Suicide cancer gene therapy using pore-forming toxin, streptolysin O

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Abstract

We cloned the streptolysin O gene from the *Streptococcus pyogenes* genome and tested the possibility of using it as an anticancer reagent. Transient transfection of the streptolysin O gene efficiently killed 293T cells after 12 hours of transfection as determined by lactate dehydrogenase release and propidium iodide uptake. No caspase activity was observed and necrosis was prominent during streptolysin O-induced cell death. Biochemical analysis of streptolysin O protein revealed that the deletion of only 5 amino acids from the COOH-terminal region of streptolysin O, which is essential for cholesterol binding activity, abolished its cell-killing activity, whereas the NH_2-terminal region was more resilient, i.e., up to 115 amino acids could be deleted without changing its cell-killing activity. We generated a streptolysin O-expressing adenovirus and injected it into human cervical cancer cell–derived tumors grown in a nude mouse model. Twenty-one days postinjection, the average size of tumors in the streptolysin O adenovirus–injected group was 29.3% of that of the control PBS-treated group. Our results show that the genes of pore-forming toxins, like streptolysin O protein, have the potential to establish a novel class of suicide gene therapeutic reagents. [Mol Cancer Ther 2006;5(6):1610–9]

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Introduction

Suicide gene therapy has received much attention from researchers and clinicians in cancer biology as an alternative therapy to conventional chemotherapy and radiotherapy (1). Typically, suicide cancer gene therapy involves the specific delivery of various cytotoxic genes, such as apoptotic factors or enzyme-prodrug combinations, to cancer cells. The subsequent expression of these genes then induces cell death. Suicide cancer gene therapies based on apoptotic factors such as p53 (2), FasL (3), Bax (4), and tumor necrosis factor–related apoptosis inducing ligand (5) have been extensively studied in athymic mice models; however, they have intrinsic limitations as anticancer gene therapeutic reagents because cancer cells evolve resistance to apoptotic insults. For example, antiapoptotic molecules like Bcl-2, Bcl-x_L, c-FILP, c-IAP, and survivins are often overexpressed in many types of cancer cells, and these confer resistance to cell death induced by apoptotic factors (6). Enzyme-prodrug systems are represented by the herpes simplex virus thymidine kinase/ganciclovir combination, the cytosine deaminase/5-fluorocytosine combination, and the cytochrome P450/cyclophosphamide combination system. These kill the targeted cancer cells by interfering with the DNA replication or transcription processes. Moreover, the toxic substances produced by these combinations can spread out to the neighboring cancer cells and induce consecutive cell death (the bystander effect). The cancer cell–killing ability of these systems is so powerful that the herpes simplex virus thymidine kinase/ganciclovir system has already reached phase I, II, or III clinical trials stage in several countries (7). The two possible drawbacks of these enzyme-prodrug systems are that a prominent bystander effect could cause unwanted toxic effects in neighboring normal cells, and that these systems tend to be less effective against cancer cells that are not actively dividing. For example, human melanoma is a slowly proliferating tumor, the doubling time of melanoma cells has been reported to be 8.6 days, as measured by the bromodeoxyuridine injection method (8). Moreover, <3% of prostate cancer cells are known to be actively dividing (9), and acute myeloid leukemia blasts do not proliferate and only a minor proportion (1%) of human leukemic cells are clonogenic progenitors (acute myeloid leukemia colony–forming units; refs. 10, 11). Therefore, we sought to develop a novel suicide gene therapeutic reagent that is powerful, can overcome the antiapoptotic resistance, and has an activity which is independent of the cell proliferation rate.

Streptolysin O is a toxin secreted by bacteria from the genus *Streptococcus* and is a prototype member of pore-forming bacterial cytolysins along with *Staphlyococcal* α-toxin and *Escherichia coli* hemolysin (12). Streptolysin O possesses a single polypeptide chain with a molecular weight of

~62 kDa. Streptolysin O binds specifically to membrane cholesterol, and oligomerizes to create a ring structure that consists of 45 to 50 units, which inserts into the membrane to make a large pore diameter of 25 to 30 nm (13, 14).

Cell biologists have exploited the pore-forming property of streptolysin O for macromolecule delivery, as evidenced by a large number of published applications (15–17). When the cell membrane is treated with streptolysin O, large membrane pores are generated and the membrane becomes permeable to extracellular DNA, RNA, peptides, and proteins. Nucleated animal cells have the ability to recover from such membrane damaged by using repair mechanisms related to those involved in the repair of complement lesions (18). Thus, using the above concept and elaborate experimental protocols, macromolecules can be transferred to the cell interior without inducing cell death. However, the continual presence of streptolysin O-induced pores in a cell membrane will result in cytolysis because of a loss of balance between influxes and effluxes across the cell membrane.

We reasoned that membrane permeabilization by streptolysin O-induced pore formation could be exploited to kill malignant tumor cells, and furthermore, because the cytolysis induced by streptolysin O pore formation causes physical damage to the cell membrane, the streptolysin O-induced cell death could overcome the antiapoptotic resistance of cancer cells and the problems associated with a low proliferation rate. Therefore, we initiated our studies with a conventional plasmid expression analysis of streptolysin O in a liposome-mediated transient transfection system. We found that transfected cells expressing streptolysin O had cell membranes that became permeable to extracellular macromolecules, and that eventually, these transfected cells died. We extended our research by developing an adenoviral expression system for streptolysin O, and by using this high-efficiency gene transfer methodology, we were able to establish the feasibility of streptolysin O-mediated gene therapy against several cancer cell lines. Furthermore, streptolysin O-expressing adenovirus strongly inhibited tumor growth in a human cervical cancer cell C33A tumor xenograft model.

Materials and Methods

Cell Lines

All cell lines, with the exception of Hep3B, which was maintained in modified Eagle's medium, were grown in DMEM supplemented with 10% fetal bovine serum (Invitrogen, Groningen, the Netherlands). HEK293 (human embryonic kidney fibroblasts), 293T (HEK 293 cell derivatives that harbor SV40 large T antigen), C33A (human cervical carcinoma cells), A549 (human lung carcinoma cells), U343 (human glioma cells), and Hep3B (human hepatocellular carcinoma cells) were purchased from the American Type Culture Collection (Manassas, VA). All cell lines were maintained at 37°C in a humidified atmosphere at 5% CO_2.

