Development of stealth transgenes for gene therapy : evaluation of cis-acting inhibitors of antigen presentation

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Citation

Chapter 4

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the Herpes Simplex Virus Thymidine Kinase gene

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Characterization of an Immuno'stealth' Derivative of the Herpes Simplex Virus Thymidine Kinase gene

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Abstract

The cellular immune response against transgene-encoded *neo*antigens is a potential hurdle in gene therapy applications where long-term expression of transgenes is desired. Here a new optimized derivative of the Herpes Simplex Virus 1-Thymidine Kinase gene is described. The HSV-TK gene is frequently used in experimental studies on gene-directed enzyme prodrug therapy. In the optimized gene, the HSV-TK coding region is fused with the codons for the Gly-Ala repeat of the Epstein-Barr virus (EBV) nuclear antigen 1 (EBNA-1) to prevent proteasomal degradation of the HSV-TK. To measure the protective effect in vitro, a model CTL epitope derived from the ovalbumin was inserted in the TK. Cells expressing the GAr-modified TK do not present TK-derived peptides in the MHC. Furthermore, conservative nucleotide substitutions were introduced, which prevent splicing, as well as mutations that render the TK-expressing cells more sensitive to ganciclovir (GCV). The GAr HSV-TK fusion protein is fully functional in vitro. This HSV-TK gene may be especially useful in those gene therapy applications where an immune response against the transgene-encoded product would frustrate the treatment.

Introduction

The cellular immune response against transgene-encoded *neo*antigens is a major setback in gene therapy applications where long-term expression of transgenes is desired. Transgene-product immunity is prominent upon adenovirus-mediated gene transfer, e.g. against the *Escherichia coli* β-galactosidase and the Tet transactivator protein in animal models. In rodents, strong immune responses against this *neo*antigen were observed following adenovirus-mediated gene transfer into liver, muscle, lung and brain, leading to local inflammation, destruction of the transduced cells, and loss of transgene expression.[1-4] The prime candidates for this target cell destruction are the antigen-specific major histocompatibility complex class I (MHC class I) restricted cytotoxic T lymphocytes (CTLs).[1,5-7] Also in primates a CTL response directed against the transgene product has been shown to occur after retrovirus-mediated gene transfer.[8,9] In a clinical trial aiming at inducing a graft-versus-leukemia response, 8 of 24 treated patients developed a specific cytotoxic CD8^{+} T cell-mediated immune response against the cells genetically engineered to express the herpes simplex virus 1 (HSV1) thymidine-kinase (TK) gene. This led to the selective elimination of the modified cells.[9]

HSV-TK is the archetypical enzyme used in gene-directed enzyme prodrug therapies (GDEPT). Its capacity to convert the antiherpetic nucleoside analogues ganciclovir and aciclovir to toxic nucleotides has been used effectively in gene therapy protocols to eradicate tumor cells and lymphocytes that expressed the HSV-TK transgene upon virus-mediated gene

transfer. Although effective, in some applications the use of the HSV-TK is limited by the induction of a cellular immune response against the HSV-TK protein.

The cellular immune response may also frustrate other cancer gene therapy applications. The TK/ganciclovir (GCV) combination is very potent and can be used to eradicate tumor cells. Here the metabolic bystander effect is enhancing its effectiveness. Transport of phosphorylated GCV via gap junctions to neighboring cells sensitizes also the neighboring non-transduced cells (reviewed by Van der Eb et al., 2004).[10] A preexisting immune response directed against HSV-TK may frustrate the efficacy especially if a significant time period exists between vector administration and the onset of GCV administration. This may be the case in those applications where the HSV-TK gene has been included in a vector as fail-safe.[11,12]

Under normal circumstances, the $CD8^+$ CTL response plays an important role in the control of virus infections, generating effector cells that kill infected cells upon recognition of viral peptides presented by MHC class-I molecules. Given the important role of CTLs in the control of virus-spread, it is no surprise that viruses have developed numerous strategies that frustrate and abrogate antigen-presentation.[13-15] In general, the viruses interfere with antigen presentation by frustrating the cell's capacity to generate or present antigenic peptides. In few cases, the inhibition blocks presentation of specific polypeptides. One such example comes from the Epstein-Barr Virus (EBV) nuclear-antigen 1 (EBNA-1). EBNA-1 is expressed in all latency programs of the virus and is indispensable for the virus as it is required for the maintenance of the viral episomes. Although EBNA-1-specific CTLs have been described in infectious mononucleosis patients and healthy carriers, they cannot efficiently recognize EBV-infected cells.[16-21]

The failure to recognize endogenously expressed EBNA-1 has been attributed to the glycine-alanine repeat (GAr) domain in the EBNA-1 sequence. It is shown that the GAr blocks proteasomal degradation in-*cis* and therefore presumably the subsequent presentation in the MHC class-I context.[22,23]

