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Transcellular transfer of active HSV-1 thymidine kinase mediated by an 11-amino-acid peptide from HIV-1 Tat

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Suicide gene therapy using herpes simplex virus type-1 (HSV-1) thymidine kinase (TK) is a widely exploited approach for gene therapy of cancer and other hyperproliferative disorders. Despite its popularity, clinical success has been so far hampered mostly by the relative inefficiency of *TK* gene transfer and its limited bystander effect. Here we report that fusion of TK to an 11-amino-acid peptide from the basic domain of the HIV-1 Tat protein (Tat11) imparts cell membrane translocating ability to the enzyme and significantly increases its cytotoxic efficacy. In cells expressing Tat11-TK, this protein is found extracellularly, associated with cell surface heparan sulfate proteoglycans, and is released into the cell culture medium. Based on its interaction with HSPGs, the protein is then internalized by neighboring, nonexpressing cells, which become susceptible to cell death when treated with the nucleoside analogue acyclovir. As a consequence, co-cultures of wild-type cells with cells expressing Tat11-TK show increased sensitivity to ACV through a mechanism involving apoptosis. Modification of TK by fusion with Tat11 might constitute an important step for the optimization of *TK* suicide gene strategy for gene therapy of cellular proliferation.
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Thymidine kinase (*TK*) from human herpes simplex virus type 1 (HSV-1) is the most extensively exploited gene for the control of cellular proliferation in gene therapy.[1] Cells expressing TK convert the nucleoside analogues acyclovir (ACV) and ganciclovir (GCV) into their phosphorylated forms, which are in turn incorporated into replicating DNA where they block further chain elongation and consequently induce cell death.[2] Prodrug gene therapy using TK has found application in several instances. In particular, clinical trials for the treatment of tumors of the central nervous system have now reached Phase III. After surgical removal of most of the tumor mass, gene transfer of *TK* into residual tumor cells is achieved by inoculation of retroviral vector packaging cells, followed by systemic administration of GCV to induce cell death in both packaging and transduced cells.[3]

In the experimental murine model, this strategy has led to a remarkable success, even beyond the expected enzymatic effect of TK in expressing cells.[4] Subsequent investigations have demonstrated that efficacy of *TK* gene therapy involves the so-called bystander effect, in which physiological channels (the gap junctions) between adjacent cells allow for transport of the activated prodrug, thus extending the TK effect.[5,6] These features of TK prodrug gene therapy have led to a number of applications for gene therapy of human brain tumors.[3,7–12] However, the results so far obtained have shown only marginal clinical benefit, mainly due to the poor rate of delivery of the *HSV-TK* gene to tumor cells.[3,10] It seems now clear that novel strategies to extend efficacy of prodrug gene therapy are required before therapeutic efficacy is attained.

In the last few years, some proteins, which present the unusual characteristic of crossing the cell membranes through noncanonical processes of secretion or internalization, have been described. This property has been recognized in some viral proteins, including HIV-1 Tat,[13,14] HSV-1 VP22,[15] and, recently, hepatitis B virus PreS2[16] and in few eukaryotic proteins, among which are basic fibroblast growth factor (bFGF),[17] interleukin-1,[18] and Antennapedia.[19] The translocating property of some of these proteins has been ascribed to a defined region, alternatively called translocating motive (TLM), protein transduction domain (PTD), or membrane-translocating sequence (MTS). For example, this module consists of a 16-amino-acid amphipatic helix in the homeodomain of Antennapedia,[19] of an 11-amino-acid hydrophobic region in FGF,[20] and of an 11-amino-acid peptide with strong basic features in HIV-1 Tat (Tat11 in the following).[14,21]

A feature of major interest is that some of these peptides (or the proteins from which they are derived) also confer

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translocating capability to other proteins when coupled or fused to them. In particular, chemical crosslinking of Tat peptides with heterologous proteins[22] or, more efficiently, production of recombinant proteins containing the PTD of Tat[21,23] facilitates the intracellular delivery of these proteins. We have recently observed that recombinant proteins fused to full-length Tat (86 amino acids) efficiently enter the cells when present in the extracellular medium and are readily transported to the nucleus in an active form.[24–26] Accordingly, Schwarze et al[23] have reported that Tat11 confers membrane crossing ability to a reporter β-galactosidase protein, which was shown to reach all body districts when administered intraperitoneally into live mice.

The molecular mechanisms by which these proteins translocate are still largely undefined and could be different. Secretion of VP22 is through a nonclassical, Golgi-independent pathway and internalization by neighbouring cells require a functional cytoskeleton;[15] the translocating region of Antennapedia is capable of directly crossing cell membranes[27] and internalization of bFGF is mediated by its interaction with specific tyrosine kinase receptors.[28] As far as HIV-1 Tat is concerned, we have recently shown

