Secretion of Thymidine Kinase to Increase the Effectivity of Suicide Gene Therapy Results in the Loss of Enzymatic Activity

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Abstract

Low efficiency of gene transfer is one of the major limitations of gene therapy. A solution to this problem may be transmission; by modification of the transgene, the gene product can be secreted and internalized by the surrounding cells. In this way more target cells can be reached, even if only a small number of cells receive the gene. In suicide gene therapy a gene is introduced into the cell that encodes for an enzyme, which converts a non-toxic prodrug into a toxic drug and thereby kills the cell. Cancer gene therapy using the Herpes Simplex thymidine kinase (HSV-TK) suicide gene is a promising treatment, and TK has been used in clinical trials with some success. However this kind of therapy has limited efficacy due to the low level of gene transfer reached. A modified TK protein, capable of migrating from the producing cell to neighboring cells, would result in a greater proportion of cells affected by the treatment.

As a first step towards transmission, we constructed a secretory form of HSV-TK by including the Igκ leader peptide in the gene. This modified gene was then cloned into an adenoviral vector and an ER export signal was added to the construct to further improve its secretion. Secretion and protein production in cancer cells transduced with the adenoviral vectors were analyzed using Western blot. The enzymatic activity of the modified proteins was determined in cell free assays and the ability of the modified TK to sensitize cancer cells to ganciclovir was tested in cell culture, to measure the effect of the modifications on functionality of the enzyme.

Addition of the Igκ leader resulted in high levels of secretion of HSV-TK, with up to 70% of the total amount of protein secreted. Inclusion of an ER export signal did not further improve secretion. The enzyme activity of the secreted TK however, was decreased when compared to native TK. Secretion of HSV-TK did not result in increased cytotoxicity in the presence of ganciclovir but decreased the sensitivity of the producing cells when compared to non secreted TK. The presence of extracellular TK did not sensitize cancer cells to TK. Although the activity of TK was reduced after secretion, cells producing the modified TK were still sensitive to GCV treatment.

This study is the first to report on secretion of TK, and provides a first step in a novel strategy to improve the efficiency of cancer gene therapy. The loss of function in secreted TK however may present a major hurdle in the development of a transmitted form of TK.
Introduction

The low efficiency of gene transfer that can be achieved with the current gene therapy vectors is one of the major problems the gene therapy field is facing. Although gene therapy approaches against many types of diseases have been developed, and many of these are very effective in vitro and in animal experiments, most fail to yield equally convincing results in clinical studies. Cancer gene therapy in particular is greatly affected by this shortcoming because the high replication rate of cancer cells causes the disease to reestablish itself when not all target cells are destroyed.

Cancer cells can be specifically eliminated by introducing a cytotoxic gene into these cells. The introduced gene can encode an enzyme able to activate an otherwise harmless prodrug (suicide gene therapy). Although some success has been reported in clinical trials, this kind of therapy is less efficient than expected from in vitro results, because the vectors used for gene transfer introduce the DNA into only a small number of tumor cells. In suicide gene therapy migration of the activated prodrug usually increases the number of cells killed. This ‘bystander effect’ however, is often too limited to result in eradication of the tumor. In many cases the impact of the bystander effect is small because the cells need to have specific properties, like the expression of gap junctions, to be affected by drug migration.

Herpes simplex virus thymidine kinase (HSV-1 TK) has been used as a suicide gene in a large number of clinical trials involving cancer patients. The nontoxic prodrug ganciclovir (GCV) can be intracellularly activated to a nucleoside analogue that causes apoptosis in cells expressing TK. The enzyme has its function inside the nucleus and is targeted there by the presence of multiple nuclear localization signals in the protein. Because the activated prodrug is a nucleoside analog, and needs to be incorporated into the replicating DNA to have an effect it is almost exclusively toxic to dividing cells. In in vitro and in animal experiments suicide gene therapy using the TK gene yielded convincing results, even eliminating tumors containing very low numbers of TK producing cells. The bystander effect thought to be responsible for this effectivity is caused by migration of the activated prodrug through gap junctions. Clinical trials with TK based gene therapy for cancer however, failed to result in total tumor elimination. This is thought to be due to the combination of low transduction efficiency caused by the presence of physiological and biological barriers to the vector and the absence of migration of activated GCV due to the low prevalence of gap junctions in the cancer cells in vivo.

