Fusion of herpes simplex virus thymidine kinase to VP22 does not result in intercellular trafficking of the protein

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Received December 15, 2006; Accepted January 22, 2007

Abstract. Suicide gene therapy is a promising approach for the treatment of cancer. Current protocols, however, suffer from low efficiency. We tried to alleviate this problem by developing a transgene that will spread from the initially transduced cell to the surrounding cells (transmission). We used herpes simplex virus (HSV) VP22 as a signal for cellular uptake of HSV-1 thymidine kinase (TK). By co-culturing naïve cells with cells producing a TK-VP22 fusion protein, we detected intercellular trafficking of this protein. We used a variety of techniques, including two-color flow cytometry and cytotoxicity assays to detect the presence of TK in the non-producing cells. We confirmed intercellular migration of VP22. We did not detect any intercellular trafficking of the TK-VP22 fusion protein, by various fixation methods or flow cytometry. In ganciclovir sensitivity assays, we found no difference between the efficiency of TK (IC_{50}=3.15±0.76 μg/ml) and TK-VP22 (IC_{50}=2.27±0.59 μg/ml). Using a cell-free enzyme activity assay we showed that fusion of TK to VP22 did not change the enzyme activity. In conclusion, we described novel and robust methods to detect intercellular trafficking. From our data we concluded that protein transmission of TK by VP22 for gene therapy is not likely to be successful. In addition, we described a useful and quantifiable assay to measure the enzymatic activity of TK and TK fusion proteins, and described some common properties of VP22 fusion proteins that may explain the different results that have been obtained by others.

Introduction

Gene therapy for cancer is a promising new approach to supplement the existing treatments for cancer that still fail to cure the majority of patients. Destroying all cancer cells is the ultimate goal in cancer gene therapy. Many proteins that are capable of killing cells have to be located inside of the cell to have an effect. Therefore, for cancer gene therapy to be effective, every cell has to receive the gene product. Although much effort has been invested in improving gene therapy vectors, current vectors are still largely ineffective because they fail to transduce a sufficient percentage of the target cells (1).

One well-explored approach in cancer gene therapy is suicide gene therapy. In this type of treatment the introduced transgene encodes a suicide enzyme. This suicide enzyme converts a non-toxic prodrug into a cytotoxic compound that kills the cell. In addition these enzymes often cause a so called bystander effect. Because the activated prodrug can spread beyond the cell producing the enzyme, a larger number of cells die, even if only a few cells receive the gene and produce the protein (2,3). In most clinical trials however, this effect has proven to be too small to compensate for the low efficiency of gene transfer (4,5).

In the presented research, we studied the suicide gene herpes simplex virus (HSV) thymidine kinase (TK) (6,7). The TK enzyme is able to phosphorylate the prodrug ganciclovir (GCV). Whereas the prodrug can freely pass cell membranes, the hydrophilic nature of the activated drug prevents this molecule from penetrating the cell membrane. This means that the activation needs to take place inside the cell (8). TK gene therapy seems very promising *in vitro* as well as *in vivo* (4), but has not yet been entirely successful in clinical trials (1,5,9-11).

Although improving the efficiency of the gene therapy vector would increase the number of cells reached by the therapy, reaching all cells will remain a problem, due to physical and immunological barriers present in tumors (12,13). Alternative techniques need to be developed to increase the number of cells affected. Spreading of the produced protein (called transmission), can be attained by introducing signal sequences in the transgene. The presence of these signals in the protein will enable it to spread to neighboring target cells. Secreted and targeted versions of suicide enzymes affect a larger number of tumor cells, if the enzyme is active outside the cancer cells (14,15). Many suicide enzymes, however, such as thymidine kinase need to be inside the cell to have an effect. In these cases, secretion alone is unlikely to be effective.

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Key words: protein transduction domain, intercellular trafficking, VP22 tegument protein, suicide gene therapy, fusion protein, HSV-TK

A number of proteins have been described that are capable of traversing the cell membrane of living cells, and enter the cytoplasm intact and active (16). Examples include the *drosophila* antennapedia protein (17), the HSV VP22 protein (18) and the HIV TAT protein (19,20). The sequences responsible for this unconventional internalization have been identified and termed protein transduction domains (PTD) or cell permeable peptides (CPP). A fusion protein consisting of a protein transduction domain and a heterologous protein often exhibits the transducing capacities of the PTD, in addition to the properties of the fusion partner (18,21,22). Although many theories have been proposed and tested, the mechanism behind the entry of these proteins is still largely unknown (23,24).