Plasmid Constructs

Genomic DNA from *Streptococcus pyogenes* 700294D was purchased from American Type Culture Collection. To clone the streptolysin O gene from this genome, PCR was done using ExTaq. Polymerase (Takara Bio, Shiga, Japan) and amplified DNA fragments were subcloned into the pcDNA3 vector. Sequence analysis revealed that the amplified streptolysin O DNA differed from the previously reported sequence (GenBank accession number, AB0505250) at six amino acid positions (Ser50 → Asn50, Ile62 → Thr62, Met69 → Thr69, Ser90 → Phe90, Val260 → Ala260, and Asp327 → Glu327). The DNA fragments of streptolysin O corresponding to amino acids 33 to 574, 106 to 574, 116 to 574, 151 to 574, 1 to 569, and 1 to 530 were amplified by PCR using ExTaq. Polymerase (Takara Bio) using pcDNA3-streptolysin O as a template and subcloned into pcDNA3 vector to generate pcDNA3-ΔN32 (tSLO), pcDNA3-ΔN105, pcDNA3-ΔN115, pcDNA3-ΔN150, pcDNA3-ΔC4, and pcDNA3-ΔC44, respectively. All inserts and vectors were cleaved with *Eco*RI and *Xho*I restriction enzymes (Promega, Madison, WI). In order to detect expressed streptolysin O derivatives using antihemagglutinin antibodies (Lab Vision, Fremont, CA), all inserts in pcDNA3 vector were further subcloned between the *Eco*RI and *Sal*I sites of pSRαHA vector. cDNA fragments encoding CrmA were amplified by PCR and subcloned into pFlag-CMV2 vector (Sigma, St. Louis, MO) to generate FL-CrmA. Bax and Bcl-x_L constructs were generated as described previously (19). The green fluorescent protein (GFP)-caspase-8 construct was kindly provided by Dr. Miyashita (National Research Institute for Child Health and Development, Japan). All plasmid inserts were verified by dideoxynucleotide sequencing. Detailed information on the primer sequences used for PCR is available on request.

Immunoblot

Cells were harvested and centrifuged at 500 × *g* for 10 minutes at 4°C. The cell pellets obtained were washed once with 1 mL of PBS and lysed with 100 μL of 2× sample buffer [20 mmol/L Tris (pH 8.0), 2 mmol/L EDTA, 2% SDS, 20 mmol/L DTT, 1 mmol/L Na_3VO_4, and 20% glycerol]. Lysates were sonicated in pulse mode, boiled for 5 minutes, centrifuged at 10,000 × *g* for 10 minutes at 4°C, and the supernatants obtained were used as whole cell lysates. Protein quantification assays were done using MicroBCA reagent (Pierce, Rockford, IL) according to the manufacturer's protocol. Typically, 50 μg of total cellular proteins were separated by SDS-PAGE and the separated proteins were then transferred to a nitrocellulose membrane which was then subjected to standard Western blot using mouse antihemagglutinin antibodies (Lab Vision), mouse anti-Bcl-x_L (Santa Cruz Biotechnology, Santa Cruz, CA), mouse anti-Bax (Santa Cruz Biotechnology), and horseradish peroxidase–conjugated secondary antibodies (Santa Cruz Biotechnology). All blotting membranes were stained with AmidoBlack (Sigma) to ensure that equal amounts of proteins had been loaded into each well.

Cell Death Assay Using Cotransfected GFP

The day before transfection, 5 × 10^5 293T cells were plated in six-well culture plates (Nalgen Nunc International, Naperville, IL). The next day, the indicated amounts

of DNA constructs were cotransfected with the pEGFPC vector (Clontech, Palo Alto, CA) to 293T cells using LipofectAMINE plus reagent according to the manufacturer's protocol (Invitrogen). After 18 to 24 hours, cells were harvested, washed once with PBS, and the intensity of green fluorescence was monitored by fluorescence-activated cell sorting (FACS) to assay cell death (20). The FACS profile of control transfection experiments, pEGFPC plus pSRαHA, showed a peak at a position $>4 \times 10^2$ on the *x*-axis and we defined GFP-expressing cells located within this region as GFP^{high} cells. In control experiments with mock vector, ~35% of total cells were GFP^{high} and the percentages of GFP^{high} cells was decreased as the transfected cells died due to cytotoxic gene expression. The percentage of dying/dead cells was also determined by monitoring the DNA content of treated cells by FACS.

Monitoring Cell Permeability

The amount of lactate dehydrogenase (LDH) released by transfected cells was measured using CytoTox 96 nonradioactive cytotoxicity assay kits (Promega). To measure the amount of propidium iodide uptake, treated cells were harvested and centrifuged at $500 \times g$ for 10 minutes at 4°C. The cell pellets obtained were washed once with 1 mL of PBS and resuspended in 300 μL of PBS. Cells were stained with propidium iodide at 0.2 μg/mL and the percentage of propidium iodide–permeable cells was measured by FACS.

Electron Microscopy

Adherent 293T cells were washed in PBS, fixed in 2.5% glutaraldehyde at 4°C for 30 minutes and postfixed in 1% OsO_4 at room temperature for 20 minutes. The cells were then dehydrated using increasing concentrations of alcohol (30%, 50%, 70% plus 1% uranylacetate, 80%, 95%, and 100%), embedded in mixtures of Epon/alcohol (1:3 for 15 minutes, 1:2 for 30 minutes, 3:1 for 30 minutes, and pure Epon for 2×30 minutes), and polymerized at 60°C for 2 days. The solidified blocks were cut into slices of 50 to 60 nm, which were then contrasted with 5% uranylacetate and Reynold's solution [80 mmol/L $Pb(NO_3)_2$, 120 mmol/L sodium citrate, 160 mmol/L NaOH] and then viewed under the electron microscope.

Biochemical Analysis of Streptolysin O-Induced Cell Death

For caspase activity assays, monolayer cells were harvested and then centrifuged at $450 \times g$ for 10 minutes at 4°C. After removing the supernatant, cell pellets were resuspended in 100 μL of cell lysis buffer [50 mmol/L HEPES (pH 7.5), 1 mmol/L DTT, 0.1 mmol/L EDTA, and 0.1% CHAPS] and lysed by repeated freezing at −70°C and thawing on ice. Lysates were then cleared by centrifugation at $15{,}000 \times g$ for 20 minutes at 4°C, and the resulting supernatants were used as cell extracts. Typically, 100 μg of cell extract was added to the caspase assay buffer [100 mmol/L HEPES (pH 7.5), 10% sucrose, 0.1% CHAPS, 10 mmol/L DTT, and 200 μmol/L DEVD-pNA] with or without 100 μmol/L of Boc-D. Mixtures were then incubated at 37°C for 4 hours and the yellowish color caused by the release of pNA was quantified using an ELISA reader at 405 nm.

To analyze DNA content, cells were harvested and washed once, as described above. The cell pellet was fixed overnight with 70% ethanol, washed once with PBS, resuspended in staining buffer (10 μg/mL propidium iodide and 0.2 mg/mL RNase A in PBS), and this cell suspension was kept at 4°C in the dark until FACS analysis.

