

# Induction of Potent TRAIL-Mediated Tumoricidal Activity by hFLEX/Furin/TRAIL Recombinant DNA Construct

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Tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) has been shown to exert selectively cytotoxic activity against many tumor cells but not normal cells. On the other hand, the ligand for the receptor tyrosine kinase Fms-like tyrosine kinase 3 (Flt3L) is a growth factor for hematopoietic progenitors and is a potent stimulating factor for dendritic and NK cells. Previously, we have demonstrated that it is possible to inhibit the outgrowth of primary tumors by the administration of an hFlex (the extracellular domain of the Flt3L) and TRAIL (amino acid residues 95–281) secreted fusion protein. Here, we report that by the insertion of a linker sequence encoding the cleavage site for the Golgi-expressed endoprotease furin between the DNA sequences encoding hFlex and TRAIL, the tumoricidal activity of the cleaved TRAIL protein generated was greatly enhanced in comparison to the hFlex/TRAIL fusion protein. Furthermore, we demonstrate that intratumoral injection of the hFlex/furin/TRAIL DNA, in conjunction with cationic liposomes, significantly suppressed the outgrowth of the human CNE-2 nasopharyngeal tumor xenografts in SCID mice. *In situ* histological examinations confirmed the expression of TRAIL in the treated tumor nodules and the induction of apoptosis was also evidenced by the presence of numerous pyknotic nuclei.

**Key Words:** TRAIL, Flt3L (hFlex), furin, fusagene, anti-tumor activity

## INTRODUCTION

A wealth of information suggests that tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) selectively induces cancer cells to undergo apoptosis while sparing normal cells in nonhuman primates. This observation has raised great interest in using TRAIL in clinical application to eradicate tumors [1,2]. The therapeutic potential of a recombinant soluble human TRAIL has been evaluated in several human cancers, including colorectal [3], glioma [4], and breast [5]. Preclinical studies with mice and cynomolgus monkeys have also provided encouraging therapeutic effect of TRAIL [6,7]. However, the possibility of applying TRAIL in clinical cancer therapy has been halted due to the reported toxicity of TRAIL to primary human hepatocytes [8,9]. It raises the concern that cancer therapy with TRAIL may result in severe side effects, including the possibility of liver damage. Thus, substantial effort has been devoted toward improving the cytolytic activities of TRAIL while reducing its potential toxicity [10]. One attempt was to employ the recombinant trimeric version of TRAIL without the addition of any exogenous sequence. It was reported that stable homotrimer TRAIL could be generated with zinc and reducing agent at neutral pH [10].

This recombinant trimeric TRAIL is postulated to be least likely immunogenic in the host and therefore could have minimal toxicity to normal human hepatocytes. However, this experimental approach is not applicable for *in vivo* gene therapy.

We have previously showed that a fusion protein denoted FETZ [11], encoding the extracellular domain of the human receptor tyrosine kinase Fms-like tyrosine kinase 3 ligand (Flt3L) (hFlex) and the N-terminus of the soluble form of human TRAIL demonstrated the expected dual function of Flt3L and TRAIL. The resulting fusion protein was therefore a potent stimulator of dendritic cells (DC) and could also induce the apoptosis of tumor cells [11]. The ability of DC to acquire antigens derived from apoptotic tumor bodies presents an attractive strategy for vaccination against tumors [12,13]. Gene therapy strategies that induce cell death *in vivo* with the concomitant stimulation of DC maturation generate strong and protective immune responses [14]. The advantage of such an approach includes the ability to prime a broad repertoire CD4<sup>+</sup> and CD8<sup>+</sup> T cell responses without prior need to identify tumor-specific antigenic epitopes and therefore could potentially be applied generically to every cancer patient independent of their HLA haplotypes [15]. Recently, we have also demon-

strated that the immunization of a tumor-bearing host with tumor-specific peptide following the expansion of DC with hFlex led to the generation of potent CTL responses that could eradicate tumor growth [16].

In this report, we describe the generation of TRAIL with potent tumoricidal activities by inserting a furin linker sequence into the DNA sequences encoding the soluble form of human TRAIL (amino acids residues 95–281) in which the homology region of the ligand is N-terminally fused with an isoleucine zipper motif and in conjunction with the DNA sequences encoding hFlex [11]. This particular design would facilitate the cleavage of hFlex/TRAIL translational product formed from a monocistronic message and provide the ability of an isoleucine zipper to facilitate TRAIL trimerization.

## RESULTS

Furin, a cellular endoprotease having the consensus recognition sequence Arg.X.Arg/Lys.Arg, is able to process diverse precursor proteins secreted via the constitutive pathway [17,18]. Furin participates in the autocrine production regulation pathway and contributes to the unchecked proliferation of cancer cells. We synthesized a 30-mer oligo encoding the DNA sequences of 10 amino acids (Gly-Arg-Arg-Ala-Arg-Try-Lys-Arg-Gly-Gly) of the furin recognition sequence [19] and cloned it in-frame between the DNA sequences encoding hFlex and TRAIL (Fig. 1). The resulting plasmid was tentatively designated *pFFT*. A second construct with the addition of the isoleucine zipper DNA sequence in *pFFT* was designated *pFFZT* (Fig. 1). The isoleucine zipper DNA sequences have been previously reported to promote the trimerization of the recombinant TRAIL [5,11].