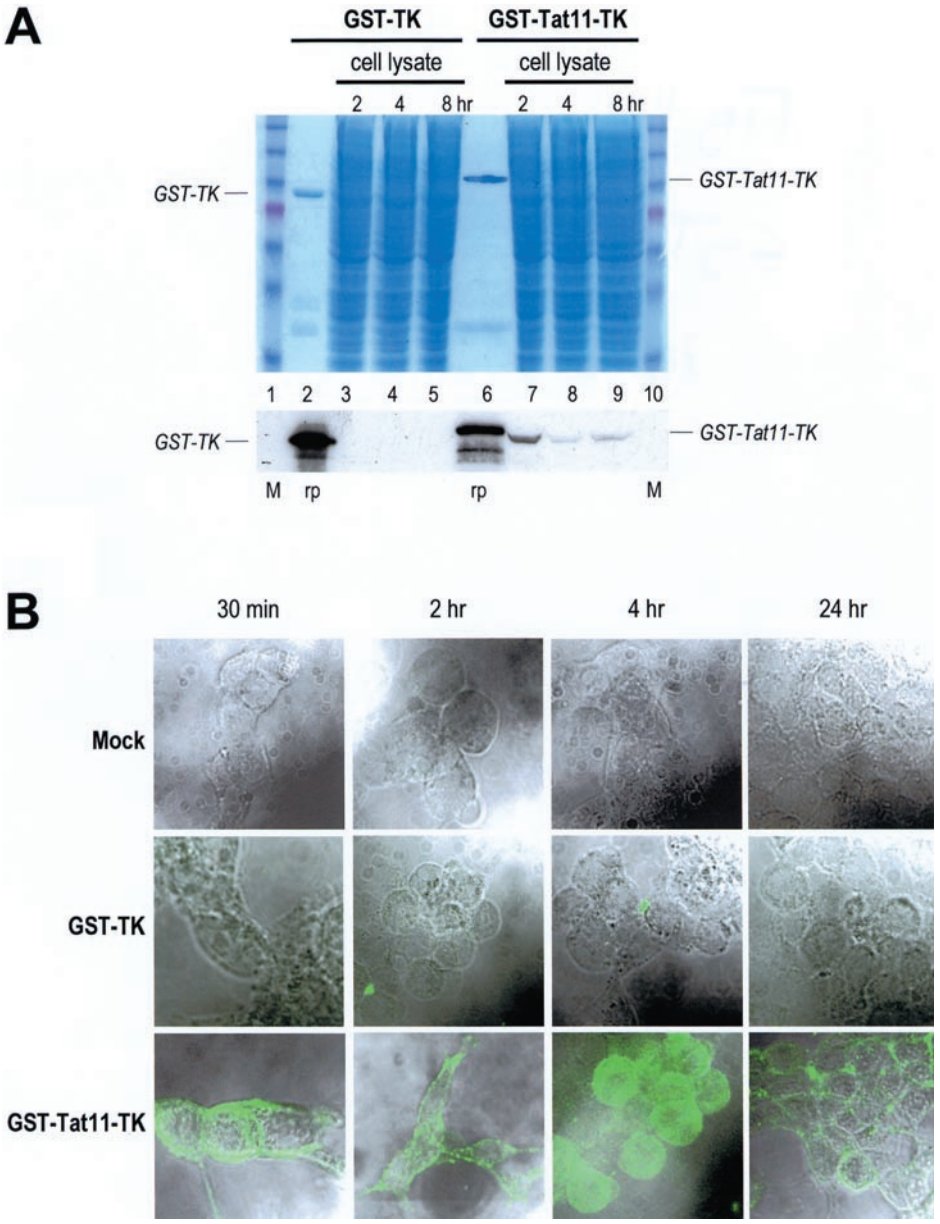

Figure 1 Internalization of recombinant TK and Tat11-TK. **Panel A:** The upper part shows a Coomassie-stained gel showing recombinant GST-TK and GST-Tat11-TK (2 μg; **lanes 2 and 6**, respectively) and total cell lysates from HEK 293 cells treated for the indicated time periods with these proteins (1 μg/mL; **lanes 3–5 and 7–9**, respectively). The lower panel shows the reactivity of the same samples with an antibody against TK. M: molecular weight markers; rp: recombinant protein. **Panel B:** Immunofluorescence of cells treated with recombinant GST-TK and GST-Tat11-TK (1 μg/mL) using an anti–TK antibody at different time points after protein addition. Mock-treated controls consisted of PBS-treated cells processed by the same procedures. Altogether, the two panels show that recombinant Tat11-TK is efficiently internalized by the cells.

that its internalization is mediated by the interaction of the basic domain of the protein with cell surface heparan sulfate proteoglycans (HSPGs) and occurs through an endocytic pathway.[26] While the presence of HSPGs is a requisite for efficient Tat internalization, cells impaired in their production are still capable of releasing the protein in the extracellular compartment through a still undefined mechanism.[26]

The ability of Tat to determine the release of fusion proteins outside the cells and to mediate their internalization by nonproducing cells is a very appealing feature when applied to the extension of the bystander effect of TK. Here we show that fusion of Tat11 to TK permits transcellullar transfer of the enzyme in co-cultures of producing and target cells, and that this process involves an extracellular step in which HSPGs play an essential role. We also show that Tat11-TK substantially improves the effect of ACV treatment in mixed populations, being additive to the canonical bystander effect, and that this protein induces cell death by the same mechanism of action of TK involving cell apoptosis.

Methods

Plasmids constructs

Constructs expressing TK and Tat11-TK were obtained either as fusions with glutathione-*S*-transferase (GST) in the prokaryotic vector pGEX-2T (Pharmacia, Uppsala, Sweden), or in the eukaryotic expression vector pcDNA3 (Invitrogen, Carlsbad, CA). The starting plasmid contained a GST-Tat-GFP cassette in vector pGEX-2T,[26] with Tat86 cloned between *Hin*dIII and *Bam*HI sites and GFP between *Bam*HI and *Eco*RI. From this plasmid, GFP was replaced with TK obtained by PCR amplification with primers containing *Bam*HI and *Eco*RI sites at their extremities, producing pGEX-Tat86-TK. In this plasmid, Tat86 was replaced with Tat11 (aa sequence YGRKKRRQRRR, preceded by an initiator methionine) produced as a pair of complementary oligonucleotides with flanking *Hin*dIII and *Bam*HI sites. Plasmid pGEX-TK was obtained by substituting the Tat86-GFP cassette with a PCR product corresponding to TK with flanking *Hin*dIII and *Eco*RI sites. To obtain the pcDNA3 versions of these two constructs, the Tat11-TK and TK cassettes were excised with *Hin*dIII and *Eco*RI and cloned into the respective sites of the vector.

Recombinant proteins

GST-TK and GST-Tat11-TK were produced in *Escherichia coli* and purified on glutathione–agarose beads as described,[25] except for the bacterial lysis step, which was performed with a cell disrupter to avoid temperature rise during sonication. Following centrifugation, glutathione–agarose beads were directly added to the supernatant; the subsequent steps were performed as described.[25] Purity and integrity of proteins eluted from beads were routinely checked by SDS-PAGE. For use, the recombinant proteins were diluted in phosphate-buffered saline and added to the cell culture medium.

Cell cultures and treatments

Human embryonic kidney 293 and mouse melanoma B16-F10 cells[29] (a kind gift of A Muro) were maintained in DMEM supplemented with 10% fetal calf serum and 50 μg/mL gentamicin. Human neuroblastoma ADF cells[30] (kindly donated by G Zupi) were cultured in RPMI 1640 supplemented with 10% fetal calf serum and 50 μg/mL gentamicin.

Proliferation experiments were typically performed by seeding 5×10^3 cells/well in 96-well plates on day -1. Recombinant protein, ACV (200 μM; Sigma, St. Louis, MO) and/or heparin (5 μg/mL; Sigma) were added on day 0 and MTT assays (Roche Molecular Biochemicals, Indianapolis, IN) were performed from day 0 at the indicated times following manufacturer's instructions. Cell culture treatment with ACV was performed by adding fresh drug every 3 days.