It would therefore benefit gene therapy in general if a strategy can be devised, that overcomes the problem of insufficient transduction of target cells by the vector.

The efficiency of gene therapy may be increased by using retargeted gene therapy vectors, since not all cancer cells express the correct receptor molecules for the vector. A significant increase in gene transfer can be achieved by redirecting the
gene therapy vector to surface molecules that are abundantly present on the target cells, either by using adapter molecules\textsuperscript{10-12} or genetic modification of the virus capsid\textsuperscript{13-15}. In addition, this technique increases the safety of gene therapy by un-targeting healthy cells. Despite the progress made in this field, reaching all target cells still remains a challenge.

Alternatively, the inherent replication ability of viral gene therapy vectors can be used to increase the number of cells reached by a gene therapy vector. The replication ability of a viral vector is usually removed in gene therapy protocols because of safety concerns. If it is reinstated however, the replication of the virus will give rise to new viral particles that can infect other cells, multiplying the effect of the therapy. This approach is particularly effective in cancer gene therapy\textsuperscript{16,17}. A tumor specific promoter can be used to restrict the expression of a gene essential for replication to tumor cells and thereby limit the replication of the virus and the lytic effect to these cells\textsuperscript{18}.

A powerful new approach to circumvent the low efficiency of gene transfer is to ensure that the protein produced from the transgene is present in or near all cancer cells, even if only a few of the cells produce the protein (transmission). When only a small number of cells produce and secrete an enzyme, a much larger number of cells can be affected by its action. If a prodrug activating enzyme is secreted from a small number of cells, an entire tumor can be eliminated by the action of the enzyme. This approach is only effective if the drug is effective when present outside of the cell\textsuperscript{19,20}. Artificial secretion will work for a number of suicide genes, but many suicide enzymes, like TK, need to be present inside the nucleus of the target cell to mediate cell death, and secretion alone will be ineffective. When TK is secreted, a loss of cytotoxicity can be expected, as the prodrug, once extracellularly activated, is unable to enter the cells. The prodrug GCV is a non-polar molecule and can enter and leave cells through the cell membrane. The phosphorylated molecule becomes more polar and is unable to pass the cell membrane passively. Therefore if TK is secreted, ganciclovir will be phosphorylated outside of the cell and the activated molecule can no longer enter the cells, thus no longer results in toxicity. As a second step however, internalization of the enzyme into the target cells can be achieved in several different ways, including receptor mediated uptake and protein transduction\textsuperscript{21}. Although protein transduction domains (PTDs), like VP22, are thought to effect secretion of the protein as well as internalization of many proteins, including TK\textsuperscript{22,23}, there is much debate about the mechanism and the efficiency of this secretion and internalization\textsuperscript{24-27}. Likely, the efficiency of protein spread achieved by these PTDs can be increased by increasing the secretion of the protein\textsuperscript{28,29}.

To construct a secretory form of TK we used the Ig\textsubscript{k} leader. The Ig\textsubscript{k} leader is a secretion signal, responsible for the very high constitutive secretion of the immunoglobulin k-chain\textsuperscript{30-32}. Because proteins containing nuclear localization signals (NLS) are often rerouted, or broken down when found in the endoplasmic reticulum (ER) by the cellular protein sorting machinery\textsuperscript{33}, we hypothesized that a more rapid export from the ER
would result in more efficient secretion of the protein. To test this hypothesis, fusion proteins including an ER-export signal were also constructed. The particular signal we used in this research has been identified by Ma et al. and Stockklausner et al. as a signal that can regulate the amount of potassium channels present on the cell surface by increasing the export from the ER$^{34,35}$.

We constructed a secretory form of thymidine kinase in order to improve the tissue distribution of TK. Secretion of this enzyme is the first step towards devising a more effective gene therapy approach, based on the intercellular spread of TK. Although internalization of the secreted enzyme still has to be achieved, various techniques can be applied to deliver the TK into the cytoplasm. For this internalization to be effective however, a high production and secretion of the TK has to be realized. In this study we show for the first time that it is possible to modify TK to reach high levels of protein outside of the cell. However, the enzymatic activity of TK is greatly reduced by passage through the secretion pathway, and further modifications to the protein are required to sustain full enzymatic activity.