In a previous study we showed that this system could be used to create a 'stealthed' version of the *E.coli*-derived LacZ gene encoding β-galactosidase. Fusions of the EBNA-1 GAr with β-gal prevented presentation of β-gal-derived peptides. This resulted in full protection of the cells against β-gal-specific CTL. The GAr-β-gal fusion protein retained its full β-gal activity. In addition, we demonstrated that the GAr fusions with the firefly luciferase and the HSV-TK yielded functional proteins [24]. Here we describe the construction and characterization of new immuno 'stealth' variants of the HSV-TK for use in experimental gene therapy.
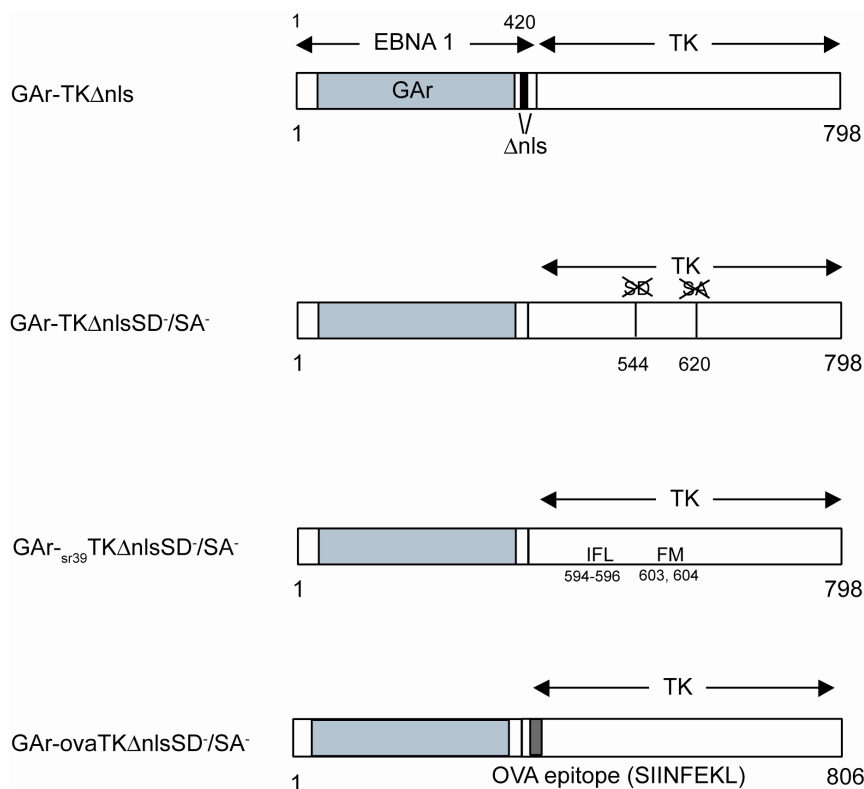
Figure 1
Schematic outline of the chimaeric Gly-Ala repeat constructs with TK. Indicated are the Gly-Ala repeat region (GAr; aminoacids 90-328), the nuclear localization signal (nls, aa 378-386, deleted from all constructs), the point mutations in the splice-donor and acceptor sites to abolish splicing (SD, SA, aa 544 and 620), the sr39TK mutations and their new aa composition (aa 594-596 and 603, 604) and the inserted OVA epitope (aa 431-438). The HSV1-TK gene starts at aa 420. Of all constructs, also variants with a deletion of the GAr region (i.c. amino acids 41 to 376) were generated.

Results

In a previous study we have shown that fusion of the HSV-TK with amino acids 1- 420 of EBNA-1, which encompasses the glycine and alanine-rich region (GAr) of EBNA-1, was compatible with enzyme function. The EBNA-1 fragment used contains the nuclear localization signal of EBNA-1 in addition to the GAr. As a result the GAr-TK fusion proteins accumulate in the nucleus, in contrast to the wild-type HSV-TK protein (fig 2). To test whether normal sub-cellular localization could be restored, the amino acids 378 to 388 encoding the nuclear localization signal (nls) were removed from plasmid pcDNA3.1-GAr-TK,[24] generating plasmid pcDNA3.1-GAr-TKΔnls. Upon transfection in 293T cells, the GAr-TKΔnls protein is indistinguishable in sub-cellular distribution from the wild-type protein. Therefore in all subsequent experiments the Δnls variants of the GAr-TK fusions were used.

