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Viral vectors carrying a marker-suicide fusion gene (TK-GFP) as tools for TK/GCV –mediated cancer gene therapy

Research Article

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Summary

A herpes simplex thymidine kinase – green fluorescent protein (TK-GFP) fusion gene was constructed to couple a marker gene to a therapeutic gene. For testing the utility of the fusion gene, it was cloned into four different viral vectors: Semliki forest virus (SFV), Sindbis virus, adenovirus and lentivirus. The produced viral TK-GFP vectors were then used to test their transduction efficiency on different tumor cells and especially the relationship between TK gene transfer and treatment result with prodrug ganciclovir (GCV). When the efficiency of the other three viral vectors were compared to adenoviral vector, a commonly used tool in cancer gene therapy, the alphaviral vectors SFV and Sindbis performed better on glioma cells, but were less efficient on renal carcinoma cells. On the other hand, an HIV-1 –based, VSV-G pseudotyped lentiviral vector was efficient on all cell lines tested, suggesting the potential of this vector type for cancer gene therapy. GCV-sensitivity studies revealed that regardless of the vector type used, the treatment result was directly proportional to the gene transfer efficiency (not to the multiplicity of infection). However, good gene transfer rate alone is not always sufficient: twenty- percent efficiency was enough to cause adequate cytotoxicity with 5 μg/ml GCV in both of the glioma cell lines, whereas even higher than 80% transduction was not enough to successfully treat Caki-2 cells under the same conditions. When the bystander effect was examined, Caki-2 cells were shown to display a much weaker effect than the glioma cells. Our results demonstrate the benefit of TK-GFP fusion gene in cancer gene therapy research and emphasize the importance of finding an appropriate vector type for each tumor, as well as testing the presence of bystander effect before continuing with TK/GCV approach.

I. Introduction

Suicide gene therapy with herpes simplex virus type 1 thymidine kinase (HSV-TK) and ganciclovir (GCV) has been one of the most widely used and well-studied paradigm in cancer gene therapy research. In this approach, HSV-TK gene is transferred to cancer cells, followed by destruction of the TK-positive cells through administration of non-toxic doses of GCV (Moolten, 1986). Another desired feature of this gene therapy approach is that some tumor cells display so called bystander effect (Moolten, 1986;Culver et al, 1992). This means that affected cells can share the cytotoxic effect of the treatment with their neighbors and therefore enhance the initial cytotoxicity. In addition to a vast number of preclinical studies *in vitro* and *in vivo*, the TK/GCV approach has been tested several times in clinical trials as

well (for review, see (Morris et al, 1999)). Despite the extensive use of this modality and encouraging results from many *in vivo* studies, we are still waiting for an actual clinical breakthrough.

A fundamental problem in gene therapy is insufficient gene transfer and it is likely to be the major reason for modest clinical success with TK/GCV suicide gene therapy. Numerous attempts have been carried out to increase the gene transfer efficiency, for example by making the TK-carrying vectors replication competent (Wildner et al, 1999). Even though improved vector systems may provide better gene transfer efficiency, the amount of gene transfer required to achieve specific results is largely unknown. Smiley and co-workers stated that, especially in the context of TK/GCV therapy, the inability to accurately quantitate the transgene transfer efficiency is "a consistent barrier to understanding the mechanisms of retroviral action" (Smiley et al, 1997). This is certainly true also in case of other vector types and many times it is not known, how extensive gene transfer efficiency is needed for a sufficient treatment result with GCV. We wanted to study this relationship and directly demonstrate the efficiency of gene transfer under various conditions and its correlation to the treatment result achieved. The most reliable way to determine this would a therapeutic protein that is easy to detect. Since antibody -based detection methods of HSV-TK are not very practical, we reasoned that by coupling a marker gene to TK could solve the problem.

To directly couple a marker gene to HSV-TK, we constructed a fusion between TK and green fluorescent protein (GFP). This construct produced a functional fusion protein that had the activities of both components, sensitizing the target cells to GCV and emitting green fluorescence under UV light (Loimas et al, 1998). After this proof-of-principle phase, we wanted to test the utility of TK-GFP by using it in viral vectors for studying the features that affect the outcome of TK/GCV cancer gene therapy. Three different TK-GFP -carrying viral vectors, Sindbis virus-, Semliki forest virus (SFV)- and lentivirus vectors were compared to respective adenovirus vector by transducing three different tumor cell lines, followed by analysis of gene transfer efficiency and its relationship to treatment result with GCV. This paper shows the utility of TK-GFP fusion construct and emphasizes the fact that the gene transfer efficiency has the major impact on the success of TK/GCV –mediated cancer gene therapy.