Materials and Methods

Construction of adenoviral vectors

Molecular biology reagents were purchased from Invitrogen (Invitrogen Corporation, Carlsbad, CA, USA). The Igκ leader was isolated from pSecTagB (Invitrogen Corporation), and the endoplasmic reticulum export signal (ER-ES, sequence: FCYENEV) was formed de novo from oligonucleotides - 5'GGCCGCATTCTGCTACGAAAACGAAGTATT 3' and 5'CGAATACTTCGTTCCTAGCAGAATGC 3') that contain both the sequence coding for the ER export signal and flanking NotI and BstBI sticky ends. The sequence of the ER export signal was based on the combined findings of Ma et al. (FCYENE)$^{34}$ and Stockklausner et al. (FxYENEV)$^{35}$. TK inserts were constructed by PCR on pcDNA3-nTK, containing the cDNA encoding for herpes simplex virus type 1 thymidine kinase$^{36}$, with custom designed primers (ATGGATCCACCATGGCTTTGATCCCTGCA and ATGGCTTTGATCCCTGCA and ATGGATCCACCATGGCTTTGATCCCTGCA and ATGGATCCACCATGGCTTTGATCCCTGCA and ATGGATCCACCATGGCTTTGATCCCTGCA) including restriction sites HindIII and NotI (underlined) The inserts were sub-cloned in the plasmid pcDNA3.1c(+) (Invitrogen Corporation). Next, the coding sequence was transferred to pAdTrack-CMV$^{37}$, including the myc and His(6) tags, using restriction sites present in front of and after the genes, resulting in pAdTrack-TK-mycHis, pAdTrack-SecTag-TK-mycHis, pAdTrack-SecTag-TK-ERES-mycHis and pAdTrack -TK-ERES-mycHis (shown in fig. 1).

Adenoviral vectors were constructed using the AdEasy system$^{37}$. All viruses were based on the shuttle plasmid pAdTrack-CMV, and include a GFP expression cassette for detection.
All viruses were produced in HEK293 cells, and crude lysate containing virus was obtained by repeatedly (3x) freezing and thawing in medium containing 2% FCS to crack the cells, followed by centrifugation to remove cell debris. Viral titer was normalized by infection of U373 cells with serially diluted lysate.

**Fig 1. Schematic representation of constructed transgenes.**

ER-ES: ER export signal (sequence FCYENEV)
Tag: myc-tag followed by His(6) tag

### Cell culture and transformation of cell culture

Green monkey kidney COS-7 cells (American Type Culture Collection (ATCC), Manassas, VA, USA) were cultured in DMEM supplemented with 5% FCS, 50 IU/ml penicillin, 50 µg/ml streptomycin (GibcoBRL, Life Technologies B.V., Breda, The Netherlands). Human glioma U373 cells (ATCC) were cultured in DMEM/F12 supplemented with 10% FCS, 50 IU/ml penicillin, 50 µg/ml streptomycin and 2 mM L-glutamine (GibcoBRL).

To infect cells with the viruses containing the TK fusion genes, cells were cultured in 6 or 24 well plates, to a confluence of 50-70%. After 24 h, medium was changed for fresh medium, containing 2% of FCS, containing an amount of virus that was previously determined to result in ~70% infection. The cells were cultured for a further 48 hours to allow for sufficient expression of the genes.