The VP22 PTD, taken from the herpes simplex virus (25), has been previously described to enable fusion proteins to migrate from the cells in which they are produced to the surrounding, non-producing cells (18). Transgenes encoding for suicide genes, including TK, fused to the VP22 PTD, have already been used successfully *in vitro* and *in vivo* to enhance the effect of suicide gene therapy (26-28). However, conflicting results have also been published concerning the ability of VP22 to cause transmission in living cells in culture and *in vivo* (29-31). The combined literature available on this subject has led us to further investigate the utility of VP22 to facilitate the transmission of TK.

Through a variety of techniques we endeavored to determine whether the VP22 PTD can be successfully used to enhance adenovirus-based thymidine kinase suicide gene therapy.

Materials and methods

Construction of plasmids and adenoviral vectors. Adenoviral vectors were constructed using the AdEasy system (32). All viruses were based on the shuttle plasmid pAdTrack-CMV, and include a green fluorescent protein (GFP) expression cassette for detection. The transgene expression is controlled by the cytomegalovirus (CMV) promoter. The resulting transgenes are depicted schematically in Fig. 1. The TK gene was isolated from pcDNA3-nTK (33) (a gracious gift from Dr G. Hospers), a pcDNA3-based plasmid bearing the cDNA for herpes simplex virus type 1 thymidine kinase, using PCR and custom-designed primers including the restriction sites. Primer sequences (restriction sites underlined) were: AT GGATCCACCATGGCTTCGTACCCCTGCCA and AT GCGGCCGCGTTAGCCTCCCCCATCTCCC. The resulting DNA fragment was either directly inserted into pAdTrack-CMV and pcDNA3.1 or first subcloned into pVP22/mycHis-2 (Invitrogen Corporation, Carlsbad, CA, USA), and subsequently into pAdTrack-CMV and pcDNA3.1, using the restriction sites shown in Fig. 1, resulting in the constructs AdTrack-TK, AdTrack-TK-VP22, pcDNA3.1-TK and pcDNA3.1-TK-VP22. As controls pcDNA3.1-VP22 and AdTrack-VP22, containing only the VP22 gene, were also constructed using the same strategy.

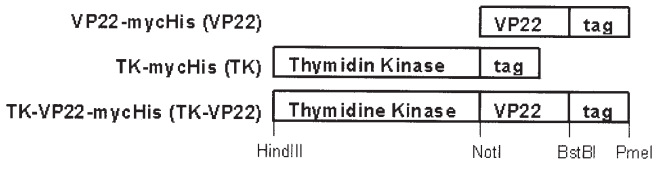

Figure 1. Schematical representation of constructed transgenes. The transgenes include a C terminal tag, consisting of the myc epitope followed by 6 histidines, for detection and purification purposes.

Viral DNA was produced in accordance with the AdEasy protocol by recombination of the shuttle plasmids with pAdEasy-1. The thus acquired DNA was transfected into HEK293 cells to produce virus. Adenoviral virus stock (crude lysate) was prepared by collecting and lysing infected HEK293 cells by repeated freezing and thawing. After centrifugation of this lysate the supernatant was used in all further experiments.

Cell culture. Human embryonic kidney cells HEK293 and glioma U373 cells [American Type Culture Collection (ATCC), Manassas, VA, USA] were cultured in DMEM/F12 (Gibco BRL, Life Technologies B.V., Breda, The Netherlands) supplemented with 10% foetal calf serum (FCS, Bio-Whittaker, Verviers, Belgium), 50 IU/ml penicillin, 50 μg/ml streptomycin and 2 mM L-glutamine (all from Gibco BRL).

Green monkey kidney COS-7 cells were cultured in DMEM (Gibco BRL) supplemented with 5% FCS, 50 IU/ml penicillin and 50 μg/ml streptomycin.

Transfection of cell lines with plasmid vectors. Cells were cultured in 6- or 24-well plates, to a confluency of 50-70%. Lipofectamine Plus (Invitrogen) was used according to the manufacturer's protocol to transform the cells with 1 or 0.4 μg respectively of the pcDNA3.1-based plasmids. After 3 h, transfection medium was replaced by a suitable volume of normal culture medium.

Adenoviral infections. Cells were cultured in 6-well plates to a confluency of 50-70%. The next day medium was changed to 1 ml of medium containing 2% FCS. Crude lysate containing virus was added to the medium, in a concentration determined prior to the experiment to result in ~90% infection, unless indicated otherwise in the results section.

Immunohistochemical detection. Cells were cultured in 24-well plates, transduced with adenoviral vector and fixed 2 days later with methanol or 4% paraformaldehyde for 10 min. After washing once with phosphate-buffered saline (PBS), the cells were stained by immunohistochemistry using a monoclonal antibody against the myc-tag (produced from hybridoma cell line Myc 9E10) (34) and a secondary antibody conjugated to peroxidase (rabbit anti-mouse IgG-HRP, DakoCytomation B.V., Heverlee, The Netherlands), followed by incubation with 3-amino-9-ethylcarbazole (AEC, Sigma-Aldrich Co., St. Louis, MO, USA).