To demonstrate the ability of furin to mediate cleavage of the fusion protein, we transfected COS7 cells with *pFFT*, *pFFZT*, or *pFETZ* plasmid DNA and studied the presence of the transgene with anti-TRAIL antibody (Ab) (Santa Cruz Biotechnology, Santa Cruz, CA, USA) using Western blot hybridization. The apparent molecular weights (MW) of the fusion proteins detected (42 or 46 kDa) following the introduction of *pFFT*, *pFFZT*, or *pFETZ* plasmid DNA constructs into COS7 cells (Fig. 2A) were in

good agreement with our previously reported fusion protein from hFlex and TRAIL [11]. The expected cleaved protein products of TRAIL and Zipper-TRAIL, having the apparent MW of 24 and 28 kDa, respectively, could also be demonstrated with the anti-TRAIL Ab (Fig. 2A). Moreover, the MW observed for TRAIL was increased by approximately 4 kDa with the addition of the isoleucine zipper (Fig. 2A). These data confirmed the presence of furin-mediated cleavage of the translational product expressed from a single cistron.

We also demonstrated furin-mediated cleavage *in vivo*. Following delivery of *pFFT*, *pFFZT*, or *pFETZ* plasmid DNA into 6- to 8-week-old BALB/c mice by the reported hydrodynamic gene delivery method [20], the respective fusion protein, as well as the cleaved TRAIL protein product, could be demonstrated in the sera of the recipient mice 24 h after the injection with the anti-TRAIL Ab (Fig. 2B, i). Reciprocally, with anti-Flt3L Ab (Santa Cruz Biotechnology), we could also confirm the presence of the expected hFlex gene product (Fig. 2B, ii). The presence of the cleaved TRAIL protein in the circulation of the recipient BALB/c mice suggests that the cleaved proteins are secreted. However, we observed that the furin-mediated cleavage was incomplete and by studying the intensities of the protein bands obtained with the Western blot hybridization, we found that the percentage of the cleavage was about 30–50% (Fig. 2B). Moreover, we consistently detected the presence of the 24-kDa cleaved TRAIL protein product with *pFFT* and the 28-kDa protein representing cleaved Zipper-TRAIL with *pFFZT* in the groups of three mice (Fig. 2C). These data demonstrated the efficient furin-mediated cleavage of the FETZ monocistronic transcript *in vivo* and are in good agreement with previous observations [19,21]. Furthermore, the specific trimerization of TRAIL following the introduction of *pFFZT* that contained the isoleucine zipper could be demonstrated using a 6% native gel. The band with the highest molecular weight (Fig. 2D, arrow) corresponded to the trimeric form of cleaved TRAIL with isoleucine zipper, whereas only the low-molecular-mass band, corresponding to the predicted monomeric form of TRAIL, could be detected with the construct *pFFT* (Fig. 2D, dotted-line arrow).

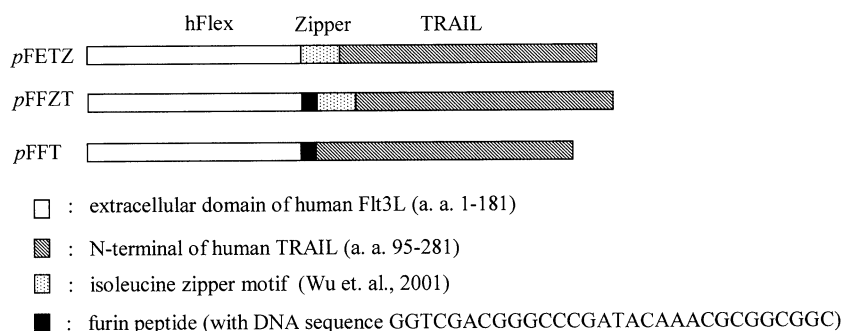
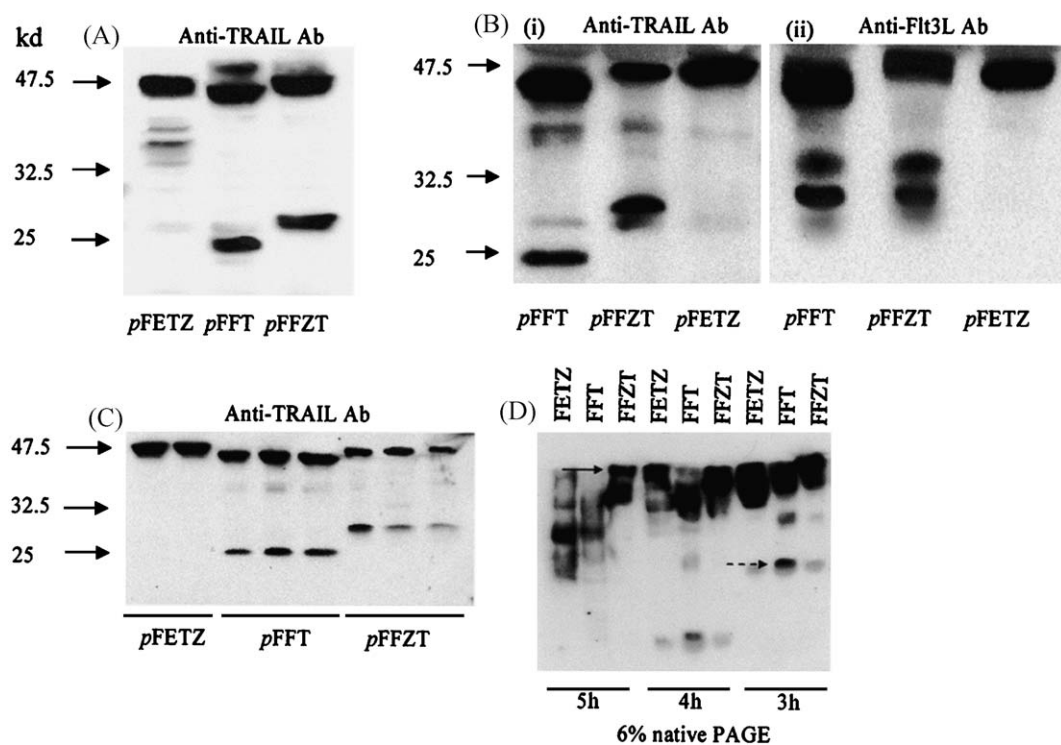