### Protein isolation and western blot

To determine the total protein production and secreted amount of protein from the constructed adenoviruses, proteins were isolated from infected cells and analyzed on Western blot. Before isolating the protein, the GFP intensity per well was determined as a measure of infection. GFP fluorescence was measured using an FL500 microplate fluorescence reader (Bio-Tek instruments Inc., Winooski, VT, USA). Protein was recovered from transduced cells and their growth medium after 48 hours by detaching the cells from culture plates by treatment with trypsin-EDTA (GibcoBRL, Life Technologies). The medium and cells were collected separately and centrifuged for 5 min at 200 x g. Pellets from both fractions were combined and resuspended in a volume of phosphate buffered saline (PBS, GibcoBRL, Life Technologies) equal to the medium volume. Samples were boiled with loading
buffer (Laemmli sample buffer, Bio-Rad Laboratories B.V., Veenendaal, The Netherlands) and 2.5 % β-mercaptoethanol before electrophoresis, separated by SDS PAGE (containing 10% acrylamide) and subsequently transferred to PVDF membrane (Bio-Rad Laboratories, Hercules, CA, USA) by Western blotting. Fusion proteins were detected by immunochemistry using a monoclonal antibody against the myc-tag (α-myc, produced from hybridoma cell line 1729 (ATCC) and a secondary antibody, conjugated to horseradish peroxidase (rabbit anti mouse IgG-HRP, Dako Cytomation B.V., Heverlee, The Netherlands). Blots were stained using 3-amino-9-ethylcarbazole (AEC staining kit, Sigma Aldrich Co., St. Louis, MO, USA) or chemo luminescence (ECL plus Western Blotting Detection system, Amersham Biosciences UK Limited, Little Chalfont, Buckinghamshire, England). The amount of protein was quantified in arbitrary units, on western blot using the GS-710 densitometer scanner and Quantity One program (Bio-Rad Laboratories) for AEC staining, or the Chemi Genius² Bio Imaging system and the GeneSnap program (Syngene, Frederick, MD, USA) for ECL. The detection range of this method and the correlation between staining intensity and amount of protein were determined by loading increasing amounts of an unrelated protein. All samples were within the linear detection range of this method. The total amount of produced protein was calculated by adding up the amounts detected in the lysate and the supernatant and corrected for the infection efficiency as measured by GFP intensity. The fraction of protein secreted was calculated by dividing the amount detected in the supernatant by the total amount of produced protein.

Cytotoxicity assays

To determine the functionality of the TK fusion proteins, U373 cells were infected with adenovirus at a concentration previously determined to yield approximately 70% infection, by adding the virus to cells in medium containing 2% FBS. After 2 hours the medium was removed and the cells detached with trypsin-EDTA. Infected cells were mixed 1:10 with naïve cells and plated in 96 well plates. After 2 days the cells were exposed to increasing concentrations of ganciclovir (Cymevene®, Roche Nederland BV, Mijdrecht, Netherlands). After a further 4 days cell viability was assayed 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 manufacturer (Promega, Leiden, The Netherlands).

Enzyme assay

The activity of the TK fusion proteins in the crude cell lysates was determined as described by Hinds and colleagues 38, using [18F]FHBG as a substrate. The volume of cell lysate for each sample was calculated such that each contained the same amount of protein, as determined by Western blot. The following standard reaction mixture was used: up to 50 µl of crude cell lysate, 20mM potassium
phosphate (pH 7.6), 40mM KCl, 25mM NaF, 5mM MgCl2, 1mM DTT, 5mM ATP and 0.5mg/ml BSA in a total volume of 400 µl. Since cell culture medium may contain free thymidine, all samples for enzyme activity assays were pre-treated with 80 units thymidine phosphorylase for 30 minutes at 37°C, to hydrolyze any thymidine that could inhibit the TK reaction with [18F]FHBG. TK activity was determined by incubating [18F]FHBG in the reaction mixture at 37°C (~0.17 nM, specific activity ~54,000 GBq / mmol). 25 µl Samples of this mixture were loaded on a Whatman DE-81 filter at different time points. The negatively charged phosphorylated [18F]FHBG is bound to these filters. Each filter was washed three times with ammonium formate and three times with 95% ethanol to remove un-reacted [18F]FHBG. Radioactivity of the filters was counted with a gamma 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 was calculated by dividing the radioactivity (cpm) of the washed filters by the radioactivity of the unwashed filters.

**Statistical analysis**

Results were tested for significance using a student’s paired t-test.

**Results**

**Construction of adenoviruses and protein production**

To investigate the secretion of TK mediated by Igκ and the ER export signal, we constructed 4 adenoviruses, depicted in fig 1; AdTrack-TK-mycHis, AdTrack-SecTag-TK-mycHis, AdTrack-TK-ERES-mycHis and AdTrack-SecTag-TK-ERES-mycHis. To verify correct protein expression, COS-7 cells were infected with the viruses. The transduced COS-7 cells stained positive when fixed and immunostained for the myc tag present at the C-terminus of the proteins, indicating the proteins were produced correctly in infected cells. The number of cells stained positive for TK corresponded closely to those containing GFP, as observed by fluorescence microscopy shortly before fixing the cells (data not shown).