II. Results

A. Viral TK-GFP vector production

After cloning the improved version of TK-GFP construct (Wahlfors et al, 2000) into different vector plasmids, viral vector preparations were produced following the standard protocols described in Materials and Methods. AdenoTGL was produced in 293 cells and the final clone obtained by plaque purification. The titer of the preparation was 1 x 10^9 pfu/ml. LentiTGL was produced in 293T cells, yielding titers of 1-2 x 10^6 iu/ml (as measured by the FACS method). SinTGL and SFVTGL production was carried out in BHK cells as described in (Wahlfors et al, 2000). The titers obtained by FACS were 1 x 10^7 – 1 x 10^8 iu/ml for both vectors.

According to our earlier results (Wahlfors et al, 2000), it is possible that formation of replication competent alphaviruses (RC V) occur at a high frequency. We therefore tested our SFVTGL and SinTGL stocks by serial passaging on BHK cells. As observed before, SFVTGL was devoid of any RC V, whereas SinTGL contained approximately one RC V per 10^4 biologically active vector particles. We have found that RC V, when low MOI is used, can spread efficiently and kill BHK cells, but the effect is much more subtle in BT4C or 9L cells and cannot be detected in human cells at all (Wahlfors et al, unpublished results). Therefore, it is unlikely that replication competent viruses in SinTGL preparations would distort our results from 9L, BT4C and Caki-2 cells.

B. Characterization and comparison of the inducible promoter expression vector

To get a rough idea of the utility of SFV-, Sindbis- and lentiviral vectors compared to the commonly used adenoviral vector in cancer cells, gene transfer efficiency and level of TK-GFP expression were determined on BT4C- and 9L cells (rat brain tumor cell lines), and Caki-2 cells (human kidney tumor cell line). Only low multiplicities of infection (MOI 0.3 and 3) were used to mimic the situation in *in vivo* gene transfer studies, where transduction efficiencies are usually quite modest. Cells were incubated for either 20 hours (SinTGL and SFVTGL) or 96 hours (AdenoTGL and LentiTGL) after transduction, followed by FACS analyses.

1. Transduction efficiency (Figure 1)

All viral vectors tested were capable to transduce all three cell lines to some extent, but the difference between rat and human cells was clear in the case of adenoviral vectors. As expected, AdenoTGL did not perform well in rat cells. MOI 0.3 yielded only 1.4 % positive BT4C cells and < 1 % positive 9L cells. On the other hand, the human renal carcinoma cell line Caki-2 was a very good target for AdenoTGL vector, which transduced 24 % of the cells, when MOI 0.3 was used. SFVTGL was relatively efficient on all three cell lines and was the most efficient vector type in 9L cells. SinTGL was poor on the human cell line Caki-2, but showed better performance in the rat cell lines and was the best vector for BT4C cells (13 % efficiency at MOI 0.3). LentiTGL was equally good for all three cell lines, being almost comparable to the best vector type in each case, especially when MOI 3 was used (51% for BT4C cells and 68% for both 9L and Caki-2 cells).

2. Expression level of TK-GFP.

Data from flow cytometer was also analyzed in terms of the mean fluorescence of the TK-GFP –positive population, indicating the level of transgene expression. In all three cell lines, the pattern was similar: both alphaviral vectors displayed a very high (yet transient) gene expression, whereas lentiviral vector yielded much lower mean fluorescence that intensified during the first week after transduction and was stable over time (results not shown). AdenoTGL-based expression was not as high as that of SFVTGL or SinTGL in the human renal carcinoma cell line Caki-2, and even lower than LentiTGL –based expression in rat glioma cells. The expression pattern of each vector type is demonstrated with FACS data from BT4C cells in **Figure 2**.

C. Ganciclovir sensitivity of the cells transduced with TK-GFP vectors

To study the relationship between gene transfer efficiency and treatment result, we selected the adenoviral vector, since it was shown to yield varying transduction rates on different target cell types (see the previous section). This experiment was carried out by transducing 9L, BT4C and Caki-2 cells with different amounts of AdenoTGL (MOI 0.1 – 10), followed by incubation in the presence of GCV (from 0.1 to 1000 µg/ml) and cell viability analysis by MTT-assay. The use of TK-GFP made possible to determine gene transfer efficiencies accurately and measure the proportion of positive cells obtained at each MOI. Therefore, we were able to precisely determine the transduction percentage needed for cancer cell destruction under clinically relevant GCV concentrations (**Figure 3**).

