Induction of Apoptosis by Gene Transfer of Human *TRAIL* Mediated by Arginine-rich Intracellular Delivery Peptides

JHENG-FONG LI[1], YULIANA HUANG[1], RONG-LONG CHEN[2] and HAN-JUNG LEE[1]

[1]*Department of Natural Resources and Environmental Studies, National Dong Hwa University, Hualien 97401, Taiwan, R.O.C.;*
[2]*Cancer Center, Chi Mei Foundation Hospital, Tainan 73657, Taiwan, R.O.C.*

Abstract. *Background: Tumor necrosis factor-related apoptosis-inducing ligand (TRAIL), a member of the tumor necrosis factor (TNF) family, has shown potent and high selective antitumor activity as a promising therapy for cancer. We have developed an arginine-rich intracellular delivery (AID) peptide-mediated system for nontoxic and efficient gene transfer in cells. Materials and Methods: To evaluate antitumor activity and therapeutic potential of TRAIL gene, a bifunctional expression plasmid was constructed encoding the secretory signal peptide of human immunoglobulin kappa (Igκ) light chain, the extracellular portion (amino acids 95-281) of human TRAIL and the humanized green fluorescent protein (GFP). Results: We demonstrated that AID peptides were able to effectively deliver TRAIL gene into human lung carcinoma A549 cells. Soluble TRAIL-GFP protein purified from media after gene delivery was further evaluated regarding selective induction of apoptosis in cells. Conclusion: AID peptide-mediated DNA transfer provides a potential and convenient tool in nonviral gene therapy.*

Numerous members of the tumor necrosis factor (TNF) and its receptor families have been characterized with important functions, such as apoptosis, in the immune system (1). TNF-related apoptosis-inducing ligand (TRAIL) is a type II transmembrane protein belonging to the TNF family (2). The *C*-terminus of TRAIL is the receptor-binding domain extending to the extracellular space, while its *N*-terminus is located in the cytoplasm. Ideal anticancer drugs are those that can specifically kill cancer cells without harming normal cells. The soluble *C*-terminus of TRAIL was reported to exert strong apoptotic activity in various cancer cell lines or tumors *in vivo* without affecting normal cells (3-5). Subsequently, this selective induction of apoptosis by TRAIL in cancer cells has highlighted it as one of the most promising candidates in cancer therapy (6, 7).

Gene therapy with *TRAIL* has recently provided an alternative approach for management of cancer therapy (8-12). It has been revealed that protein therapy in mice with tumor required prolonged administration and high doses of recombinant TRAIL protein (12). In addition, production of functional TRAIL protein was relatively difficult due to its physical properties and the various purification procedures required (12, 13). To overcome these weaknesses, gene therapy has provided another choice. Viral carriers, such as adenovirus (8, 9, 11) and lentivirus (10), have been preferentially applied in soluble *TRAIL*-mediated gene therapy. However, nonviral carriers, such as liposome (12), offer several advantages, such as simplicity, amendment and safety, in soluble TRAIL-mediated gene therapy.

Protein transduction is a term used to describe the delivery of a biologically active protein into living cells or animals mediated by a protein transduction domain (PTD) (14, 15). PTDs are very promising tools for the delivery of therapeutic macromolecules, such as proteins and nucleic acids (16-19). Transactivator of transcription (TAT) protein of human immunodeficiency virus type 1 was initially discovered to be able to cross the plasma membrane (20, 21). The basic region between amino acids 47-57 of TAT was responsible for cellular entry (22). These PTDs which can deliver protein cargoes into cells are also named cell-penetrating peptides. Since then, the list of available PTDs and cargoes has grown dramatically, and the number continues to increase (17, 23). We have demonstrated that a group of PTDs which are innocuous arginine-rich intracellular delivery (AID) peptides can efficiently deliver noncovalently conjugated proteins (24-28) or RNA (29) into living cells or tissues. In addition, AID peptides were able to directly deliver plasmid DNA through cell wall/membrane and into nuclei of plant cells noncovalently (30).

Correspondence to: Han-Jung Lee, No. 1, Sec. 2, Da-Hsueh Road, Shoufeng, Hualien 97401, Taiwan, R.O.C. Tel: +886 38633642, Fax: +886 38633260, e-mail: hjlee@mail.ndhu.edu.tw

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In this study, we constructed a secretable TRAIL-green fluorescent protein (GFP) expression plasmid and characterized its expression of human TRAIL protein. AID peptides were demonstrated to effectively deliver this *TRAIL* gene into human lung carcinoma A549 cells. This administration led to the expression of TRAIL-GFP fusion protein in cells and culture media. Soluble TRAIL-GFP protein concentrated and purified from media was further evaluated regarding selective induction of apoptosis in cells.

Materials and Methods

Plasmid construction. The pcDNA-TRAIL plasmid containing a coding region of amino acids 95–281 of human *TRAIL* cDNA fragment (GenBank Accession No. BC032722) (2) was amplified from a template pEGFP-TRAIL plasmid (Addgene, Cambridge, MA, USA) with primers *TRAIL95* (5'-ACCTCTGAGG AAACCATTTCTACAGTTCAAGAA-3') and *TRAIL*281 (5'-GCCAACTAAAAAGGCCCCGAAAAAACTGGCTTCATG-3') into the pcDNA3.1/V5-His-TOPO vector (Invitrogen, Carlsbad, CA, USA) by PCR. The pcDNA-antiIgκ plasmid was amplified from annealing of primers Igκ-U (5'-TTAAGCTTAGAAGGAGA ACCatggaagccccagctcagcttctcttcctcctgct-3') and Igκ-D (5'-AAGGATCCTCTCCGGTGGTATCTGGGAGCCAGAGTAGCAGG AGGAAGAGAAGCTG-3') encoding the secretory signal peptide of human immunoglobulin kappa (Igκ) light chain (GenBank Accession No. X72489) into the pcDNA3.1/V5-His-TOPO vector. A silent mutation occurred at the 16th amino acid (proline) of the Igκ peptide during PCR. The pcDNA-Igκ-TRAIL plasmid was generated by insertion of a DNA fragment coding for a copy of the Igκ signal peptide of the pcDNA-antiIgκ plasmid into the pcDNA-TRAIL plasmid at *Hind*III and *Bam*HI sites.