FIG. 1. Schematic representation of the various DNA constructs employed in this study.



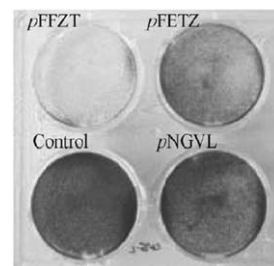
**FIG. 2.** Western blot analysis. (A) Expression of pFETZ-, pFFT-, and pFFZT-encoded proteins in COS7 cells following transfection. The presence of fusion proteins and cleaved protein products was detected using anti-TRAIL Ab. (B) Expression of pFFT, pFFZT, and pFETZ *in vivo*. pFFT-, pFFZT-, and pFETZ-encoded proteins could be detected in sera collected from mice injected with pFFT, pFFZT, or pFETZ with (i) anti-TRAIL Ab and (ii) anti-Flt3L Ab. (C) pFFT-, pFFZT-, or pFETZ-encoded proteins were consistently detected in sera collected from groups of mice injected with pFFT, pFFZT, or pFETZ, respectively. (D) Analysis of FFT, FFZT, and FETZ protein by 6% native PAGE. Cleaved form of TRAIL was assembled in stable trimers upon addition of isoleucine zipper sequence (arrow) compared to the monomeric cleaved TRAIL (dotted-line arrow).

To determine the optimal time course of *TRAIL* gene expression following the delivery of the pFETZ or pFFZT plasmid, we studied the level of TRAIL in the sera of recipient BALB/c mice following a single injection of either pFETZ or pFFZT plasmid. The results indicated that the expression of TRAIL peaked at 24

h after delivery of 10 µg plasmid DNA via hydrodynamic injection. At the peak, the TRAIL protein reached a concentration of 30–35 µg/ml in the sera and then steadily declined. The serum concentration of TRAIL remained in the range of 10–300 ng/ml at day 7.

**TABLE 1.** Comparison of the cytotoxicity of pFFZT and pFETZ on human cancer cell lines

Cell line	Cell viability (crystal violet assay)	
	FFZT	FETZ
CNE-2	27%	61%
CNE-1	40%	76%
I-K-1	51%	77%
HepG2	46%	69%
Hep3B	45%	66%
Hela	49%	75%
MDA-23 1	35%	78%
Jurkat	46%	72%
A549	68%	82%
U87	40%	72%



Human CNE-2 cells

Cells were incubated for 6 h with sera containing FFZT and FETZ at the TRAIL concentration of 1 µg/ml. The mouse serum was obtained and diluted from pFFZT or pFETZ-injected mice. (Inset) Induction of apoptosis of CNE-2 cells following *in vitro* transfection of CNE-2 cells with pFFZT, pFETZ, or pNGVL DNA complexed with NCC4 liposomes. CNE-2 cells were incubated with NCC4 liposomes as the control.

To determine the cytolytic ability of the sera, we employed a panel of tumor cells including the human nasopharyngeal carcinoma cells CNE-2, CNE-1, and HK-1 cells; human hepatocellular carcinoma cells HepG2 and Hep3B; human cervical cancer HeLa cells; human breast carcinoma MDA-231 cells; human Jurkat cells; human A549 lung carcinoma cells; and human U87 glioma cells (Table 1). We detected the presence of toxicity using the annexin V apoptosis detection kit. We determined cell viability by crystal violet assay. Among the human tumor cell lines tested, sera of recipient BALB/c mice collected 24 h after hydrodynamic delivery of the *pFFZT* exhibited much more potent cytotoxicity toward the panel of cell lines studied in comparison to the sera collected from mice injected with *pFETZ* (Table 1). The serum collected from control vector *pNGVL*-treated mice had little

effect on the viability of the different cell lines tested (data not shown).