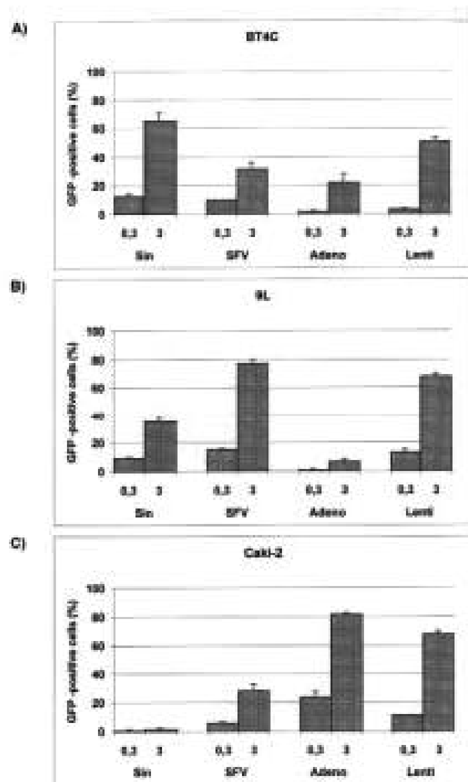

Figure 1. Transduction efficiencies with different viral vectors. BT4C (A), 9L (B) and Caki-2 (C) cell lines were transduced with Sindbis-, SFV-, adenoviral- and lentiviral vector carrying TK-GFP gene. Two different multiplicities of infection, 0.3 and 3, were used. Proportion of GFP –positive cells (%) was determined by FACS analysis 20 h (SinTGL, SFVTGL) or 96 h (AdenoTGL, LentiTGL) post-transduction. The results are mean of three different transductions, standard deviation markers are indicated.

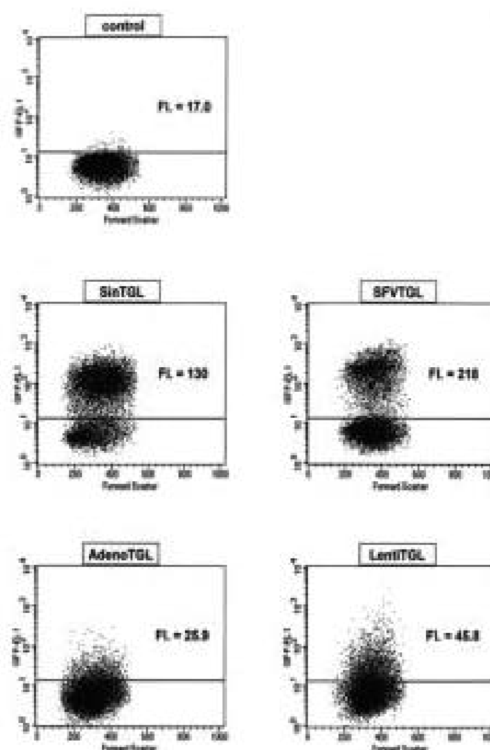

Figure. 2. TK-GFP expression levels with different viral vectors. BT4C cells transduced with different viral vectors were analyzed by FACSCalibur flow cytometer to obtain dot blot: forward scatter (X-axis) versus GFP fluorescence (Y-axis). Control = untreated BT4C cells. The numeric value of the mean fluorescence intensity of the GFP positive population (Fl.) is indicated in each panel.

As demonstrated in the previous experiment, low MOIs of AdenoTGL transduced rat glioma cells, especially 9L cells at low rate. This can be seen as low or moderate transduction efficiency, even at MOI 3 or 10 (25% or 61% in BT4C cells and 5% or 15% in 9L cells, respectively). On the other hand, Caki-2 cells were very permissive to adenoviral vectors and the respective efficiencies on these cells were 72% and 89%. As shown in **Figure 3.**, the cytotoxicity of GCV correlates well with the actual transduction efficiency.