Protein isolation and Western blot analysis. To verify correct expression and to compare the concentration of the fusion proteins, cell-free protein samples were prepared from infected cells. Cells were detached from culture plates by treatment with trypsin-EDTA (Invitrogen). The culture medium and cells were collected separately and subsequently centrifuged

for 5 min at 200 x g. The supernatant from the medium was used as the medium fraction of the isolated protein. Pellets from both fractions were combined, re-suspended in a volume of PBS, lysed by freezing in liquid nitrogen and cleared by centrifugation at 16,000 x g. The resulting supernatant was used as the cell lysate fraction. For Western blotting the samples were mixed with loading buffer (Laemmli sample buffer, Bio-Rad Laboratories B.V., Veenendaal, The Netherlands), boiled for 5 min and separated on SDS-PAGE gel and subsequently transferred to polyvinylidene fluoride (PVDF) membrane (Bio-Rad) by Western blotting. Fusion proteins were detected by immunohistochemistry using a monoclonal antibody against the myc-tag and a secondary antibody, conjugated to peroxidase (rabbit anti-mouse IgG-HRP, DakoCytomation B.V.). Blots were stained using AEC. The amount of protein was quantified in arbitrary units on Western blots using the GS-710 densitometer scanner and Quantity One program (Bio-Rad Laboratories). The detection range of this method and the correlation between staining intensity and the amount of protein were determined by loading increasing amounts of an unrelated myc-tagged protein. All samples were within the linear range of this method.

Flow cytometry. To determine whether TK fused to VP22 could be transmitted to neighboring cells, U373 cells were infected with the constructed adenoviruses to start production of the different fusion proteins. After 2 h the cells were washed 3x with PBS, and then detached with trypsin-EDTA (Gibco BRL). Naïve cells were also trypsinized, and naïve and infected cells were mixed in equal amounts and co-cultured for 2 days before preparing them for flow cytometry.

Prior to labeling cells for flow cytometry analysis, cells were detached with trypsin-EDTA, fixed with 4% paraformaldehyde and permeabilised with 0.2% Triton X-100 (Sigma-Aldrich) in PBS. Subsequently, cells were stained with a monoclonal antibody against the myc-tag and a secondary antibody, conjugated to R-phycoerythrin (RPE) (rabbit anti-mouse IgG-RPE, DakoCytomation B.V.). Both GFP fluorescence of the infected cells and RPE fluorescence of the TK-containing cells were detected by flow cytometry (Coulter EPICS Elite flow cytometer). The flow cytometer was calibrated using samples that contained only GFP fluorescence, only myc-tagged protein stained with RPE, or both.

Because the exact amount of infected cells was expected to vary between the samples, all data were expressed as the number of RPE-positive cells divided by the number of GFP-positive cells.

Based on the literature and previous experiments (results not shown) we expected the fixation and permeabilization procedure to cause protein to leak out of the cells. This protein might have subsequently bound to cells that did not contain any protein. This effect would have caused an overestimation of the intercellular spread of the protein. To correct for this effect, a separate control was performed for each sample. In this control sample, the cells were not grown together, but mixed immediately before the fixation procedure.

Because this leaching effect was not equal in all samples, due to the variations in protein production, size and charge, the transmission was corrected for this difference by expressing it as the part of the spreading effect that was not caused by leaching during fixation.

Prodrug sensitivity assay. U373 cells were infected with virus in 24-well plates. After 2 h the cells were washed with PBS and detached with trypsin-EDTA, then mixed 1:10 with naïve cells and plated in 96-well plates. GFP expression of each sample was measured using an FL500 microplate fluorescence reader (Bio-Tek instruments Inc., Winooski, VT, USA), to verify that all samples were infected by the adenoviruses to the same extent. Medium was changed to complete medium containing 0-1000 μg/ml ganciclovir (Cymevene, Roche Nederland B.V., Woerden, the Netherlands). Cell survival was appraised after 4 days, using the CellTiter 96 assay (based on the mitochondrial conversion of 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, MTS), performed as recommended by the manufacturer (Promega, Leiden, The Netherlands).