Furthermore, transfection of CNE-2 cells with the *pFFZT* DNA plasmid resulted in strong cytotoxicity of the transfected cells in comparison to cells transfected with the *pFETZ* plasmid (Table 1, inset). We normalized the data by routinely cotransfecting cells with the *pFFZT* or *pFETZ* plasmid together with the *pNGVL*-Luciferase plasmid and utilized the subsequent RLU obtained for normalization between the various samples. The samples normally showed very similar RLU/ $\mu\text{g}$ , thus indicating the transfection efficiency was very similar between the plasmids. Cell viability was not affected when the CNE-2 cells were transfected with the control *pNGVL* vector.

As the human Jurkat cells are well established as a model for studying TNF-related apoptosis, we chose

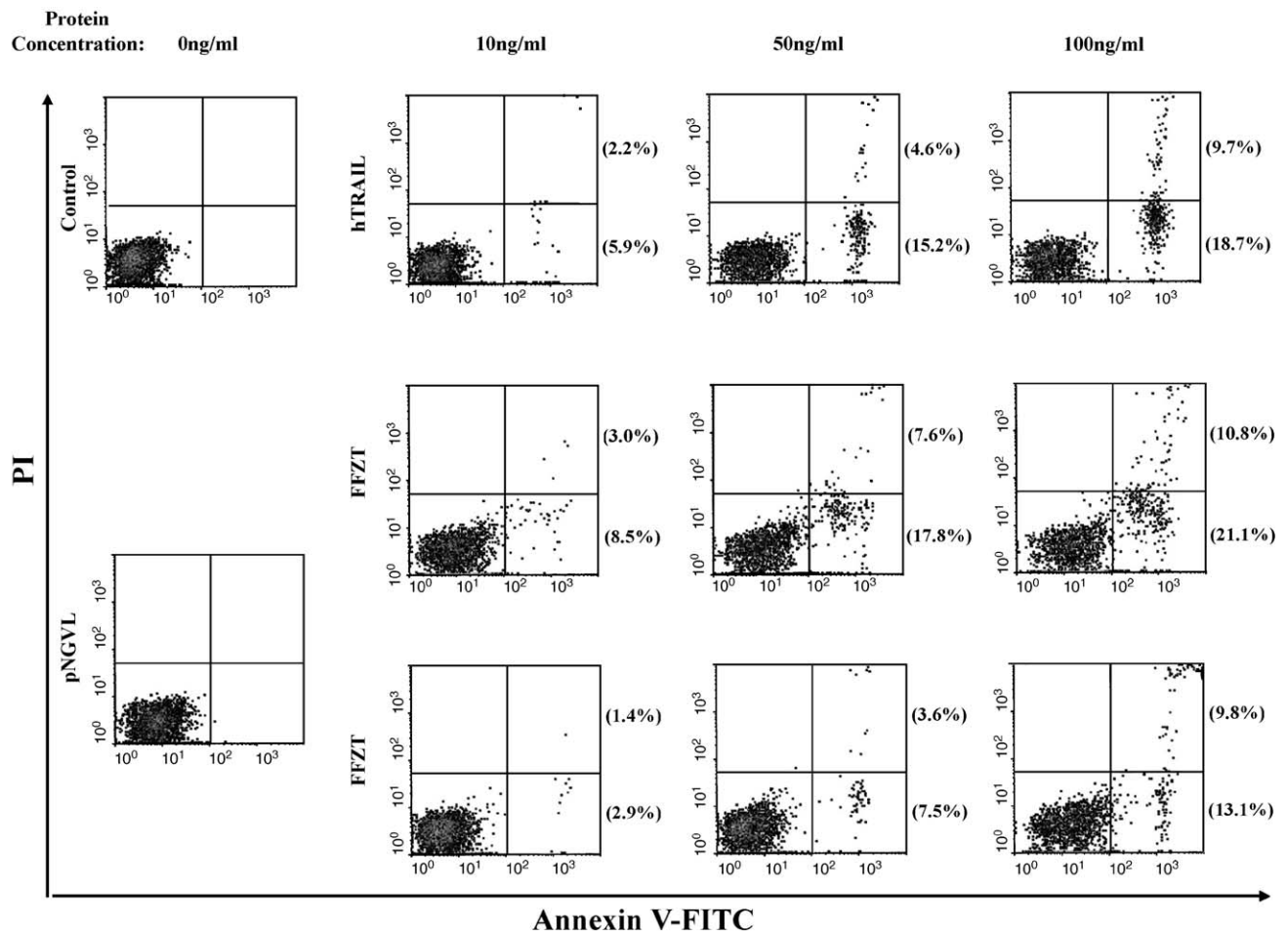


FIG. 3. Ability of cleaved TRAIL to induce the killing of Jurkat cells. Jurkat cells were incubated for 6 h with sera of mice injected with *pNGVL*, *pFFZT*, *pFETZ*, or human recombinant protein at the indicated concentrations. Cells were stained with annexin V and propidium iodide (PI) (Apoptosis Detection Kit II; BD PharMingen, San Diego, CA, USA) for 15 min and analyzed by flow cytometry.

