Gene Transfer–Mediated Pre-mRNA Segmental Trans-splicing As a Strategy to Deliver Intracellular Toxins for Cancer Therapy

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Abstract

Virus-mediated transfer of genes coding for intracellular toxins holds promise for cancer therapy, but the inherent toxicity of such vectors makes them a risk to normal tissues and a challenge to produce due to the intrinsic dilemma that expression of toxin molecules kills producer cells. We employed pre-mRNA segmental trans-splicing (STS), in which two engineered DNA fragments coding for 5′ “donor” and 3′ “acceptor” segments of a toxin gene, respectively, are expressed by viral vectors. When co-delivered to target cells, the two vectors generate two toxin pre-mRNA fragments which are spliced by the target cell machinery to produce functional mRNA and toxin. To test this approach, we used an enzymatic fragment of Shigatoxin1A1 (STX1A1) known to provoke apoptotic cell death. Two adenovirus vectors, Shigatoxin1A1 donor (AdSTx1A1Do) and Shigatoxin1A1 acceptor (AdSTx1A1Ac), respectively, were used to deliver the STX1A1 gene fragments. HeLa, HEp2, and A549 cells transfected with AdSTx1A1Do and AdSTx1A1Ac had a dose-dependent reduction in viability and inhibition of protein synthesis. Intratumoral injection of AdSTx1A1Do and AdSTx1A1Ac into preexisting HeLa, HEp2, and A549 tumors in immunodeficient mice revealed significant inhibition of tumor growth. There was no evidence of liver damage, suggesting that there was no leakage of vector or toxin from the site of injection following intratumoral injection of AdSTx1A1Do and AdSTx1A1Ac. These results suggest that the obstacles preventing gene transfer of intracellular toxins for local cancer therapy could be overcome by pre-mRNA segmental trans-splicing. (Cancer Res 2005; 65(1): 254-63)

Introduction

Although there is considerable interest in the use of gene transfer vectors to deliver intracellular toxins as potential therapeutic agents for cancer, the development of this strategy has been hampered by the inherent toxicity of viral vectors coding for toxins (1). In this regard, not only is it a difficult challenge to produce such vectors (attempts to produce a vector coding for an intracellular toxin have been thwarted because the toxin kills the producer cells), but if such vectors inadvertently escape from the tumor they would be inherently dangerous to normal tissues (2).

To circumvent this challenge, we have employed a strategy called “segmental trans-splicing” (STS), a process in which two engineered individual DNA fragments coding for 5′ and 3′ fragments of pre-mRNA of a toxin gene are delivered to mammalian cells (3).

Materials and Methods

Cell Culture. The human cervical carcinoma cell line HeLa, human epidermoid carcinoma cell line HEp2, and the lung carcinoma cell line A549 [American Type Culture Collection (ATCC), Manassas, VA; CCL-2, CCL-23, and CCL-185, respectively] were cultured as described in the ATCC instructions. All media and supplements were from Life Technologies (Gaithersburg, MD) unless otherwise noted.

Adenovirus Vectors Expressing Shigatoxin Fragments. Shigatoxin1A1 (Stx1A1; Genbank #AE005174) is an enzymatic fragment of Shigatoxin1 (Stx1) derived from the pathogenic E. coli O157:H7 strain (ATCC 700927; ref. 6). Stx1A1 acts as a specific N-glycosidase, cleaving a single adenine residue from 28S rRNA, thereby provoking the inhibition of protein synthesis and cell death (7–9). For eukaryotic cytoplasmic expression of Stx1A1 fragments from adenovirus vectors, the bacterial signal sequence was eliminated and an optimized Kozak motif and ATG (start) codon were added. Based on a report suggesting that Stx1A2, the linker region between Stx1A1 and Stx1B (the targeting domain), inhibits the activity of Stx1A1, the Stx1A2 region was deleted (10, 11). A synthetic Stx1A1 gene was constructed that encodes the native amino acid sequence but uses human-preferred codons (12). In addition, Stx1A1 coding sequences were selectively changed so they would not be inappropriately recognized by the splicing apparatus. The final sequence encoding Stx1A1, comprised of the start codon followed by amino acids 2 to 245, was inserted into pcDNA3.1, and sequenced through ligation sites to confirm the identity of each clone. All plasmids were prepared with Mini or Maxi kits (Qiagen, Valencia, CA), according to the manufacturer’s protocol.

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The engineered trans-splicing junction of the humanized Stx1A1 gene was located between G374 and G375 (Fig. 1, asterisk). The donor and acceptor STS constructs were designed so that the resulting pre-mRNAs would hybridize, and the nuclear spliceosome apparatus would trans-splice the resulting hybridized pre-mRNAs into mature, humanized Stx1A1 mRNA. Final elements in the plasmid Stx1A1 STS constructs used to create the adenovirus vectors included: (1) donor (pStx1A1Do, 5'-3') containing the cytomegalovirus immediate early promoter/enhancer, the 5' portion of Stx1A1 cDNA, the 5' portion of vascular endothelial growth factor (VEGF) intron 6, a unique hybridization domain and poly-adenylation signal; and (2) acceptor (pStx1A1Ac; 5'-3') containing the cytomegalovirus promoter/enhancer, and hybridization domain complementary to the hybridization domain of the donor construct, the 3' portion of VEGF intron 6, the 3' fragment of Stx1A1 cDNA, and a polyA signal from bovine growth hormone. Specific details of the trans-splicing sequence elements have been previously described (3).