Western blots of cell lysates of infected cells showed 2 bands, at approximately 45 and 40 kDa, corresponding to two different isoforms of the TK protein. Mainly full length protein was detected. The fusion proteins containing a secretion signal or ER export signal showed bands with a slightly higher molecular weight, as expected (Fig 2). From these results we concluded that the different proteins were expressed correctly from the viruses. Unexpectedly, the bands observed in the medium fractions of the secreted proteins sTK and sTK-E run slower, indicating a higher molecular weight.
Secretion of Thymidine Kinase

**Fig 2. TK fusion proteins are correctly expressed.**

COS-7 cells were infected with adenoviral constructs. Protein was isolated from the cells and medium after 2 days. TK fusion proteins were analyzed on Western Blot with α-myc and secondary antibody linked to HRP and stained with AEC. All bands shown are from the same experiment, but irrelevant lanes were removed from the picture for clarity. Neg: Mock infected, TK: AdTrack-TK-mycHis, sTK: AdTrack-SecTag-TK-mycHis, TK-E: AdTrack-TK-ERES-mycHis, sTK-E: AdTrack-SecTag-TK-ERES-mycHis

**Quantification of secretion and production**

U373 and COS-7 cells were infected with the adenoviral constructs to quantify the amount of protein secreted and produced. Proteins from either medium or cell lysate were separated on SDS PAGE gel and analyzed using Western blotting and immunochemistry.

For quantification the intensities of the bands of the two isoforms of TK were added together.

Cells transduced with a virus encoding TK fused to the Igκ secretion signal showed a 5.3 fold increase in secretion (increase from approximately 20% to 70%, measured as fraction of total protein produced) compared to unmodified TK (p<0.003), while protein production was unaffected.

Addition of the ER export signal did not further increase or decrease secretion. Adding the ER export signal to the otherwise unmodified TK however decreased the production of protein to 29% of unmodified TK (p<0.005). The results of secretion and protein production experiments in U373 and COS-7 cells were comparable, and therefore combined in these results. The results of quantification on Western blot are summarized in Fig 3.
Chapter 3

**Fig 3. Igκ-TK is secreted from the producing cells upon transfer of the TK fusion gene.**

U373 or COS-7 cells were infected with adenoviral constructs. Protein was isolated from cell lysate and medium 2 days after infection. TK fusion proteins were detected by Western blot with α-myc and secondary antibody linked to HRP and stained with AEC. The amounts of protein in medium and cell lysate were quantified using the Quantity One program. Results are the mean of 8 separate experiments and the error bars represent the standard error of the mean. TK: AdTrack-TK-mycHis, sTK: AdTrack-SecTag-TK-mycHis, TK-E: AdTrack-TK-ERES-mycHis, sTK-E: AdTrack-SecTag-TK-ERES-mycHis

* indicates a significant difference from unmodified TK with p<0.003.

** indicates a significant difference from unmodified TK with p<0.005.

**Cytotoxicity assay**

While the protein levels show that the leader-modified TK enzyme is produced and secreted, it is important to ensure that the protein is still active in converting the prodrug. To verify the activity of the constructs, cytotoxicity assays were performed. All cytotoxicity experiments were performed with populations of cells of which 10% was infected with one of the adenoviral constructs. In all our cytotoxicity experiments we expected and found a much larger fraction of the cells killed. This additional cell death beyond the expected 10% is caused by the bystander effect. The cells producing TK transfer some of the activated GCV to their neighbours through gap junctions, resulting in a large number of cells killed in addition to the producer cells.

Because the constructs containing the ER export signal showed no increased secretion, these were not included in further experiments.

When U373 cells infected with the adenoviral constructs were exposed to ganciclovir, their viability dramatically decreased over naïve cells and cells infected with a control virus. Whereas the mean IC$_{50}$ of ganciclovir for cells infected with control virus was above 1000 µg/ml, the IC$_{50}$, linearly intrapolated from the graph for TK was $2.91 \pm 0.67$ µg/ml and $74.3 \pm 33$ µg/ml for SecTag-TK (Fig 4). Results with COS-7 cells were comparable. The difference between TK and sTK was significant (p < 0.05) at all GCV concentrations. The difference in IC$_{50}$ was also significant (p < 0.05). Thus the ability of secreted TK to sensitize the cells to GCV is decreased when compared to unmodified TK. This effect was anticipated, as TK present outside of the cells is not expected to have an effect on cell viability.
Fig 4. The cytotoxic effect of ganciclovir is decreased by secretion of TK.

