Tumor-targeted delivery of biologically active TRAIL protein

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The tumor necrosis factor (TNF)-related apoptosis-inducing ligand (TRAIL) is a potent inducer of tumor cell apoptosis, but concerns of considerable liver toxicity limit its uses in human cancer therapy. Here, we show that i.v. injected Escherichia coli DH5α (E. coli DH5α) specifically replicates in solid tumors and metastases in live animals. E. coli DH5α does not enter tumor cells and suits for being the vector for soluble TRAIL (sTRAIL), which induces apoptosis by activating cell-surface death receptors. With the high ‘tumor-targeting’ nature, we demonstrate that intratumoral (i.t.) and intravenous injection of sTRAIL-expressing E. coli DH5α results in the tumor-targeted release of biologically active molecules, which leads to a dramatic reduction in the tumor growth rate and the prolonged survival of tumor-bearing mice. TRAIL delivery by E. coli DH5α did not cause any detectable toxicity to any organs, suggesting that E. coli DH5α-delivered sTRAIL protein therapy may provide a feasible and effective form of treatment for solid tumors.

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Introduction

The tumor necrosis factor (TNF)-related apoptosis-inducing ligand (TRAIL) is a type-II transmembrane protein that was initially identified based on homology of the extracellular domain to CD95 L, TNF and LTα.1,2 The membrane-bound and the soluble extracellular domains of TRAIL (amino acids 95–281 or 114–281) induce apoptosis in a wide variety of tumor cell lines. Approximately two-thirds of tumor cell lines tested are sensitive to the cytotoxic effects of TRAIL in vitro.3,4 suggesting that TRAIL may be exploited as a powerful antitumor agent. Repeated intravenous administration of recombinant, biologically active TRAIL (rTRAIL) can induce apoptosis in a wide variety of tumor cell lines and metastases in live animals.16,17 It has been known for more than 60 years that anaerobic bacteria can selectively grow in tumors.18–21 Several approaches to developing anaerobic bacteria for tumor therapies have been described. The anaerobic species Bifidobacterium longum and Clostridium novyi could selectively grow in the hypoxic environment of large solid tumors. Recently, Salmonella typhi-murium with attenuated lipid-A was evaluated in a phase-I clinical trial.26,27 To overcome toxicity, Zhao et al.28–30 mutagenized S. typhi-murium and selected an amino-acid auxotrophic strain, which selectively grew in and killed tumor cells. Non-pathogenic Escherichia coli is a facultative anaerobic bacterium that naturally resides in the digestive tracts of humans and other animals. There have been no reports to date describing the use of E. coli as an anticancer protein-delivery agent to repress tumor growth in vivo.31,32

Gene therapy may enable continuous production of TRAIL in substantial amounts for a relatively long period of time. This strategy would overcome the necessity for repeated injections of recombinant TRAIL and may also result in increased antitumor effect. The transfer and expression of TRAIL into cells by adenovirus or adenovirus-associated virus induces apoptosis and apoptotic bystander effects in several human cancer cell lines in vitro and in xenograft models of human tumors.13–15 However, adenovirus or adenovirus-associated virus-based vectors may cause hepatotoxicity through innate and cell-mediated immune responses, and have less targeting to the tumor site when systemically administrated.16,17

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Minimizing side effects while maximizing tumor targeting is the greatest challenge of current tumor therapeutic protocols. Here, we show that intravenously (i.v.) injected E. coli specifically replicates in solid tumors and metastases in live animals. With this high ‘tumor-targeting’ nature, we describe a novel treatment of
injecting TRAIL-expressing *E. coli* so that it can grow loco-regionally inside tumors and release the biologically active soluble TRAIL (sTRAIL) protein at relatively high levels, thereby achieving maximal therapeutic effects while sparing potential systemic side effects.

### Materials and methods

**Cell lines and cell culture**

Murine B16-F10-luc^+^ melanoma cells and human NCI-H460 lung tumor cells were obtained from Xenogen (Hopkinton, MA, USA) and ATCC (Rockville, MD, USA), respectively. B16-luc^+^ were grown in Dulbecco’s modified Eagle’s medium (Invitrogen, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum, 1% non-essential amino acids, L-glutamine, and sodium pyruvate (all from Hyclone, Ogden, UT, USA); MEM vitamin solution (Invitrogen); and 1% penicillin and streptomycin. NCI-H460 cells were grown in RPMI-1640 medium supplemented with 10% fetal bovine serum, and 1% penicillin and streptomycin. All the cells were cultured at 37°C in a humidified atmosphere of 5% CO₂. H460 cells were transfected with pcDNA3.1-Luc^+^ vector containing firefly luciferase using Lipofectamine 2000 (Invitrogen). Stable clones expressing luc were isolated in the presence of 300 μg ml⁻¹ genitcin (G418) and the clone with the highest level of luc expression (as determined by bioluminescence) was selected by using D-luciferin and an In Vivo Imaging System (IVIS) (Xenogen).

**Bacterial strains and plasmids**

*E. coli* DH5α were routinely grown in LB medium and cultured in a shaker at 37°C. The plasmid pMK *lux*ABCDE, also called pXen-1 (Xenogen), which contains bacterial luciferase, was electroporated into DH5α and positive transformants were screened with an IVIS. The clone with the highest bioluminescence was grown overnight at 37°C in LB medium containing 100 μg ml⁻¹ ampicillin. The bioluminescent clones were stored at −80°C and thawed before use. Bacterial concentration was estimated spectrophotometrically by determining absorbance at 600 nm, and cell numbers were verified by plating dilutions of inoculum onto LB agar plate (1 OD ≈ 8 (10^8^ c.f.u. ml⁻¹)^⁻¹^).