Enzyme assay. The activity of the TK fusion enzymes in crude cell lysates was determined as described by Hinds and colleagues (35), using [18F]FHBG as a substrate. The amount of myc-tagged protein was determined by Western blotting, and the amount of cell lysate for each sample was calculated such that each contained the same amount of TK. The following standard reaction mixture was used: 25-100 μl of crude cell lysate, 20 mM potassium phosphate (pH 7.6), 40 mM KCl, 25 mM NaF, 5 mM $MgCl_2$, 1 mM dithiothreitol (DTT), 5 mM adenosine triphosphate (ATP) and 0.5 mg/ml bovine serum albumin (BSA) in a total volume of 400 μl. This mixture was treated with thymidine phosphorylase for 45 min at 37˚C to remove any thymidine that might be present in the samples and compete with the tracer. TK activity was determined by incubating [18F]FHBG in the reaction mixture at 37˚C (~0.17 nM, specific activity ~54,000 GBq/mmol). At different time points, 50-μl samples of this mixture were loaded on a Whatman DE-81 filter. Phosphorylated and therefore negatively charged [18F]FHBG was bound to these filters. Each filter was washed three times with ammonium formate and three times with 95% ethanol to remove unreacted [18F]FHBG. Radioactivity of the filters was counted with a γ–counter. At the end of the experiment, 50 μl of reaction mixture was loaded on a filter, and the activity of this filter was measured without washing (reflects both unchanged and phosphorylated [18F]FHBG). The unwashed filter was used to normalise the activity bound on the washed filters. [18F]FHBG phosphorylation as a fraction of the original amount of tracer was calculated by dividing the radioactivity (cpm) of the washed filters by the radioactivity of the unwashed filters. The initial conversion rate of the reaction was calculated by determining the derivative of the curve.

Statistical analysis. Results were tested for significance using the Student's paired t-test.

Results

Verification of recombinant proteins. To verify correct expression of the fusion proteins from the constructed

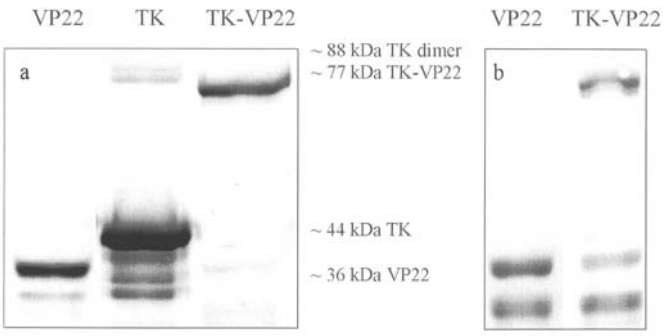

Figure 2. Verification of protein production. U373 cells were infected with AdTrack-TK, AdTrack-TK-VP22 or AdTrack-VP22. Protein was isolated from these cells after 48 h. Proteins were separated and visualized by Western blot analysis using monoclonal antibodies against either the myc-tag or TK. (a) Intact proteins; (b) protein samples stored at 4˚C showing breakdown of the VP22 fusion protein.

plasmids and adenoviruses, COS-7 cells were transfected with the constructed plasmids, or U373 cells infected with the constructed adenoviruses. The protein samples obtained from these cells showed correct expression of the proteins. Correct detection of TK by the anti-myc antibody was verified by detection with anti-TK antibody, which showed the same band (results not shown). Predominantly full-length protein was formed, but some breakdown products were also detected on the Western blot. Fig. 2 shows that some breakdown product can be observed in the TK-VP22 that has the same size as VP22. By using an anti-TK antibody, we confirmed that some of the TK-VP22 fusion product was cleaved between the two proteins, a phenomenon that has been previously reported for C-terminal VP22 fusion proteins by other authors (26,36,37). The amount of cleaved fusion protein increased over time when the protein was stored at room temperature (results not shown), indicating this is indeed a breakdown effect, and is not caused during protein production.

Immunohistochemical observations of transmission of VP22 and TK-VP22. To directly visualize the intercellular spread of the fusion protein, COS-7 or U373 cells were transfected with plasmids pcDNA3.1-TK, pcDNA3.1-TK-VP22 and pcDNA3.1-VP22, fixed after 48 h and immunostained for the myc-tag present in all fusion proteins. All transfections yielded an efficiency of ~5-10%. A distinct difference was visible between the different transfections, but also between different methods of fixation. Cells transfected with the VP22 plasmid that were fixed with methanol, showed a staining pattern with single heavily stained cells, surrounded by many cells of which only the nucleus was lightly stained (Fig. 3a), comparable to the staining pattern described in the literature for cells producing VP22. We did not observe a similar pattern for TK (Fig. 3b) or the fusion protein (Fig. 3c). In these samples we found only single stained cells. The localization of the VP22 fusion protein within the producing cells matched with earlier reports (38). When transfected cells were fixed with paraformaldehyde, only single cells could be found that contained the protein in all samples (Fig. 3d and f). Based on this experiment, and data from the literature, we concluded that no transmission of TK-VP22 occurred, or the amounts of protein internalized in the surrounding cells were too low to detect with this technique.