U373 or COS-7 cells were infected with adenoviral constructs and two days after infection the sensitivity of the cells to GCV was measured. Cell viability was determined using the MTS assay, and viability of the cells at 0 µg/ml GCV was set at 1. Data are the mean of 6 separate experiments and the error bars represent the standard error of the mean. Control: Infected with irrelevant adenovirus, TK: AdTrack-TK-mycHis, sTK: AdTrack-SecTag-TK-mycHis.

**Enzyme activity assay**

To ascertain whether the enzymatic activity of the secreted TK is compromised by secretion, cell free enzyme activity assays were performed on protein isolated from cell lysates and medium. The experiments where performed with equal amounts of either wild type TK or TK including the Igκ leader. The results were plotted and the initial reaction speed was calculated by linear regression. The enzymatic activity of TK was 0.82 +/- 0.08 %/min and that of sTK (cellular fraction) 0.26 +/- 0.03 %/min (fig. 5a).

In a similar experiment, using a lower amount of protein the enzymatic activities of the medium and cellular fractions of sTK were compared. Although the cellular fraction of sTK retains part of its activity (1.6%/min), the secreted fraction totally looses the ability to phosphorylate the tracer (fig. 5b).
**Fig 5. Secretion decreases the enzymatic activity of TK.**

Equal amounts of protein were incubated with [18F]FHBG. At various time points the amount of phosphorylated tracer was measured. TK: cellular fraction of cells infected with AdTrack-TK-mycHis, sTK C: cellular fraction of cells infected with AdTrack-SecTag-TK-mycHis, sTKM: medium fraction of cells infected with AdTrack-SecTag-TK-mycHis. Experiments were performed in duplo and error bars represent the standard deviation.

**Fig 6. Stability of thymidine kinase in solution at 37°C.**

Equal amounts of lysate, containing TK, were incubated at 37°C for 1 to 16 hours. Subsequently the samples were analyzed for enzymatic activity and visible breakdown by Western blot.

- a: Western blot of TK protein stored at 37°C, probed with antibodies against the myc tag.
- b: enzyme activity of TK stored at 37°C.
- c: Western blot of TK stored in spent culture medium at 37°C, probed with antibodies against the myc tag.
- d: enzyme activity of TK stored in culture medium.

0: stored at -20°C, 1: stored 1 h at 37°C, 16: stored 16 h at 37°C, TK+medium: stored 48h at 37°C, in used culture medium. Experiments were performed in duplo and error bars represent the standard deviation.
Secretion of Thymidine Kinase

Because the decrease of activity of the secreted TK could be the result of breakdown of the protein in the medium, the stability of TK was tested. When cell lysate containing TK was incubated at 37°C for 1-16 hours, no significant breakdown or loss of enzymatic activity was detected by western blot (fig 6a) or enzyme assay (fig 6b). When TK was incubated in culture medium that was previously used to culture cells (spent medium) the protein lost more than 80% of it’s enzymatic activity (fig 6d) although little breakdown was apparent on Western blot (fig 6c).

Discussion

Intercellular spread of suicide enzymes can increase the effectivity of suicide gene therapy for cancer. Secretion of the suicide enzyme is the first step towards intercellular spread. In this article we describe the construction of a secretory thymidine kinase. Although this TK variant is secreted in adequate amounts, the secreted enzyme has severely decreased enzymatic activity.

Gene therapy is a promising therapy for many diseases, but expression of the transgene is mostly restricted to the needle tract, when injected directly at the target site. Unless the infected cells function as factories, or many injections are used, the therapeutic effect is limited. Especially in cancer gene therapy this is a major problem because all cells need to be reached for the therapy to be effective. In suicide gene therapy, usually a so called ‘bystander effect’ is observed as a result of migration of the activated drug. However, in vivo the bystander effect is too limited to overcome the lack of effectivity of gene transfer, because most tumor cells do not express gap junctions which are needed for this transfer of activated drug molecules.

To solve this problem the transgene may be altered to be secreted so that when only a few cells receive the gene, many surrounding are affected by the therapy. The validity of this concept for cancer gene therapy has been shown using carboxyl esterase, carboxypeptidase and β-glucuronidase, which are suicide enzymes that activate a prodrug. If a small number of cells produce and secrete one of these enzymes, the activated drug will enter and kill all surrounding cancer cells. This effect can even be enhanced by targeting the enzyme to a specific receptor present on the cancer cells, to concentrate the enzyme near the target cells and at the same time preventing the enzyme from leaving the tumor and causing toxicity elsewhere.