The pSTBlue-GFP plasmid was generated by amplification of the pR9-GFP plasmid (31) with primers GFP3 (5'-ACCATGAGCA AGGGCGAGGAACTGTT-3') and GFP4 (5'-CTTGTACAGCTCGT CCATGCCATGT-3') coding for the humanized GFP (GenBank Accession No. U50963) without its original stop codon into the pSTBlue-1 TA cloning vector (Novagen, Madison, WI, USA) by PCR. The pcDNA-antiGFP plasmid containing a coding region of the GFP without stop codon was constructed by amplification of the pSTBlue-GFP plasmid with primers GFP10 (5'-CTGATAT CACCATGAGCAAGGGCGAGGAACTGTT-3') and GFP11 (5'-TTCTCGAGTTCTTGTACAGCTCGTCCATGCCATGT-3') into the pcDNA3.1/V5-His-TOPO vector. Finally, the pcDNA-Igκ-TRAIL-GFP plasmid was generated by insertion of the stop-codon-less GFP fragment released by digestion of restriction enzymes *Eco*RV and *Xho*I from the pcDNA-antiGFP plasmid into the pcDNA-Igκ-TRAIL plasmid. All constructs were confirmed by DNA sequencing.

Cell culture. Human lung carcinoma A549 cells (CCL-185; American Type Culture Collection, Manassas, VA, USA) were cultured as previously described (28). Cells were plated at a density of 3×10^5 per 30-mm petri dish and incubated for 24 h prior to further assays.

Transient DNA transfection. Plasmid DNA was transfected into cells with either the jetPEI transfection reagent (Polyplus-transfection, San Diego, CA, USA) according to the manufacturer's instructions (26, 29) or the AID peptide-mediated DNA delivery method (30). For the latter, both 7.14 μg of a synthetic nona-arginine (SR9) peptide, an AID peptide (24), and 5 μg of the pcDNA-Igκ-TRAIL-GFP plasmid were mixed at an N/P (NH_3^+ per PO_4^-) ratio of 3 in 50 μl of phosphate-buffered saline (PBS) for 15 minutes. Cells were treated with SR9/DNA mixture at room temperature for 15 minutes followed by washing with PBS several times. Subsequently, 1 ml of fresh culture medium was added to the petri dish and incubated for 24-48 h in an incubator at 37˚C. Reverse transcription-polymerase chain reaction (RT-PCR). Total RNAs were extracted by the NucleoSpin RNA II kit (Clontech, Mountain View, CA, USA) according to the manufacturer's instructions. The one-step RT-PCR was conducted as previously described (32). In brief, amplification was performed with 5 μl of extracted total RNAs in a total volume of 12.5 μl containing 15 pmol of *TRAIL*95 and *TRAIL*281 primers, 4 μl of 5× the reaction buffer, 0.5 μl of RNase inhibitor (20 U), 2 μl of dNTP mixture (final concentration 1 mM) and 1 μl of RevertAid H-Minus M-MuLV reverse transcriptase (Fermentas, Glen Burnie, MD, USA) at 42˚C for 60 minutes (for RT), then at 70˚C for 10 minutes, followed by 30 cycles of PCR (32).

Western blot analysis. Western blot analysis was employed as previously described (33, 34). Briefly, the culture medium of jetPEI/pcDNA-Igκ-TRAIL-GFP transfected cells was collected by centrifugation at 3,000 rpm for 3 minutes at 4˚C to remove cell debris. The expressed protein containing a hexa-histidine tag was purified by one-step metal chelation chromatography as previously described (35). The protein content in culture medium was concentrated by Amicon Ultra (Millipore, Billerica, MA, USA), separated by SDS-PAGE on a 10% polyacrylamide gel at 120 V for 2 h and semi-dry transferred onto a PVDF membrane (GE Healthcare, Little Chalfont, Buckinghamshire, UK) at 100 V for 1-2 h according to the manufacturer's instructions. After dairy milk blocking, the membrane was incubated with the anti-GFP-HRP conjugated antibody (Abcam, Cambridge, MA, USA) for 1 h at room temperature under agitation. The membrane was then incubated with the chemiluminescent HRP-substrate (Millipore) for 10 minutes and detected by Luminescent image analyzer LAS-3000 (Fujifilm, Tokyo, Japan).

Cell viability assay. The sulforhodamine B (SRB) colorimetric assay was used as previously described (26). The absorbance of dye solution was measured at 510 nm using a DU800 spectrophotometer (Beckman Coulter, Fullerton, CA, USA).

Terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) assay. For TUNEL assay, cells were transfected by the pcDNA-Igκ-TRAIL-GFP plasmid with either jetPEI reagent or SR9 peptide for 3 days. Cells were fixed with 2 ml of 4% (w/v) formaldehyde in PBS on ice for 15 minutes, washed with PBS 3 times and fixed for 1 h with 2 ml of cold 70% ethanol at –20˚C. Cells were then washed with PBS 3 times and incubated in 150 μl of the DNA labeling solution [30 μl of terminal deoxynucleotidyl transferase (TdT) reaction buffer, 2.25 μl of TdT enzyme, 24 μl of Br-dUTP and 93.75 μl of H_2O] of the ApoBrdU Red DNA Fragmentation Kit (BioVision, Mountain View, CA, USA) for 1 h at 37˚C. Cells were rinsed with the rinse buffer and then dried by aspiration twice. Subsequently, cells were incubated in 200 μl of the antibody solution (10 μl of anti-BrdU-Red antibody and 190 μl of H_2O) for 30 minutes in the dark at room temperature. Finally, cells were analyzed by a TCS SL confocal microscope system (Leica, Wetzlar, Germany) as previously described (24). For the GFP

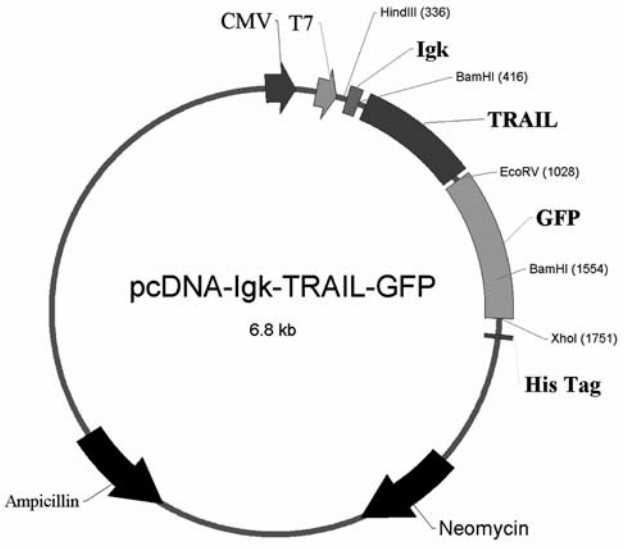

Figure 1. *Schematic structure of the pcDNA-Igκ-TRAIL-GFP plasmid. The pcDNA-Igκ-TRAIL-GFP plasmid contains a coding region of the signal peptide (Igκ), the extracellular domain of TRAIL, the humanized GFP and hexa-histidine (His) tag under both controls of cytomegalovirus (CMV) and T7 promoters in the pcDNA3.1/V5-His-TOPO expression vector (Invitrogen).*

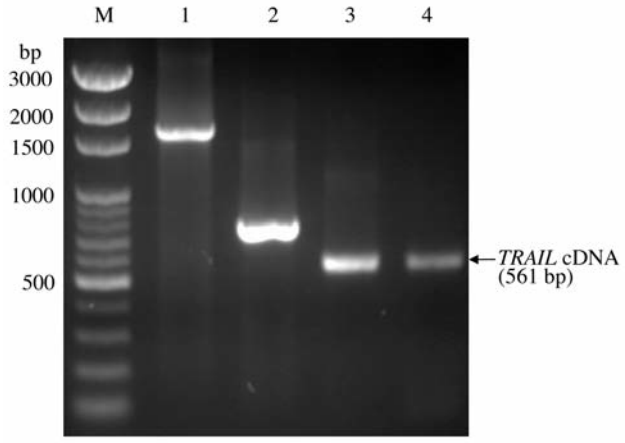

Figure 2. *RT-PCR analysis of TRAIL transcripts. Commercial 100 bp DNA ladder (Hopegen, Taichung, Taiwan) served as the DNA marker (lane M). PCR products with 1,591, 731 and 561 bp separated by DNA electrophoresis on a 1% agarose gel were generated from amplification of the template pcDNA-Igκ-TRAIL-GFP plasmid with T7 and BGH reverse (lane 1), GFP10 and GFP11 (lane 2) as well as TRAIL95 and TRAIL281 (lane 3) primers, respectively. The RT-PCR product with 561 bp was generated from amplification with TRAIL95 and TRAIL281 primers of transcripts in jetPEI/pcDNA-Igκ-TRAIL-GFP transfected cells (lane 4).*

channel, excitation at 488 nm and emission at 500-540 nm were set using band-pass filters. For the red fluorescent protein (RFP) channel, excitation at 543 nm and emission at 550-650 nm were set using band-pass filters as previously described (26).