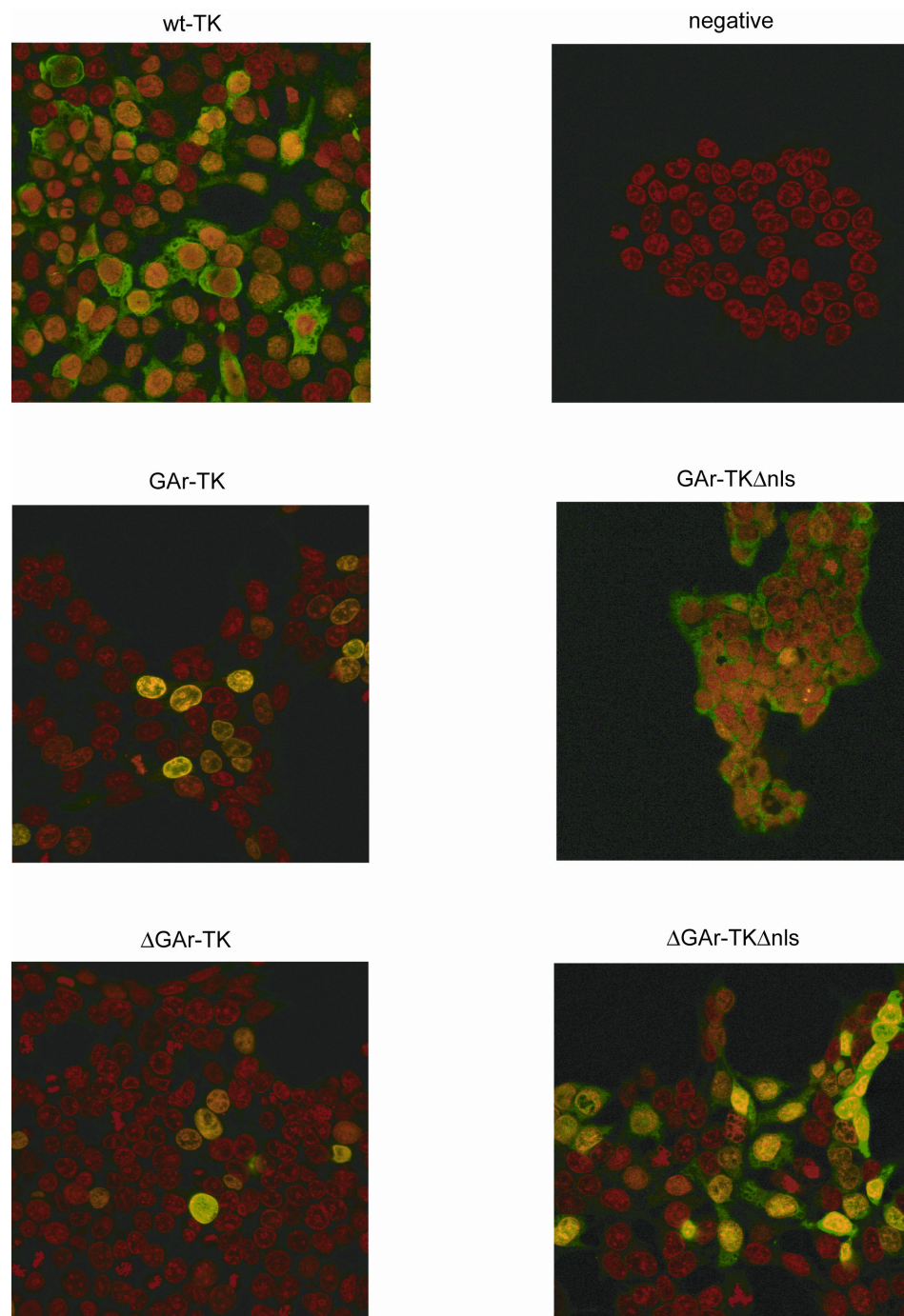Characterization of an immuno'stealth' derivative of the herpes simplex virus thymidine kinase gene

Figure 2.
Sub-cellular localization of the different TK proteins. 293T cells were transfected with wt-TK, GAr-TK, GAr-TKΔnls, ΔGAr-TK or ΔGAr-TKΔnls. Cells were incubated with rabbit anti-HSV-TK antiserum and a FITC-conjugated goat-anti-rabbit secondary antibody. The nucleus was stained with Propidium Iodide and the cells were analyzed by a confocal laserscan microscopy. Deletion of the nls but not the GAr restores the wt-TK distribution pattern.

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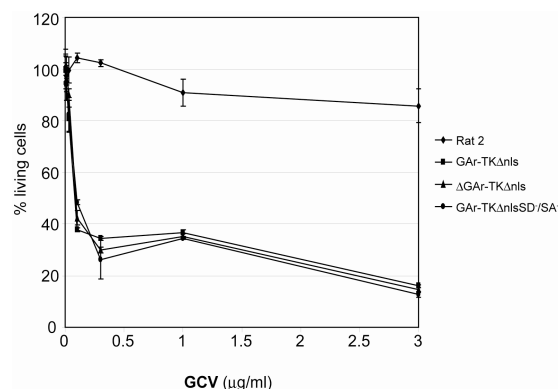
Figure 3
Deletion of the nls, GAr or SD/SA sites do not alter GCV sensitivity. Rat2 cell lines containing different TK constructs were seeded 3000 cells per well, 0.6 cm², with different concentrations GCV. After 4 days cell viability was assessed with the WST-1 colorimetric assay.