Immunocytochemistry with an anti–TK antibody (a kind gift of M Janicot) was performed on 293 cells treated with GST-TK or GST-Tat11-TK by fixing the cells with paraformaldehyde 4% in PBS for 15 minutes, followed by 5-minute incubation in PBS/0.1 M glycine. After washing

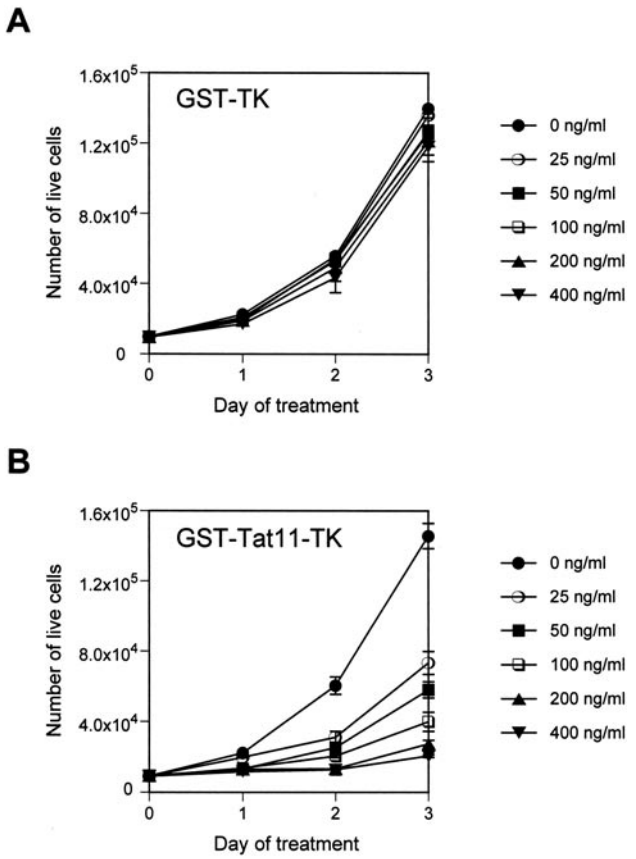

Figure 2 Effect of recombinant TK and Tat 11-TK on cell proliferation. HEK 293 cells in 96-well plates were incubated with the indicated amount of recombinant, purified GST-TK **(Panel A)** or GST-Tat11-TK **(Panel B)** in the presence of 200 μM ACV. Cellular proliferation was measured in triplicate every day by the MTT assay; results are expressed as number of live cells after conversion using a previously set calibration curve. Treatment with Tat11-TK (but not with TK) confers dose-dependent ACV sensitivity to the culture.

with PBS, chamber slides were incubated for 5 minutes in PBS/Triton 0.1% and, after extensive wash with PBS/1% BSA/0.1% Tween 20, the primary antibody was added for 1 hour at 37°C. After an additional extensive washing step with PBS/1% BSA/0.1% Tween 20, slides were incubated with the secondary antibody for 30 minutes at 37°C. Finally, slides were washed in PBS, mounted, and observed by confocal microscopy.

Stable transfectants were obtained by selection with geneticin (Gibco BRL Life Technologies, Paisley, Scotland, UK) after transfection with pCDNA3-based constructs. Clones (24 for each type) were obtained by limiting dilution of recently selected pools and characterized for ACV sensitivity and TK production by Western blotting.

For the experiment shown in Figure 3, TK and Tat11-TK clones were expanded in 10-cm plates. At about 80% confluence, the medium was replaced by Optimem without serum and cultures were kept for 3 days at 37°C. After removing the medium (used as supernatant fraction), cells were washed once in PBS, scraped off the plates, and resuspended in 0.2 mL of heparinase buffer (BSA 1%, glucose 0.1%, gelatin 0.2% in PBS) with 30 μU of heparinase III (Sigma). Finally, the cells were pelletted and the supernatant (heparinase-released fraction) and pellet