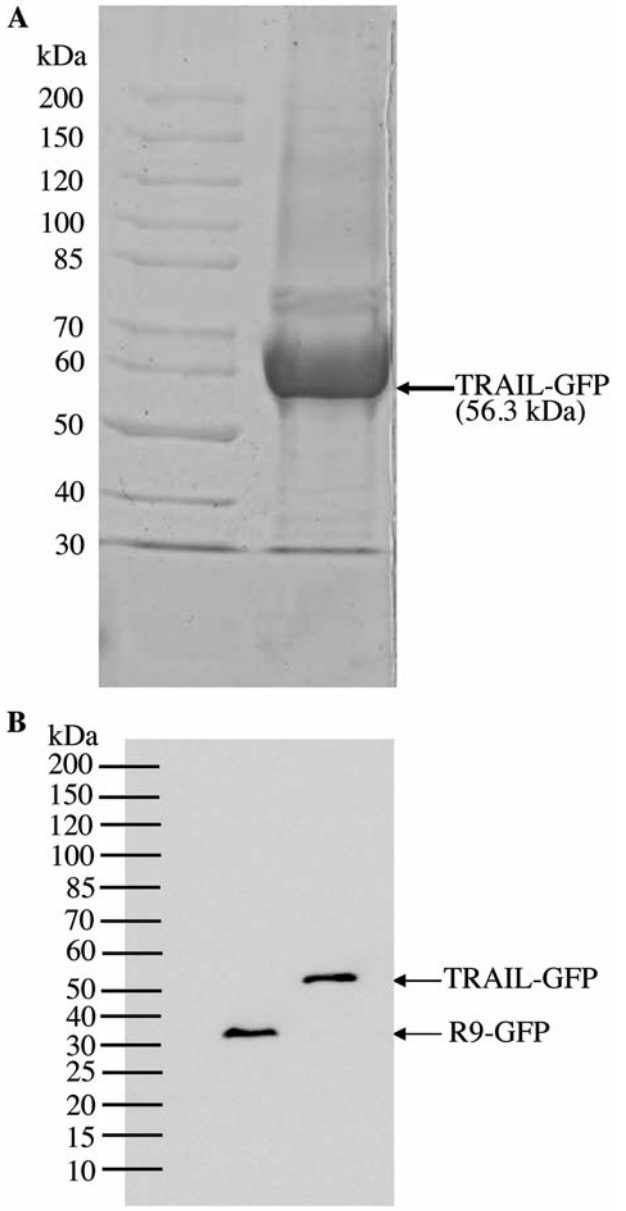

Figure 3. *Western blot analysis of the secretory TRAIL-GFP protein. A: TRAIL-GFP protein product with a calculated molecular mass of 56.3 kDa purified from jetPEI/pcDNA-Igκ-TRAIL-GFP transfected cell culture medium was analyzed by SDS-PAGE. B: The purified TRAIL-GFP protein was detected by Western blot analysis. R9-GFP protein purified from Escherichia coli with a calculated molecular mass of 32.6 kDa served as a positive control.*

Flow cytometry. Flow cytometric analysis was applied as previously described (28). Human A549 cells were seeded at a density of 1×10^5 per 30-mm petri dish and then incubated overnight in 2 ml of culture medium. Cells were transfected by the pcDNA-Igκ-TRAIL-GFP plasmid over 1-4 days, washed with PBS, fixed overnight with 3 ml of cold 70% ethanol at –20˚C and then washed with PBS. Cells were completely broken up by pipetting twice. One

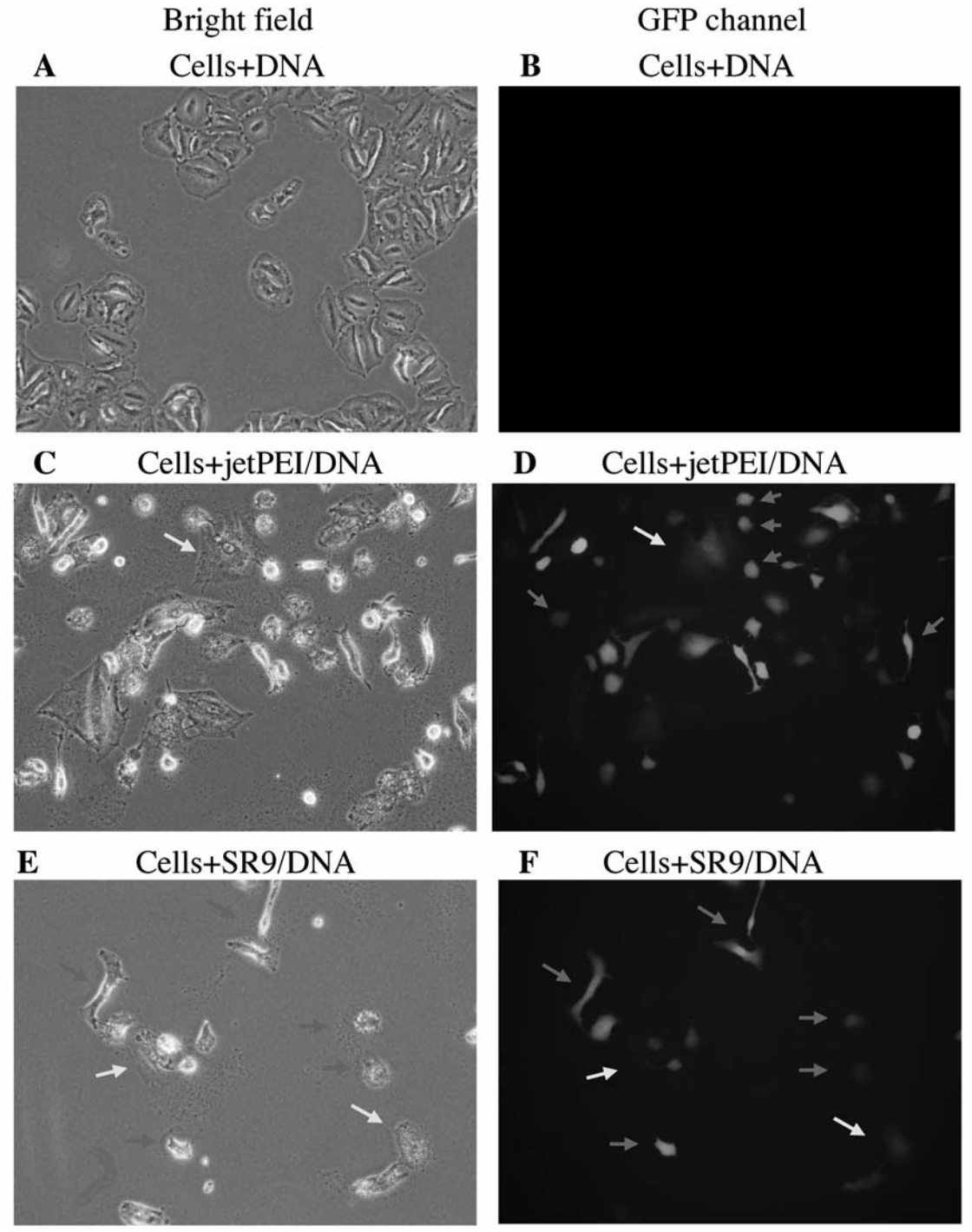

Figure 4. *Confocal microscopy of gene expression in AID peptide-mediated DNA transfected cells. Human A549 cells treated with the pcDNA-Igκ-TRAIL-GFP plasmid (A and B), jetPEI/pcDNA-Igκ-TRAIL-GFP (C and D), or SR9/pcDNA-Igκ-TRAIL-GFP complexes (E and F) were detected in the bright-field (A, C and E) or GFP channel (B, D and F) using a confocal microscope. Bright arrows show non-apoptotic cells which contain intact nuclei and have fish-scale-like cell morphology, while dark arrows indicate apoptotic cells which have cell shrinkage and apoptotic bodies.*

ml of propidium iodide (PI) solution (final concentration of 20 μg/ml of PI, 0.1% Triton-X 100 and 0.2 mg/ml of RNase A) was added to stain cells for 1 h. Finally, cells were analyzed by a Cytomics FC500 flow cytometer (Beckman Coulter).