Construction of Modified Shuttle Vectors and the Generation of Recombinant Adenoviruses

The stuffer DNA fragment flanked by two loxP sequences was amplified from the pDNR-CMV plasmid (Clontech) using ExTaq. Polymerase (Takara Bio) using the primers: 5′-gatccctctagagctagcgtcagtgagcgagg-3′ and 5′-gatccctctagagtcgcggccgcttaacttcg-3′ (*Xba*I sites are underlined). The product was cloned into the *Xba*I site of pCA14 shuttle vector to generate the pCA14-loxP construct. The GFP gene from pEGFPC1 plasmid (Clontech) was amplified using ExTaq. Polymerase (Takara Bio) using the primers: 5′-gatcccgaattcatggtgagcaagggcgagg-3′ and 5′-gatagacccgtcgactcaacttgtacagctcgtccatgc-3′ (*Eco*RI and *Sal*I sites are underlined). The resulting DNA fragment was cloned between the *Eco*RI and *Sal*I sites of pCA14-loxP shuttle vector to generate pCA14-loxP-GFP. cDNA encoding the deletion mutant of streptolysin O (tSLO) was excised from pcDNA3-tSLO plasmid using *Eco*RI and *Xho*I restriction enzymes and cloned between the *Eco*RI and *Sal*I sites of pCA14-loxP shuttle vector to generate pCA14-loxP-tSLO. All inserts in plasmids were verified by dideoxynucleotide sequencing. The pCA14-loxP-GFP and pCA14-loxP-tSLO constructs were then linearized by *Xmn*I and *Pvu*I digestion, respectively, and the adenoviral vector vmdl324Bst (obtained from S.B. Verca, University of Fribourg, Switzerland) containing the Ad5 genome deleted of E1 and E3 regions was linearized by *Bst*BI digestion. Linearized pCA14-loxP-GFP or pCA14-loxP-tSLO were cotransformed into *E. coli* BJ5183 together with the *Bst*BI-digested vmdl324Bst for homologous recombination. To verify the respective homologous recombinants, plasmid DNA, purified from overnight *E. coli* culture, was digested with *Hin*dIII and the digestion pattern was analyzed. Proper homologous recombinant adenoviral plasmid DNA was digested with *Pac*I and transfected into 293 cells to generate Ad-loxP-GFP and Ad-loxP-tSLO viruses. Cre-expressing adenovirus, AdCreM2, was purchased from Microbix Biosystems Inc., Toronto, Ontario, Canada. Viruses were propagated in 293 cells and purified using standard methods (21). After viral generation, PCR amplification and DNA sequencing, using primers specific for GFP or streptolysin O, confirmed the correctness of the viruses. The titer (multiplicity of infection, MOI) used in this study was determined by the absorbance of the dissociated virus at 260 nm, where 1 absorbance unit is equivalent to 10^{12} viral particles per milliliter. The particle-to-infectious unit ratio was 100:1.

Functional Analysis of GFP and Streptolysin O-Expressing Adenoviruses *In vitro*

Cells grown in 24-well plates to 30% to 70% confluence were infected with Ad-loxP-GFP or Ad-loxP-tSLO with or without AdCreM2 virus at the indicated MOIs. After 3 days, GFP expression was monitored under blue light excitation using an Axiovert 100 inverted epifluorescence microscope

(Carl Zeiss, Thornwood, NY) and the percentage of GFP-expressing cells was determined by FACS. The viability of infected cells was measured using a Cell Counting Kit-8 (Dojindo Molecular Technologies, Kumamoto, Japan). Briefly, 100 μL of WST-8[2-2(-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium, monosodium salt] solution was added to each 24-well cell culture. After 3 hours of incubation at 37°C, the colored supernatant was transferred to a 96-well microplate and absorbance at 450 nm was measured using a microplate reader. All assays were done in triplicate. The DNA content of infected cells was analyzed by FACS, as described above.

Treatment of Tumor-Bearing Nude Mice with Adenoviral Vectors

C33A cells (1×10^7) were injected into the flanks of 6- to 8-week-old male nude mice (Charles River Japan, Inc., Yokohama, Japan) to establish human tumor xenografts. When tumor volumes reached 5 to 10 mm in diameter, mice were randomized into three groups and the Ad-loxP-GFP or Ad-loxP-tSLO viruses at 5×10^8 plaque-forming units in 50 μL of PBS were coinjected directly into the tumors with AdM2Cre viruses at 2.5×10^8 plaque-forming units in 25 μL of PBS, twice on alternating days. There was no appearance of any leakage of viral solution into surrounding tissue. Control tumors were injected with PBS only. Tumor growth was measured twice or thrice times weekly until the end of the study by measuring the length and width of each tumor using a caliper. Tumor volumes were estimated using the following formula: volume = 0.523 L w^2.

Results

Transient Expression of Streptolysin O-Induced Cell Death in 293T Cells

Streptolysin O is a typical exotoxin that is produced by group A streptococci. It is secreted by bacteria, binds to cholesterol-containing membranes, and produces pores. Because streptolysin O toxin acts outside of the plasma membrane, it is uncertain whether streptolysin O expressed in mammalian cells possess cytolytic activity and kills host cells. To test this possibility, we initially cloned the streptolysin O gene obtained from the genomic DNA of *S. pyogenes* into the mammalian expression vector, pcDNA3. Sequence analysis revealed that it differed at six amino acids from the reported streptolysin O sequence (GenBank accession number, AB050250; Materials and Methods). The cloned streptolysin O gene was further subcloned into pSRαHA expression vector in order to detect the expressed streptolysin O by Western blotting with antihemagglutinin antibody (Materials and Methods). However, we could not detect a western band from the hemagglutinin-tagged full-length streptolysin O protein (data not shown), and thus, we used a truncated version of streptolysin O, which lacked the 32 NH_2-terminal amino acids of full-length streptolysin O (tSLO, 542 amino acids).

When the tSLO gene was transiently transfected into 293T cells, the cells became small in size and detached from culture plates (Fig. 1A). Transfection with proapoptotic Bax produced a similar morphology, but antiapoptotic Bcl-x_L and control mock vector did not change the cell morphology (Fig. 1A). We cotransfected a GFP reporter with each expression plasmid and measured the fluorescence intensity by FACS to estimate the viability of the transfected cells. As shown in Fig. 1B, the expression of death-inducing Bax or tSLO gradually lowered the fluorescence intensity in transfected cells as the amount of expression plasmid was increased, whereas mock vector and Bcl-x_L expressions maintained similar levels of fluorescence intensity independent of the amount of expression plasmid added. Therefore, streptolysin O expressed in 293T cells was found to be cytotoxic despite differences between the bacterial and mammalian expression systems. The expression level of all expression plasmids were monitored by Western blotting (Fig. 1C).