Detection of transmission of TK-VP22 by flow cytometry (Fig. 4). The amount of protein transmitted to non-producer cells might be too small to detect using conventional fixation and immunostaining procedures (39). Therefore we used flow cytometry to assess the transfer of protein from producer cells to non-producing cells. Each cell was analysed for GFP fluorescence whose presence indicated infection with the adenovirus, and for RPE fluorescence which indicated the presence of the myc-tagged fusion protein. Cells that contained RPE but no GFP must have received the protein from other cells.

All experimental data were given as the number of RPE-positive cells divided by the number of GFP-positive cells to

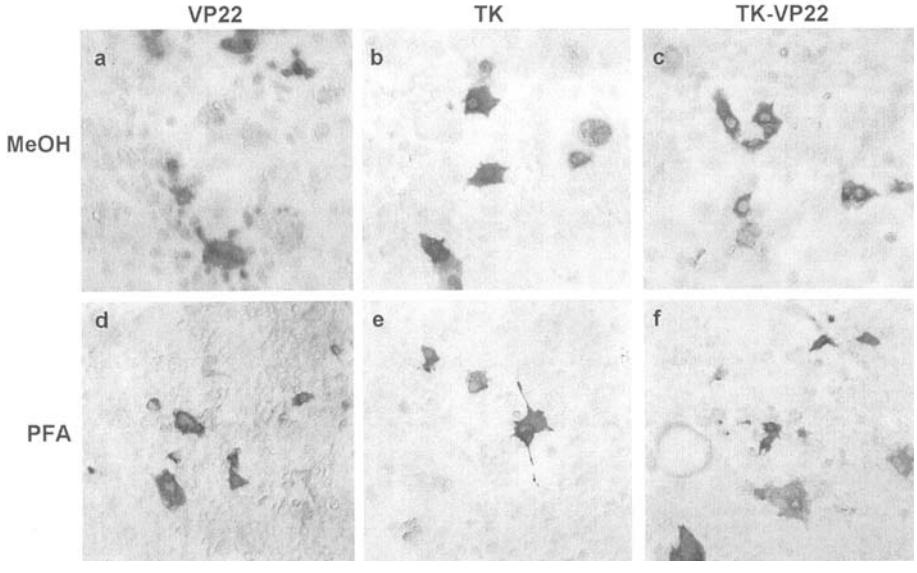

Figure 3. Intercellular spread of TK-VP22 is not observed in cell culture by immunohistochemical staining. COS-7 cells were transfected with pcDNA3.1-TK, pcDNA3.1-TK-VP22 or pcDNA3.1-VP22. After 2 days the cells were fixed with either methanol (a-c) or paraformaldehyde (d-f) and stained for presence of the myc-tag. (a,d) VP22; (b,e) TK; and (c,f) TK-VP22.

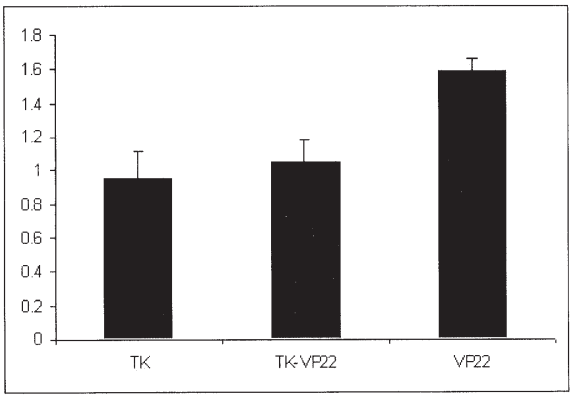

Figure 4. Intercellular spread of TK-VP22 was not observed in cell culture by flow cytometry staining. U373 cells were infected with AdTrack-TK, AdTrack-TK-VP22 or AdTrack-VP22. These cells were then mixed with naïve cells 1:10. After 2 days the cells were stained for the myc-tag, using RPE and analysed by flow cytometry for GFP and RPE fluorescence. Values are presented as the number of RPE-positive cells divided by the number of GFP-positive cells and corrected for leakage during fixation by dividing the spread after co-culture by the spread without co-culture (leaching). These data are the mean of 2 experiments and the error bars represent the standard error.