In order to determine the sufficient gene transfer efficiency for satisfactory treatment result, we used the following criteria: what is the gene transfer percentage, where 5 µg/ml GCV destroys 60 – 80% of the transduced cells? This ganciclovir concentration is comparable to the standard human dose 5 mg/kg/day and thus is relevant to the dose response for *in vivo* settings. In BT4C cells, this was achieved at MOI 2 - 3, yielding about 20% positive cells. Nine L cells were showing barely satisfactory cytotoxicity only at MOI 10, which was due to the fact that these cells are somewhat resistant to adenoviruses (the transduction efficiency was 15%, although 5 times more AdTGL was used for 9L cells than for BT4C cells). Nevertheless, in both rat glioma cell lines, the cell destruction was remarkably higher than the actual transduction percentage, suggesting an efficient bystander effect present in these cells.

Despite high adenovirus vector –mediated gene transfer efficiency, Caki-2 cells were not killed effectively with 5 µg/ml GCV. These cells were clearly more resistant to GCV, since the control (non-transduced) cells are not fully destroyed in 1000 µg/ml GCV. This may reflect lower proliferation activity of Caki-2 cells compared to the glioma cell lines. When observing cytotoxicity with GCV concentration 10 µg/ml (**Figure 3**, panel C), it appears that only about 20% more cells were destroyed than expected on basis of the proportion of TK-GFP positive cells. For example, MOI 0.6 yielded 24% positive cells and about 40% of the cells died (the respective numbers at MOI 3 in BT4C were 25% and >90%). These results indicate that Caki-2 cells displayed a weak bystander effect in comparison with the rat glioma cells.

We also tested the ability of other viral vector types to induce cell death upon GCV administration. BT4C or Caki-2 cells were subjected to low MOI (0.3) of LentiTGL, SFVTGL or SinTGL followed by treatment with increasing concentrations of GCV. This experiment revealed similar, transduction efficiency-dependent and vector-independent cytotoxic effect that was again weaker in Caki-2 cells than in BT4C cells (results not shown).

D. Bystander effect in BT4C, 9L and Caki-2 cells

Results from GCV sensitivity experiments indicated that there are differences in the level of bystander effect present in the cell lines studied. We decided to test the extent of bystander effect in a way where the proportion of TK-GFP positive cells remains the same and the density of the cells changes. In this experiment, when certain threshold density is reached, the cell-cell contacts start to form, manifesting the bystander effect. In comparison with the "classical" bystander analysis (where increasing percentage of TK-positive cells at the same density is used), this method is only qualitative and tells, whether the bystander effect exists or not. However, this assay can also reveal the presence of a cell contact -independent effects,

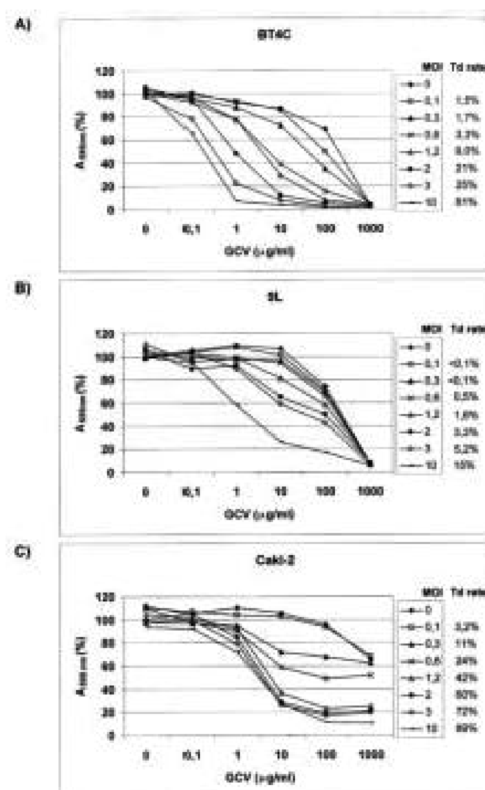

Figure. 3. Ganciclovir sensitivity of the cells transduced with TK-GFP vectors. BT4C (A), 9L (B) and Caki-2 (C) cell lines were transduced with indicated multiplicities of infection (MOI) of AdenoTGL, followed by determination of transduction rate (Td rate, percent of GFP positive cells). Transduced cells were then subjected to increasing concentrations of ganciclovir (GCV) for 5 days, followed by cell viability analysis using MTT-assay. The results are shown as percent of the A_{595nm} –value from the assay, the negative control (non-transduced cells) being 100%. Each data point is mean of three different analyses, standard deviation markers have been omitted for clarity.