Adenovirus vectors were created to deliver the two fragments of the Stx1A1 gene. The recombination adenovirus vectors Shigatoxin1A1 donor (AdStx1A1Do), Shigatoxin1A1 acceptor (AdStx1A1Ac) and null (AdNull), are replication-deficient, E1, E3 serotype 5 vectors, with the expression cassette in the E1 position. The Stx1A1 donor and acceptor expression cassettes were developed as adenovirus vectors using the pAdEasy system (Stratagene, La Jolla, CA). The AdNull vector is identical to AdStx1A1Do or AdStx1A1Ac, except that it lacks an expression cassette. The vectors were propagated in 293 cells (human embryonic kidney, ATCC), purified by two rounds of cesium chloride density gradient ultracentrifugation, dialyzed, and stored at -70°C as previously described (13, 14). All vector doses were

**Figure 1.** Schematic illustration of the genomic configuration and strategy for STS of Stx1A1 genes. The genomic organization of the Stx1A1 gene (enzymatic unit of Stx1) is shown at the top, with the scale of the diagram indicated by a size bar. The signal peptide, linker (Stx1A2) and binding unit (Stx1B) were removed to ensure uniquely intracellular expression of Shigatoxin activity. Humanization of Stx1A1 was used for generation of STS adenovirus vectors. In the STS constructs, the 5' fragment is termed the donor and the 3' fragment is the acceptor. AdStx1A1Do construct contains (5'-3') cytomegalovirus immediate early promoter/enhancer, 5' portion of Stx1A1 cDNA, 5' portion of VEGF intron 6 and hybridization domain. The adenovirus AdStx1A1Ac contains cytomegalovirus promoter/enhancer, hybridization domain complementary to the hybridization domain of the donor construct, 3' portion of VEGF intron 6, 3' fragment of Stx1A1 cDNA, and poly-adenylation signal. *, location of the trans-splicing junction. Below the constructs are shown schematics of the predicted pre-mRNAs, with the splice donor, branch point, and splice acceptor sites, as well as the complementary hybridization domains. The final STS spliced mRNA is as indicated at the bottom. The location of primer pairs (PF and PR) used for PCR are indicated.
expressed in particle units (pu) determined spectrophotometrically as described by Mittereder et al. (15).

Adenovirus enhanced green fluorescent protein (eGFP) vectors were prepared as previously described (16). To ensure that HeLa, HEp2, and A549 cell lines were equally responsive to infection with adenovirus vectors, the cells were infected with adenovirus eGFP at a dose of 10^5 pu/cell, and analyzed after 24 and 48 hours by fluorescent microscopy and flow cytometry.

**In vitro Expression of Stx1A1 mRNA by Adenovirus-Mediated STS.** To evaluate the expression of segmental trans-spliced Stx1A1, HeLa cells were seeded at 1.5 \times 10^5 cells/well in 24-well plates. After overnight culture, cells were counted and infected with the adenovirus vectors alone or in combination. All infections were done at a total dose of 8,000 pu/cell for 90 minutes. Sixteen hours after infection, total RNA was isolated using Trizol (Invitrogen, Carlsbad, CA) and reverse-transcribed (SuperScript First-Strand Synthesis System for reverse transcription-PCR, Invitrogen) after oligo dT priming. PCR was done with Platinum Taq DNA polymerase (Invitrogen). To detect the Stx1A1 mRNA production in vitro from adenovirus-mediated STS, primers PF and PR, which span the trans-splicing junction (Fig. 1) were used. Primer sequences were as follows: primer PF (CCGGATCCGCGCATTTGTTG-CCACCTGTGACTCTCAAG) and primer PR (CCAGTTCAGGAGATCATGAGCTCCGCGGGTC). PCR amplification (30 cycles) was carried out at 94°C for 30 seconds, 62°C for 30 seconds, and 72°C for 1 minute followed by final extension at 72°C for 5 minutes, and analyzed by agarose gel electrophoresis. To assess the fidelity of adenovirus-mediated STS, the reverse transcription-PCR products were purified by gel extraction kit (QiaQuick gel extraction kit; Qiagen), and sequenced.

**Morphologic Assessment.** To assess the impact of adenovirus-mediated STS of Stx1A1 on morphology of target cells in vitro, HeLa cells were seeded at 10^5 cells/well in 24-well plates. After overnight culture, cells were counted and infected with adenovirus vectors AdNull, AdStx1A1Do + AdNull, AdStx1A1Ac + AdNull or AdStx1A1Do + AdStx1A1Ac. Ratios of mixed vectors were 1:1. All infections were done for 90 minutes with the total infection dose of adenovirus maintained at 10^5 pu/cell. Sixteen hours later, morphology was analyzed by phase-contrast using an Olympus IX70 microscope (Olympus, Tokyo, Japan).

**Cell Viability.** To estimate the impact of adenovirus-mediated STS of Stx1A1 on cell viability in vitro, the CellTiter 96 AQueous One Solution Cell Proliferation Assay Kit (Promega, Madison, WI) was used according to the manufacturer's instructions. HeLa, HEp2, or A549 (3 \times 10^5 each) were seeded in 96-well culture plates. After overnight culture and counting, each cell line was infected with adenovirus vectors AdNull, AdStx1A1Do + AdNull, AdStx1A1Ac + AdNull or AdStx1A1Do + AdStx1A1Ac. Ratios of mixed vectors were 1:1. Uninfected cells (medium only, negative control) or incubation with 100 ng/mL Shigatoxin protein (positive control) were analyzed at the same time. At the indicated day after infection (0, 1, 2, 3, or 4), the cells were washed once in culture medium, and 20 L of reagent solution was added to each well of the 96-well plate. After incubation at 37°C in a humidified 5% CO2 incubator for 1 hour, the absorbance at 490 nm was recorded using a 96-well plate reader (model 680, Bio-Rad, Hercules, CA). Data points for the negative control (medium) at day 4 were set to 100, and all other measurements were normalized to that control for purposes of comparison.