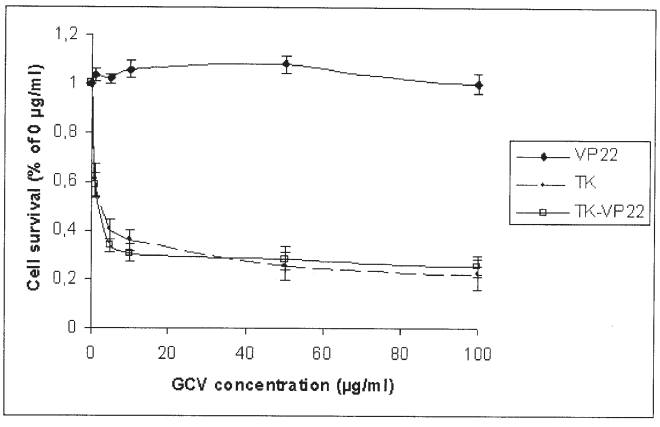

Figure 5. The TK-VP22 fusion protein did not increase the sensitivity of U373 cells to GCV when compared to wild-type TK. U373 cells were infected with AdTrack-TK, AdTrack-TK-VP22 or AdTrack-VP22. These cells were then mixed with naïve cells 1:10. After 2 days the cells were exposed to increasing concentrations of GCV. Four days later cell viability was assayed using MTS. Data are means of 5 separate experiments and error bars represent the standard error.

gain a representative quantification for this transfer. The data were also corrected for transfer of proteins occurring during fixation as described in Materials and methods. When infected and naïve cells were mixed after fixation, ~40% of the cell population was observed to be positive for RPE fluorescence. This number increased to 60% when the cells were mixed before fixation. This increase indicated that some transfer of the protein occurred during fixation of the cells and the number of positive cells might have been over-estimated. After correction for this leaching, no transmission effect was found for TK or for the TK-VP22 fusion protein. The VP22 protein, however, did exhibit a 59% increase in transmission, when compared to TK.

Table I. The TK-VP22 fusion protein did not increase the sensitivity of U373 cells to GCV when compared to wild-type TK.[a]

Protein produced in producer cells	IC_{50} ± SEM of GCV at 10% producer cells (μg/ml GCV)
No protein	>1000
VP22	>1000
TK	3.15±0.76
TK-VP22	2.27±0.59

[a]IC_{50} values were determined by linear interpolation from the data represented in Fig. 5. There was no statistical difference between TK and TK-VP22 (paired Student's t-test, p=0.12).

The ability of TK and TK-VP22 to sensitize cells to GCV. To test whether the addition of VP22 to TK resulted in more effective oncolysis in cell culture, naïve cells were co-cultured with cells producing this protein or unfused TK, and the viability of the cultures was determined after exposure to GCV.

An extensive bystander effect was observed for all cells expressing TK or a TK fusion protein; the presence of 10% producer cells in culture led to a 40-70% decrease in cell viability. No difference in cytotoxicity was observed between TK and TK-VP22 (p>0.28 for all points). The results of these experiments are summarized in Fig. 5 and Table I.

In these cytotoxicity experiments, the infection efficiency was measured by expression of the GFP in each sample. When the infection efficiency was equal, so were the cytotoxic effects of TK and TK-VP22. On Western blots made from the same cells, however, the TK-VP22 samples showed fainter bands than TK (results not shown), indicating a lower amount of protein with a higher activity in the case of the TK-VP22 fusion protein, when compared to native TK. This prompted us to investigate the relative enzymatic activities of TK and TK-VP22.

Enzyme activity assays (Fig. 6). Because we found less protein to have the same effect in cytotoxicity assays, we expected the addition of VP22 to TK to have an effect on either enzymatic activity or stability of the protein. To determine the enzymatic activity of the TK fusion proteins, enzymatic assays were performed, using [18F]FHBG as a substrate.

When equal amounts of protein (as determined by Western blotting) were incubated with the substrate, the activity of the fusion protein was not significantly different (initial speed TK, 5.4%/min; TK-VP22, 5.1%/min).

Effect of external addition of TK-VP22 protein on the GCV sensitivity of U373 cells. To investigate the possibility that secretion might be the rate limiting step in transmission of VP22 fusion proteins, lysates were prepared from U373 cells infected with the adenoviral vectors, and thus producing TK or TK-VP22 protein. These lysates were transferred to naïve cells to determine whether TK-VP22 outside of the cell could sensitize cells to GCV by uptake of the protein.

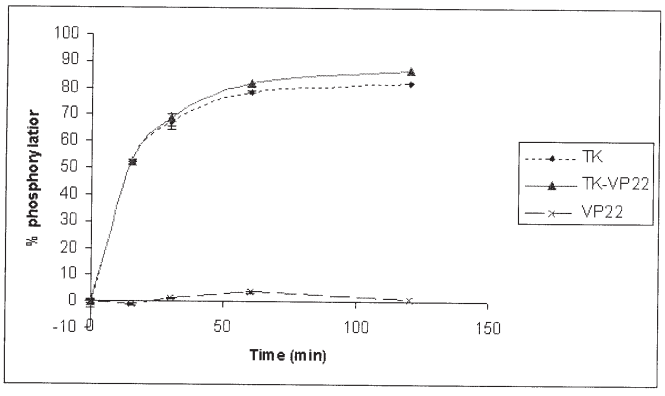

Figure 6. Enzymatic activity of TK and TK-VP22. Equal amounts of enzyme were incubated with [18F]FHBG. At different time points samples were taken and analysed for phosphorylated tracer. The experiment was performed in duplicate. Error bars indicate the standard deviation of the data.

