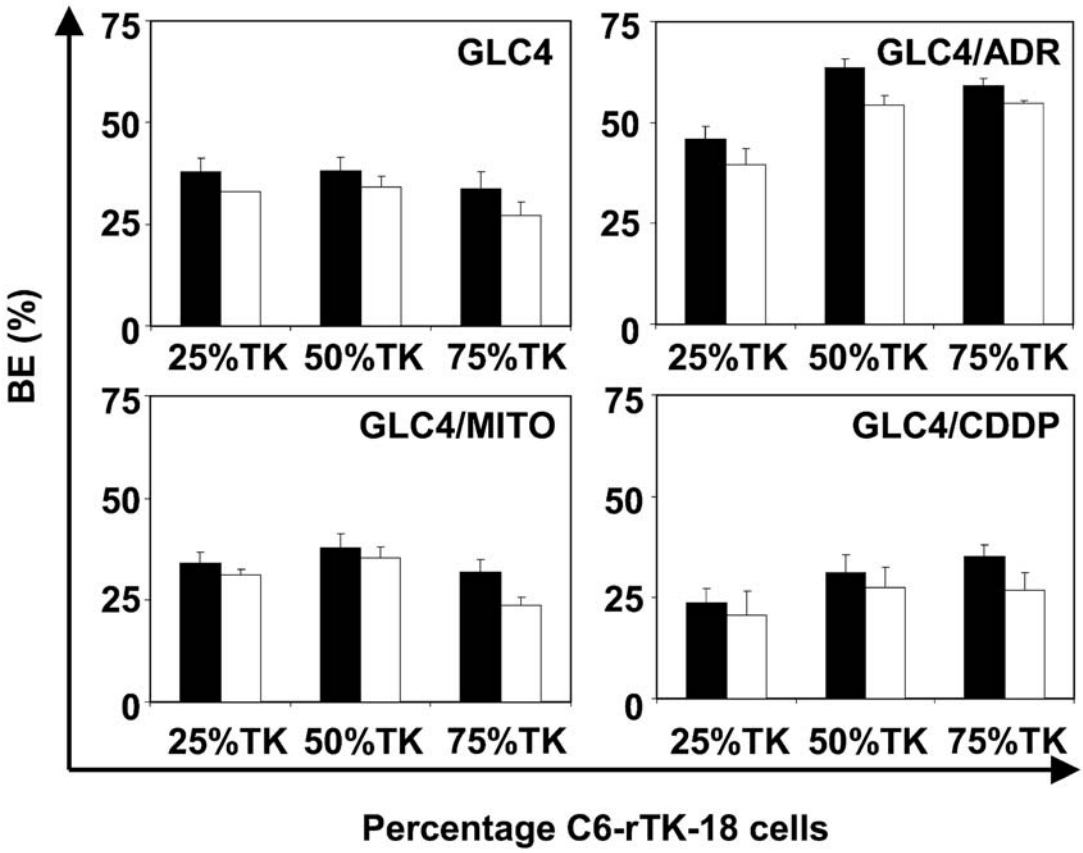

Figure 5. *Influence of AGA on the HSV-tk/GCV-induced BE in a panel of GLC_4 lines which differ in chemoresistance. Different mixtures of C6-rTK-18 cells and GLC_4, GLC_4/ADR, GLC_4/MITO and GLC_4/CDDP were plated and incubated for three days without (black) or with (white) 18-AGA (75 μM). Survival was measured by MTA (n≥3). Error bars represent the standard error of the mean.*

inhibited cell growth by 50%) of the resistant GLC_4 subline by the IC_{50} of the parental GLC_4 cell line. At least three independent experiments were performed, each in quadruplet.

Bystander effect. To determine the BE, the MTA was used. In a total volume of 200 μl per well, different mixtures of C6-rTK-18 cells and GLC_4 cells (0, 25, 50, 75, 100% C6-rTK-18 cells with 100, 75, 50, 25, 0% GLC_4 cells, respectively) were plated. To achieve a linear relationship between formazan crystal formation and cell count, sufficient cell-cell contact and an optimal BE, 5,000 cells per mixture for GLC_4 and 10,000 cells per mixture for the resistant GLC_4 sublines, were incubated for 72 h in the presence or absence of GCV (25 μg/ml). After 72 h, the MTA was performed as described above. At least three independent experiments were performed, each in quadruplet.