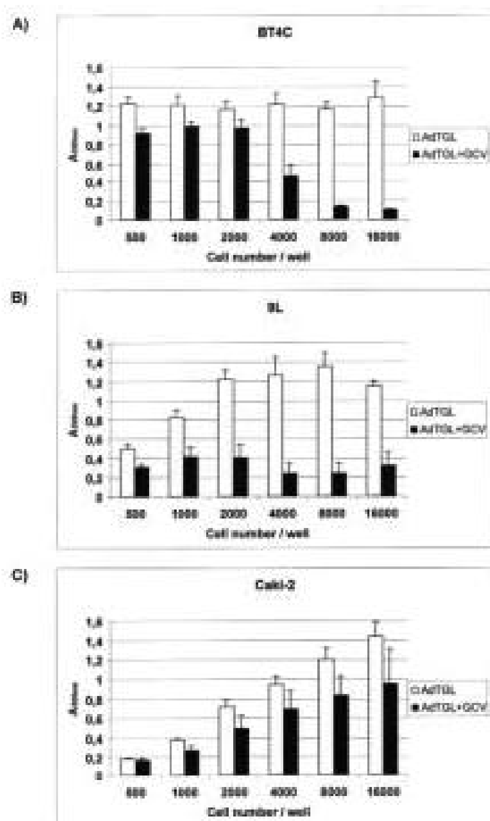

Figure 4. Bystander effect in the cell lines. BT4C (A), 9L (B) and Caki-2 (C) cell lines, containing 20% of TK-GFP positive cells, were split in 96-well plates using the indicated densities (X-axis). The cells were then grown with (AdTGL+GCV) or without (AdTGL) 5 µg/ml GCV for five days, followed by cell viability analysis using MTT-assay. The results are shown as A_{595nm} –values from the assay and they are means of three different analyses, standard deviation markers are indicated. The presence of bystander effect is demonstrated as higher proportion of cytotoxicity than the original 20% (of TK-GFP cells present in the cell population). The threshold density of all the cells (i.e. cells starting to form physical contacts) was between 2000 and 4000 cells per well.

where the GCV-induced cytotoxicity is amplified by indirect contact. The idea of this type of analysis has been described before (Samejima and Meruelo, 1995).

In this experiment (**Figure 4**), the proportion of TK-GFP positive cells (created by AdenoTGL transduction) in each population was set to 20% and the cells were incubated in different cell densities in the presence of 5µg/ml GCV for five days. The negative controls (non-transduced cells treated with GCV) did not reveal any significant cytoxicity of ganciclovir *per se* (results not shown). As expected, the bystander effect was obvious in BT4C cells, where the amount of living cells decreased when the cell density increased. This effect was not seen before the cells grew close enough to each other (between 2000 and 4000 cells per 96-well plate well), suggesting that these cells displayed the classical, cell contact –dependent bystander effect. In addition, 9L cells displayed a strong bystander effect, but it was detected also in low cell densities, where there were no cell-cell contacts. As shown in panel B of **Figure 4**, the cell destruction was slightly amplified after the threshold cell density (2000-4000 cells) was reached, yet the difference was not statistically significant. This indicates that the dominant form of bystander effect in 9L cells is cell contact -independent, but the cell contact –mediated effect may be present as well. As opposed to BT4C and 9L rat glioma cells, Caki-2 human renal carcinoma cells didn't show any significant increase of cell death, when the number of cell-cell contacts increased (**Figure 4**, panel C). These data confirm that the bystander effect in Caki-2 cells is weak in comparison to rat glioma cells.

III. Discussion

To assess the extent of gene transfer efficiency sufficient to provide a satisfactory TK/GCV –mediated cancer gene therapy, we created a TK-GFP fusion construct, TGL. This fusion was shown to make the target cells fluorescent under UV light and sensitize them to GCV at concentrations similar to native TK (Loimas et al*,* 1998). After the functionality of the fusion construct was verified, it was cloned into several viral vectors. In this paper, we report how these TGL –carrying viral vectors were used *in vitro* to study the features of TK/GCV suicide gene therapy approach. We had two major tasks in this research: 1. To get a rough idea how well three viral vector types (SFV, Sindbis and lentivirus) perform on model tumor cell lines compared to first generation adenoviral vector. 2. With the aid of TK-GFP –carrying viral vectors to find out, what is the true transduction efficiency needed for sufficient destruction of different cancer cells with GCV and what is the exact role of bystander effect in the treatment outcome.