**Inhibition of Protein Synthesis.** To assess inhibition of protein synthesis by adenovirus-mediated STS of Stx1A1, cells grown in 24-well plates were analyzed for [3H]leucine incorporation, using HeLa, HEp2 and A549 cells seeded at 10^5 cells/well in 24-well plates (17). After overnight culture and counting, each cell line was infected with adenovirus Stx1A1 STS vectors at a dose of 10^5 pu/cell. Results for the negative control (medium) were set to 100, and all other measurements were normalized to that control for purposes of comparison (percentage of control).

**Induction of Apoptosis.** To detect cell death via apoptosis induced by adenovirus-mediated STS of Stx1A1, a cell death detection ELISA assay kit (Cell Death Detection ELISA plus, Roche Applied Science, Nutley, NJ) was used according to the manufacturer's instructions. HeLa, HEp2, and A549 cell lines were seeded at 5 \times 10^5 in 96-well plates in culture medium. After overnight culture and counting, each cell line was infected with adenovirus vectors AdNull, AdStx1A1Do + AdNull, AdStx1A1Ac + AdNull (1:1), AdStx1A1Do + AdStx1A1Ac (1:1), or AdStx1A1Do + AdStx1A1Ac (1:1). To assess the dose response of AdStx1A1Do + AdStx1A1Ac on induction of apoptotic cell death, we also prepared cells infected with AdStx1A1Do + AdStx1A1Ac + AdNull at ratios of 1:1:8, 1:8:1, and 8:1:1. All infections were done for 90 minutes with a total dose of adenovirus maintained at 10^5 pu/cell. Each cell line was also treated with Shigatoxin protein (100 ng/mL), and cisplatin (20 \muM) as positive apoptotic controls (13). Results for the negative control (medium) were set to 10, and all other measurements normalized to the control for purposes of comparison.

**Loss of Cells.** To assess the impact of adenovirus-mediated STS of Stx1A1 on cell number, in vitro cell counts were determined daily. HeLa and A549 cells were seeded at 2.5 \times 10^5 in 6-well plates. After overnight culture and determination of total cell number, each cell line was infected with adenovirus vectors as described above at a dose of 10^5 pu/cell for 90 minutes. Cells were harvested using 0.05% trypsin, 0.53 mmol ethylenediamine tetraacetic acid (pH 7.4), on the indicated days after infection, and the number of viable cells determined by the trypan blue exclusion method, using a final concentration of 0.2% for 5 minutes and counted using a hemocytometer.

**Suppression of Tumor Growth by STS of the Stx1A1 Gene In vivo.** Six- to eight-week-old female BALB/c nu/nu mice, and BALB severe combined immunodeficient mice (SCID) mice were obtained from Jackson Laboratory (Bar Harbor, ME) and/or Taconic Laboratory (Germantown, NY). To evaluate the expression of segmental trans-spliced Stx1A1 mRNA in vivo, tumors were established in BALB/c nu/nu mice by s.c. injection of 5 \times 10^5 A549 cells and allowed to grow for 7 days. When tumors were established, adenovirus Stx1A1 STS vectors were administered directly into the tumor. Twenty-four hours later, tumors and livers were harvested and total RNA extracted from approximately 100 mg of tissue by homogenization in Trizol (Invitrogen) and reverse-transcribed as described above. To detect the in vivo mRNA production of Stx1A1 by adenovirus-mediated STS, reverse transcription-PCR was done with primers PF and PR, as described for the in vitro analysis of Stx1A1 mRNA expression (above). Reverse transcription-PCR products were electrophoresed and purified using a gel extraction kit (QiaQuick gel extraction kit; Qiagen), and sequenced.

To assess the effect of adenovirus-mediated STS of Stx1A1 on suppression of tumor growth in vivo, human tumor cells (10^7 HeLa, 10^7 HEp2, or 5 \times 10^6 A549 cells) were injected s.c. into the flanks of immunodeficient mice (HeLa and HEp2 into BALB SCID, A549 into BALB/c nu/nu mice). Seven days later, tumors of comparable size (approximately 40 mm^3 for HeLa and HEp2, and 30 mm^3 for A549) were established. The tumors were then injected with adenovirus STS vectors or PBS (pH 7.4), intratumorally in an 80 \muL volume thrice (days 0, 3, and 10). Tumor size was measured in a blinded fashion with calipers every 2 to 3 days and recorded as the product of width \times length (tumor area, mm^2).

**Assessment of Systemic Toxicity.** To evaluate systemic toxicity which might be induced by the adenovirus vector–based STS Shigatoxin delivery strategy administered to the tumors, liver damage was assessed based on the knowledge that > 90% of adenovirus vectors that reach the systemic circulation localize to the liver (18–20). To accomplish this, the levels of serum alanine aminotransferase and serum aspartate aminotransferase were measured in BALB/c nu/nu mice bearing injected A549 cell tumors receiving intratumoral injection of AdStx1A1Do and AdStx1A1Ac. Mice were bled from the tail vein (100 \muL) pre-intratumoral, 2, and 5 days post-intratumoral administration of AdStx1A1Do and AdStx1A1Ac. The blood samples were centrifuged (3,000 \times g, 20 minutes) and sera were collected. The levels of alanine aminotransferase and aspartate aminotransferase in each sample were analyzed (IDEXX Laboratories, West Sacramento, CA).