Immunohistochemistry for MRP4 and MRP5 expression. Acetone-fixed cytospins were air-dried and washed three times with phosphate-buffered saline (PBS). For MRP4, the samples were incubated in 0.025% hydrogen peroxide in PBS for 30 min to block endogenous peroxidase and washed again. Samples were incubated for 1 h at room temperature with a monoclonal antibody against MRP4 (1:100) or MRP5 (1:50) in 2% BSA in PBS (2%BSA/PBS). All samples were incubated with rabbit anti-mouse (MRP4) or rabbit anti-rat (MRP5) biotinylated IgG (1:150 in 1% BSA/PBS) followed by horseradish peroxidase conjugated streptavidin (1:500 in 1% BSA/PBS), each for 30 min. AEC was used as chromogen, which provided a red-stained color in areas of MRP4 or MRP5 expression. Sections were counterstained with haematoxylin. We used cytospins of Sf9 insect cells transfected with human MRP4 or HEK293 cells (human embryonic kidney cells) transfected with human MRP5 as positive controls and untransfected HEK 293 cells as negative control. The optimal dilution for immunostaining was obtained using the positive controls.

Western blot analysis of Cx43 expression. Total cellular extracts were prepared in 0.1% Triton X-100 in PBS containing protease inhibitors and 1mM sodium vanadate. After protein determination, the extracts were diluted with Laemmli SDS loading buffer (final concentration 50 mM Tris-HCl pH 6.8, 10% glycerol, 2% SDS, 100mM DTT) and boiled for 5 min at 95˚C. Twenty micrograms of proteins were loaded to SDS-polyacrylamide gel. After electrophoresis, the proteins were

transferred onto a nitrocellulose membrane. The membranes were blocked in 5% milk in TBS-T buffer (Tris-buffered saline (50 mM Tris-HCl pH 7.5, 150 mM NaCl), including 0.1% Tween 20) and then probed with primary rabbit anti-Cx43 antibody and horseradish peroxidase conjugated swine anti-rabbit antibody. The secondary antibody was visualized by using the ECL PlusTM chemoluminescense detection system obtained from Amersham Biosciences according to the instructions of the manufacturer. After detection, the membranes were stained with colloidal gold to verify the equality of protein loading.

Modulation of the BE by blocking GJIC. To elucidate the role of GJIC in inducing the BE, different mixtures of C6-rTK-18 cells and the various GLC_4 cells (0, 25, 50, 75, 100% C6-rTK-18 cells with 100, 75, 50, 25, 0% GLC_4 cells, respectively) were plated in a total volume of 200 µl per well, as described above. Treatment consisted of continuous incubation with the long-term GJIC blocker AGA (75 µM, dissolved in DMSO) followed by addition of GCV (25 µg/ml) after 5 h. After a total incubation time of 72 h, the MTA was performed. DMSO-treated cells were used as control. At least three independent experiments were performed, each in quadruplet.

Statistical analysis. For each experiment mean optical densities (OD) were calculated. For the situation with and without GCV, expected OD's (ED) were determined assuming absence of growth interference of both lines: $ED_{x\%}=(OD_{0\%}*(100\%-x\%)+OD_{100\%}*x\%)$, where OD0% represents the OD of 100% HSV-tk-negative cells, OD100% represents the OD of 100% HSV-tk-positive cells, and x% is the percentage of HSV-tk-positive cells. Subsequently, the ED's of the GCV-treated mixtures were corrected for growth interference by calculating a growth factor (GF). We calculated the percentage Px% of C6-rTK-18 cells that had survived in each well with GCV from the OD's of the 100% C6-rTK-18 wells. The value of Px% was defined as: $x\%*(OD_{(100\%+GCV)}/OD_{(100\%-GCV)})$.

For $P_{x\%} < 25\%$ (in our experiments the remaining percentage of GCV-treated HSV-tk-positive cells was always below 25%), we could determine the accompanying OD's and ED's by interpolating the 0% and 25% data of the GCV-untreated mixtures using linear regression. The GF for $P_{x\%}$ could then be calculated as:

$$GF(P_{x\%}) = \frac{OD_{(0\%\,-\,GCV)} + \frac{P_{x\%}}{25} * (OD_{(25\%\,-\,GCV)} - OD_{(0\%\,-\,GCV)})}{ED_{(0\%\,-\,GCV)} + \frac{P_{x\%}}{25} * (ED_{(25\%\,-\,GCV)} - ED_{(0\%\,-\,GCV)})}$$

Differences in GCV sensitivity, BE and BE after modulation with the GJIC blocker were considered significant if $p \leq 0.05$ (unpaired two-sided Student's *t*-test).