Our results from transduction efficiency analyses were in line with existing published data. First, type 5 human adenoviruses are good vectors for human cells but for many rodent cells, high MOIs were needed to obtain similar transduction efficiency. Secondly, alphaviral vectors (especially Sindbis virus) may not be promising vectors for all human tumor cells based on low transduction efficiency of Caki-2 in this report and similar results with HeLa (human cervical carcinoma) and HepG2 (human hepatoma) cells (Wahlfors et al*,* 2000). Thirdly, lentiviruses appeared to be promising vectors for cancer gene therapy, as they worked equally well on every tumor cell type tested. As many solid tumors contain large masses of quiescent or very slowly dividing cells, lentiviral vectors could be suitable for *in vivo* cancer gene therapy studies. The actual reasons for different transduction rates with different cell lines were not studied, but it is likely that the viral receptor status of the cells plays a role.

It is highly unlikely that the differences in transduction efficiency in the tested cell lines are relevant to *in vivo* situation. The purpose of these experiments was solely to map the potential of certain viral vector types that have not been tried on tumor cells extensively and may be useful for further cell culture and animal work. We are aware of the fact, for example, that different viral vectors are titrated using different target cells and transduction protocols. Therefore, their efficiencies cannot be compared directly and readers are asked to be cautious about the conclusions that can be made based on the results shown in **Figure 1**.

Varying gene expression levels (for example, in rat glioma cell line BT4C, see **Figure 2**) apparently reflect the nature of the vectors and the strength of the promoter used. Alphaviral vectors typically display a very powerful expression due to the replication cycle that amplifies the subgenomic sequence (which is transgene in recombinant vectors). Therefore, alphaviral expression is usually significantly higher than from any known promoter that utilizes eukaryotic RNA polymerase II. Promoters present in the adenoviral- and lentiviral vectors (hEF1 and CMV, respectively) are considered strong, but when low MOIs are used, multiple transduction events per cells are rare and most of the positive cells contain a few or only one TK-GFP expression unit. This is especially relevant explanation for the adenoviral vector (MOI 0.3) that yields only a few percent transduction efficiency on rat BT4C cells.

The studies that determine transduction efficiency of different viral vectors can naturally be carried out with vectors that contain GFP alone. Therefore, the genuine utility of the TKGFP fusion gene system is demonstrated in GCV –sensitivity assays (**Figure 3**). In this experiment, similar multiplicity of infection with the TK-GFP vector on different cell lines yielded very different transduction efficiencies. If this kind of analysis was carried out using a vector carrying TK only, the actual gene transfer efficiency would have been difficult to determine and the reasons for treatment outcome would be more difficult to explain.

The ganciclovir sensitivity experiments revealed a strict correlation between gene transfer efficiency and cytotoxicity, but the strength of bystander effect also appeared to play major role. AdenoTGL -transduced BT4C glioma cells were destroyed efficiently with low concentrations of GCV, because the transduction rate was high enough and bystander effect strong in these cells. The other glioma cell line 9L did not respond as well, although the bystander effect was strong. This was clearly attributable to modest transduction efficiency of adenoviral vectors. The third case was human renal carcinoma cell line Caki-2, which was very permissive to adenoviral vectors (almost 90% transduction rate with MOI 10) and displayed a weak bystander effect. In addition, these cells were responding poorly to low GCV concentrations, regardless that most of the cells were TK-GFP positive. It is possible that this is due to low proliferative activity of this cell line.