**Statistical Analysis.** Results are expressed as mean ± SD, except for tumor areas in the in vivo tumor growth suppression experiments, in which
results are expressed as mean ± SE. Statistical comparisons were made using the unpaired two-tailed Student’s t test.

**Results**

**Cell Death Mediated by Adenovirus Stx1A1 STS In vitro.** As a prelude to targeting tumor cells in vivo, it was necessary to show that Stx1A1 gene segments delivered by STS could be co-expressed in target cells, combine to form the functional Stx1A1 gene product and kill cells. To determine whether adenovirus-mediated STS of Stx1A1 was able to direct the synthesis of intact Stx1A1 mRNA, HeLa cells were infected with adenovirus Stx1A1 STS vectors alone or in combination. RNA isolated from the infected cells was analyzed by reverse transcription-PCR using primers (PF and PR, Fig. 1), which would only produce a product if trans-splicing had occurred (Fig. 2A). Lanes 1 to 4 show the result of omission of reverse transcriptase from the reverse transcription-PCR reaction, and as expected, no products were observed in RNA from cells infected with AdNull, AdStx1A1Do + AdNull, AdStx1A1Ac + AdNull, or AdStx1A1Do + AdStx1A1Ac, respectively. When reverse transcriptase was included, no amplification was detected from cells infected with AdNull, AdStx1A1Do + AdNull, or AdStx1A1Ac + AdNull (lanes 5-7). However, RNA from cells infected with AdStx1A1Do + AdStx1A1Ac showed the anticipated product of 621 bp (lane 8), as did the control plasmid (lane 9). To assess the fidelity of Stx1A1 STS, the reverse transcription-PCR products from cells infected with AdStx1A1Do and AdStx1A1Ac (lane 8) were purified and sequenced (Fig. 2B). The observed sequence across the splice junction was identical to that of the intact Stx1A1 gene, indicating that adenovirus-mediated STS can produce intact Stx1A1 mRNA in HeLa cells.

To evaluate the cytotoxic potential of adenovirus-mediated STS of Stx1A1 in vitro, the HeLa, HEp2, and A549 cell lines were analyzed for their response to infection. HeLa and HEp2 cells have receptors for Shigatoxin protein and are sensitive to external Shigatoxin protein, whereas A549 cells do not express Shigatoxin receptors, and are therefore resistant to external Shigatoxin protein (18, 21–23). All of these cell lines can be readily infected by

**Figure 2.** Adenovirus-mediated Stx1A1 STS produces intact Stx1A1 mRNA in vitro and in vivo. **A**, evaluation of Stx1A1 STS in vitro by reverse transcription-PCR. Analysis was carried out on total RNA from HeLa cells with primers PF and PR (Fig. 1) 16 hours after infection with the adenovirus vectors (8,000 pu). To show that RNA and not DNA was amplified, a set of control reactions were done without the addition of reverse transcriptase; these controls are shown in lanes 1 to 4. Lane 1, null adenovirus vector (AdNull); lane 2, AdStx1A1Do + AdNull; lane 3, AdStx1A1Ac + AdNull; lane 4, AdStx1A1Do + AdStx1A1Ac. In the presence of reverse transcriptase, trans-spliced Stx1A1 mRNA yields a reverse transcription-PCR product of 621 bp. Lanes 5 to 8 show the results of reverse transcription-PCR amplification as in lanes 1 to 4, but with reverse transcriptase included: lane 5, AdNull; lane 6, AdStx1A1Do + AdNull; lane 7, AdStx1A1Ac + AdNull; lane 8, AdStx1A1Do + AdStx1A1Ac. Lane 9 shows reverse transcription-PCR (including reverse transcriptase) of intact humanized Stx1A1 plasmid (positive control). Reverse transcription-PCR products were visualized on ethidium bromide agarose gels. **B**, sequence of the reverse transcription-PCR product of Stx1A1 STS isolated from HeLa cells infected with AdStx1A1Do + AdStx1A1Ac (A, lane 8). The data shows the sequence around the splice junction (asterisk in Fig. 1) in the forward orientation. The observed sequence is identical to the expected sequence. **C**, reverse transcription-PCR analysis of Stx1A1 expression in tumor cells from mice receiving intratumor injections with Stx1A1 donor and acceptor adenoviruses. A549 flank tumors were established in BALB/c nude (nu/nu) mice by s.c. injection of 5 × 10⁶ A549 cells and allowed to grow for 7 days. Adenovirus Stx1A1 STS vectors or PBS were delivered intratumorally. Animals received AdStx1A1Do + AdStx1A1Ac (total 5 × 10¹⁰ pu); AdStx1A1Do + AdNull (total 5 × 10¹⁰ pu); AdStx1A1Ac + AdNull (total 5 × 10¹⁰ pu); AdNull alone (5 × 10¹⁰ pu), or PBS. Tissues were harvested after 24 hours. Reverse transcription-PCR controls were as described above for A, and are shown in lanes 1 to 4: lane 1, AdNull; lane 2, AdStx1A1Do + AdNull; lane 3, AdStx1A1Ac + AdNull; lane 4, AdStx1A1Do + AdStx1A1Ac. In the presence of reverse transcriptase, trans-spliced Stx1A1 mRNA yields an reverse transcription-PCR product of 621 bp. Lanes 5 to 8 show the results of reverse transcription-PCR amplification as in lanes 1 to 4, but with reverse transcriptase included: lane 5, AdNull; lane 6, AdStx1A1Do + AdNull; lane 7, AdStx1A1Ac + AdNull; lane 8, AdStx1A1Do + AdStx1A1Ac. Lane 9, reverse transcription-PCR (including reverse transcriptase) of intact humanized Stx1A1 plasmid (positive control).
adenovirus vectors, with more than 95% of cells infected at a dose of 10^3 pu/mL, as confirmed by flow cytometry after GFP adenovirus infection (not shown).