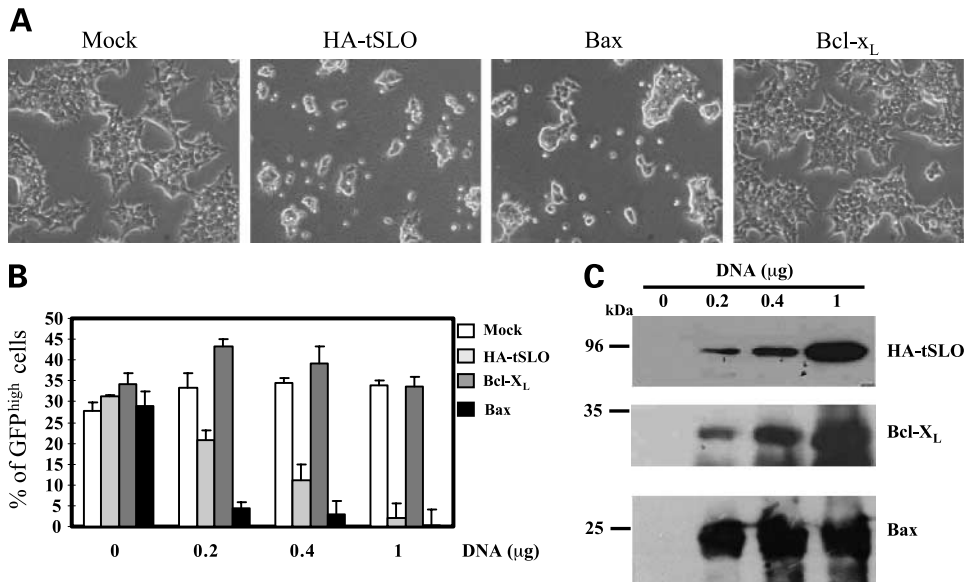

Figure 1. Overexpression of the streptolysin O gene induces cell death in 293T cells. The streptolysin O gene from *S. pyogenes* was subcloned into pSRα expression vector and transiently transfected into the 293T cell line. **A**, after 16 h of transfection, cell morphology was monitored under the microscope. Cells were transfected with 500 ng of the expression construct. **B**, GFP reporter plasmid was cotransfected with the indicated amounts of each expression construct, and cell viability was assessed by measuring the intensity of green fluorescence by FACS after 24 h of transfection. *Columns,* mean of triplicate experiments; *bars,* +1 SD. **C**, the expression of each transfected gene in **B** was monitored by Western blot analysis using antibody against hemagglutinin peptide, Bcl-x_L, or Bax antibody.

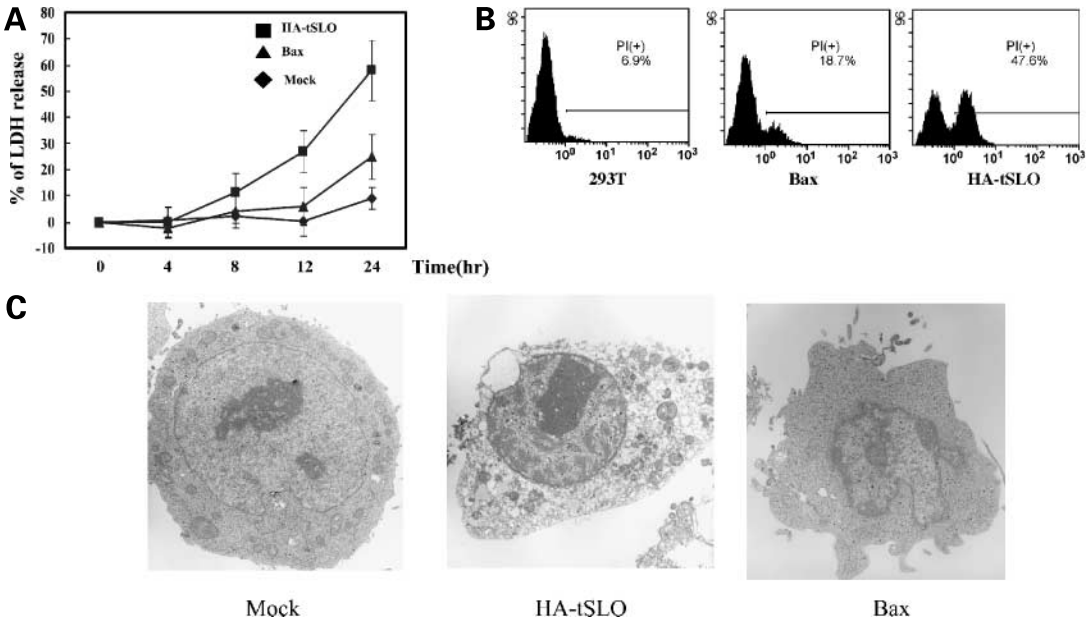

Figure 2. Streptolysin O expression increases plasma membrane permeability and disrupts the plasma membrane. **A**, the release of cytosolic LDH into culture medium by membrane-permeabilized cells was monitored using a CytoTox 96 nonradioactive cytotoxicity assay kit. 293T cells were transfected with 500 ng of each expression construct and the culture media was harvested at the indicated times. *Points,* mean of triplicate experiments; *bars,* ±1 SD. **B**, the uptake of propidium iodide dye by transfected 293T cells was monitored by FACS. Five hundred nanograms of each expression construct was used for transfection and the culture was harvested after 16 h. **C**, the morphologies of dying cells were monitored using an electron microscope. Cells grown in six-well culture plates were transiently transfected with 500 ng of each expression construct. After 16 h of transfection, cells were treated as described in Materials and Methods, and cell morphology was observed under the electron microscope.

The Integrity of the Plasma Membrane Is Disrupted by tSLO Expression

Streptolysin O toxin secreted by bacteria creates large pores in the target cell plasma membrane, and thus, large molecules that normally cannot pass through the membrane are able to freely pass. We wanted to determine whether the observed cytotoxicity in tSLO-transfected cells was the result of membrane pores generated by the expressed tSLO. Thus, we monitored the level of LDH release from the cytosol of transfected cells (Fig. 2A). LDH has a molecular weight of ~125 kDa, and cannot freely traverse the plasma membrane, thus, the LDH release assay has been widely used to measure the level of cell lysis caused by membrane attack by perforin, complement system, or pore-forming toxins.

As shown in Fig. 2A, the expression of control mock vector caused a release of <10% of LDH, and Bax induced the release of 23% after 24 hours of transfection. In contrast, the level of LDH release due to tSLO expression was higher (~60%) indicating that the plasma membrane is readily permeabilized by tSLO. Moreover, the release of LDH was more rapid than that of Bax. Bax induces cell death via apoptotic pathways, which usually retain an intact plasma membrane until the late phase. In contrast, the membrane integrity of tSLO-transfected cells was rapidly disrupted, which implies that a cell death pathway other than apoptosis is activated during tSLO-induced cell death. This rapid disruption of plasma membrane integrity was also monitored using a propidium iodide uptake experiment (Fig. 2B). tSLO-expressing cells were readily stained by exogenous propidium iodide dye, which cannot penetrate intact cell membranes. Bax-expressing cells were also stained with propidium iodide but the level of staining was significantly lower than that of tSLO-expressing cells.