Statistical analysis. Results are expressed as means±standard deviations. Statistical comparisons between the control and treated groups were performed by the Student's *t*-test. Means and standard deviations were calculated for each sample assayed at least in

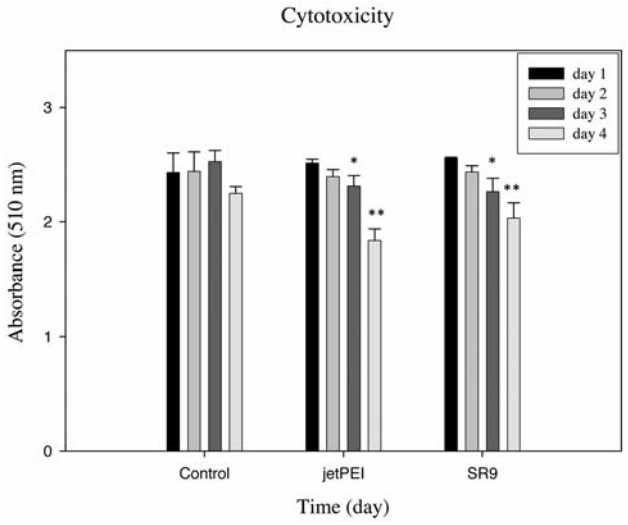

Figure 5. *Cytotoxicity assay of DNA transfected cells. Cell viability of A549 cells transfected with the pcDNA-Igκ-TRAIL-GFP plasmid alone (control), jetPEI/pcDNA-Igκ-TRAIL-GFP, or SR9/pcDNA-Igκ-TRAIL-GFP complexes at a time course was assessed by SRB assay.*

triplicate. The level of acceptable statistical significance was set at $p<0.05$ (*) or 0.01 (**).

Results

Plasmid construction and in vitro expression of a bifunctional TRAIL-GFP fusion protein. The pcDNA-Igκ-TRAIL-GFP plasmid was constructed to include a coding region of a bifunctional TRAIL-GFP fusion protein under both controls of cytomegalovirus (CMV) immediate-early and T7 promoters in a bifunctional shuttle vector, able to replicate in both eukaryotes and prokaryotes (Figure 1). The expression of the pcDNA-Igκ-TRAIL-GFP plasmid in eukaryotic and prokaryotic systems produced an open reading frame encoding the secretory signal peptide of human Igκ, the extracellular domain (amino acids 95–281) of TRAIL, the humanized GFP, the V5 epitope and a hexa-histidine tag. Sequencing analysis confirmed the correct and in-frame fusion of *TRAIL-GFP* at the DNA level (data not shown).

Human A549 cells were transiently transfected with the pcDNA-Igκ-TRAIL-GFP plasmid by the jetPEI transfection reagent. The expression of *TRAIL* mRNA was confirmed by RT-PCR. As shown in Figure 2, a specific *TRAIL* cDNA with 561 bp corresponding to a coding region of amino acids 95-281 of human TRAIL was amplified by *TRAIL*95 and *TRAIL*281 primers from transcripts in transfected cells. SDS-PAGE and Western blot analysis proved the expression of TRAIL-GFP protein with a calculated molecular mass of 56.3 kDa (Figure 3).

AID peptide-mediated gene delivery. Human A549 cells were treated with the pcDNA-Igκ-TRAIL-GFP plasmid alone as a negative control (Figure 4A and B), jetPEI/pcDNA-Igκ-TRAIL-GFP complex (Figure 4C and D), or SR9/pcDNA-Igκ-TRAIL-GFP complex (Figure 4E and F) and incubated 72 h after transfection. No cells were visible observing the GFP channel when cells were treated with DNA plasmid alone (Figure 4B). In contrast, strong green fluorescence was detected in cells treated with jetPEI/DNA (Figure 4D) and SR9/DNA complexes (Figure 4F). Compared to pre-apoptotic cells (bright arrows), several cells displayed the most characteristic features of apoptosis, namely shrinkage and pyknosis, resulting from irreversible chromatin condensation during the early process of apoptosis (dark arrows). These results demonstrated that AID peptides are able to facilitate specific gene delivery in cells.

Cytotoxicity of TRAIL protein. A549 cells were transfected with the pcDNA-Igκ-TRAIL-GFP plasmid alone or plasmid plus either jetPEI reagent or SR9 peptide (Figure 5) to investigate cytotoxicity of the bifunctional TRAIL-GFP fusion protein. Cell viability of A549 cells was assessed by SRB assay. The survival fractions of cells significantly decreased after 2 days of treatment. These results indicated that the secretory TRAIL-GFP protein expressed from the pcDNA-Igκ-TRAIL-GFP plasmid caused apoptosis in human lung carcinoma cells.