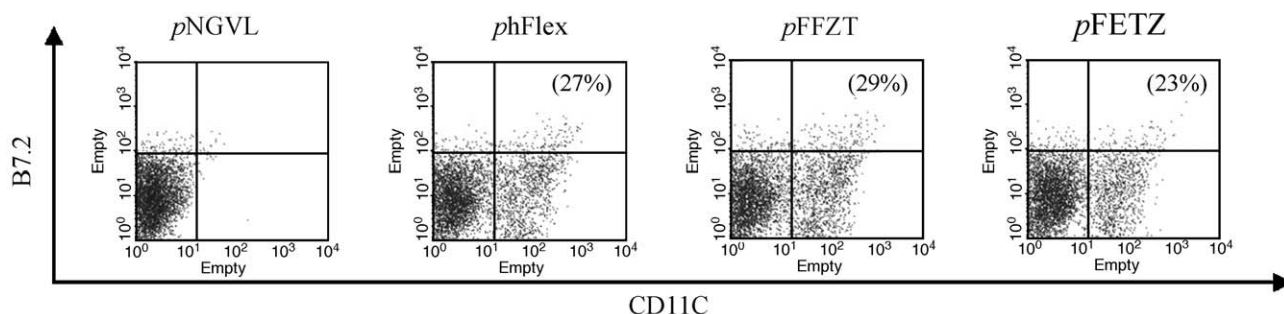


FIG. 4. DC population in the spleen after double injection of *pNGVL*, *phFlex*, *pFFZT*, and *pFETZ* genes at days 1 and 5. Spleen cells were harvested on day 11 and stained with FITC-conjugated anti-CD11c and PE-conjugated anti-B7.2 Abs (BD PharMingen). The stained cells were analyzed by flow cytometry. The data are representative of three mice in two independent experiments.

them for our subsequent cytolytic studies. We incubated Jurkat cells for 6 h with sera collected from recipient BALB/c mice 24 h following the injection of *pNGVL*, *pFETZ*, and *pFFZT*, as well as the human TRAIL recombinant protein (Biomol, PA, USA). We diluted the various sera in RPMI medium at different concentrations of 10 to 100 ng/ml. Following incubation, we stained the Jurkat cells with annexin V and propidium iodide (PI) and analyzed them by flow cytometry. As shown in Fig. 3, *pFFZT* induced 21% of the treated Jurkat cells to undergo apoptosis (annexin V<sup>+</sup>). This is in comparison to the 13% annexin V<sup>+</sup> obtained with *pFETZ*. The observed apoptosis of Jurkat cells induced by the various sera was dose-dependent (Fig. 3). It is apparent that the apoptotic activities of the cleaved form of TRAIL, induced by *pFFZT*, were enhanced in comparison to its chimeric counterpart. Moreover, we observed that the anti-tumor apoptosis induced by FFZT was slightly better than that of the recombinant TRAIL protein (Fig. 3).

Next, we compared the ability of the sera of recipient mice collected 24 h following injection of *pNGVL*, *phFlex*, *pFETZ*, or *pFFZT* to stimulate the expansion of DC. We gave BALB/c mice two injections of these various plasmid DNAs separately on days 1 and 5, respectively. The level of DC at day 11, determined previously to be the peak for DC expansion following systemic delivery of *phFlex*, in the spleen of *pFFZT*-injected mice increased significantly in size and cellularity [11,16,22]. When analyzed by flow cytometry, the levels of CD11c<sup>+</sup>B7.2<sup>+</sup> cells obtained with *pFFZT* were comparable to those following injection of *phFlex* or *pFETZ* plasmid DNA. There was about 30-fold enhancement of the DC expansion in spleens of mice receiving the *pFFZT* plasmid DNA compared with the control vector *pNGVL*-treated mice (Fig. 4). These data are consistent with the interpretation that hFlex, following cleavage by furin, could maintain its ability to stimulate the expansion of DC progenitor cells *in vivo*.

To determine whether the enhanced *pFFZT*-tumoricidal activity could be translated into the efficient rejection of tumors *in vivo*, we established the SCID mouse tumor xenograft model using the TRAIL-sensitive human CNE-2 nasopharyngeal carcinoma cells for our studies. We inoculated SCID mice subcutaneously with the human nasopharyngeal carcinoma cells. When the tumor nodules reached a size of between 4 and 5 mm, we injected tumor-bearing mice intratumorally with the *pFFZT*, *pFETZ*, or *pNGVL* gene complexed with the cationic liposome NCC-4 [23]. As demonstrated in Fig. 5, tumor grew