To differentiate Bax-induced cell death and tSLO-induced cell death more clearly, the ultrastructures of transfected cells were monitored under the electron microscope (Fig. 2C). After 16 hours of transfection, Bax-expressing cells had a shrunken appearance, shed fragmented cell debris in a budding manner, and maintained a dense cytoplasm. However, tSLO-expressing cells showed many vacuoles and a less dense cytoplasm. Thus, we concluded that tSLO induces nonapoptotic cell death by creating pores in the plasma membrane.

Biochemical Analysis of tSLO-Induced Cell Death

Caspases are proteolytic enzymes that are activated during various forms of cell death. To test the possibility that caspases are involved in tSLO-induced cell death, we measured cellular caspase activities using the caspase-3-specific peptide substrate DEVD-pNA. The classical proapoptotic proteins, Bax or caspase-8, activated cellular caspase-3, whereas treatment with the pan-caspase inhibitor, Boc-D, prevented this activation. In contrast, tSLO did not activate cellular caspase-3, indicating that tSLO-induced cell death does not involve caspase-3 activation (Fig. 3A).

As tSLO-induced cell death seems to be independent of caspases, we tested the hypothesis that tSLO-induced cell

death should not be blocked by the overexpression of potent antiapoptotic molecules, which is a mechanism commonly used by neoplastic cells to prevent apoptosis (ref. 6; Fig. 3B). Bax-induced cell death was inhibited by the overexpression of Bcl-x_L and caspase-8-induced cell death was inhibited by the overexpression of CrmA. However, tSLO-induced cell death was not affected by the overexpression of either of these antiapoptotic molecules. Even the pan-caspase inhibitor Boc-D did not affect tSLO-induced cell death. These data show that streptolysin O protein expressed within 293T cells kills them via a necrotic pathway.

Streptolysin O Deletion Study

Several deletion mutants of streptolysin O were generated in order to identify the region responsible for the observed cell death (Fig. 4A). All deletion constructs were fused to hemagglutinin-tag to enable the expressed protein to be detected with antihemagglutinin antibody. Full-length streptolysin O protein fused with hemagglutinin-tag permeabilized cell membranes and killed transfected cells (Fig. 4C). However, we could not detect the hemagglutinin-streptolysin O protein by Western blotting with antihemagglutinin antibody, suggesting that the end of the NH_2-terminal region of streptolysin O is rapidly cut out during the posttranslational processing (Fig. 4B). Deletions up to 115 amino acids from the NH_2 terminus induced cell death when expressed in 293T cells (Fig. 4C). However, when 150 amino acids from the NH_2 terminus were removed, the cell-killing activity of streptolysin O disappeared almost completely, indicating that these terminal 150 amino acids are essential for streptolysin O-induced membrane permeabilization (Fig. 4C). However, in contrast to the NH_2 terminus, deletions of only five amino acids from the COOH terminus abolished the cell-killing activity of streptolysin O (Fig. 4C). The strict requirement for an ''intact COOH-terminal region'' is explained by the identification of this region as a cholesterol-binding region (12). Thus, our deletion study emphasizes the importance of cholesterol binding on the action of streptolysin O and provides indirect evidence that streptolysin O protein expressed in mammalian cells behaves like its bacterial counterpart.

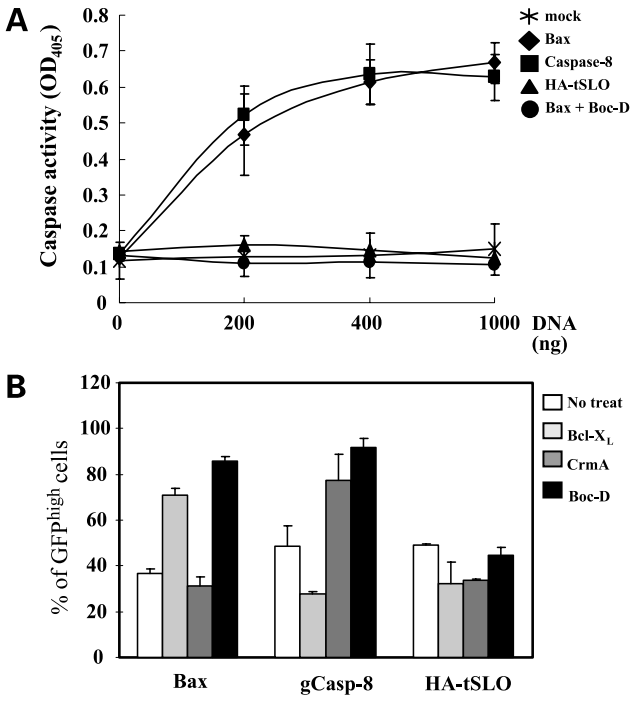

Figure 3. Biochemical analysis characterized streptolysin O-induced cell death as necrosis. **A**, 293T cells were transfected with the indicated amounts of each DNA construct and incubated for 24 h. Cell lysates were prepared and the activity of cellular caspase-3 was assayed as described in Materials and Methods. Pan-caspase inhibitor Boc-D was added to the culture where indicated at a final concentration of 100 μmol/L. **B**, the inhibition of cell death by cellular antiapoptotic protein Bcl-x_L, viral antiapoptotic protein CrmA or peptide pan-caspase inhibitor Boc-D was assayed by measuring the green fluorescence emitted by coexpressed GFP reporter protein by FACS. Cells were transfected with 500 ng of Bax, GFP-caspase-8, or hemagglutinin-tSLO expression construct. The expression construct for Bcl-x_L or CrmA (500 ng) was cotransfected with each cell death inducing DNA construct or pan-caspase inhibitor Boc-D was added to the culture where indicated at a final concentration of 100 μmol/L. *Columns*, mean of triplicate experiments; *bars*, ±1 SD.