It has been demonstrated that the HSV-TK coding region contains cryptic splice-donor and splice-acceptor sites flanking the codons for the catalytic domain.[31] These were removed from the GAr-TKΔnls construct by conservative mutagenesis as described previously to generate the construct GAr-TKΔnlsSD⁻/SA⁻. This prevented splicing as described previously. Indeed, no spliced product could be detected by RT-PCR after transfection of this plasmid (data not shown). Therefore in all subsequent experiments the SD⁻/SA⁻ variants of the GAr-TK gene were used.

To verify the functionality of these plasmids, these were transfected into Rat2 cells and the resulting clones were tested for GCV sensitivity. Cells were seeded in 96-well plates and different concentrations GCV were added to the medium. After 5 days the viability of the cells was determined. As shown in figure 3, all the cell lines containing the various GAr, nls and SD-/SA- modifications were sensitive to the GCV, indicating that the plasmids encode functional TK variants.

So far no immunodominant CTL epitopes have been identified in the HSV-TK. To test the presentation of antigenic peptides by of the various TK constructs, a model CTL epitope was inserted in the N-terminal region of TK (Fig. 1). It has been shown that this part of the protein is dispensable for TK activity.[32] The ovalbumin epitope SIINFEKL (OVA 257-264) was used.[33,34] This allowed the use of an established CTL clone, B3Z [29], to test antigen presentation in vitro.

We first inserted the coding regions for GAr-TKΔnlsSD⁻/SA⁻ and ΔGAr-TKΔnlsSD⁻/SA⁻ into a lentiviral vector. Subsequently, the OVA epitope was inserted creating pGAr-ovaTKΔnlsSD⁻/SA⁻ and pΔGAr-ovaTKΔnlsSD⁻/SA⁻, respectively. Lentiviral particles were generated by the quadruple transfection technique and quantified by p24 ELISA. Vectors with and without GAr region could be grown to similar titers (data not shown). To transfer the variant TK genes into B16 cells (H2-K^b), we exposed the cells to approximately 80 ng of p24/10⁵ cells for each vector. PCR analysis of DNA isolated from the transduced cell populations did not reveal evidence for rearrangements or deletions in the GAr region. Western-blot analysis revealed the presence of proteins of the expected sizes (Fig 4).

Characterization of an immuno'stealth' derivative of the herpes simplex virus thymidine kinase gene

Figure 4
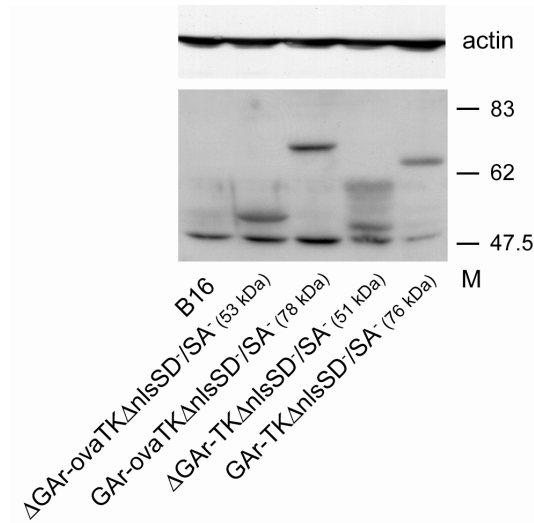All modified TK genes are efficiently expressed. B16 cells and B16 cells expressing GAr-TKΔnls, ΔGAr-TKΔnls, GAr-ovaTKΔnls or ΔGAr-ovaTKΔnls were lysed and protein extracts were size fractionated by SDS-PAGE. The western blot was analyzed with a polyclonal rabbit anti-HSV-TK antibody. All bands are at the expected size and no smaller products are visible. The bands migrating at 48 and 60 kDa are resulting from the cross reactivity in the assay with a B16-derived cellular protein and the immunoglobulin light chains, respectively.

Moreover, the GAr containing proteins reveal a homogeneous band, demonstrating the integrity and the stability of the vectors with the GAr element. The GCV-sensitivity assay did not reveal differences between the TK variants with and without the OVA peptide (Fig 5). This demonstrates that insertion of the OVA epitope into the N-terminal part of TK does not significantly affect its capacity to convert GCV.