Thymidine Kinase is the most commonly used transgene in clinical trials involving suicide cancer gene therapy. Although TK showed to be effective in reducing tumors in animal experiments, clinical trials were less convincing, most likely because the vector failed to penetrate into the tumor, and only a small number of tumor cells were affected. The bystander effect proves insufficient, probably because the bystander effect of TK is dependent on gap junctions and not all tumors express gap junctions. Gene therapy protocols utilizing TK would greatly benefit if a form of TK can be developed that is able to be secreted from the
producing cell and taken up by the neighboring cells. As a first step in the development of such an enzyme, we aimed to construct a secretory form of TK.

The Igκ leader sequence was cloned upstream of the TK gene in an adenoviral vector in order to construct a secretory thymidine kinase, and the resulting virus was used to introduce the gene into eukaryotic cells. In this particular case the secretion of the enzyme did not lead to increased effectivity in eliminating cancer cells, because the activated prodrug will not enter cells and therefore activity of the enzyme outside of the cells does not lead to cell death. To reach increased efficiency of cell kill, the enzyme should be further modified to allow for specific uptake by the target cells, as discussed below.

When the protein produced from the modified TK genes was analyzed by Western blot we observed 2 distinct bands at approximately 45 kDa. The presence of the second band is probably caused by a splicing variant of TK that has been previously described. A small size difference can also be observed as expected between TK and the TK containing the Igκ leader. However, the Igκ leader cannot account for the difference in size between the protein present within and outside of the cells, because the leader is removed during secretion. Furthermore, the difference in size is larger than expected and larger than the difference observed in cell lysate.

Since the Igκ leader directs the protein into the ER and the Golgi system, the difference is most likely caused by a difference in post-translational processing in the cytoplasm and the Golgi system.

The amount of protein produced by the cells varied substantially between experiments and the two cell lines, due to the variations in infection efficiency, leading to high SEM values in fig. 3. When the amount of protein on Western blot was quantified it was clear that a large fraction of the TK protein was found in the medium when the secretion signal was included. Whereas only 20% of the total amount of wild type TK produced was found outside of the cells, 70% of the Igκ-TK was present in the medium. The small amount of TK found in the medium of the cells expressing wild type TK is most likely due to cell lysis of the producing cells.

In contrast to our expectations, addition of the ER export signal did not increase the secreted fraction. This failure of the ER export signal to increase the secretion can probably be explained by the fact that the signal was identified in a membrane bound molecule. Possibly this signal needs to have interaction with a membrane associated part of the protein sorting machinery, that is not possible for a protein without a membrane bound component like TK. Additionally, when the total protein recovery was considered, the amount of protein found in the cells transfected with construct containing the ER export signal was much lower than in the cells that received the other constructs. This decrease could be due to a decrease in production, or more likely, an increase in degradation of the protein. Degradation of the protein may be caused by rerouting of the protein to proteasomes during production. Breakdown of proteins in proteasomes is common in the case of miss-folded or otherwise defective proteins and can in this case be due to conflicting sorting signals present in the protein. Whether this is the case...
could be tested by exposing the producing cells to proteasome inhibiting substances like MG132. Degradation of protein due to conflicting sorting signals might be circumvented by the removal of the nuclear localization signals from the TK gene, for example by point mutation in the coding region. However, since the ER export signal does not increase the secretion of TK, and the detrimental effect on protein recovery is not present in the Igκ-TK protein we did not pursue this approach. Moreover, mutations in the NLSs might have detrimental effects on the enzymatic function of the protein⁶ due to the overlap of the active site and the nuclear localization signals.