Generation of Ad-loxP-tSLO Adenovirus

In order to examine the possibility that the streptolysin O gene can be used as an anticancer gene therapeutic reagent, we generated a replication-deficient adenovirus that can express the tSLO gene. Because tSLO is a cytotoxic protein, we used a Cre-inducible system that is widely used for toxic gene expression to circumvent the difficulties of virus packing in the 293A cell line (Fig. 5A). Recombinant adenoviral genomes encoding loxP-GFP or loxP-tSLO were constructed and transfected into a 293A cell line, as described in ''Materials and Methods.'' Ten days posttransfection into the 293A cells, the cytopathic effect became evident. Cell culture supernatants were then harvested and centrifuged to obtain a clear culture supernatant. Aliquots were analyzed by PCR to confirm the presence of recombinant adenovirus encoding loxP-GFP or loxP-tSLO (data not shown). We named these adenoviruses, Ad-loxP-GFP and Ad-loxP-tSLO, and further amplified and purified them, using standard CsCl gradient methods (Materials and Methods). To test the functionality of the purified adenovirus, we infected the human cervical carcinoma C33A cell line and monitored the green fluorescence emitted by GFP. Infection with only Ad-loxP-GFP, and not Cre virus (AdM2Cre), showed a slight increase in the number of green-colored cells, indicating that the loxP system is leaky. When C33A cells were coinfected with Ad-loxP-GFP and AdM2Cre, GFP overexpression was induced and >80% of the cell population was positive for the green color (Fig. 5B). This control experiment confirmed the functionality of the purified adenovirus and of the Cre-loxP system. We also tested the functionality of Ad-loxP-tSLO in C33A cells. As shown in Fig. 5C, coinfection with Ad-loxP-tSLO and AdM2Cre caused marked C33A cell death. The control Ad-loxP-GFP virus in combination with AdM2Cre showed an intermediate level of cytotoxicity, and Ad-loxP-GFP or Ad-loxP-tSLO without AdM2Cre showed little cytotoxicity at a MOI <10. All these combinations showed remarkable cytotoxicity with MOIs >20 in the C33A cell line.

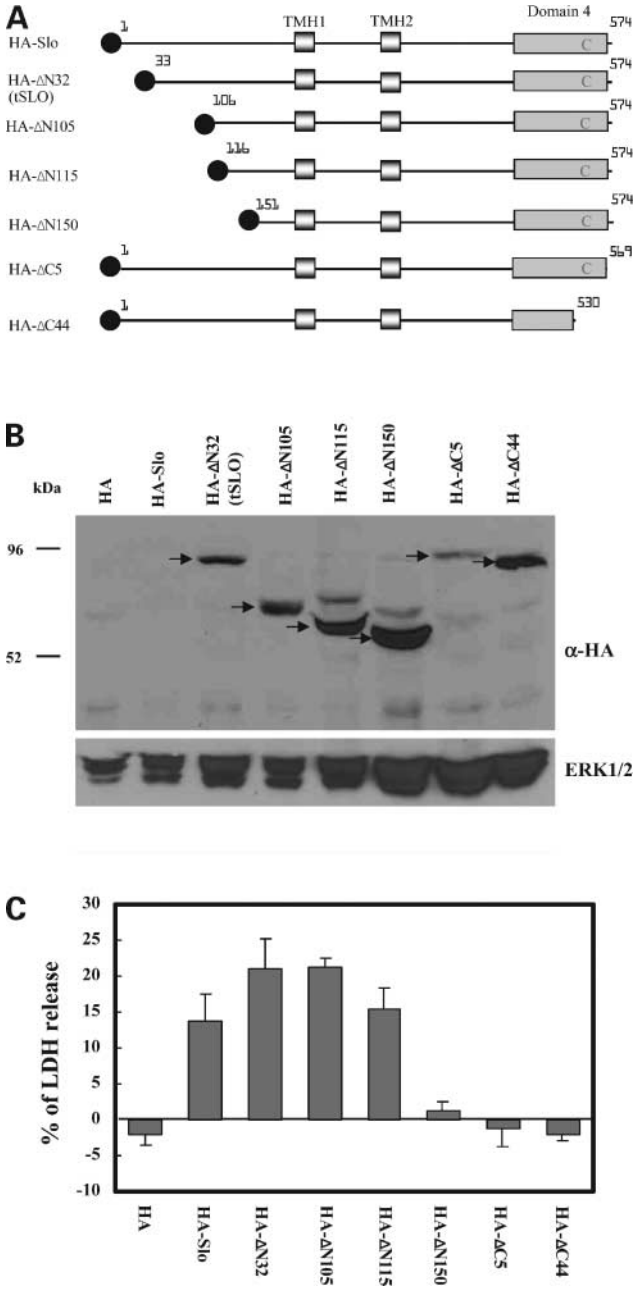

Figure 4. Deletion study of streptolysin O protein showed a strict requirement for an intact COOH terminus. **A**, a diagram of the deletion constructs used in this study. All deletion constructs were fused to hemagglutinin tag at the NH_2 terminus. Locations of transmembrane helixes 1 and 2 (*TMH1* and *TMH2*), domain 4, and the cholesterol binding domain of streptolysin O are indicated. **B**, the expression of each deletion construct was monitored by Western blot analysis using antihemagglutinin antibody. Cells grown in six-well culture plates were transfected with 500 ng of each DNA construct, incubated for 24 h and harvested for Western blot analysis. After one set of Western blot with antihemagglutinin antibody, the blotting membrane was stripped and reblotted with antibody against ERK1/2 to monitor the amount of protein loaded onto the protein gel. *Small arrows,* the deletion mutant detected using its molecular weight. **C**, the degree of plasma membrane permeabilization was monitored using an LDH release assay kit as described in Materials and Methods. Cells were transfected and harvested as in **B**. *Columns,* mean of triplicate experiments; *bars,* ±1 SD.