The morphologic changes in HeLa cells in response to adenovirus-mediated Stx1A1 STS were analyzed (Fig. 3A–F). No changes were observed in mock-infected cells (medium) or cells infected with AdNull, AdStx1A1Do + AdNull, or AdStx1A1Ac + AdNull (Fig. 3, C, D, and E, respectively). However, dramatic morphologic changes, including cytoplasmic shrinkage and decreased cell numbers, were observed in the cells infected with AdStx1A1Do + AdStx1A1Ac (Fig. 3F), which were similar to those in the cells treated with Shigatoxin protein (positive control, Fig. 3F).

To assess the dose response of AdStx1A1Do + AdStx1A1Ac on HeLa cell viability, cells were infected with AdStx1A1Do + AdStx1A1Ac + AdNull at ratios of 1:1:48 or 1:1:8. The morphologic change and cell viability, cells were infected with AdStx1A1Do + AdStx1A1Ac + AdNull at ratios of 1:1:48 or 1:1:8. The morphologic change and decrease of cell numbers occurred in a dose-dependent manner (not shown) with the 1:1 ratio of AdStx1A1Do + AdStx1A1Ac yielding the most significant decrease in cell viability (Fig. 3F). The morphologic changes observed in infected HeLa cells occurred from 8 to 36 hours after infection, with all cells dislodged from the culture plates by 48 hours. HEp2 cells infected with AdStx1A1Do + AdStx1A1Ac showed similar morphologic changes (not shown). A549 cells infected with AdStx1A1Do + AdStx1A1Ac became quiescent, but did not show any cytotoxic changes over the period studied (not shown).

The response of HeLa, HEp2, and A549 cells to adenovirus-mediated Stx1A1 STS as a function of time was assessed (Fig. 3G–K). Assessment of cell viabilities revealed complete killing of HeLa (Fig. 3G), and significantly reduced cell viability in HEp2 and A549 (Fig. 3H and I). To assess the dose response of AdStx1A1Do + AdStx1A1Ac on cell viability, the cells were infected with AdStx1A1Do + AdStx1A1Ac + AdNull (1:1:8) at the same total virus dose. Reducing the titer of AdStx1A1Do + AdStx1A1Ac in this manner resulted in reduced cell death at all time points studied, demonstrating a dose-dependent response for adenovirus-mediated Stx1A1 STS (not shown). The effect of adenovirus-mediated STS of Stx1A1 on cell growth in vitro was assessed by cell counting (Fig. 3J and K). The number of HeLa cells infected with AdStx1A1Do + AdStx1A1Ac decreased rapidly, with complete cell death 2 days after infection. A549 cells infected with AdStx1A1Do + AdStx1A1Ac showed suppression of cell growth, but little change in cell number 3 days after infection. These results were consistent with the results of morphologic and cell viability analyses.

Inhibition of protein synthesis 8 hours after infection with AdStx1A1Do + AdStx1A1Ac was studied in HeLa, HEp2, and A549 cells. Protein synthesis was significantly impaired in all cell lines (Fig. 4A–C). No inhibition of protein synthesis was observed in mock (medium only, control), AdNull, AdStx1A1Do + AdNull, or AdStx1A1Ac + AdNull-infected cells. The degree of impairment of protein synthesis in HeLa and HEp2 cells infected with AdStx1A1Do + AdStx1A1Ac was similar to those seen in cells treated with intact Shigatoxin protein. A549 cells showed no response to treatment with Shigatoxin protein, likely due to the lack of an external receptor for Shigatoxin (23). Significantly, despite the lack of an external receptor, protein synthesis in A549 cells was inhibited by the internal generation of Shigatoxin by STS following infection with AdStx1A1Do + AdStx1A1Ac.

To determine whether cell death induced by the STS-mediated delivery of STx1A1 was occurring via apoptosis, a DNA fragmentation ELISA assay was used to assess HeLa, HEp2, and A549 cells infected with the adenovirus STx1A1 STS vectors. As a positive control for apoptotic cell death in A549 cells, the cells were treated with cisplatin ([sp-4-2]-diaminedichloroplatinum (20 μM)), previously reported to kill cells in this manner (24). HeLa and HEp2 cells infected with AdStx1A1Do + AdStx1A1Ac revealed dose-dependent apoptotic DNA fragmentation (Fig. 4D and E). The degree of relative apoptotic DNA fragmentation on the cells infected with AdStx1A1Do + AdStx1A1Ac at the maximum dose [AdStx1A1Do + AdStx1A1Ac (1:1)] was similar to that seen in HeLa and HEp2 cells treated with Shigatoxin protein. However, A549 cells infected with AdStx1A1Do + AdStx1A1Ac revealed no apoptotic DNA fragmentation (Fig. 4F), suggesting that more than one mechanism of cell killing may be operative in Stx1A1 STS. Because the process of STS involves the formation of a double-stranded RNA intermediate, it is conceivable that the double-stranded RNA-regulated serine/threonine protein kinase could be induced, leading to apoptosis independently of Shigatoxin A (25, 26). This phenomenon was ruled out in control experiments using cDNA for GFP with hybridization domain sequences identical to those used in the current study. Delivery to the liver of trans-splicing GFP vectors revealed liver GFP expression without any increases in serum glutamic-oxaloacetic transaminase or serum glutamic-pyruvic transaminase, and no evidence of apoptosis (not shown). From these data, we conclude that the double-stranded RNA-regulated serine/threonine protein kinase system had not been activated and apoptosis observed in the reported experiments was the direct result of intracellular Shigatoxin expression.