To test the presentation of the OVA peptide, the variant-TK expressing cell populations were exposed to B3Z CTL hybridoma cells. In these cells, recognition of the OVA epitope induces the expression of an IL2-promoter that drives expression of a LacZ gene. The accumulation of LacZ was assayed by CPRG assay (Fig. 6). In the absence of the OVA peptide no β-gal activity can be detected. Expression of ΔGAr-ovaTKΔnlsSD⁻/SA⁻ leads to a strong induction of the reporter. However, if the GAr region is linked to the epitope as in the GAr-ovaTKΔnlsSD⁻/SA⁻ expressing cells, the peptide is not presented as is evident from the lack of induction of the β-gal reporter. This demonstrates that the GAr repeat can prevent presentation of the OVA epitope.

To enhance the applicability of the GAr-modified TK we introduced the activating mutations of the sr39 mutant described by Black et al. in GAr-TKΔnlsSD⁻/SA⁻ to generate GAr-TK $_{sr39}$ΔnlsSD⁻/SA⁻. The sr39 mutant was isolated after semi-random mutagenesis and has a Km value of 14.3-fold lower then wt-TK.[35,36] However, under the conditions used here we could show no enhancement for GCV sensitivity (data not shown).
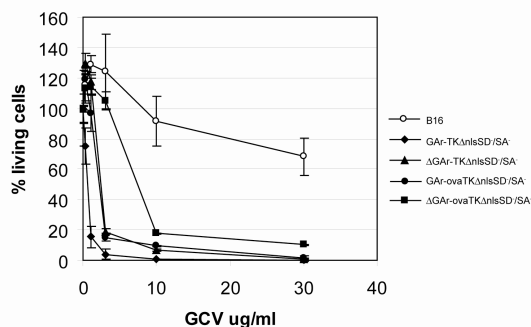
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Figure 5
Insertion of the OVA epitope does not essentially alter GCV sensitivity at higher GCV concentrations. B16 cell lines containing different TK constructs were seeded 3000 cells per well, 0.6 cm^2, with different concentrations GCV. After 4 days cell viability was assessed with the WST-1 colorimetric assay.

Discussion

Herpes Simplex Virus-derived thymidine kinase (HSV-TK) is the archetypical enzyme used in gene-directed enzyme prodrug therapies (GDEPT). Its capacity to convert the antiherpetic nucleoside analogues ganciclovir and acyclovir to toxic nucleotides has been used effectively in gene therapy protocols to eradicate tumor cells and lymphocytes that expressed the HSV-TK upon virus mediated gene transfer. Although effective, the use of the HSV-TK is limited by the induction of a cellular immune response against the HSV-TK protein. Upon infusion of HSV-TK-expressing T lymphocytes, 8 of 24 recipients developed an immune response against the transgene product. One avenue to circumvent this problem is to develop new 'human' suicide genes.[9,37,38]

As an alternative approach to evade the antigen-specific immune response we evaluated the use of the Glycine-Alanine repeat (GAr) region of the nuclear antigen 1 (EBNA-1) of the Epstein Barr Virus (EBV) to protect HSV-TK derived peptides from being presented. Here we provide evidence that fusing the GAr region of the EBNA-1 to HSV-TK reduces the presentation of transgene-product-derived peptides in vitro. Furthermore, we introduce some extra features that could be optimizing the TK gene even more. Fusions of the entire N-terminal sequence (aa 1- 420) of EBNA-1 with TK lead to a markedly changed subcellular localization TK. We were able to restore the normal cell distribution pattern of the modified-TKs by deleting the nls from the EBNA-1 derived sequences. In addition, we introduced mutations to prevent splicing, something that is known to occur in up to 5% of the cells expressing TK after retrovirus-mediated gene transfer. Previously we have shown that fusing the GAr with TK does not alter the effectiveness of the enzyme. In this study we show that the mutations we made to restore the distribution pattern and to prevent the splicing maintained the enzymatic activity of the encoded TK. Furthermore, we could show that
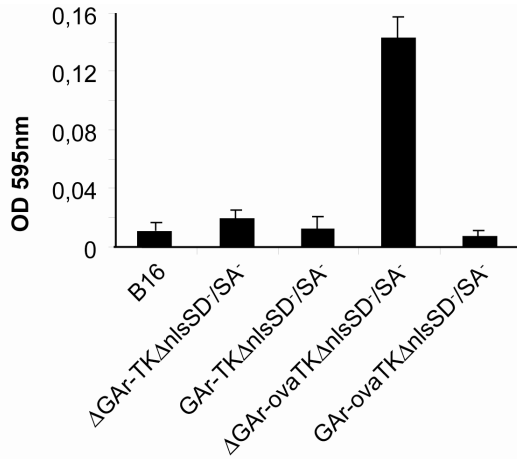
Figure 6
GAr protects the OVA epitope from being recognized. Target cells were seeded 6250 per 0.6 cm^2 well and 50000 B3Z cells were added (n = 3). After o/n incubation CPRG was added and OD was measured at 595 nm. Depicted is one representative experiment of four experiments. The B3Z cells react on the presence of the OVA epitope in absence of the GAr and OVA epitope. The presence of the GAr completely abolishes the recognition.