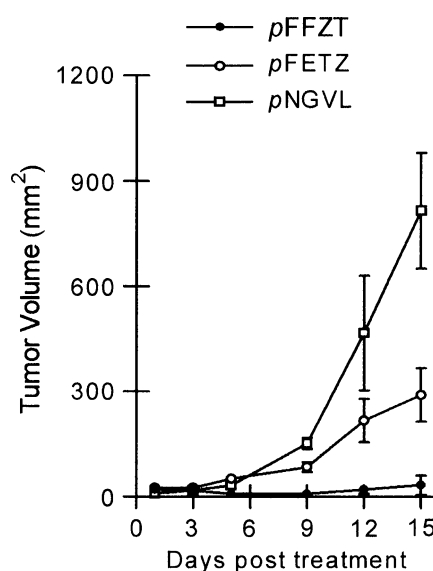
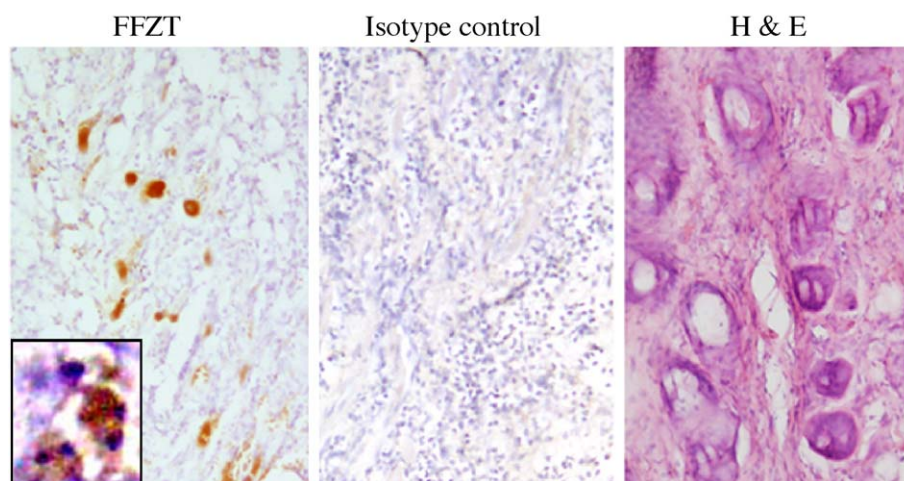


FIG. 5. Inhibition of the growth of CNE-2 tumor in SCID mice mediated by *pFFZT* or *pFETZ*. Mice were inoculated sc with CNE-2 cells ( $2 \times 10^6$  cells/mouse). When the tumor nodules reached a size of between 4 and 5 mm, they were intratumorally injected with 10  $\mu$ g *pFFZT* or *pFETZ* DNA complexed with the cationic liposomes NCC-4 in 20  $\mu$ l Hepes buffer. Mice in the control group were injected with 10  $\mu$ g *pNGVL* DNA complexed with the NCC-4 cationic liposomes.



**FIG. 6.** Detection of TRAIL-induced apoptosis with immunohistochemical stains. Cationic liposomes complexed with *p*FFZT were injected directly into tumor nodules of mice induced by sc injection of human CNE-2 cells. Forty-eight hours following injection of the DNA/liposome complexes, tumor sections of the injected nodules were prepared and FFZT expression was detected by rabbit anti-TRAIL polyclonal AB (original magnification  $\times 100$ ; inset  $\times 400$ ). Anti-rabbit Ig G was employed as the isotype control ( $\times 100$ ). H&E staining was also performed on the section ( $\times 100$ ).

steadily up to day 15 in the group of tumor-bearing mice treated with the *p*NGVL control vector. This was similar to the group of untreated tumor-bearing mice (data not shown). Tumor-bearing mice treated with the *p*FETZ/liposome complexes gave a moderate but noticeable suppression of tumor growth (Fig. 5). The growth of tumor in mice treated with *p*FFZT was significantly suppressed, with no detectable tumor in 2 of the 10 treated mice by day 9. In addition, the sizes of the tumors in the 8 mice treated with *p*FFZT also regressed and were much smaller than the tumors of mice in the *p*FETZ-treated group. Thus, *p*FFZT demonstrated a much more potent anti-tumor effect in comparison to *p*FETZ with this animal model.

When we performed immunohistochemistry staining using anti-TRAIL Ab on tumor sections from mice treated with cationic liposome *p*FFZT DNA complexes, TRAIL expression could be demonstrated in tumor sections obtained 48 h following intratumoral injection of the cationic liposome *p*FFZT DNA complexes (Fig. 6). TRAIL expression in *p*FFZT-treated tumor sections showed strong staining. Moreover, the tumor sections studied displayed many cells with condensed nuclei, an observation that is consistent with cells undergoing apoptotic condensed nuclei (Fig. 6 inset).

## DISCUSSION

To explore the potential of using TRAIL for cancer therapy, various recombinant versions of TRAIL have been tested to direct the specific toxicity of TRAIL to the malignant cells without damaging normal cells. A polyhistidine-tagged recombinant version of human TRAIL (residues 114–281) was found to induce apopto-