In vivo Suppression of Tumor Growth with Adenovirus Stx1A1 STS. To determine whether adenovirus-mediated STS of Stx1A1 would produce intact Stx1A1 mRNA in vivo, RNA was isolated from A549 tumors 24 hours after virus administration and analyzed by reverse transcription-PCR (Fig. 2C). As expected, omission of reverse transcriptase from the reverse transcription-PCR reaction resulted in no products observed in tumors injected with AdNull, AdStx1A1Do + AdNull, AdStx1A1Ac + AdNull, or AdStx1A1Do + AdStx1A1Ac (lanes 1–4, respectively). When reverse transcriptase was included, no amplification was detected from tumors injected with AdNull, AdStx1A1Do + AdNull, AdStx1A1Ac + AdNull, or AdStx1A1Do + AdStx1A1Ac (lanes 5–7). However, RNA from tumors injected with AdStx1A1Do + AdStx1A1Ac (lane 8) showed the anticipated product of 621 bp, as did the control plasmid (lane 9). To assess the fidelity of Stx1A1 STS in vivo, the reverse transcription-PCR product from the tumors injected with AdStx1A1Do and AdStx1A1Ac (lane 8) was purified and sequenced. As for the in vitro analysis, the observed sequence across the splice junction was identical to that of the intact Stx1A1 gene, indicating that adenovirus-mediated STS could produce intact Stx1A1 mRNA following intratumoral injection of AdStx1A1Do and AdStx1A1Ac (not shown).

To assess the ability of adenovirus-mediated Stx1A1 STS to suppress the growth of tumors in vivo, HeLa, HEp2, and A549 tumors were established in appropriate host mice by s.c. injection of cells and allowed to grow for 7 days. Adenovirus Stx1A1 STS vectors or PBS were then injected directly into the tumors, and tumor size was monitored over time. HeLa, HEp2, and A549 tumors injected with PBS, AdNull, AdStx1A1Do + AdNull, or AdStx1A1Ac + AdNull showed similar growth curves (Fig. 5A; P > 0.1, all comparisons, days 2 to 22). However, all tumors injected with AdStx1A1Do and AdStx1A1Ac showed a marked suppression of growth [P < 0.05, days 14 to 26 in HeLa cells (Fig. 5B); days 10 to 22 in HEp2 (Fig. 5B); days 14 to 22 in A549 (Fig. 5C)], demonstrating the ability of adenovirus-mediated STx1A1 STS to suppress tumor growth in vivo.
Figure 3. Effect of adenovirus-mediated STS of Stx1A1 on cell viability and growth. A–F, viability of HeLa cells was analyzed by brightfield microscopy 18 hours after infection with the vectors. A, medium alone, negative control; B, Shigatoxin protein (100 ng/mL, positive control); C, AdNull alone; D, AdStx1A1Do + AdNull; E, AdStx1A1Ac + AdNull; and F, AdStx1A1Do + AdStx1A1Ac. The total dose of adenovirus was maintained at 10^4 pu/cell, with the ratios of mixed vectors at 1:1. All panels are at the same magnification; bar = 50 μm. G–I, cell viability analysis. G, HeLa; H, HEp2; and I, A549 cells. Total virus doses were 10^4 pu/cell. AdNull was used as a negative control and was mixed with the trans-splicing vectors to maintain the total viral dose as described for Fig. 2. For each panel, data is shown for infection with AdNull (○), AdStx1A1Ac + AdNull (△), AdStx1A1Do + AdNull (□), AdStx1A1Do + AdStx1A1Ac (●), no infection [medium only, negative control (■)] or incubation with Shigatoxin protein (□) 100 ng/mL, positive control. Ratios of mixed vectors were 1:1. Cell viability was assessed using the CellTiter 96 assay. J–K, cell growth analysis. J, HeLa; and K, A549 cells. Data points are shown for infection with AdNull alone, AdStx1A1Ac + AdNull, AdStx1A1Do + AdNull, AdStx1A1Do + AdStx1A1Ac, no infection [medium only, negative control] or incubation with Shigatoxin protein (100 ng/mL, positive control). Total virus doses were 10^4 pu/cell. X-axis, time after transfection; Y-axis, absorbance relative to negative control (medium) at 490 nm. All data points are mean ± SD, n = 4.
One potential concern in the use of viral vectors that mediate the delivery of a toxin is the possibility of leakage of the AdStx1A1Do and AdStx1A1Ac vectors from the site of injection to remote sites where normal, healthy cells might be damaged. Several observations indicated that this did not occur. First, no systemic toxicity (reflected by animal death) was observed following intratumoral injection of these vectors, nor were there differences among the groups in general alertness, state of the skin coat, food intake, or body weight during observation (not shown). Because adenovirus vectors have great affinity for liver cells following i.v. administration, we analyzed levels of the serum liver enzymes alanine aminotransferase and aspartate aminotransferase to evaluate the risk of leakage after local administration of adenovirus-mediated Stx1A1 STS. Stx1A1 gene expression was not detected in the livers of injected animals (not shown), and liver enzymes were not elevated 5 days after intratumoral injection of adenovirus Stx1A1 STS vectors (Table 1).