Detection of apoptotic effect with TUNEL assay. The TUNEL assay is a common method for detecting DNA fragmentation that results from apoptotic signaling cascades (11). Cells transfected with jetPEI/pcDNA-Igκ-TRAIL-GFP (Figure 6A-F) and SR9/pcDNA-Igκ-TRAIL-GFP complex (Figure 6G-L) were detected by TUNEL assay. The expression of the bifunctional TRAIL-GFP fusion protein was observed in cells in the GFP channel (Figure 6A and G) and at a magnification of ×400 (Figure 6D and J) using a confocal microscope. Red fluorescence was detected in apoptotic cells which were stained with anti-BrdU-Red antibody in the RFP channel (Figure 6B and H) and at a magnification of ×400 (Figure 6E and K). Yellow fluorescence was detected in some cells expressing the secretory TRAIL-GFP fusion protein and undergoing cell apoptosis in merged images of GFP/RFP channels (Figure 6C and I) and at a magnification of ×400 (Figure 6F and L). These data suggest that TRAIL-GFP protein expressed from the pcDNA-Igκ-TRAIL-GFP plasmid in cells after gene delivery did indeed induce efficient apoptosis in transfected cells.

Flow cytometry. PI is a fluorescent molecule which binds to DNA by intercalating between the bases. Flow cytometric analysis was used to evaluate changes of DNA content in the cell cycle. Cells transfected with jetPEI/pcDNA-Igκ-TRAIL-