insertion of the OVA epitope did not overtly alter the capacity of TK to convert GCV. When we monitored for presentation of the OVA epitope using B3Z indicator cells, we observed a clear difference between ovaTK with or without the GAr, indicating that fusing the GAr to TK does indeed frustrate presentation even in highly stable enzymes as TK. So far, we have been unable to test the effect of the GAr on TK presentation in mice. We did not see significant differences in tumor take between naive mice and mice that were vaccinated with a first generation adenovirus vector that carriers the HSV-TK gene, upon challenge with B16 cells, B16 GAR-TKΔnls cells, and B16 ΔGAR-TKΔnls cells (data not shown). These data suggest that expression of the HSV-TK does not lead to the induction of antigen-specific CTL in C57/BL6 mice.

Although an EBNA-1 derived glycine and alanine-rich sequence of only 8 amino acids in length was found to significantly inhibit proteasomal degradation of instable reporter protein,[39,40] it remains to be established if such short sequences are sufficient to fully prevent antigen-presentation and CTL-mediated cell destruction. We will therefore direct further research into the reduction of the repeat length while maintaining the beneficial effect of the GAr in our system.

A recent study demonstrated that the GAr does not fully prevent the generation of antigenic peptides of full-length EBNA-1.[41] This is attributed to the formation of so-called defective ribosomal products or DRiPs. Several differences between the latter study and ours should be noted. The full length EBNA-1 was studied in human B-cells and lymphoblastoid cell lines (LCLs). Whereas specific lysis is observed in the GAr-containing EBNA-1, more efficient lysis could be obtained when the GAr was deleted from EBNA-1, confirming a stealthing effect of the GAr in the natural context. This is in accordance with the data from Lee et al.,[42] who also noted a partial protective effect of GAr in its natural context.

Furthermore, deletion of the GAr greatly reduces the half-life of EBNA-1 in B cells. This is consistent with a negative effect of the GAr on the efficiency of proteasomal degradation.[22] In addition, we have studied the effect of the GAr with HSV-TK (this study), LacZ and luciferase,[24] and GFP (M.O. & A.Z., unpublished data). These proteins are rather stable already and therefore may require less stabilization by the GAr, to sufficiently inhibit antigenic-peptide formation.

Nonetheless, our data describe an immuno 'stealthy' HSV-TK gene. We demonstrated that the 'stealthy' variant could confer GCV sensitivity to the transduced cells. It also evades recognition by established CTL. However, formal proof of its efficacy in humans awaits demonstration of the protective effects against human TK-directed CTL. This could be tested with CTL isolated from patients that have received TK-modified cells and mounted a TK-specific CTL response. We kindly invite anyone who has such material to initiate such studies using the vectors described here.

Materials and Methods

Construction of the GAr fusion genes

The HSV-TK gene containing plasmids are all derived from pGAr-TK.[24] To create pΔGAr-TK, pGAr-TKΔnls, pΔGAr-TKΔnls, pGAr-TKΔnlsSD⁻/SA⁻ and pGAr-TK $_{sr39}$ΔnlsSD⁻/SA⁻ the QuikChange Site-Directed Mutagenesis Kit (Stratagene Europe, Amsterdam, The Netherlands) was used according to the manufacturer's protocols with the primers listed in Table 1.

First, the leucine-rich nuclear localization signal (nls) was deleted from the pcDNA3.1-GAr-TK with primer set 1 creating pcDNA3.1-GAr-TKΔnls. Following the manufacturer's suggestion both primers were chosen to have completely complementary sequences. In the sequence, the mark indicates the junction. Primer set 2 was used to delete the GAr (from amino acids 41 to 376) from the pcDNA3.1-GAr-TK, yielding pcDNA3.1-ΔGAr-TK. To obtain pcDNA3.1-ΔGAr-TKΔnls that lacks both the nls and the GAr, primer set 3 was used on plasmid pcDNA3.1-GAr-TKΔnls to remove the GAr.

The plasmid lacking the cryptic splice-donor and splice-acceptor sites (SD⁻/SA⁻) was created from pcDNA3.1-GAr-TKΔnls. First primer set 4 was used to insert conservative changes that remove the splice-donor site (the mutation is shown in bold). After mutagenesis the presence of this mutation was verified by DNA sequence analysis. The resulting plasmid was used as template to create the pcDNA3.1-GAr-TKΔnlsSD⁻/SA⁻ by mutagenesis with primer set 5 (the mutation that is introduced is shown in bold) to inactivate the cryptic splice-acceptor site. A similar strategy was used to create pcDNA3.1-ΔGAr-TKΔnlsSD⁻/SA⁻ by starting with plasmid pcDNA3.1-ΔGAr-TKΔnls.