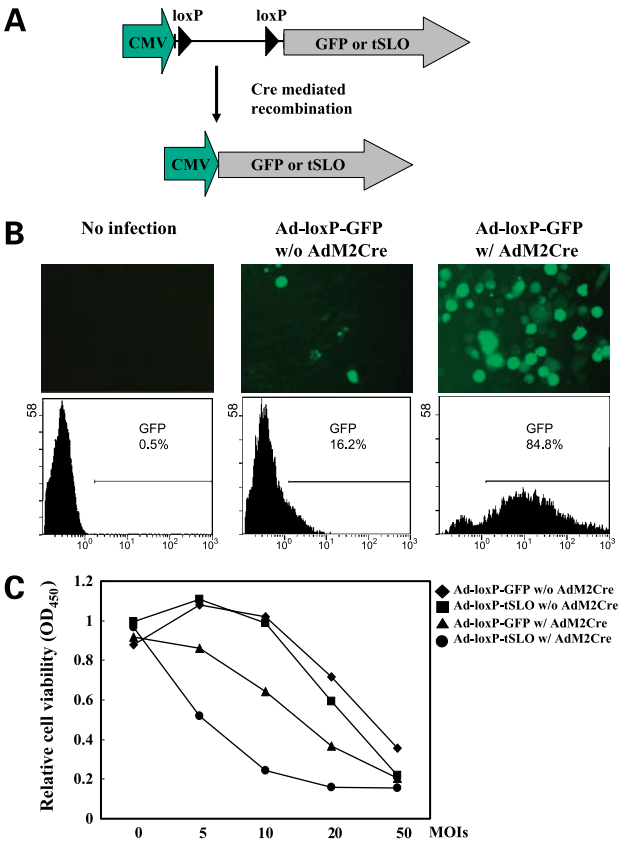

Figure 5. Generation of Cre-inducible streptolysin O expressing adenovirus and functional testing. **A**, diagram of how the modified shuttle vector works for the inducible expression of GFP or streptolysin O protein. **B**, C33A cells grown in 24-well culture plates were infected with 10 MOIs of Ad-loxP-GFP with or without 5 MOIs of AdM2Cre, incubated for 3 d, and harvested. The Cre-inducible expression of GFP was monitored by fluorescence microscopy and FACS. **C**, the cell death induced by the Cre-inducible expression of streptolysin O protein was measured by viability assay as described in Materials and Methods. C33A cells grown in 24-well culture plates were infected with the indicated MOIs of Ad-loxP-GFP or Ad-loxP-streptolysin O with or without AdM2Cre, incubated for 3 d, and then viability reagent was added to the cultures. The ratio of AdM2Cre to loxP-Ad-GFP or loxP-Ad-streptolysin O was 1:2 at all MOIs.

The Antitumor Effect of Ad-loxP-Streptolysin O Adenovirus *In vitro*

The time-dependent anticancer effect of Ad-loxP-tSLO adenovirus was monitored in several cell lines. Six days after coinfection with Ad-loxP-tSLO (20 MOI) and AdM2-Cre (10 MOI), >90% cell death was observed in C33A and A549 cells (Fig. 6), whereas the control PBS group did not show any remarkable cytotoxic effect. However, coinfection with Ad-loxP-GFP (20 MOI) and AdM2Cre (10 MOI) produced an intermediate level of cytotoxicity in these cells, suggesting that the expression of GFP and/or Cre protein exerts a toxic effect in these cell lines. There have been reports that expression of GFP or Cre proteins are toxic to cell lines under specific experimental conditions (22–25). When MCF-7 and PC-3 cells were used as target cells, ~60% cell death was observed for the same viral dose

and time as in C33A and A549 cells (Fig. 6). In contrast to C33A and A549 cells, coinfection with the control Ad-loxP-GFP (20 MOI) and AdM2Cre (10 MOI) viruses showed little cytotoxicity in MCF-7 and PC-3 cells. We measured the expression level of GFP by FACS and found a strong correlation between the level of GFP expression and the level of cytotoxicity (data not shown). Thus, differences in the level of cell death in each cell line can be explained by differences in the sensitivity of each cell line to viral infection. We cannot exclude an alternative possibility that low-level contamination with wild-type recombinant-competent adenovirus induces the observed background cell death.

Antitumor Effect of Ad-loxP-tSLO Adenovirus *In vivo*

We next evaluated the antitumor effect of tSLO-expressing adenovirus by injecting adenoviruses into tumors established from human cervical cancer xenografts, C33A, in nude mice. Tumors were generated by the s.c. injection of cells into flanks, and when tumors reached an average of 90 to 100 mm^3, 5×10^8 plaque-forming units of Ad-loxP-tSLO and 2.5×10^8 plaque-forming units of AdM2Cre were coinjected directly into tumors on days 0 and 2. As shown in Fig. 7, control tumors, which were treated with PBS, increased to an average size of 1,643 $\pm$ 467 mm^3 21 days after virus injection, whereas tumor growth was significantly inhibited in mice coinjected with the replication-incompetent Ad-loxP-tSLO/AdM2Cre. More specifically, the average tumor size in the Ad-loxP-tSLO/AdM2Cre–treated animals was 481 $\pm$ 184 mm^3 21 days after coinjection, which is >70% reduction in average tumor size ($P < 0.001$, versus PBS treated mice, $n = 8$). Control tumors, which received Ad-loxP-GFP/AdM2Cre, showed an intermediate level of growth inhibition, which is consistent with the *in vitro* data. However, Ad-loxP-tSLO/AdM2Cre–treated tumors were still 47% smaller 21 days after coinjection ($P < 0.05$, versus Ad-loxP-GFP/AdM2Cre–treated mice, $n = 8$). Throughout the course of this study, no systemic toxicity, such as diarrhea, loss of weight, or cachexia was observed.

Discussion

Toxins have the ability to kill cells efficiently, and thus, many toxins have been examined as potential anticancer agents, as immunotoxins or in viral gene therapy. Diphtheria toxin is one of the best-studied molecules as a suicide gene therapeutic reagent. It efficiently ADP-ribosylates elongation factor 2, and thus, blocks the translational machinery of target cells (26). It is estimated that a single molecule of diphtheria toxin can kill target cells (27), and many studies have successfully used its toxicity to eradicate target cancer cells (28–32). Plant-derived ricin and pseudomonas exotoxin use a mechanism similar to that of diphtheria toxin to kill target cells, and have been examined as effective anticancer reagents (33, 34). However, all toxins that have been tested as suicide gene therapeutic reagents are known to act ''inside'' of cells.

Conversely, pore-forming toxins act ''outside'' the cell membrane naturally, and thus, they have been used as immunotoxins or native proteins for anticancer agents rather than as suicide gene therapeutic reagents (35–37). We were not sure whether a representative pore-forming toxin, streptolysin O, synthesized using eukaryotic expression machinery, could function ''inside'' the cell membrane. Our initial trial with streptolysin O DNA-lipid complexes in the 293T cell line showed that intracellular streptolysin O had significant cytotoxicity (Fig. 1). Biochemical analysis of this streptolysin O-induced cytotoxicity revealed that the plasma membranes of streptolysin O-expressing cells were permeabilized, and that cytoplasmic contents leaked out of cells, which