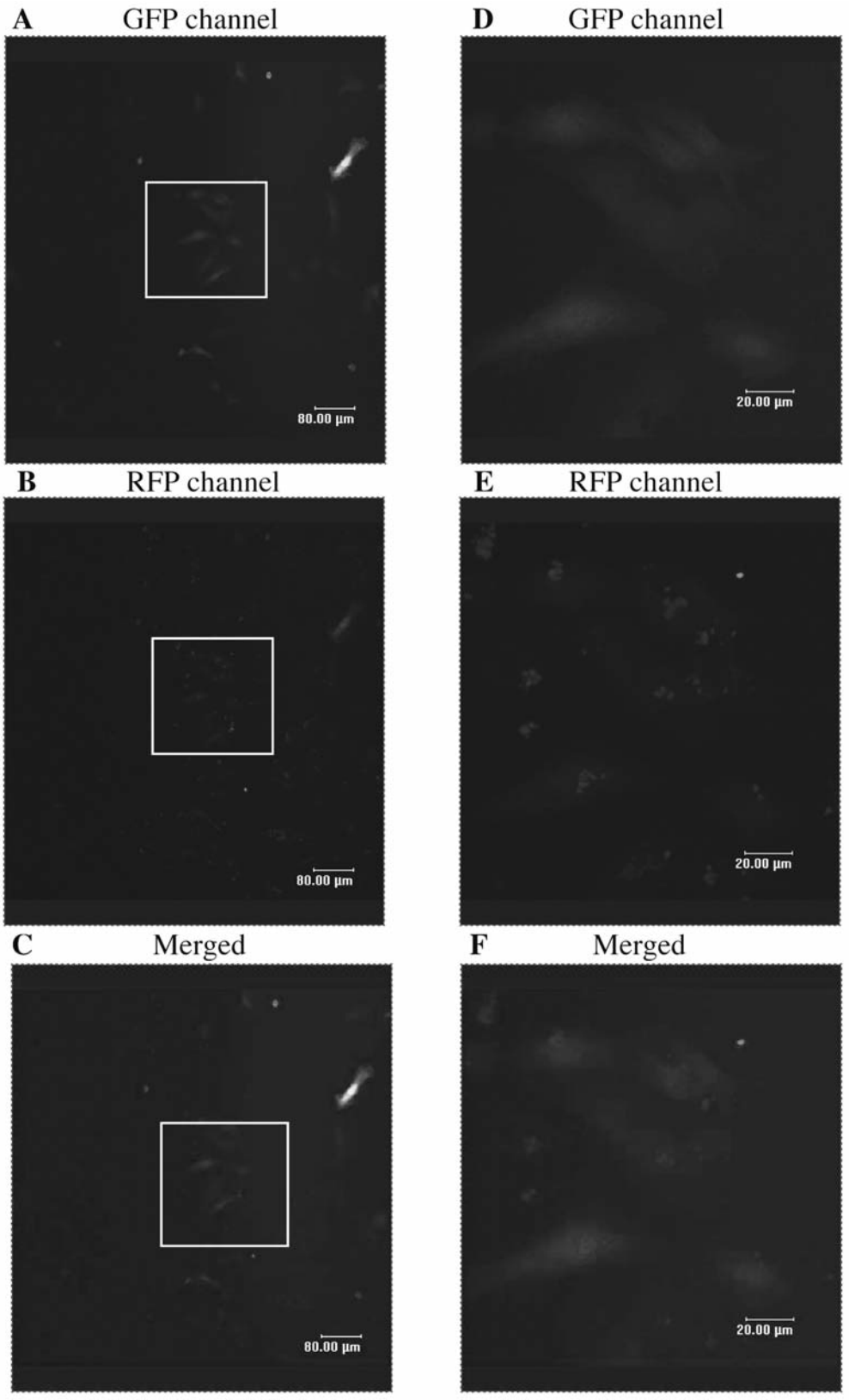

Figure 6. *continued*

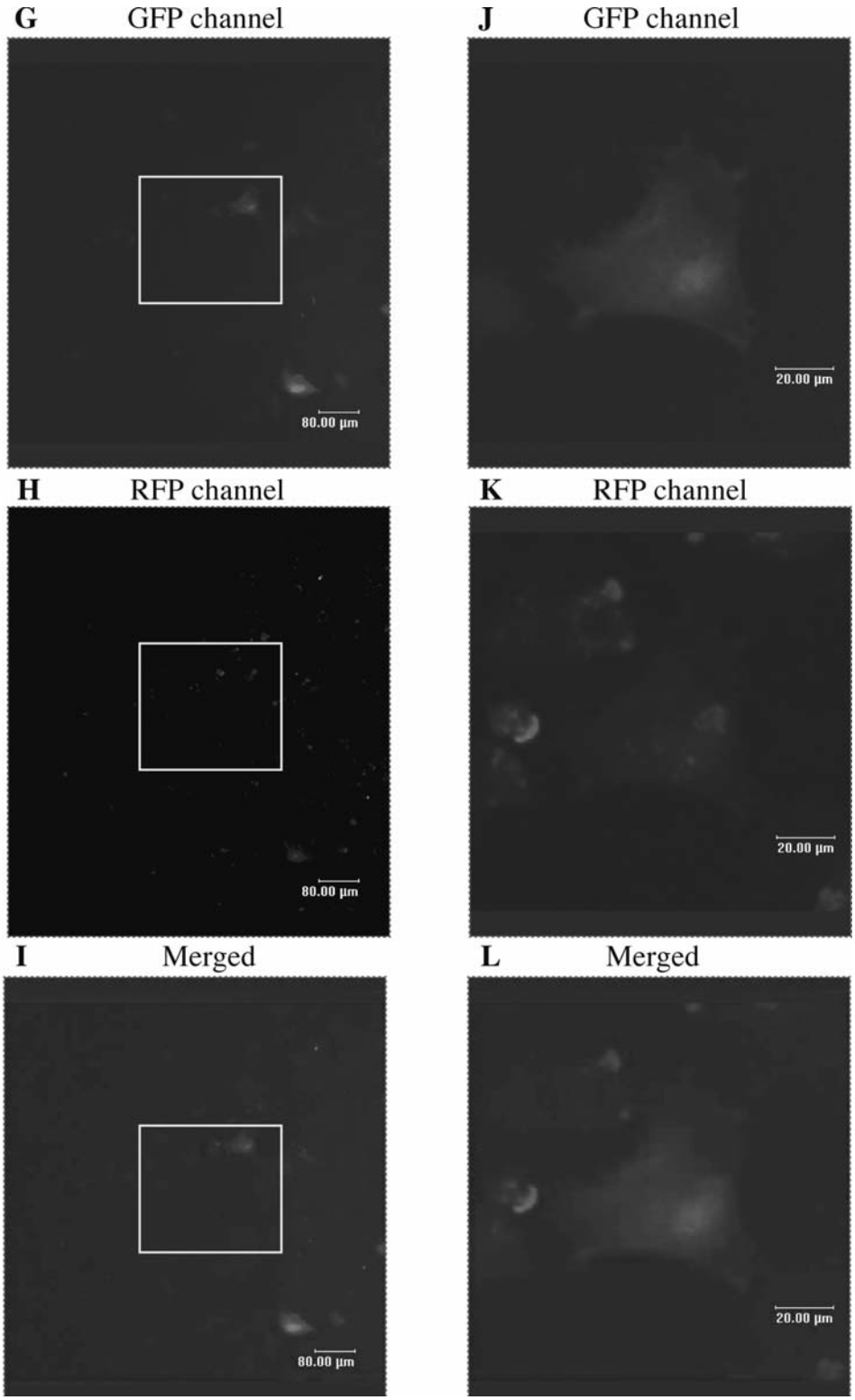

Figure 6. *Confocal microscopy of induction of apoptosis in DNA transfected cells by TUNEL assay. A549 cells treated with either jetPEI/pcDNA-Igκ-TRAIL-GFP (A–F), or SR9/pcDNA-Igκ-TRAIL-GFP complexes (G–L) were detected at a magnification of ×400 in the GFP (A, D, G and J at 500–540 nm), RFP (B, E, H and K at 550–650 nm), or merged GFP/RFP channels (C, F, I and L) by a confocal microscope in TUNEL assay. Scale bar is 80 μm. Areas in A–C and G–I are shown at higher magnification in D–F and J–L with a scale bar of 20 μm, respectively. Green fluorescence represents the location of TRAIL-GFP fusion protein, while red fluorescence indicates apoptotic cells recognized by anti-BrdU-Red antibody.*