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Table 1: Primers used for the construction of the TK mutants.

Primers		Sequence 5'-3'
1	Δnls-$_{fw}$	GGTCGTGGACGTGGAGAA|CAGTCATCATCATCCGGG
	Δnls-$_{rv}$	CCCGGATGATGATGACTG|TTCTCCACGTCCACGACC
2	ΔGAr-$_{fw}$	GGGGGTGATAACCATGGA|GGAGAAAAGAGGCCCAGG
	ΔGAr-$_{rv}$	CCTGGGCCTCTTTTCTCC|TCCATGGTTATCACCCCC
3	ΔGAr$_{2-fw}$	GGGGGTGATAACCATGGA|GGAGAACAGTCATCATCATCC
	ΔGAr$_{2-rv}$	GGATGATGATGACTGTTCTCC|TCCATGGTTATCACCCCC
4	SD$^-$$_{fw}$	CCGCCTCGACCA**A**GGTGAGATATC
	SD$^-$$_{rv}$	GATATCTCACC**TT**GGTCGAGGCGG
5	SA$^-$$_{fw}$	CAGCATGACCCCCCA**A**GCCGTGCTGGCGTTC
	SA$^-$$_{rv}$	GAACGCCAGCACGGC**TT**GGGGGGTCATGCTG
6	OVA-$_{fw}$	CGCGAGCATCATTAATTTCGAGAAGCTGGC
	OVA-$_{rv}$	CGCGGCCAGCTTCTCGAAATTAATGATGCT
7	sr39$_{1-fw}$	CCGGCCCTCACC**A**TC**T**TC**C**TCGACCGCCATGGG
	sr39$_{1-rv}$	CCCATGGCGGTCGA**G**GA**A**GA**T**GGTGAGGGCCGG
8	sr39$_{2-fw}$	CGCCATCCCATCGCC**TTC A**TGCTGTGCTACCCGGCC
	sr39$_{2-rv}$	GGCCGGGTAGCACAGCA**T**G**AA**GGCGATGGGATGGCG
9	TK splice-$_{fw}$	CTCGACCAGG|GCCGTGCT
	TK-$_{rv2}$	CGACGAAGCTTAGTTAGCCTCCCCCATCTCCCG

The mark in primer sets 1-3 indicated where the nls or the GAr was deleted. The bold and underlined letters in the other primers indicate mutations. The mark in primer 9 indicates the junction of the spliced TK.

In the resulting plasmids, the ovalbumin (OVA)-derived CTL epitope (SIINFEKL, 257-264) was inserted in frame in the thymidine kinase coding region. The plasmids were digested with *Mlu*I. A unique site for this enzyme is present in the region of the TK gene coding for the N-terminus. Primer set 6 was annealed generating *Mlu*I overhangs, and the double-stranded product was cloned into the *Mlu*I site of the (Δ)GAr-TKΔnlsSD$^-$/SA$^-$ plasmids, creating pcDNA3.1-(Δ)GAr-OVA-TKΔnlsSD$^-$/SA$^-$.

All PCR products were characterized by restriction analysis and the presence of the mutations was verified by DNA sequence analysis. For the lentiviral system, the complete GAr-TKΔnlsSD-/SA$^-$ and ΔGAr-TKΔnlsSD$^-$/SA$^-$ coding regions were excised by *Spe*I and *Xho*I digestion and cloned into the pRRL vector digested with the same enzymes,[25] yielding pRRL-GAr-TKΔnlsSD-/SA$^-$ and pRRL-ΔGAr-TKΔnlsSD$^-$/SA$^-$.

On the latter plasmids, primer set 7 was used to create the mutations of the TK$_{sr39}$ at amino acids 594-596 (Fig 1). After confirmation of the presence of these mutations, primer set 8 was used on this template to create the mutations of the TK$_{sr39}$ at the amino acids 603 and 604, yielding pRRL-GAr-TK$_{sr39}$ΔnlsSD-/SA$^-$ and pRRL-ΔGAr-TK$_{sr39}$ΔnlsSD$^-$/SA$^-$. The PCR products were confirmed by restriction analysis and sequencing for the presence of the mutations and the GAr.

Cell lines

The 293T cells [26], B16 cells [27], and the thymidine-kinase deficient Rat2 [28] cells were cultured in DMEM (Gibco) containing 8% (vol/vol) fetal bovine serum, 100 IU of penicillin per ml, 100 µg of streptomycin per ml and 0.2% glucose. The B3Z indicator cells [29] were cultured in IMDM (Gibco) containing 8% (vol/vol) fetal bovine serum, 100 IU of penicillin per ml, 100 µg of streptomycin per ml, 28 µM β-mercaptoethanol and 500 µg hygromycin B per ml.