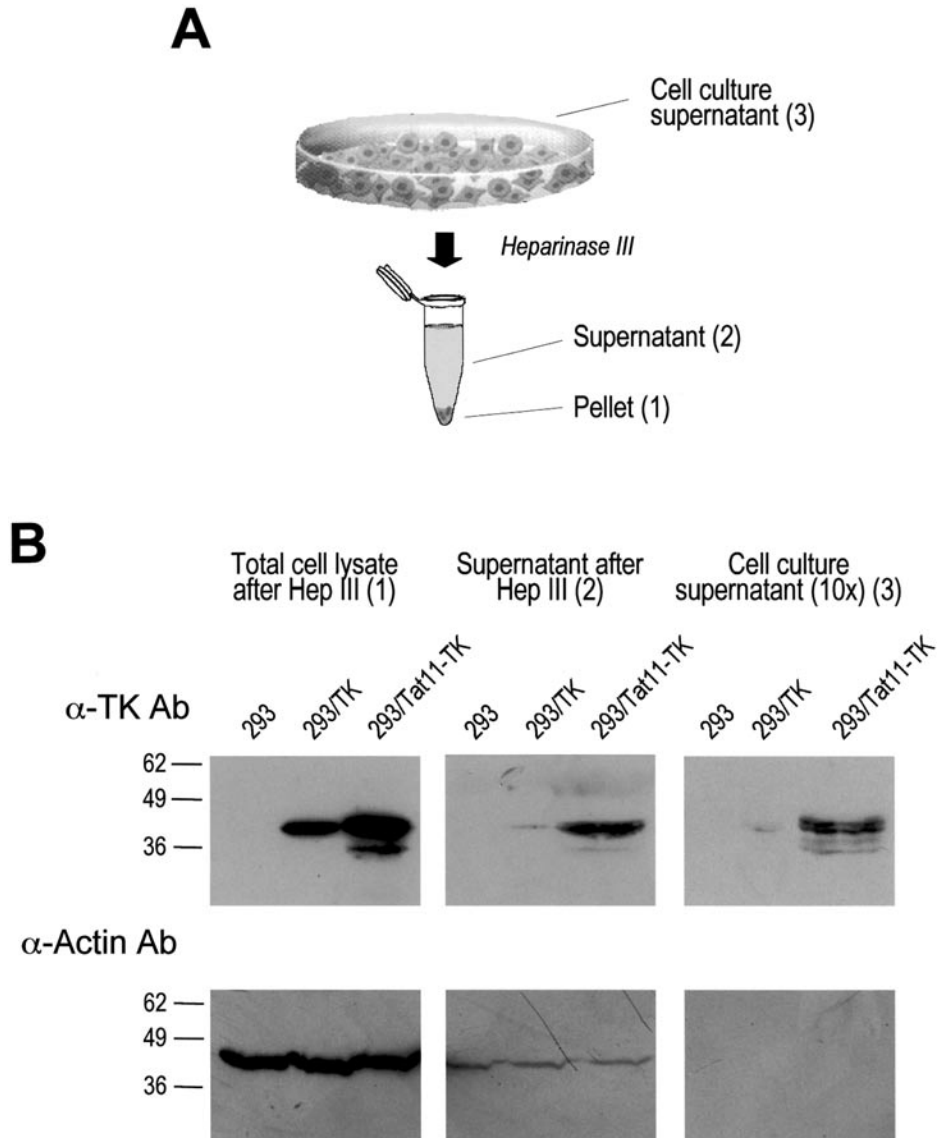

Figure 3 Extracellular export and release of Tat11-TK. **Panel A:** Supernatant from control, TK- and Tat11-TK–expressing cells were harvested before cell treatment with heparinase III (Hep III). After treatment, cells were pelleted. Samples 1, 2, and 3 represent total cell lysates after Hep III treatment, membrane-associated proteins released by Hep III treatment, and cell culture supernatants before treatment, respectively. **Panel B:** Aliquots of samples obtained after treatments 1, 2, and 3 for each cell type were probed by Western blotting using antibodies against TK **(upper row)** or actin **(lower row)**. Samples 1, 2, and 3 correspond to 1/25, 1/5, and 1/10 of the total amount of protein obtained after the various treatments, respectively. The results indicate that TK is only present in the cellular extract, whereas a consistent fraction of Tat11-TK is found outside of the cells, partially released in the supernatant and mostly concentrated on cell surface HSPGs.

were prepared for SDS-PAGE and Western blotting. The anti–actin antibody (A2066) was purchased from Sigma and recognizes an 11-amino-acid epitope in the C-terminal region of actin.

The experiments involving treatment with mitomycin C (Sigma) were performed by adding the drug at 2.5 μg/mL for 3 hours prior to seeding cells in 96-well plates for co-cultures.

Cytofluorimetry

Cells (2.5×10^6) were seeded in 10-cm plates and grown for 48 or 72 hours after addition of ACV. After trypsinization, 1×10^6 cells were analyzed for membrane reactivity to annexin V using the kit manufacturer's instruction (Roche Molecular Biochemicals). DNA content was analyzed by incubating cells after ethanol fixation with RNAse A (250 μg/mL) and propidium iodide (10 μg/mL) in PBS for at least 1 hour at 37°C. Cytofluorimetric analysis was performed in a FACScalibur (Becton Dickinson, San Jose, CA) instrument acquiring 3×10^4 events for each sample.

Results

Fusion of HSV-1 TK to an 11-amino-acid peptide from HIV-1 Tat permits its intracellular delivery

We obtained two plasmid vectors expressing fusion proteins of GST with either TK or TK preceded by 11 amino acids from the basic domain of Tat (Tat11) that permit cellular internalization.[21] The recombinant proteins GST-Tat11-TK and GST-TK were purified on a glutathione–agarose column and added to the culture medium of growing HEK 293 cells. After 2, 4, and 8 hours from protein addition, cells were trypsinized and cell-associated protein visualized by Western blotting using anti–TK antibody. Figure 1A upper panel shows Coomassie staining of a preparation of the two recombinant proteins (lanes 2 and 6) and total cell lysates from cells treated with these proteins (1 μg/mL). The lower panel of the same figure shows immunoreactivity of these preparations to the anti–TK antibody. The recombinant protein was clearly visible in cell lysates from cells treated with GST-Tat11-TK (lanes 7–9) but not from cells treated with GST-TK (lanes 3–5).

To directly visualize protein internalization, HEK 293–treated cells were also analyzed by confocal microscopy after immunocytochemistry with the same antibody. As shown in Figure 1B, intracellular fluorescence was detected only in cells treated with GST-Tat11-TK. The localization pattern of the internalized protein indicates significant uptake already at 30 minutes from protein addition, with subsequent nuclear accumulation at 4 hours. After 24 hours, residual fluorescence was detected at the periphery of the cells. The localization pattern and kinetics of the internalized protein were similar to those already observed for recombinant GST-Tat86.[26]

To assess functionality of the internalized protein, HEK 293 cells were treated with scalar amounts of GST-Tat11-TK or GST-TK together with the prodrug ACV. Cellular proliferation was monitored daily by the MTT assay, a colorimetric measure that directly correlates with the number of metabolically active cells in a population. As shown in Figure 2, even large amounts of TK did not have any effect on cellular proliferation. In contrast, GST-Tat11-TK sensibly impaired cell growth even at its lowest dose (25 ng/mL). Altogether, these results indicate that the GST-Tat11-TK protein is uptaken by cells through a mechanism mediated by Tat11, and that it exerts its enzymatic activity intracellularly in a dose-dependent manner.

Tat11-TK is released by mammalian cells and associates with cell surface proteoglycans

We have recently observed that an additional property of full-length Tat is to be released by producing cells and to mediate export of fused proteins of larger size.[26] Therefore, we set out to investigate whether this might be again a property imparted to the protein by the same basic domain. For this purpose, we constructed eukaryotic expression