sis *in vitro* in isolated human, but not in nonhuman hepatocytes [8]. A second variant contains amino acid residues 95–281 of TRAIL fused to a “modified” yeast Gal4 leucine zipper to promote the trimerization of the ligand [5]. It was noted that normal human keratinocytes (KCs) were also sensitive to this fusion protein preparation [24]. A third version contains residues 95–281 of TRAIL fused to an amino-terminal “Flag” epitope tag. Crosslinking of this tagged protein with anti-Flag antibodies enhances its activity against certain cell lines, such as Jurkat, but again is toxic to the normal human primary culture [25]. These various versions of recombinant TRAIL proteins were also shown to induce apoptosis in normal human brain cells, including neurons, oligodendrocytes, astrocytes, and microglial cells [26]. A fourth recombinant version has been reported, consisting of residues 114–281 of TRAIL, which lacks exogenous sequence tag [10]. This nontagged recombinant TRAIL, with the addition of zinc and reducing agent to optimize for the formation of stable, soluble, homotrimer purified protein at neutral pH, could induce the apoptosis of cancer cells with minimal toxicity for hepatocytes [10]. Moreover, this recombinant TRAIL did not trigger the apoptosis of more than 5% of normal human KCs or melanocytes and yet could effectively induce the apoptosis of transformed cells [27]. This recombinant TRAIL appears to be the least likely to be immunogenic in human patients and is probably the most preferred recombinant TRAIL version for clinical application. An attractive alternative to deliver recombinant therapeutic proteins is to introduce the gene that encodes the specific ligand, resulting in the continuous production of large amounts of thera-

peutic protein for a prolong period of time [11]. This could couple with targeted gene delivery to direct gene expression locally to the tumor.

The recombinant TRAIL construct reported in the present study allows the trimerization of the cleaved TRAIL in the presence of the isoleucine zipper (Fig. 2D). This isoleucine zipper motif exhibits a high degree of specificity to form trimers as engineered in the *pFFZT* construct, and the trimerization of the cleaved TRAIL product enhanced the tumoricidal activities. In comparison, constructs without the isoleucine zipper motif yielded the monomeric form of TRAIL (Fig. 2D). It is likely that the homotrimer TRAIL could reduce the multimerization of death receptors and therefore be unable to deliver a signal that could surpass the threshold necessary for the activity of apoptosis in the normal cells. The added advantage of expressing a biologically active form of TRAIL without fusion to heterologous sequences is that the product would be less immunogenic and would therefore be a more suitable for multiple dosing.

We have adopted a strategy to express multiple gene products encoded in a single cistron that is subsequently cleaved and processed into the various active components [19]. The extracellular domain of the human Flt3L gene and the DNA encoding amino acid residues 95–181 of the human TRAIL gene were linked by the furin recognition peptide sequence to achieve the objective of generating multiple gene products at the tumor site. Furin, a ubiquitous endoprotease localized within the Golgi apparatus, is highly conserved among eukaryotic cells. Our present results demonstrated furin-mediated cleavage of the recombinant Flt3L/TRAIL translational product into two biologically functional gene products (Fig. 2C). The anti-tumor cytolytic activities of the recombinant TRAIL generated from the hFlex/furin/TRAIL construct were very potent and could reverse the growth of tumor xenografts in SCID mice following intratumoral delivery of the construct (Fig. 5). The potential toxicity of TRAIL could be further reduced by the incorporation of cell-type-specific promoters or peptide recognition sequence in addition to the ubiquitous furin sequence employed in the present study. With these cell-type-specific promoters or peptide recognition sequence, it is anticipated that the translational products encoded by the construct will be preferentially cleaved only by enzymes specifically present in cancer cells. Our present report provides a new approach to engineering recombinant TRAIL in the homotrimeric form that could offer a potential benefit for a selected group of patients whose tumors are highly responsive to TRAIL.

## MATERIALS AND METHODS

**Cell lines.** Human TRAIL-sensitive human tumor cell lines MDA-231 (mammary adenocarcinoma), HeLa (cervical cancer), HepG2 and Hep3B

(hepatocellular carcinoma), U87 (glioblastoma), Jurkat (T lymphoma), and A549 (lung adenocarcinoma) were obtained from American Type Culture Collection (Manassas, VA, USA) and grown in DMEM or RPMI medium supplemented with 10% fetal bovine serum (FBS), glutamine, and nonessential amino acids. The cell lines CNE-2, CNE-1, and HK-1 (nasopharyngeal carcinomas) were cultured in RPMI containing 10% FBS, 100  $\mu$ l/ml streptomycin and penicillin, and 2 mM glutamine as previously described [28].

**Animals.** Female BALB/c mice were purchased from the animal center of the National University of Singapore (Singapore). CB17 SCID mice were purchased from Charles River Laboratories (Wilmington, MA, USA). Mice were age-matched at 6–8 weeks at the onset of each experiment. All of mice were housed in the pathogen-free animal facility of the Singapore National Cancer Center according to the guidelines published by the NIH.

**Construction of recombinant hFlex/TRAIL plasmids with the insertion of the furin recognition sequence.** The published plasmids denoted *pFETZ* and *pFET* [11] were digested with *NheI* enzyme and a 30-mer oligo encoding the DNA sequence of 10 amino acids (Gly-Arg-Arg-Ala-Arg-Try-Lys-Arg-Gly-Gly) of the furin recognition sequence was synthesized and cloned in-frame into the *NheI* site between the DNA sequences encoding hFlex and TRAIL. The presence of open reading frames in the various DNA constructs was confirmed by DNA sequencing analysis.