The BE was calculated according to the following formula:

$$BE = \frac{((ED_{(x\%\,+\,GCV)} * GF) - OD_{(x\%\,+\,GCV)})}{ED_{(x\%\,+\,GCV)} * GF} * 100\%$$

The BE was considered significant if the OD was outside the 95% confidence intervals of the (corrected) ED's.

Results

GCV sensitivity of GLC_4 cell lines. Figure 1 shows the sensitivity of the GLC_4 cell lines for GCV. In comparison to the GLC_4 cell line, a significant decrease in GCV sensitivity was found for all the resistant sublines of GLC_4. The resistance factors for the GLC_4/ADR, GLC_4/MITO and GLC_4/CDDP cell lines were 1.52 (SEM: 0.18; $p<0.04$), 1.86 (SEM: 0.10; $p<0.001$) and 2.48 (SEM: 0.54; $p<0.05$), respectively.

Bystander effect. The BE in the various GLC_4 cell lines is shown in Figure 2. A significant BE was found in all GLC_4 cell lines ($p\leq0.05$). Compared to the GLC_4 cell line, the BE was significantly higher for the GLC_4/ADR cell line when it was combined with 50% or 75% C6-rTK-18 cells ($p<0.05$). In addition, the BE was significantly lower in the GLC_4/CDDP cell line when it was combined with 25% C6-rTK-18 cells ($p<0.02$).

MRP4/MRP5 expression. Figure 3 shows the protein expression of the nucleoside analogue efflux pumps MRP4 (A) and MRP5 (B). No protein expression of MRP4 or MRP5 was found in any of the GLC_4 cell lines, although MRP4 and MRP5 were identified in the positive controls (only a representative example of the GLC_4 parental cell line is shown).

Cx43 expression. The expression of Cx43 in C6-rTK-18 and in the different GLC_4 cell lines is depicted in Figure 4. Cx43 protein expression was detected in C6-rTK-18 and in all four GLC_4 cell lines. No clear differences in expression between the various GLC_4 cell lines could be observed.

Effect of inhibition of GJIC on the BE. Modulation of GJIC with the long-term GJIC blocker AGA did not lead to a significant decrease of the BE in any of the GLC_4 cell lines, as compared to the BE that occurred without co-incubation with AGA (Figure 5).

Discussion

HSV-tk/GCV gene therapy is one of the most often studied suicide gene/prodrug systems to date and is currently under investigation in various clinical trials. Despite promising preclinical results, the anti-tumor effect in these trials is less convincing. Preclinical research is, therefore, focusing on factors that could improve the outcome of this approach, *e.g.* by increasing transfection efficiencies and tumor-specific targeting.

We hypothesized that certain changes, due to previous chemotherapeutic treatment of patients, might have resulted in a changed sensitivity to the HSV-tk/GCV gene therapy. If

so, treatment and anti-tumor effects might be improved by preselection of patients on the basis of their treatment history. Therefore, we tested if chemoresistance leads to changes in the HSV-tk/GCV-induced BE in a human small cell lung cancer cell line, GLC_4, and its sublines with *in vitro* acquired resistance to adriamycin (GLC_4/ADR), mitoxantrone (GLC_4/MITO) and cisplatin (GLC_4/CDDP).

Our results showed that resistance to adriamycin, mitoxantrone and cisplatin led to about a 2-fold decrease in sensitivity to GCV of the resistant GLC_4 sublines, as compared to the GLC_4 parental cell line. Multiple studies have already established that the occurrence of chemoresistance has a variety of causes. In our cell line model it has been shown before that, among other factors, adriamycin resistance led to an increase in GSH expression (14) and an increase in DNA repair (11), mitoxantrone resistance led to an increase in GSH expression (unpublished data) and cisplatin resistance led to an increase in GSH expression (15) and an increase in DNA repair (13). The resistance to GCV might, therefore, be due to the increased intracellular gluthathione levels or the increase in DNA repair in the chemoresistant GLC_4 cells. Lai *et al.* have shown that intracellular glutathione levels can influence the sensitivity for nucleoside analogues (16), whereas Tomicic *et al.* (17) have demonstrated the role of DNA repair in GCV resistance. However, in these cell lines GCV sensitivity depends on the activity of endogenous TK. Substantial cell kill was only obtained with GCV doses that are probably not clinically applicable.