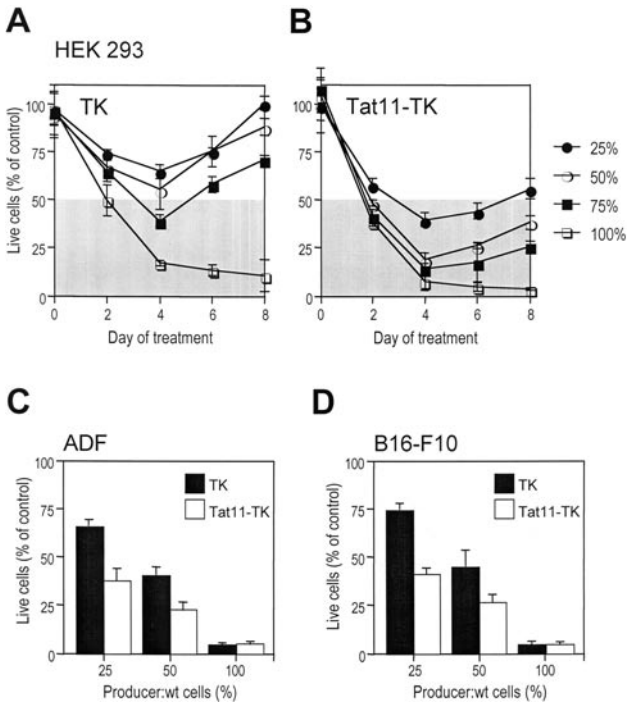

Figure 4 Proliferation of co-cultures of wild-type cells mixed with TK– or Tat11-TK–expressing cells. **Panels A and B:** Mixtures containing the indicated percentages of HEK 293 cells expressing TK **(Panel A)** or Tat11-TK **(Panel B)** cultured with wild-type cells were grown in the presence of ACV in 96-well plates. Proliferation was measured in triplicate at the indicated times by the MTT assay. Data on each day are expressed as percentage of live cells relative to control on the same day. **Panels C and D:** Viability of co-culture experiments with human neuroblastoma ADF cells and mouse melanoma B16-F10 cells expressing TK or Tat11-TK with the respective wild-type cells. Co-cultures were performed with 25%, 50%, and 100% producer cells and analyzed on day 6 after seeding. Proliferation was measured in triplicate by the MTT assay. Data are expressed as percentage of live cells relative to control on the same day. For all three analyzed cell types, cultures containing Tat11-TK–producing cells are remarkably more sensitive to ACV than cultures containing the corresponding proportions of TK-producing cells.

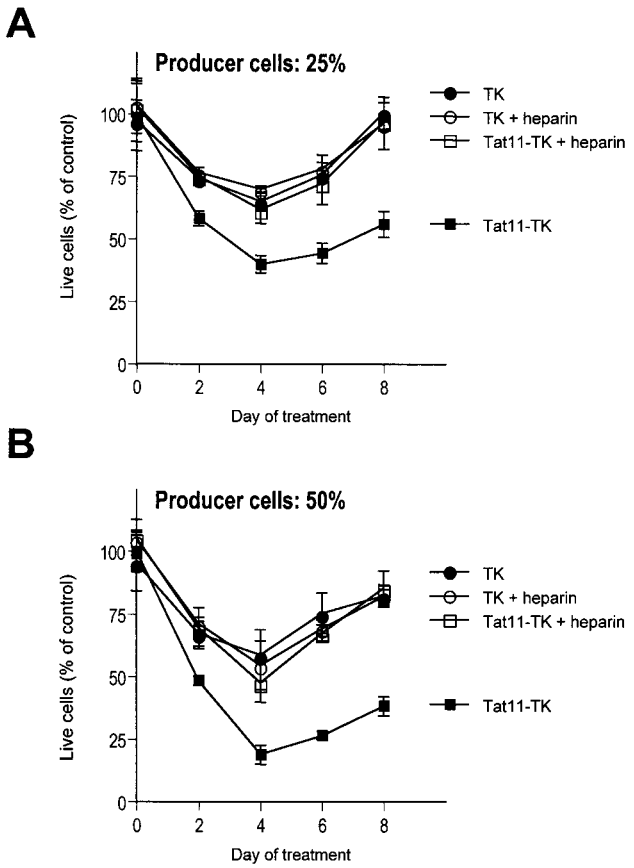

Figure 5 Effect of heparin on the proliferation of co-cultures of wild-type cells with TK- or Tat11-TK–producing cells. Co-cultures were set up containing wild-type cells mixed with 25% or 50% TK- or Tat11-TK–expressing cells (**Panels A and B**, respectively). Co-cultures were grown in the presence of ACV and with or without 5 μg/mL heparin. Proliferation was measured in triplicate at the indicated times by the MTT assay. On each day, data are expressed as percentages of live cells relative to control on the same day. The presence of heparin has no influence on the effects of TK cell on cell proliferation, whereas it abolishes the increased efficacy of Tat11-TK, blocking the component due to intercellular translocation.

vectors encoding TK or Tat11-TK and obtained stable HEK 293 cell clones constitutively expressing these proteins. To assess the levels of expression and possible extracellular release of these proteins, cells were kept in serum-free medium for 72 hours, the medium was collected, and the cells treated with heparinase III in order to solubilize the proteins associated with the cell surface (Fig 3A). The concentrated tissue culture supernatant, the heparinase-released fraction, and the total lysate from heparinase-treated cells were resolved by SDS-PAGE and reacted with anti–TK antibody. As shown in Figure 3B, upper panel, lanes 1–3, both Tat11-TK and TK were present at similar levels inside the cells. By contrast, only the modified enzyme was also visualized both in the cell culture supernatant after concentration (lanes 7–9) and in the fraction associated with the cell surface (lanes 4–6). As a control (Fig 3B, lower panel), we observed that another abundant cytoplasmic protein, α-actin, was not released from the cell into the medium (lanes 7–9) and was only minimally appreciable after lyase treatment (lanes 4–6), possibly as a consequence of marginal unspecific leakage.

We conclude from this experiment that the 11-amino-acid basic peptide of Tat is also sufficient to confer to TK the capacity of being exported from the cells that produce it. A fraction of the secreted protein is released into the medium, whereas the rest is found associated with HSPGs on the cell surface.