**Transfection.** For *in vitro* transfections, 3  $\mu$ g of the recombinant plasmid DNA was mixed with 8  $\mu$ l of Lipofectamine (Gibco BRL, Gaithersburg, MD, USA). COS7 cells ( $5 \times 10^5$  cells/well) or CNE-2 cells were plated in six-well plates 24 h prior to transfection. COS7 cells were transfected with DNA/Lipofectamine complexes. CNE-2 cells were transfected with 3  $\mu$ g DNA complexed with 20  $\mu$ l cationic liposomes NCC-4 in 1 ml of Hepes buffer for 4 h. Transfection was further carried out in OptiMEM (Gibco BRL) for 20 h before proceeding to the crystal violet assay. For *in vivo* transfections 10  $\mu$ g of the recombinant DNA in 1.7 ml of 0.9% NaCl was injected into each mouse (16–18 g) via the tail vein with a 271/2-gauge needle with a short interval (6–8 s).

**Western blot.** Cells were lysed in buffer (50 mM Tris, pH 8.0, 150 mM NaCl, 0.02% Na azide, 0.1% SDS, 1% NP-40, 0.5% sodium deoxycholate, 10  $\mu$ g/ml leupeptin, and 2  $\mu$ g/ml aprotinin) on ice for 30 min. The cell supernatant collected from transfected COS7 cells and mouse serum collected from BALB/c mice injected with different genes were centrifuged for 10 min at 14,000g. The samples were diluted in 5 $\times$  SDS sample buffer, heated for 5 min, and subjected to 10% SDS-PAGE using standard procedures. For processing in the 6% native PAGE, 1  $\mu$ l serum was diluted in sample buffer containing no SDS and loaded into the gel. Electrophoresis was performed at 4°C for 3, 4, and 5 h in the same blot. The proteins were electrophoretically transferred from the gel onto a nitrocellulose membrane. The blocking of nonspecific protein binding was achieved by preincubating the membrane at 4°C overnight with buffer containing 5% milk (5% nonfat dry milk, 0.5% Tween 20, 50 mM Tris, 150 mM NaCl). The membrane was then incubated with 5  $\mu$ g/ml rabbit anti-TRAIL polyclonal antibody or goat anti-Flt3L polyclonal antibody (Santa Cruz Biotechnology) for 1.5 h at room temperature. Following extensive washing with washing buffer, the membrane was incubated with HRP-conjugated goat anti-rabbit Ig G (1  $\mu$ g/ml) or donkey anti-goat Ig G (Santa Cruz Biotechnology) for 45 min at room temperature. The membrane was washed extensively and incubated in the substrate solution (Santa Cruz Biotechnology) for 1 min and then autoradiographed for 30 s.

**Quantification of the cleavage of the fusion protein by ELISA.** One microliter of mouse serum was diluted 50- to 250,000-fold for the ELISA using the Flt3L ELISA kit (R&D Systems, Minneapolis, MN, USA), following the manufacturer's instructions. The same dilution sample was also

measured using TRAIL ELISA prepared by coating 96-well plates with anti-TRAIL polyclonal antibody overnight following the standard procedure. The recombinant TRAIL protein (R&D Systems) was used as a standard control to assay the concentration of TRAIL.

**Comparison of the cytotoxicity of the cleaved form of TRAIL and its fusion protein on human cancer cell lines.** Different cells were plated at  $2.5 \times 10^4$  cells per well in 96-well plates and allowed to adhere overnight. Cells were incubated for 6 h with sera obtained either from pFFZT- or from pFETZ-injected mice. The concentration of TRAIL was determined by ELISA with anti-TRAIL Ab and diluted with RPMI or DMEM to 1  $\mu$ g/ml. The viability of cells was assessed using the crystal violet assay.

**Flow cytometry.** Jurkat cells were used for studying the apoptosis profile when incubated for 6 h with sera of mice injected with pNGVL, pFFZT, pFETZ, or recombinant human TRAIL protein (Biomol) at the indicated concentrations. Cells were stained with annexin V and PI (Apoptosis Detection Kit II, BD PharMingen) for 15 min and analyzed by FACScan (Becton–Dickinson). Jurkat cells were treated with RPMI or sera collected from pNGVL-treated mice as the control.

The DC expansion by cleaved FFZT and FETZ was analyzed using FACScan as described [11].

**Inhibition of growth of human CNE-2 tumor in SCID mice mediated by pFFZT or pFETZ.** Female SCID mice were subcutaneously inoculated with  $10^6$  CNE-2 cells in 50  $\mu$ l Hanks' solution (Gibco BRL). One week after inoculation of tumor cells, mice were injected with 10  $\mu$ g pFFZT or pFETZ DNA complexed with the cationic liposomes NCC-4 in 20  $\mu$ l Hepes buffer into the tumor site. Mice in the control group were injected with 10  $\mu$ g pNGVL plasmid DNA complexed with the NCC-4 cationic liposomes. The tumor growth rates were determined by plotting the tumor size vs time after tumor inoculation. Tumor size represents the product of two perpendicular diameters.

**Detection of TRAIL expression in tumor site by immunohistochemistry staining.** Cationic liposomes complexed with the pFFZT plasmid DNA (10  $\mu$ g) were injected directly into tumor nodules of mice induced by sc injection of human CNE-2 cells. Forty-eight hours following injection of the DNA/liposome complexes, tumor sections of the injected nodules were prepared and TRAIL-specific expression was determined using anti-TRAIL Ab. Anti-rabbit IgG was employed as the isotype control.

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