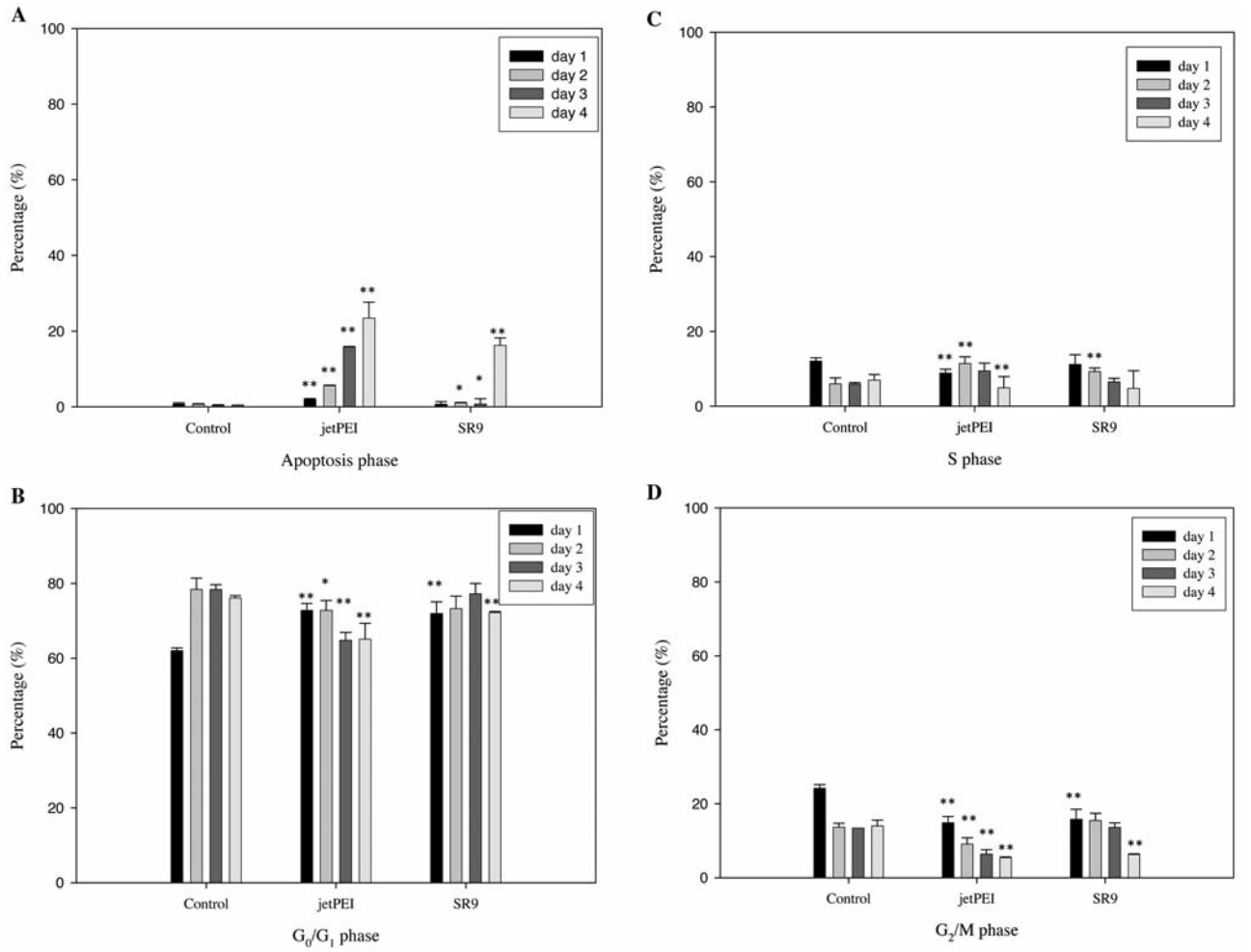

Figure 7. *Arrest of cell cycle in S–G_2/M phase by propidium iodide (PI)-mediated flow cytometric analysis. A549 cells transfected with the pcDNA-Igκ-TRAIL-GFP plasmid alone (control), jetPEI/pcDNA-Igκ-TRAIL-GFP, or SR9/pcDNA-Igκ-TRAIL-GFP complexes were stained with PI and analyzed by flow cytometry over 4 days, indicating apoptosis (A), G_0/G_1 (B), S (C) and G_2/M (D) phases, respectively.*

GFP and SR9/pcDNA-Igκ-TRAIL-GFP complex were quantitatively analyzed for 4 days by flow cytometry (Figure 7). The apoptotic phase of the cell cycle was clearly increased after transfection (Figure 7A). In addition, the proportion of cells in the S phase decreased (Figure 7C), while those in the G_2/M phase significantly decreased after transfection (Figure 7D), indicating that TRAIL-GFP protein induced efficient apoptosis by arresting the cell cycle in the S–G_2/M phases.

Discussion

In this study, our results demonstrated that AID peptides can facilitate gene delivery in cells and cause apoptosis. Efficient and safe delivery of nucleic acids is a fundamental prerequisite for successful gene therapy in humans (19). Numerous gene delivery systems have been developed. However, they have exhibited major drawbacks which have limited their clinical applications. In general, viral carriers are highly efficient but can cause problems of high immunogenicity and insertional mutagenesis. On the other hand, nonviral carriers have low efficiency and high toxicity. JetPEI is a commercial reagent for delivery of DNA into cells, but it is expensive and can only be applied *in vitro* (17). This has led scientists to explore the possibility of using the TAT-based system for the delivery of nucleic acids. Our data are in concert with previous studies indicating the TAT-based system was able to delivery DNA *in vivo* (36) and *in vitro* (37-39). In conclusion, we have established a more efficient AID-based system to transport the functional *TRAIL* gene into cells and induce apoptosis.

The best characterized feature of TRAIL is the induction of apoptotic signaling (6, 7). The extracellular domain of TRAIL can be cleaved by metalloproteinases to form a soluble cytokine. Protein sequence analysis revealed that two

short highly conserved motifs of this extracellular portion of TRAIL have the characteristics of a TNF family member. A recent model of apoptotic TRAIL signaling proposed that trimeric TRAIL binds to its five distinct receptors (TRAIL-Rs) (7). Upon ligand binding, the receptor becomes trimerized and activated. The activated receptor recruits the adapter protein and pro-caspase molecules to form a death-inducing signaling complex (DISC). Conversion of pro-caspases into their active forms then activates downstream caspases and induces apoptosis (7). Interestingly, TRAIL induced apoptosis in many tumor cells, but did not kill normal cells (3–5). This selective induction of apoptosis in tumor cells is a good starting point in the application of TRAIL in cancer therapy.

Apoptosis is a genetically controlled mechanism that is essential for the maintenance of tissue homeostasis, proper development and the elimination of unwanted cells (7). Pyknosis is the irreversible condensation of chromatin in the nucleus of a cell undergoing programmed cell death and is the most characteristic feature of apoptosis. In general, the apoptotic cell appears as a round or oval mass and becomes smaller in size because of cell shrinkage. The cytoplasm of an apoptotic cell is dense and organelles are more tightly packed (Figure 4). In addition, our results showed that the proportion of apoptotic cells (sub-G_0/G_1 phase) increased gradually, while those of mitotic cells (G_2M phase) decreased gradually post-transfection (Figure 7). We suspected that TRAIL induced apoptosis by arresting the cell cycle in the S-G_2/M phase, in agreement with previous studies (40-43).

In conclusion, AID peptides were able to effectively deliver the pcDNA-Igκ-TRAIL-GFP plasmid into human A549 cells. The expression of the secretory TRAIL-GFP fusion protein in cells after gene transport induced efficient apoptosis in transfected cells. AID peptides can facilitate gene delivery and may be able to provide a more convenient and effective method for gene therapy of cancer.

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