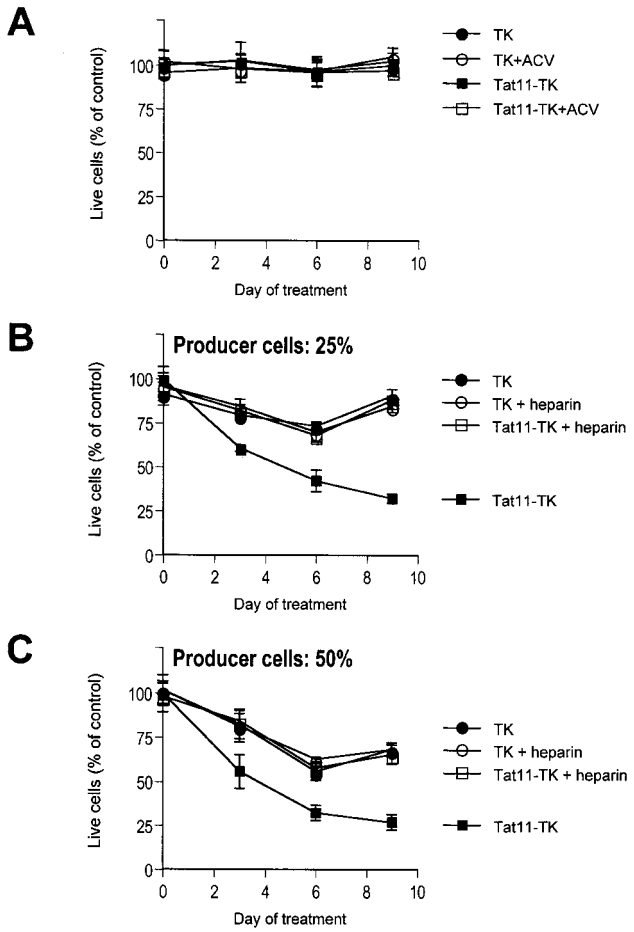

Figure 6 Prolonged bystander effect of Tat11-TK after cell treatment with mitomycin C. **A:** Cells treated with mitomycin C do not proliferate and become insensitive to ACV treatment. Cells expressing TK or Tat11-TK were treated with 2.5 μM mitomycin C for 3 hours and then ACV was added. Data (average of three measurements) were obtained every 3 days by the MTT assay and are expressed as percentages of live cells relative to control. The absolute number of live cells remained constant during the whole duration of the experiment. **B and C:** Long-lasting inhibition of cellular proliferation by mitomycin-treated Tat11-TK cells. Co-cultures containing 25% **(Panel B)** or 50% **(Panel C)** cells expressing either TK or Tat11-TK, and treated with mitomycin C in the presence or absence or heparin, were started. All co-cultures were treated with ACV. Data (average of three measurements) were obtained every 3 days by the MTT assay and are expressed as percentages of live cells relative to controls. Co-cultures with mitomycin-treated Tat11-TK cells show impaired proliferation up to day 9; the increased effect with respect to TK is abolished by heparin, again suggesting transcellular translocation of the protein. The limited decrease observed in cell proliferation of TK co-cultures compared to the curves shown in **Panel A** might be attributable to a limited canonical (gap junction–mediated) bystander effect of TK.

Tat11-TK exerts its cytotoxic activity beyond producer cells

After demonstrating that Tat11-TK can be both uptaken into and secreted from HEK 293 cells, we wanted to check whether the two steps could be joined together in a cell culture system. For this purpose, we co-cultured either TK- or Tat11-TK–expressing cells (producers) with wild-type cells (targets) at different ratios. The mixtures were grown in the presence of ACV and cell proliferation was measured every 2 days by the MTT assay. Figure 4 reports the proliferation data of such co-cultures. Data are expressed as percentage of live cells relative to control cultures (100% wild-type cells, 0% producer cells) at the same day, and are averaged over three measures.

As expected, both TK and Tat11-TK pure cultures were equally sensitive to ACV and died out by day 8. In the mixtures between wild-type and TK cells, the decline in cell growth was approximately proportional to the number of TK-expressing cells (Fig 4A). For example, in cultures containing 25% of TK-expressing cells, this decline was about 25% on day 4. This indicates that only cells expressing the enzyme were selected by the drug. By contrast, the percentage of live cells in mixed cultures containing Tat11-TK producers was remarkably lower than expected from the effect of TK alone at all the investigated cell ratios. Again on day 4, in the 25% co-culture, the decline in viability was about 60% relative to control. These results indicate that fusion of TK to Tat11 extends the cytotoxic activity of the enzyme to nonproducing co-cultured cells.

In order to ascertain whether the increased functionality of the modified TK protein was effective also in other cell types, we obtained clones expressing TK or Tat11-TK from human ADF neuroblastoma and mouse B16-F10 melanoma cells. In both cases, the selected clones expressed comparable amounts of the two proteins as assessed both by Western blotting and immunofluorescence (data not shown). Figure 4C and D shows viability of co-cultures containing different ratios of producer/wild-type cells on day 6 after beginning of ACV treatment. In all cases, expression of Tat11-TK led to significantly increased cytotoxicity of mixed cultures. For both HEK 293 (Fig 4A and B) and B16-F10 cells (Fig 4D), the classic TK bystander effect was minimal because the experiments were performed with cells seeded at low density. In contrast, in the case of ADF cells, which were grown at higher density, a bystander effect was detectable because the number of dead cells in the co-cultures with unmodified TK exceeded the number of TK-expressing cells (Fig 4C). Also in this case, however, co-cultures containing Tat11-TK showed increased ACV sensitivity, thus indicating that the Tat11 modification improves TK functionality over canonical bystander effect.

Intercellular transduction of Tat11-TK is inhibited by heparin

The intercellular translocation of recombinant Tat, Tat peptides, and Tat fusion proteins is strongly inhibited by heparin, a soluble analogue of heparan sulfate glycosaminoglycans (GAGs).[31–33] To understand whether transfer of the cytotoxic effect of Tat11-TK to nonexpressing cells was mediated by extracellular passage of the protein, we set up a series of co-culture experiments in the presence of heparin. As shown in Figure 5, the presence of this drug at 5 μg/mL did not impact on proliferation of the TK co-cultures. In contrast, the extended bystander effect observed in the Tat11-TK co-cultures was completely abolished by the presence of the heparin. This effect was observed at all