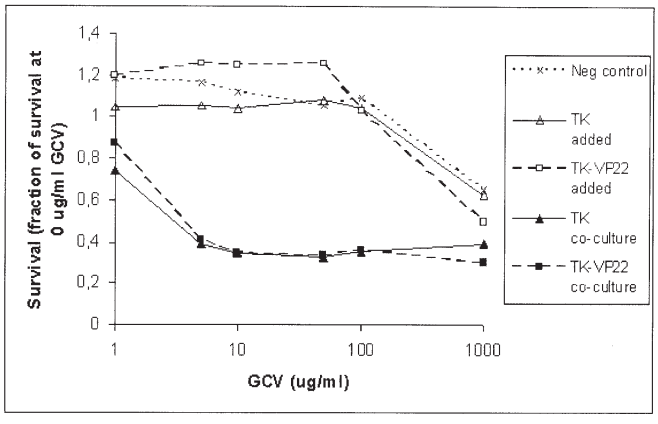

Figure 7. Exposure of cells to TK-VP22 fusion protein did not result in sensitivity to GCV. Cell lysate from U373 cells producing either TK or the TK-VP22 fusion protein was added to the medium of naïve U373 cells. After 24 h the cells were exposed to increasing concentrations of GCV. Four days later cell viability was assayed using MTS. For reference, the data from the positive control experiment (non-lysed cells) were also included in this graph.

To verify the functionality of the protein obtained from the producer cells, a small fraction of the producer cells from each sample was not lysed but plated 1:10 with naïve cells to test their vulnerability to GCV.

The naïve cells were exposed to the lysate for 1-24 h and subsequently fixed and stained for the myc-tag or exposed to GCV. Fixed cells from all time points, exposed to either TK or TK-VP22, stained positive for the myc-tag when exposed to the proteins, but the staining seemed to be membrane bound rather than cytoplasmic or nuclear. Similar staining results were obtained when using bacterially produced TAT-ß-galactosidase (data not shown). When the cells were washed and exposed to GCV 1-24 h after addition of the protein, no increase in cell death was observed for the fusion construct over wild-type TK or over untreated control (Fig. 7). The separately plated cells that were not lysed showed efficient sensitization to GCV, indicating that these cells indeed produced functional TK-containing proteins. Apparently, these proteins were not internalized from the medium by naïve cells. No difference in sensitization to GCV was found between the different time points of incubation with the fusion protein-containing lysate. From these results we conclude that either no TK or TK-VP22 was taken up into non-producing cells, or that the internalized amount was too low to have any effect on cell viability.

Discussion

We investigated the usefulness of VP22 fusion proteins to increase the efficiency of cancer gene therapy. In this study we describe results that illustrate the lack of intercellular trafficking of a TK-VP22 fusion protein, after gene transfer by an adenoviral vector. Using various techniques such as flow cytometry and cytotoxicity experiments, we were unable to demonstrate detectable levels of TK-VP22 fusion protein in cells that did not receive the gene. In addition, we described common properties of VP22 fusion proteins that might explain why the results of various authors indicated improved efficacy of TK when fused to VP22, while others failed to detect intercellular migration of TK-VP22 fusion proteins.

Because of their ability to enter cells indiscriminately, protein transduction domains (PTDs), in particular HSV-1 VP22, are apparently ideal candidates as fusion partners for a suicide enzyme that has to be internalized into cells. Although promising results were obtained by some authors, even in *in vivo* models (26,27), controversy has been rising around whether these PTDs actually enter living cells when linked to heterologous proteins (29,40). It has even been implied that VP22 internalization could be entirely attributed to fixation artefacts (41). However, although only few groups have been able to convincingly demonstrate intercellular trafficking in live cells (42,43), ample evidence is present that shows transmission by indirect means, especially the apoptotic death of cancer cells after exposure to apoptosis-inducing peptides fused to VP22 (43-47). Most authors now agree that at least a fraction of VP22 fusion proteins are internalized into the cells, most probably by constituous endocytosis (48). In some cases enough of the proteins escape from the endocytotic vesicles to have an effect on the cell.

While TK-VP22, and other suicide enzyme fusion proteins are reported to increase the efficiency of tumor eradication in tumor-bearing mice (26,28,49-51), other authors report an absence of any effect of VP22 on the migration of TK and the effectness of the therapy (31,40,45). In our experiments we found VP22 protein in non-producing cells by immunohistochemical staining of fixed cells. However, this observation was previously found to be attributed to a fixation artefact (30). We obtained comparable results in flow cytometry analysis of a partially transduced cell population. We could not detect any TK-VP22 fusion protein in non-producing cells in these experiments. From these results we assume that VP22 on its own does spread from the producing cell to the naïve cells. However, this effect was not found for the TK-VP22 fusion protein, indicating that at least part of the transduction qualities of VP22 were lost when fused to TK.