To determine the HSV-tk/GCV-induced BE, we used 25 μg/ml GCV in each experiment. With this concentration, we aimed at maximal cell kill of C6-rTK-18 cells and minimal cell kill of GLC_4 cells. Interestingly, all GLC_4 cell lines showed a significant HSV-tk/GCV-induced BE when mixed with various percentages of C6-rTK-18 cells. Previously published results also showed a sufficient BE between xenogenic cells (18). The use of xenogenic cells with human tumor cells could be of clinical interest, because xenogenic cells could increase the BE by preclinical manipulation *in vitro* and *via* induction of an immune response. A contribution of the immune system to the induction of the BE has been demonstrated before (19).

One of the problems in clinical trials with HSV-tk/GCV gene therapy is to obtain sufficient transfection of tumor cells. Hence, in our cell line model, a higher percentage of HSV-tk-positive cells did not correlate with a higher BE in the GLC_4/MITO cell line. As compared to the GLC_4 parental cell line, the BE was significantly increased for GLC_4/ADR, if mixed with 50% or 75% C6-rTK-18 cells, but significantly decreased when GLC_4/CDDP cells were mixed with 25% C6-rTK-18 cells. Because of the reduced sensitivity of the resistant GLC_4 cells, one would expect a smaller BE for all resistant cell lines. Hence, factors other than GCV sensitivity may have a larger contribution to the BE.

Differences between cell lines in the HSV-tk/GCV-induced BE could be due to differences in the expression of drug efflux pumps, as demonstrated by Adachi *et al.* (8). We determined whether the differences in HSV-tk/GCV-induced BE that we had found were due to differences in expression of MRP4 and MRP5, two nucleoside analogue drug efflux pumps. However, with the antibodies used, no detectable expression of MRP4 and MRP5 was observed. It seems, therefore, unlikely that changes in the expression of these drug efflux pumps could explain the differences seen in our cell line model.

The differences in the HSV-tk/GCV-induced BE could also be due to differences in Cx's or in GJIC. GJIC and the expression of Cx's are important determinants of the BE (4). Cx43 is a gap junction protein that is commonly expressed on epithelial cells of the lung (20). Neoplastic transformation of lung tumor cells is associated with a decline in GJIC and a decline in Cx43 expression (21). Interestingly, an increase in sensitivity to adriamycin was seen after transfection of an ovarian carcinoma cell line with Cx43 (22). In addition, up-regulation of GJIC with cyclic adenosine monophosphate increased adriamycin sensitivity (23). In our study, all cell lines expressed Cx43 and no obvious differences in protein expression were observed. The latter was not due to differences in protein loading. Additionally, no difference in BE was obtained after modulation of GJIC with the long-term GJIC blocker AGA. Hence, in our cell line model, chemoresistance did not lead to differences in expression of Cx43 and the obtained BE seems not to be induced *via* GJIC.

Although, GJIC is the most acknowledged contributing factor to the HSV-tk/GCV-induced BE, there are previously published *in vitro* studies in which no effect was seen on the BE by modulation of GJIC in cell lines derived from lung cancer (24). Drake *et al.* (25), demonstrated a Cx-independent BE which was induced *via* transport of GCV metabolites into the extracellular medium. Additionally, the BE could also be induced *via* the transport of apoptotic vesicles (3). Further experiments have to be performed to elucidate the underlying mechanisms of the differences in HSV-tk/GCV-induced BE between our various chemoresistant cell lines.

In conclusion, our results show that chemoresistance can increase (GLC_4/ADR) or decrease (GLC_4/CDDP) the HSV-tk/GCV-induced BE in a SCLC cell line model. The differences found cannot be explained by the expression of MRP4, MRP5 and Cx43. Also, the BE did not depend on GJIC in any of the four GLC_4 cell lines. In the future, treatment and anti-tumor effects might be improved by preselection of patients for HSV-k/GCV gene therapy on the basis of their treatment history.

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