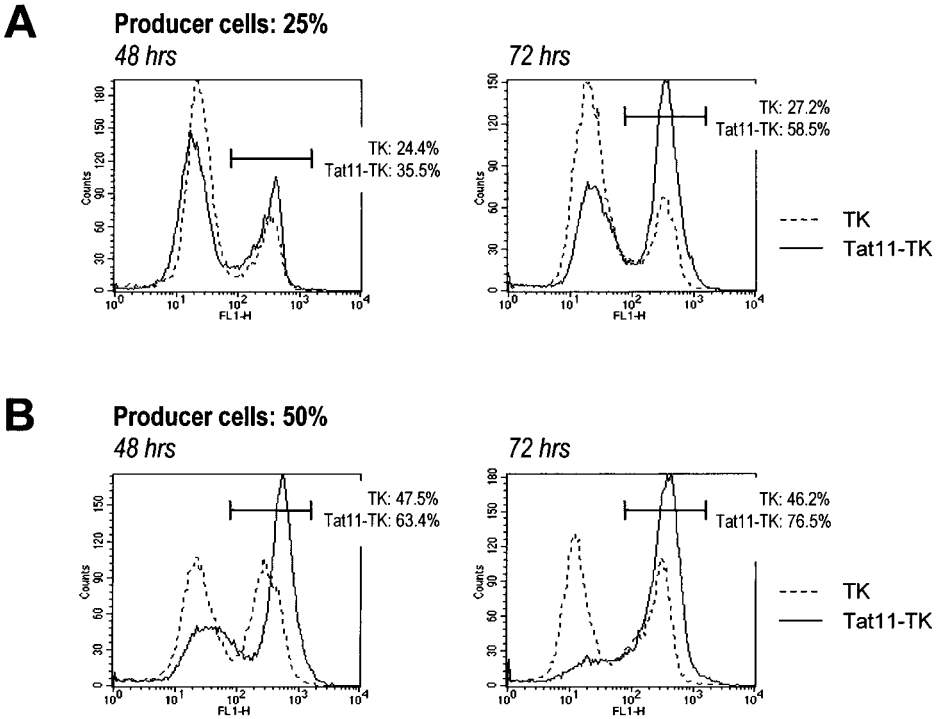

Figure 7 Membrane reactivity to annexin V of co-cultures with TK- or Tat11-TK–expressing cells. Co-cultures containing 25% **(Panel A)** or 50% **(Panel B)** cells expressing either TK or Tat11-TK were grown for 48 or 72 hours and then analyzed for reactivity to annexin V. The percentages of apoptotic, annexin V–positive cells (gated) are indicated.

producer/target cell ratios (of which 1:2 and 1:4 are shown in Fig 5).

These results are in agreement with the observation (Fig 3) that a large fraction of Tat11-TK is found associated with cell surface HSPGs, and indicate that transfer of this protein from producer to target cells involves an extracellular passage.

Treatment of Tat11-TK cells with mitomycin C confers long-lasting conditional cytotoxicity to co-cultures

The co-culture proliferation profiles of Figures 4A and B, and 5 show a decline during the first 4 days followed by a rebound at later time points. This is most likely explained by the fact that producing cells are themselves sensitive to ACV and are cleared out of the cell population after 4 days. Confirmation to this interpretation is provided by the experiment in Figure 6, in which co-cultures are set up with producing cells treated with mitomycin C. This antibiotic at low dosage does not induce cell death, while it stops cellular proliferation, rendering producing cells insensitive to the action of ACV (Fig 6A). Nevertheless, the metabolic activities of cells remain unaffected, allowing for continuous production/release of the enzyme.

On the basis of this observation, we set up co-culture experiments of wild-type cells with TK or Tat11-TK producers treated with mitomycin C. Shown in Figure 6B and C are the proliferation profiles of co-cultures containing 25% and 50% producer cells, respectively. Although TK-producing cells can persist for the entire duration of the experiment, the unmodified TK they express is not sufficient to maintain an active control of the population: After day 6, the amount of growing cells increases. By contrast, in the case of the Tat11-TK co-cultures, the increased cytotoxic effect is well evident for the whole duration of the experiment, indicating that persistence of producer cells despite treatment with ACV results in the extension of the cytotoxic effect to target cells for a longer period of time.

Again, addition of heparin to the Tat11-TK co-cultures restores the growth pattern of TK-containing co-cultures, at both the qualitative (shape of the curve) and quantitative (values in the curve) levels. This observation indicates that the long-lasting effect is achieved, thanks to the diffusible nature of Tat11-TK.

The enhanced bystander effect of Tat11-TK involves apoptosis

Data obtained by the MTT assay provide a measure of the proliferation of a cellular population, but are not informative about the fate of the cells. In the presence of ACV, cells expressing TK are known to undergo cell cycle arrest and subsequent apoptosis.[34–36] To confirm that extension of Tat11-TK–mediated cytotoxic effect to nonproducer cells also involves an apoptotic mechanism, we analyzed cellular DNA fragmentation and membrane annexin V reactivity of co-cultures. Wild-type cells were cultured with TK- and Tat11-TK–expressing cells at different ratios and analyzed by cytofluorimetry at 48 and 72 hours after addition of ACV. Co-cultures with both TK and Tat11-TK producers show a remarkable proportion of cells reactive to annexin V (Fig 7) and a DNA profile displaying a prominent sub-G1 peak (Fig 8). At both time points, but more visibly at 72 hours, both annexin V reactivity and propidium iodide staining reveal that the percentage of cells undergoing apoptosis is higher in the Tat11-TK co-cultures. For example, in the

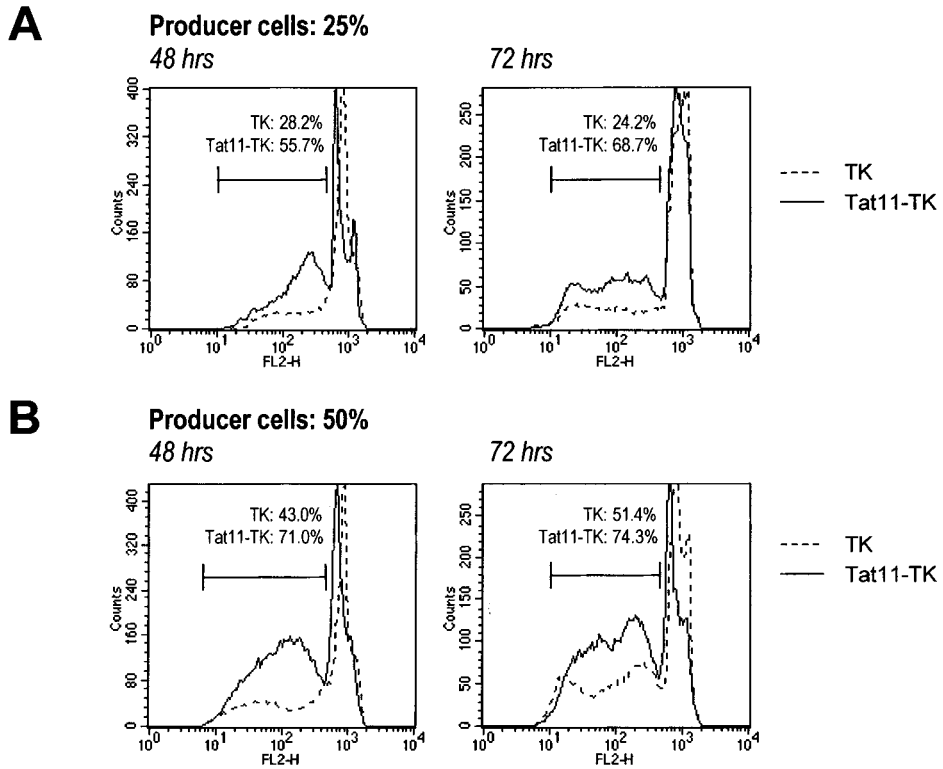

Figure 8 Analysis of DNA fragmentation in co-cultures with TK- or Tat11-TK–expressing cells. Co-cultures containing 25% **(Panel A)** or 50% **(Panel B)** cells expressing either TK or Tat11-TK were grown for 48 or 72 hours. Cells were then fixed in ethanol, treated with RNAse, and stained with propidium iodide. DNA profiles were evaluated as described in Ref. [59]. The gated peaks show the percentage of cells with fragmented DNA.