We also investigated the sensitivity to GCV using a mixed cell population containing a small percentage of producer cells. In these experiments no difference was

observed between cells producing TK or TK-VP22. When comparing the results for TK to TK-VP22, it seems that no intact uptake of the protein from the medium occurred. This was further confirmed by the observation that protein obtained from cell lysate and transferred to naïve cells, did not sensitize the cells to GCV. This indicates that there was no uptake, or that internalization was so low that it was undetectable by these means.

We concluded that, in our experiments, fusing VP22 to TK did not transfer the effect of the PTD to the fusion protein. If such an effect did exist, then it was too small to have the desired effect of increasing the efficiency of gene therapy.

These results are in compliance with some of the literature, as it seems that fusing a protein to VP22 is not a sure way to give it transduction properties. From the combined literature it seems that the protein that is fused to VP22 has a definite effect on the transduction qualities that are not determined by protein size alone (52-54). Even studies performed by the same authors, but with a different protein yielded contrary results (36,44). The orientation of the protein in relation to the VP22 molecule is also a factor and can entirely abolish any transduction effect (45). Furthermore, the linker used to connect VP22 and the fusion partner may affect both the translocation properties and the function of the linked protein. We used a short linker consisting of three alanine residues, to generate a flexible connection.

In our experiments, cells expressing TK-VP22 seemed to produce less protein than those expressing TK but were equally susceptible to GCV. This observation, in combination with previous reseach that indicates VP22 may modify the enzymatic activity of TK (55), prompted us to investigate the activity of the enzymes in a cell-free enzyme activity assay. Indeed, VP22 has been reported to fundamentally alter the attributes of the fusion protein in some cases. The effects vary from aggregation of the protein (45,56) to loss of function (57-59) and even the repression instead of induction of a promoter in the case of a VP22-p53 fusion protein (36).

When equal quantities of protein were tested for their ability to phosphorylate [18F]FHBG (a radioactively labelled thymidine analogue), we found comparable activity for TK and TK-VP22. We concluded that the seemingly lower production of protein was caused by cleavage of the fusion protein. When the protein was cleaved between TK and VP22, as we observed if the protein was improperly stored or broken down, lower amounts of full-length protein were detected on the Western blot. This effect of C-terminal VP22 fusion proteins was previously described for various other proteins (26,36,37). Therefore, this may be a commonly occurring phenomenon, resulting in underestimation of the amount of protein produced in a particular cell line. This apparently may have implications for research involving VP22 fusion proteins. Detection of the full-length protein only may result in an underestimation of the amount of protein produced in the VP22 fusion-producing line.

VP22 has been reported to increase the effectivness of TK therapy in tumors in mice that consist of cancer cells and a smaller number of producer cells (18). These experiments were performed with stably transduced cells, and these cell lines were selected for equal expression of the full-length protein. The authors contributed the greater effect of the therapy to the transmission of TK-VP22. They did not however take into account the possibility that VP22 might influence the activity or the stability of TK, or that unlinked TK may also be produced in these cells.

There are many difficulties associated with VP22-induced intercellular trafficking and VP22 fusion proteins. It is therefore difficult to predict the effect that fusing a suicide enzyme to VP22 might have. In general the VP22 mechanism of transmission might be more suited to traffic smaller proteins, like p53 (57,60,61), that do not interfere with VP22 function, but still exhibit potent anti-cancer activity. Yet in most cases the utility of VP22 to increase the efficacy of cancer gene therapy will have to be judged on a case by case basis. To generate a fusion protein that incorporates both the ability to migrate to neighbouring cells after production and to retain the function of the original protein, it may be necessary to screen many different fusion proteins with variations in orientation and linker composition.

From the combined results described here, and previous research that indicates failure to traffic heterologous proteins, we conclude that TK-VP22 fusion proteins, and possibly other PTD fusion proteins as well, are unlikely to enhance gene therapy by effecting intercellular spread of the gene product.

Transmission of the gene product is a desirable property in almost every gene therapy setting. It is therefore imperative to keep investigating ways to construct a protein that spreads to the surrounding cells. Many more options can be explored, including different PTDs (16), conventional secretion (14,15), and the use of target-specific ligands (62), antibodies (63,64) or bacterial toxins (64,65) to achieve internalization of the therapeutic protein.

Acknowledgements

The authors wish to thank Dr G. Hospers for the gift of pcDNA3-nTK, containing the cDNA of HSV-1 thymidine kinase.

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