Figure 4. Inhibition of protein synthesis and induction of apoptosis by Stx1A1 trans-splicing in vitro. A-C, inhibition of protein synthesis (A) HeLa, (B) HEp2; and (C) A549. Cells were infected with either AdNull, AdStx1A1Ac + AdNull, AdStx1A1Do + AdNull, AdStx1A1Do + AdStx1A1Ac, or exposed to Shigatoxin protein (100 ng/mL, positive control), or medium only (negative control). The virus dose was as described for Fig. 2. Incorporation of [3H]leucine by the cells was measured after 8 hours. Results for the negative control (medium) were set to 100, and all other measurements normalized to that control. D-F, apoptotic DNA fragmentation. D, HeLa cells; E, HEp2 cells, and F, A549 cells. Cells were infected with adenovirus Shigatoxin donor and acceptor STS vectors at doses described for Fig. 2. AdNull was used as a negative control and also mixed with the trans-splicing vectors to maintain total viral dose. Black columns, dose dependency of apoptosis with AdStx1A1 vectors, after mixing with AdNull at ratios of 1:1:0, 1:1:8, and 1:1:48. Twenty hours after infection, cells were analyzed using a Cell Death Detection ELISA. Uninfected cells are shown as a negative control (medium, white column), and Shigatoxin protein (100 ng/mL) or cisplatin (CDDP, 20 μM; gray column) as positive controls. HeLa and HEp2 cells are sensitive to external Shigatoxin protein; A549 is known to be resistant to external Shigatoxin protein (18, 21–23). All data are normalized to the negative control (medium). Y-axis, relative DNA fragmentation; X-axis, treatment groups.
Discussion

Targeted killing of cancer cells by the expression of a plasmid-encoded intracellular toxin has been shown using the diphteria toxin A–chain in vitro (27) and Pseudomonas exotoxin in vivo (28), but gene transfer via plasmid vectors is a relatively inefficient process. Theoretically, the ability to use viral vectors to direct the expression of toxic genes to cancer cells using vector-mediated gene transfer of intracellular cytotoxin or apoptosis-inducing genes should be an effective strategy to add to the armamentarium against tumors. However, the generation of viral vectors carrying intracellular toxins has been difficult due to the fact that expression of the toxin kills the packaging cells during generation of the vector. The present report describes a new strategy, called segmental trans-splicing, which provides a solution to this problem by generating the toxic gene product from two fragments of a toxic gene that, by themselves, are innocuous.

STS is a pre-mRNA, spliceosome-mediated trans-splicing-based paradigm for gene transfer, in which two engineered individual DNA fragments coding for 5′ and 3′ fragments of pre-mRNA of a gene are delivered to mammalian cells (3). Conventional trans-splicing gene transfer targets endogenous mRNA with low efficiency, leading to limited production of the trans-spliced product. STS overcomes this obstacle by generating two separate pre-mRNAs at high concentration which bind through unique binding sequences. The feasibility of STS has been shown in vivo using α-cobratoxin, a secreted neurotoxin that binds irreversibly to postsynaptic nicotinic acetylcholine receptors (29). Plasmid-mediated STS of α-cobratoxin yielded correctly trans-spliced α-cobratoxin mRNA and functional α-cobratoxin protein with high efficiency in vitro and in vivo (3). In this study, the speed with which the LD₅₀ was achieved in animals receiving trans-splicing toxin plasmids was evidence of the very high efficiency of STS, which we ascribe to the high relative abundance of the donor and acceptor pre-mRNAs driven by strong cytomegalovirus promoters. Their interaction is governed by Cot hybridization rules, e.g., more RNA and equal

![Figure 5](https://example.com/figure5.png)

**Figure 5.** Effect of adenovirus Stx1A1 STS on tumor growth in vivo. A, HeLa. Tumors were established in BALB SCID mice by s.c. injection of 10⁷ HeLa cells (n = 6 mice per group) and allowed to grow for 7 days. Adenovirus Stx1A1 STS vectors or PBS were delivered thrice intratumorally (arrows; days 0, 5, and 10). Animals received AdStx1A1Do + AdStx1A1Ac (total 5 × 10¹⁰ pu); AdStx1A1Do + AdNull (total 5 × 10⁹ pu); AdStx1A1Ac + AdNull (total 5 × 10¹⁰ pu); AdNull alone (5 × 10⁹ pu), or PBS. B, HEp2. Tumors were established in BALB SCID mice by s.c. injection of 10⁷ HEp2 cells (n = 6 mice per group) and allowed to grow for 7 days. Adenovirus Stx1A1 STS vectors or PBS were then delivered intratumorally as described for A. C, A549. Tumors were established in BALB/c nu/nu mice by s.c. injection of 5 × 10⁶ A549 cells (n = 6 mice per group) and treated 7 days later with adenovirus Stx1A1 STS vectors or PBS as in A and B. X-axis, time after the first adenovirus inoculation. All data are presented as mean ± SE, with a minimum of n = 6/data point.
amounts of RNA leads to faster interaction, and thus higher efficiency (30). This distinguishes STS from traditional transsplicing into an endogenous pre-mRNA, the concentration of which is limited by cellular regulatory elements.