25% producer/target cell ratio at 72 hours, the percentage of cells with DNA fragmentation is 24.2% for TK cells *vs* 68.7% for Tat11-TK cells. Altogether, these results indicate that fusion with Tat11 extends TK conditional cytotoxicity to nonexpressing cells by induction of an apoptotic program.

Discussion

Suicide gene therapy represents an appealing possibility for the treatment of pathologic conditions of cellular hyperproliferation, including cancer (for recent reviews, see Refs. [1,37]), intimal hyperplasia following balloon angioplasty,[38–40] and undesired T-cell alloreactivity.[41–43] Among the various enzyme/prodrug combinations that have been developed for this purpose, cell transduction with HSV-1 TK followed by treatment with GCV/ACV constitutes the basis for over 60 clinical trials. With special reference to gene therapy of cancer, this approach has so far brought very limited clinical success, especially because of the unsatisfactory delivery of the suicide gene by the currently available gene therapy vectors.[3,10,12] While the bystander effect could, at least in principle, partially compensate for the poor delivery, it is not infrequent that cancer cells reduce expression of connexins — the proteins that form gap junctions, which permit diffusion of the toxic nucleotide product to neighboring cells.[44] Several approaches have been tested to overcome these problems, including co-expression of TK and connexins,[45–47] enhancement of the catalytic activity of TK,[48] or co-delivery of TK with genes encoding for cytokines increasing the anti–tumoral response,[49–52] for proteins inhibiting cell cycle progression,[53] for the enzyme guanylate kinase,[54] or for caspase 3.[55]

An alternative (and complementary) possibility to increase *TK* suicide gene therapy is to extend its functionality to nonexpressing cells by fusing the enzyme with proteins capable of mediating its extracellular release and uptake. One of these proteins is the VP22 protein of HSV-1, which has been originally shown to be exported from the cytoplasm of expressing cells and subsequently imported into neighboring cells, where it accumulates in the nucleus.[15] Fusion of TK to VP22 generates a protein of ~130 kDa, which maintains TK catalytic activity and extends GCV conditional cytotoxicity to nonexpressing cells in cell culture.[56,57]

In this work, we show that tagging TK with only 11 amino acids from HIV-1 Tat confers trafficking capacity to the enzyme. This was observed in terms of both cellular internalization using a recombinant GST fusion protein and extracellular release, by analyzing cells constitutively expressing Tat11-TK. The molecular basis for the unusual trafficking property imparted by this short peptide is still largely obscure. Cellular internalization of Tat is mediated by its interaction with cell surface HSPGs. Through its basic domain, Tat binds to heparin and HS but not to other GAGs, with binding affinity depending on charge density, extent of sulfation, and GAG structure.[31–33] We have recently observed that cells in which biosynthesis of HSPGs is genetically impaired fail to efficiently internalize the wild-type protein, and that substitution of the arginine residues with alanines impairs cellular internalization.[26] Thus, it is conceivable that Tat entry into the cells is mediated by binding of the Tat basic domain to cell membrane HSPGs followed by endocytosis of these molecules as it normally occurs for their physiological recycling.

Co-culture of HIV-1 Tat-expressing cells with cells containing an HIV-1 LTR-reporter gene cassette results in promoter transactivation, thus indicating that functional Tat is released from expressing cells and internalized by neighboring cells. Extracellular release also occurs in cells genetically impaired in HSPG production, clearly indicating that it occurs following a molecular route independent of HSPGs;[26] consistently, the process is not inhibited by drugs blocking secretion, indicating that it is not a Golgi-mediated event.[58] The work described in this manuscript provides experimental evidence that the 11 amino acids in the basic domain of Tat are also sufficient to mediate extracellular export of larger fusion proteins. Cells expressing Tat11-TK contain a relatively large amount of protein attached to cell surface HSPGs and a smaller fraction is released into the extracellular medium. As shown by the co-culture experiments and by the effect of heparin, this protein is readily available for internalization by neighboring cells in an HSPG-dependent fashion.

Fusion of Tat11 to TK extends the conditional cytotoxic effect of TK to a larger number of co-cultured cells, which do not express the enzyme and target these cells for apoptosis. Based on the data obtained by analysis of cell viability and on those from detection of apoptotic markers, we can roughly estimate that two to three times more cells in the mixed population undergo a cytotoxic effect than those expected on the basis of intracellular expression of TK alone. The inhibitory effect of soluble heparin on this enhanced cytotoxicity of Tat11-TK allow for a clear distinction between the canonical bystander effect, mediated by gap junctions and insensitive to the soluble GAG, and the enhanced effect of Tat11-TK, which is therefore independent — and hence additive — to the bystander effect itself. The inhibitory function of heparin also supports the notion that the intercellular trafficking of Tat11-TK indeed passes through an extracellular compartment.

Extended cytotoxicity is prolonged over time if Tat11-TK producer cells are themselves rendered insensitive to the toxic effect of TK and, therefore, express and release the protein for longer times. For example, in the experiments shown in Figure 6, despite that only 25% of the seeded cells was producing Tat11-TK, these were still able to control proliferation of the entire population, being mitotically inactivated. This is an additional advantage for some gene therapy applications in which the fusion protein might be released by nondividing tissues to take advantage of the prolonged expression of the therapeutic gene.

Finally, the use of Tat11-TK instead of TK appears to be additive to any other amelioration that could be introduced to improve suicide gene therapy using this enzyme, including modification of the TK amino acid sequence, co-expression of other genes, or improvement of the delivery system. Additionally, it is expected that the enhanced bystander effect of Tat11-TK involving a larger number of cells might also potentiate the spontaneous immune reaction elicited by tumor cells undergoing necrosis or apoptosis *in vivo*.

Ongoing experiments on animal models of induced tumorigenesis will better clarify these issues.

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