      Stable Rat2 cell lines expressing the different TK variants were made by transfection of 10 cm dishes with 9 µg TK construct and 1 µg pRSVneo using the Ca-Phosphate coprecipitation technique.[30] After 48 h, medium was replaced with medium containing 800 µg G418 sulphate (Geneticin, Gibco) per ml. After elimination of the G418-sensitive cells, the cultures were maintained on medium with 200 µg G418 per ml and used as polyclonal cultures. The cells were evaluated for the presence of the TK protein by immunofluoresence and ganciclovir (GCV) sensitivity assays.

      B16 cell lines stable expressing TK variants were made by transduction with lentiviruses at 1 ng p24/5000 cells o/n in the presence of 8 µg/ml polybrene. The next day, cells were seeded sparsely and monoclonal cell lines were isolated and expanded. Monoclonal cell lines were assayed for TK expression by western analysis and GCV sensitivity.

Virus production

Lentiviruses were produced using the quadruple transfection technique as described.[25] Briefly, 293T cells (60-70 % confluent) were transfected using the Ca-Phosphate co-precipitation technique. In a Ø 10 cm dish, 3.5 µg pCMV-VSVG, 6.6 µg pMDLg/RRE, 5 µg pRSV-REV and 10 µg transfer vector plasmid were added. After 48 h and 72 h, the virus-containing supernatant was harvested by collecting the medium and filtrating through a 45-µm filter (Pall Gelman, Portsmouth, UK). The virus-containing supernatant was stored at –80ºC until use. The p24 amounts were determined by ELISA (Gentaur, Brussels, Belgium) and served as a surrogate measurement of the transducing titer, by assuming that 1 ng p24 is the equivalent of 2500 transducing particles.

Enzymatic assays

Ganciclovir sensitivity assays to detect HSV-TK activity were performed in Rat2 cells or B16 cells as indicated. Briefly, 3000 cells were seeded per 0.6 cm^2 well, with different concentrations of GCV (Cymevene®, Roche diagnostics, Almere, The Netherlands) in triplicate. Cultures were grown for 4 days at 37ºC, 5% CO_2. Cell viability was assessed with the WST-1 colorimetric assay (Roche) according to the manufacturer's description.
Chlorophenolred-β-D-galactopyranoside (CPRG, Calbiochem) assays to detect presentation of OVA, target cells were seeded 12000-6000-3000 per 0.6 cm^2 well and 50000 B3Z cells

were added. After o/n incubation at 37ºC, 5% CO_2, CPRG was added and OD was measured at 595 nm.

Western analyses

B16 cells or B16 cells expressing GAr-TKΔnls, ΔGAr-TKΔnls, GAr-ovaTKΔnls or ΔGAr-ovaTKΔnls were lysed in RIPA buffer (50 mM Tris pH 7.5, 150 mM NaCl, 0.1% SDS, 0.5% DOC, 1% NP40) and protein extracts were size fractionated by SDS-PAGE, transferred to Immobilon-P nitrocellulose membranes and probed with rabbit anti-HSV-TK antiserum (1/250 diluted), kindly provided by Dr M Janicot (Aventis-Gencell, Vitry-Sur-Seine, France). After incubation with a peroxidase-conjugated goat-anti-rabbit secondary antibody, the proteins were visualized by enhanced chemiluminescence (Amersham Pharmacia Biotech, Roosendaal, The Netherlands).

RT-PCR

293T cells were transfected with GAr-TKΔnls or GAr-TKΔnlsSD⁻/SA⁻ using the Ca-Phosphate coprecipitation technique. After 48 h, RNA was extracted using the Absolutely RNA®, RT-PCR miniprep kit (Stratagene Europe, Amsterdam, The Netherlands) according to the manufacturer's protocol. Superscript II (Clontech) was used to reverse transcribe 4 μg of RNA (DNaseI treated). After reverse transcription, a PCR was performed with primer set 9. The forward primer was chosen to overlap the junction after splicing occurred, to show exclusively spliced, and therefore inactive, TK. The PCR products were analyzed on a 1% agarose slab gel.

Localization study

293T cells were transfected with wt-TK, GAr-TK, GAr-TKΔnls, ΔGAr-TK or ΔGAr-TKΔnls with the Ca-Phosphate coprecipitation technique. After 48 h, cells were fixed with methanol and subsequently incubated with rabbit anti-HSV-TK antiserum (1/250 diluted). After incubation with a FITC-conjugated goat-anti-rabbit secondary antibody and Propidium Iodide staining of the nucleus, the cells were analyzed by a Confocal LaserScan Microscope (CLSM, Leica DM IRBE, Leica microsystems B.V., Rijswijk, The Netherlands) using the Leica confocal software.

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