Adenoviral vector was used in the GCV sensitivity experiment to demonstrate the differences in treatment efficacy caused by different gene transfer rates. In order to test whether TK-GFP behaves differently in other viral vectors, SFVTGL, SinTGL and LentiTGL were tested in BT4C and Caki-2 cells using the same GCV sensitivity protocol. It turned out that regardless of the vector type carrying TK-GFP, the GCV-induced cytotoxicity was dependent on the gene transfer efficiency. We didn't observe an effect of the higher TK-GFP expression level from alphaviral vectors or lower levels from the lentiviral vector. This in accordance with a report about variable effect of TK/GCV approach in different tumor cells. Beck and co-workers show that TK expression level is insignificant to the treatment result and there are other factors governing the susceptibility of the cell to TK/GCV effect (Beck et al, 1995). Another study by Chen et al (Chen et al, 1995) that used retrovirus-TK -transduced 9L cells, demonstrated that cell clones containing different TK enzyme levels give a similar GCV sensitivity. On the other hand, a study with C6 glioma cells (Shewach et al, 1994) concluded that increasing MOI (of retrovirus TK) increases the GCV sensitivity of the target cells. Alltogether, these findings suggest that the level of TK expression plays a role in the efficiency of this form of cancer gene therapy, but it is limited to situations, where GCV concentration and the proportion of TK-positive cells are low. We think that TK expression level is unlikely to be a factor in the primary TK-transduced cells, but may plays a major role in the fate of the neighboring cells i.e. the effect of the TK expression level is transmitted through the bystander effect.

The bystander effect in our experiments was detected in changing the cell density instead of the proportion of TK positive cells. This method can reveal both cell contact –mediated and cell contact –independent effects. In our studies (see **Figure 4.**), we found three different types of bystander effect: a strong, cell contact -mediated (BT4C cells), a weak, cell contact -mediated (Caki-2 cells), and a mixed type, with both cell contact -dependent and cell contact –independent components (9L cells). Although Princen and co-workers have recently shown that bystander effect in 9L is strictly dependent of cell-cell contacts (Princen et al, 1999), their experimental approach was different and does not rule out the possibility of mixed-type bystander effect in these cells. In addition, Bai et al. have shown the presence of cell contact –independent, apoptosis-mediated bystander mechanism in another rat glioma cell line C6 (Bai et al, 1999).

Nine L cells have been studied extensively as targets for TK/GCV suicide gene therapy and their bystander effect has been reported. Based on the dye transfer results by Princen et al, it is likely that at least part of the effect is gap junction -mediated (Princen et al, 1999). Also, connexins 26 and 43 have been shown to be expressed in

9L cells, yet overexpression of connexin 43 further enhanced the bystander effect in these cells (Estin et al, 1999). There are some published TK gene transfer studies on BT4C cells (Poptani et al, 1998;Sandmair et al, 1999) and the most recent one is indicating that there is a strong bystander effect in these cells (Sandmair et al, 2000). However, none of the papers indicated, whether the effect is connexin dependent. The same applies to Caki-2 cells that have not been studied as targets for TK/GCV –mediated gene therapy.

Altogether, our results demonstrate that it is important to select the correct viral vector for each tumor type, and that the bystander effect must be present in the target cancer for optimal cell killing. Based on the data presented here, we suggest that TK/GCV approach should be considered only if the tumor cells have an efficient bystander effect and the viral vector in use can give at least 20% gene transfer efficiency. As shown in Caki-2 cells with a weak bystander effect, transduction efficiency as high as 90% is not enough at clinically relevant GCV concentration ranges.

More importantly, our studies demonstrate the utility of TK-GFP as a tool in cancer gene therapy. In this paper, we have shown that viral vectors carrying this fusion construct can give valuable information about the features of TK/GCV –mediated cancer cell destruction in cell cultures. We are currently carrying out experiments in an animal model to seek answer to the most crucial question: how much gene transfer can we get *in vivo* with different viral vectors and is that enough for a sufficient GCV treatment result?

IV. Materials and Methods

A. Plasmid constructs and viral vectors

The plasmid pETLGB containing TK-GFP fusion gene has been described earlier (Loimas et al, 1998). Due to a suboptimal emission wavelength for FACS analyses, the construct was modified by replacing the original GFP by the improved GFP from plasmid pGreenLantern (Life Technologies, Gaithersburg, MD) (Wahlfors et al, 2000). Shortly, the stop codon of the HSV1-TK was removed and a sequence encoding 11 amino acid linker (LEU ARG ASP PRO MET ALA ARG ALA ALA ALA THR) was added in-frame between the TK and GFP open reading frames. The second generation TK-GFP fusion gene (TGL) was subsequently cloned into following viral vectors using standard procedures: adenoviral vector pAVC2 (Ramsey et al, 1998) (the resulting vector: AdenoTGL), HIV-1 based lentiviral vector pHR' (Naldini et al, 1996) (the resulting vector: LentiTGL), Sindbis virus pSinRep5 (Hahn et al, 1992) (the resulting vector: SinTGL) and Semliki forest virus vector pSFV1 (Liljestrom and Garoff, 1991) (the resulting vector: SFVTGL). The expression of TGL is driven by 26S subgenomic promoter in both alphaviral vectors SFV and Sindbis, whereas the promoter in the adenoviral vector is hEF1 (from human elongation factor 1 alpha –gene) and CMV (cytomegalovirus early promoter) in the lentiviral vector.