From assays that measure enzymatic activity of TK it is clear that although TK is secreted when the Igκ leader is added, the protein is less active after its passage through the cellular secretion machinery. Whereas the enzyme recovered from the cells infected with the sTK construct shows a 70% decrease in enzyme activity when compared to wild type TK, the activity of the secreted TK recovered from the medium decreased to background levels. Although wild type TK also lost a large part of its activity when incubated in culture medium, this deactivation does not account for the loss of function of the secretory form in the cell lysate. Based on this result and the observed difference in mobility of the protein on Western blot, we speculate that, although no obvious methylation or glycosylation sites are present in TK (ProSite: http://www.expasy.org/prosite/), the decline in function was caused by differential post-translational processing or folding of the protein in the cytosol and the ER or Golgi apparatus. Loss of function by glycosylation in the ER has been previously described for β-glucuronidase⁴⁵. Human TK activity has been described to be regulated by phosphorylation⁴⁶, making it likely that HSV TK activity can also be altered by post-translational modifications. This theory could be tested by inhibiting specific types of post-translational modifications using chemicals.

However, cytotoxicity experiments show that at least part of the enzymatic activity is retained. We expected a loss of cytotoxicity of the secretory TK, as the prodrug, once activated is unable to enter the cells. Fig. 4 shows that if the cells express secretory TK the number of cells killed at any given concentration of GCV is lower than when the cells produce normal wild type TK. In addition, the viability graph for cells producing the secretory TK becomes horizontal at a higher viability, indicating that the total amount of enzyme activity present in the cells becomes limiting for the number of cells that can be killed.

Although a large fraction of the enzyme is secreted, the cells are still sensitized to GCV, to a much higher extend than one would expect when only comparing the enzyme activity. Possibly a fraction of the protein, produced from the second translation initiation site, does not enter the secretion pathway, because it does not contain the Igκ leader. In fig. 1 this incomplete protein can be recognized, at the same molecular weight as the truncated version of the wild type TK, indicating it does not contain the Igκ leader. This fraction, not containing the Igκ leader, and thus not entering the ER, remains functional and sensitizes the cells to GCV.
resulting in an apparent 70% loss of activity, which is in fact caused by modification of 70% of the protein in the ER.

Therefore it is obvious from these experiments that, although the bystander effect is larger when more enzyme is present in the cells, only a low amount of protein per cell is necessary to kill the producing cells and the neighboring cells. The protein secreted from the cells however, is non-functional. Only investigation into the cause of this loss of function and subsequent modification of the enzyme to make it impervious to this effect may enable the use of TK in transmission enhanced gene therapy.

Secretion of an enzyme can be beneficial to suicide gene therapy, in cases where activity of the transgene outside of the cell is sufficient. Carboxylesterase and β-glucuronidase are examples of suicide genes where the activated prodrug can freely traverse biological membranes\textsuperscript{20,44}. The majority of suicide enzymes however need to be inside the cell to be effective, like TK, and secretion alone will not increase the number of affected cells. When a fragment responsible for internalization, or preferentially cancer cell specific internalization, is also included in the produced protein, the resulting fusion protein will be able to be internalized and have its effect in neighboring cells. Many candidate molecules for cancer specific targeting have already been identified. For example, cancer specific ligands have been identified that are transported into the cell by their receptor\textsuperscript{47} and antibodies specific for cancer are already in use in immuno-radiation and immunotoxin treatment\textsuperscript{48,49}. Another promising approach could be the use of protein transduction domains (PTDs). PTDs are peptides that have been implicated to be capable of ferrying proteins into cells without a specific receptor, like the HIV TAT-PTD, HSV VP22 and the antennapedia protein from \textit{Drosophila}\textsuperscript{50}. A fusion protein of TK and VP22 was already used to enhance the distribution of TK in mouse models of cancer\textsuperscript{22,23}. Addition of a secretion signal might increase this distribution even further\textsuperscript{51}. Even bacterial toxins have been exploited to deliver protein cargos specifically into cancer cells\textsuperscript{49,52}.

For this approach to be effective, the enzyme will have to be transported inside the cell intact and enzymatically active. When proteins are taken up by cells, they will usually be routed to lysosomes, where they are broken down. Therefore it is of great importance to select the method of uptake carefully.

In summary, we have for the first time demonstrated the correct expression and secretion of a secretory form of HSV-1 thymidine kinase. Although the protein was secreted, the secreted protein lost its enzymatic function. This loss of function requires further investigation in order to develop a transmitted thymidine kinase to improve cancer gene therapy.
Reference List


23. Liu CS, Kong B, Xia HH, Ellem KA, and Wei MQ. VP22 enhanced intercellular trafficking of HSV thymidine kinase reduced the level of ganciclovir needed to cause suicide cell death. *J Gene Med* 2001; 3(2).