Successful application of STS to the destruction of tumors requires that cells be infected by both the donor and acceptor vectors to produce toxin. High local doses or multiple injection sites could minimize the impact of the two-vector requirement. Although the use of two vectors is a restriction, there are potential advantages to using more than one vector. First, the ability to use two different promoters to enhance the specificity of expression, e.g., using liver-specific and cancer-specific promoters in the two vectors, ensure that only cycling hepatic cells would express the toxin. Second, capsid-modified vectors or even different vector types can be employed to enhance delivery and specificity to specific tumors. Targeted vectors might permit systemic administration with associated improvements in the safety profile of this therapeutic modality.

Cancer Therapy Using Stx1. Shigatoxin was chosen as the model toxin for this study based on the fact that it had not been delivered previously via gene transfer vector as an intracellular toxin. Previous attempts to use Stx1 to kill cells either exploited direct injection of Stx1 into tumors or used fusion products to target the protein to specific cells. Intratumoral administration of Stx1 protein significantly improved survival in a tumor xenograft model in nude mice using a human malignant meningioma (31) and a human astrocytoma (32). VEGF121 was able to kill endothelial cells expressing VEGFR-2 in a dose-dependent manner in vitro (33). Another study showed that a Stx1A1-human CD4 fusion protein selectively killed cells infected with HIV type 1 (25). Stx1 protein was used ex vivo to purge B cell lymphomas expressing the Stx1A1 receptor from stem cell grafts in a SCID mouse lymphoma model (34). Despite the success of these models, the use of intact Stx1 protein, alone or fused with another protein, is complicated by the fact that any cell, tumor, or normal, that comes into contact with the toxin could be killed. In contrast, the use of STS gene transfer to deliver Stx1A1, stripped of its binding domain and retaining only the intracellular enzymatic moiety, permits precise delivery of the toxin. The Stx1A1 gene is expressed inside the cell and kills only that cell. Without the binding domain, any Stx1A1 that escapes the cell when lysis occurs cannot gain entry into other cells. Thus, only the vector-targeted cells are affected by the expression of the toxin and systemic effects are minimized. It should be noted that the STS gene transfer approach would be amenable to any intracellular toxin.

Relevance of Apoptosis Induced by Stx1. Cellular changes that inhibit apoptosis play an essential role in tumor development (35). Many chemotherapeutic drugs function via the activation of apoptotic mechanisms leading to tumor cell death, and factors that impair programmed cell death contribute to the resistance of the tumor cells to drug treatment (35). Furthermore, apoptotic cell death likely provides tumor antigens to the immune system in an efficient manner (36). In the context of these considerations, cancer therapies that function in part by inducing apoptosis of malignant cells, may develop several antitumor host response mechanisms against tumors (35).

The current concepts of Shigatoxin action suggest that it may signal apoptosis in different cell types via different mechanisms including, inhibition of protein synthesis, interaction of Stx1B chains with the Gb3 receptor on target cells, and enzymatically induced damage of DNA (37). Stx1 protein has been shown to induce apoptosis in a variety of cells including human lung epithelial cells, human renal cortical epithelial cells, THP-1 human monocytic cell lines, HEp2 cells, Vero cells, HeLa cells, and endothelial cells from several organs treated with tumor necrosis factor-α (18, 22–24, 38–43). The activation of apoptotic pathways by caspase 3 and 8, and the down-regulation of the antiapoptotic proteins Mcl-1 or FLICE-like inhibitory protein have been observed following exposure to Stx1 protein (44, 45). The mechanism by which Stx1 initially triggers activation of apoptosis is not fully understood. Although Stx1 acts as ribosome-inactivating protein, removing a specific adenine from 28S rRNA with an attendant inhibition of protein synthesis, blockage of protein synthesis by cycloheximide does not induce apoptosis or enhance the effect of Stx1 in endothelial cells, and thus the inhibition of translation alone does not seem to be sufficient to induce programmed cell death (46). One report suggested that recombinant Stx1B (the cell-targeting element of Stx1) could induce apoptosis in Burkitt’s lymphoma cells, although at much higher concentrations than with the holotoxin (46, 47). In contrast, the cloned Stx1B subunit is not toxic in HeLa cells or Vero cells, even at high concentrations (18, 44). In this report, the Stx1A1 activity was generated in the target cells internally, thereby circumventing all external signaling and activation of ancillary pathways.

Cancer Therapy Using Adenovirus-Mediated STS. In this study, we employed an immunodeficient mouse model implanted with human tumors to evaluate the effect of Stx1A1 on killing tumor cells in vivo. In a clinical study, Stx1A1 might be used in combination with immunoactivation (48–50). In the context that Stx1A1 delivered by STS induced apoptosis in many tumor cell types, and that induction of apoptosis in tumors induces host antitumor immune processes, this combination should cause programmed cell death and induce enhanced antitumor immune responses resulting in the elimination of tumors.

Intratumoral administration of adenovirus for STS of Stx1A1 showed no liver damage, confirmed by the lack of Stx1A1 mRNA expression in liver and no elevation of serum liver enzymes. In
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Awards
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Gene Transfer–Mediated Pre-mRNA Segmental Trans-splicing As a Strategy to Deliver Intracellular Toxins for Cancer Therapy

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