All the vectors were grown and purified according to manufacturers instructions (Sindbis and SFV, Invitrogen and Life Technologies, respectively) or the original publication: adenoviral vectors (Graham and Prevec, 1992), lentiviral vectors with VSV-G envelope and pCMV_R8.2 helper construct –derived virus particles (Naldini et al, 1996; Zufferey et al, 1997). Sindbis viruses were produced using DH-BB packaging RNA and SFV viruses with conditionally infective helper2 packaging RNA (Berglund et al, 1993). For details of alphaviral vector production, see (Wahlfors et al, 2000).

AdenoTGL vector preparation was titered on 293 cells, using the standard plaque assay method (Graham and Prevec, 1992). Titers of alphaviral and lentiviral vectors were determined with FACS analysis (FACSCalibur, Becton Dickinson, San Jose, CA, USA) by counting the proportion of GFP positive cells after viral transduction. Briefly, 1, 10 and 100 µl of viral supernatant was used for transduction of known number of BHK cells (alphaviral vectors) or 293T cells (lentiviral vectors) as described below. After appropriate incubation period, the transduced cells were analyzed by flow cytometry (triplicate samples, 10 000 events per sample). The titer was calculated from the percentage of fluorescent cells, using the value from transduction volume that yields 10-20% positives.

B. Cell lines

BT4C rat glioma cells (Laerum et al, 1977), 9L rat gliosarcoma cells (ATCC CRL 2200) and Caki-2 human renal carcinoma cells (ATCC HTB-47) were grown in high-glucose Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum, 2 mM glutamine, 2 mM sodium pyruvate and 50 µg/ml gentamicin at 37 °C in the presence of 5 % CO_2. BHK and 293T cells were grown as described in (Wahlfors et al, 2000).

C. Transduction of cultured cells

Viral transductions were carried out as follows: the target cells were split into 24-well plates (50 000 / well). After 20 hours of incubation, the cells were washed once with PBS and incubated with viral vectors (MOI 0.3 or 3.0) for 1 hour, followed by addition of fresh medium. Flow cytometry was carried out 20 hours (SinTGL and SFVTGL) or 96 hours (AdenoTGL and LentiTGL) post-transduction to determine the number of positive cells and the level of TK-GFP expression.

D. GCV sensitivity of transduced cells

Alphaviral vectors: The cells were split into 96-well plates (2 500 / well), incubated for 20 hours, washed once with PBS (containing Ca^{2+} and Mg^{2+}), incubated with viruses for 1 hour and exposed to different concentrations of GCV (0.1-1000µg/ml) for five days. To determine cell viability, MTT-assay was performed according to manufacturer's instructions (Cell Proliferation Kit I, Roche Diagnostics, Indianapolis, IN). Adeno- and lentiviral vectors: The cells were split into 6-well plates (200 000 / well), incubated for 20 hours, washed once with PBS and incubated with viruses for 1 hour. After 3 days incubation the cells were split into 96-well plates (2500 / well), incubated for 20 hours and exposed to GCV. FACS analysis was performed to determine the proportion of GFP positive cells i.e. the transduction efficiency with

each MOI used. After five days incubation with GCV, MTT-assay was carried out.

E. Bystander effect studies

For bystander experiment, the cells were transduced with a high MOI of AdenoTGL as described above and FACS analysis was performed three days post-transduction. Parental (non-transduced) 9L cells with or without GCV treatment were used as negative controls. Based on flow cytometry, the proportion of TK-GFP positive cells in each transduced culture was set to 20%. The cells were the divided into 96-well plates in different densities (500-32000 cells/well) in order to obtain cultures with varying extent of cell-cell contacts. After incubation with 5µg/ml GCV for 5 days, cell viability was measured by MTT assay. The bystander effect was demonstrated by a higher degree of cytotoxicity than the 20% that was based on the presence of positive cells in the culture.

F. Statistical analysis

Bystander effect experiments were analyzed with two-tailed *t* test. Differences were considered significant when the probability (P) was <0.05.

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