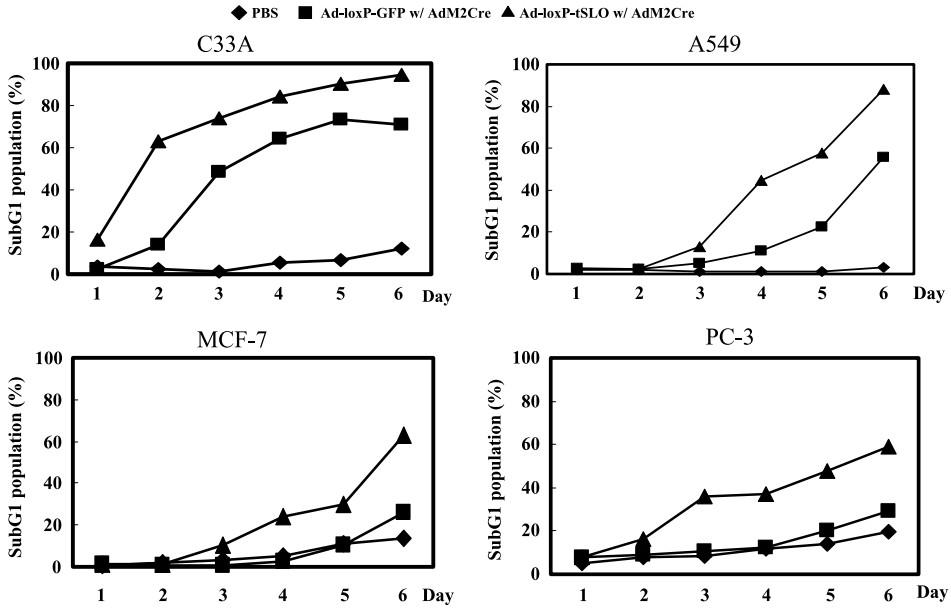

Figure 6. Streptolysin O-expressing adenovirus kills various cancer cell lines effectively. Four different cancer cell lines were grown in 24-well culture plates and infected with 10 MOIs of Ad-loxP-GFP or Ad-loxP-streptolysin O along with 5 MOIs of AdM2Cre. Infected cells were harvested after the indicated times and the percentages of dying/dead cells containing subgenomic DNA were measured by staining cellular DNA with propidium iodide dye as described in Materials and Methods. PBS-treated cells viewed as negative controls.

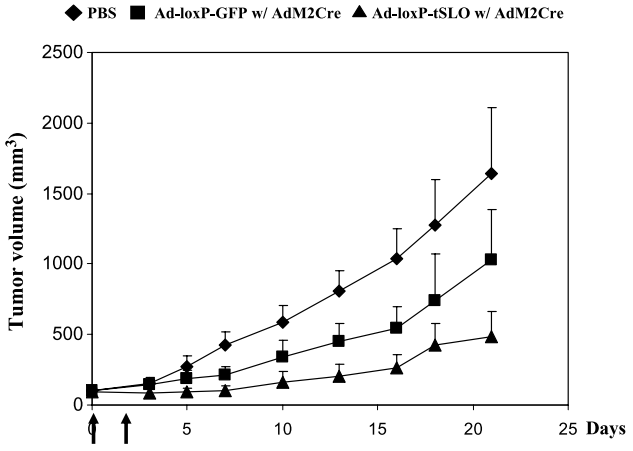

Figure 7. Streptolysin O-expressing adenovirus slows down the growth of human tumors derived from a C33A xenograft in immunodeficient mice. C33A cells were injected into the flanks of 6- to 8-week-old male nude mice to establish human tumor xenografts. Three weeks after cell injection, the volumes of tumors reached ~100 mm^3, and at this time, Ad-loxP-GFP or Ad-loxP-streptolysin O were coinjected directly into tumors with AdM2Cre as described in Materials and Methods. Virus-free PBS (75 μL), a negative control, was injected in the same manner. Tumor volume was measured every 2 to 3 d and data from eight different mice per group were analyzed. *Arrows,* times of adenovirus injection on days 0 and 2. *Points,* mean of each group of mice ($n = 8$); *bars,* +1 SD.

eventually died due to plasma membrane disintegration. No caspase activation was observed in the present study, indicating that cells died from necrosis. These findings coincide with the cell death induced by native streptolysin O toxin and suggest that streptolysin O protein synthesized within eukaryotic cells acts in a similar way to native streptolysin O toxin. Our deletion analysis of the streptolysin O protein further supports this viewpoint (Fig. 4). The COOH-terminal region of streptolysin O is the cholesterol-binding region, and the structure of this region has been suggested to be very compact. Only a single amino acid deletion in this region disabled the cholesterol-binding property of streptolysin O (38). In contrast, the NH_2-terminal region of streptolysin O is more tolerant and deletions of >100 amino acids retained streptolysin O function (38). These structural properties were repeated by streptolysin O protein synthesized within eukaryotic cells (Fig. 4). Up to 115 amino acids of the NH_2-terminal region were found to be dispensable for streptolysin O-induced cytotoxicity, whereas the deletion of only 5 amino acids from COOH-terminal region markedly depressed the cytotoxicity.

The biochemical properties of streptolysin O-induced cell death have several interesting features that could be potentially advantageous for treating cancer cells. First, cellular caspases remained at a basal level during streptolysin O-induced cell death, which implies that cellular ATP has been depleted to a level that can't support the activation of cellular caspases. Most anticancer reagents damage DNA and initiate DNA damage–induced apoptosis or necrosis, and whereas these anticancer reagents are effective against sensitive cancer cells, some cancer cells are less sensitive to these drugs. The depletion of ATP from cells proved to be a crucial method of killing cancer cells, as ATP is a key cellular metabolite. Several studies have found that ATP-depleting agents significantly enhanced the anticancer activity of chemotherapeutic reagents, and that ATP-depleting peptides or polymers are effective against drug-resistant cancer cells (39–41). Second, expressed streptolysin O damages the plasma membrane directly, and thus, the antiapoptotic machinery developed during cancer cell evolution is unable to inhibit streptolysin O-induced cancer cell death. Third, as streptolysin O-induced cell death is not dependent on the cellular proliferation rate, cancer gene therapy based on the streptolysin O gene may be effective against tumors with low proliferation rates, such as prostate cancer.

It is worth mentioning that the use of the streptolysin O gene for tumor cell–killing offers some advantages over the use of intact streptolysin O toxin protein. First, streptolysin O protein needs to oligomerize to create pores in the cell membrane, which is difficult if intact streptolysin O protein or antibody conjugated to streptolysin O protein is injected into a patient. Second, humans have antistreptolysin antibodies that clear injected streptolysin O protein and cause a deleterious inflammatory response, which has proven to be one of the most difficult problems associated with the use of pore-forming toxins as anticancer reagents. Third, streptolysin O binding to the cell membrane is not tumor-specific, and thus, if intact streptolysin O protein is injected into a patient, it will also attack normal cells. The streptolysin O-expressing adenovirus used in the present study is not specific against tumors, and thus, we created this specificity by intratumorally injecting the adenovirus. However, the virus can be modified to target tumor cells either by attaching tumor-specific promoters upstream of the streptolysin O gene or by changing the adenoviral fiber proteins to bind to tumor cell surface proteins (42, 43). It would be promising to integrate the streptolysin O gene with oncolytic viruses. This strategy may decrease the virus titer required, which is important because the adenovirus itself has significant side effects when used at a high titer, as shown by our results and those of others (44).

In summary, we report the successful use of the streptolysin O gene as an anticancer reagent in both cell culture and in a C33A xenograft nude mouse model. In this study, prototype adenovirus was used to deliver the streptolysin O gene into a tumor, but obviously, the streptolysin O gene can easily be implemented into other cancer-specific gene delivery systems. We believe that other kinds of pore-forming toxins, which are abundant in nature, should also be tested as potential anticancer gene therapies.

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