A sense primer (5’-GGGATCCGACCTCAGAGGAACCATTTC-3’) and antisense primer (5’-CCGCGTCGAGTTAGCAGTAAAAGGC-3’) were used to amplify a 561-bp DNA fragment representing the sTRAIL from mammary library by PCR. The products were gel-purified, digested with *BamH*I and *Xho*I restriction enzymes, repurified and inserted into the *BamH*I and *Xho*I sites of the prokaryotic expression vector pGEX-KG (Amersham Biosciences, Piscataway, NJ). The resulting vector pGEX-sTRAIL was electroporated into *E. coli* DH5α and the resulting clone was termed as *E. coli* DH5α (sTRAIL). Protein expression was induced by 0.1 mM isopropyl-β-thiogalacto pyranoside in culture and protein production was analyzed by Coomassie brilliant blue (CBB) staining.

**Recipient animals and tumor models**

Female BALB/c and nude mice were housed under aseptic conditions in micro-isolator cages. The animals used in the studies were approximately 4–6 weeks of age and weight ranged between 20 ± 5g and 18 ± 2g. All studies involving mice were approved by the institute’s Animal Care and Use Committee.

B16-luc^+^ or H460-luc^+^ tumor cells (1 × 10⁶ or 1 × 10⁷, respectively) were injected subcutaneously into the dorsal flanks of the BALB/c and nude mice and tumor growth was monitored twice a week by *in vivo* imaging or external caliper measurement. Mice bearing subcutaneous melanoma tumors and subcutaneous lung tumors were grown about 7–10 days until the tumor size was approximately 200 mm². A total of 5 × 10⁵ B16 cells were injected into nude mice through the tail vein and extensive lung metastases occurred within 2 weeks after cellular implantation.

**Bioluminescence imaging**

B16-F10 melanoma and H460 lung carcinoma xenografts were established as above. Luminescent bacteria were grown and harvested at late logarithmic phase, washed and diluted with sterile normal saline and injected via the tail vein into tumor-bearing or non-tumor-bearing mice at various doses ranging from 1 × 10⁶ to 1 × 10¹⁰ c.f.u. per mouse. To analyze bacterial luciferase activity and trace bioluminescent bacteria, luminescence was quantified from the *in vivo* signals emitted from the dorsal or ventral views of each mouse prior to killing and from *ex vivo* images taken of excised tissue immediately after being killed. Total photon emission from different parts within the images of each mouse was quantified at different time points using the Living Image software (Xenogen).

For recording tumor growth by *in vivo* imaging, the animals were injected intraperitoneally with D-luciferin at 150 mg kg⁻¹ in Dulbecco’s phosphate buffered saline and anesthetized with 1–3% isoflurane. The mice were then placed on a warmed stage inside a light-tight camera box with continuous exposure to 1–3% isoflurane. Imaging time ranged from 1 s to 3 min, depending on the tumor model and time points. Low levels of light emitted from the bioluminescent tumors were detected by an IVIS, and were integrated, digitized and quantified as photons/second using the Living Image software.

**Cytotoxicity assay**

H460 cells were seeded in 24-well plates at a density of 1 × 10⁵ cells per well in 500μl of complete culture medium. After 12–18 h of adherence, the cells were treated with *E. coli* DH5α (sTRAIL) and *E. coli* DH5α (empty vector) at various multiplicities of infection (MOIs) (50:1, 100:1, 200:1 and 400:1) in fresh culture medium containing 5% penicillin and streptomycin to inhibit bacterial growth. Cell morphology was observed by light microscopy after 12 h of incubation. For blocking assay, H460 cells were incubated with 0 to 200 ng of death receptor4 (DR4):Fc or TNF receptor:Fc (both from Alexis Biochemicals, San Diego, CA, USA) per milliliter in addition to *E. coli* DH5α (sTRAIL) or *E. coli* DH5α.
(empty vector) at an MOI of 100:1 in the same setting, and the results were documented as photographs. Unless otherwise specified, E. coli DH5α (empty vector) was used as vector control and the cell culture medium as mock control.

Cells plated in 12-well dishes were infected with E. coli DH5α (sTRAIL) at different MOIs and stained with crystal violet. The medium was removed 12 h after co-incubation and the cells were fixed for 3 min in 4% paraformaldehyde at room temperature prior to crystal violet staining. Fixed cells were washed with phosphate-buffered saline and incubated for 3 min in 1% crystal violet in 70% ethanol. Cells were rinsed three times with water, air-dried and photographed. Cells in a duplicate plate were trypsinized and stained with trypan blue and the percentage of viable cells was counted by light microscopy.

Flow cytometry
H460 cells were infected with E. coli DH5α (sTRAIL) at an MOI of 200 for 2, 4 or 8 h. The cells were harvested by trypsinization, followed by washing with cold phosphate-buffered saline containing 10% fetal bovine serum and fixation in 70% ethanol. Cells were then stained with 10 μg ml⁻¹ propidium iodide and analyzed using a FACScan instrument.

Bacterial infection
In order to study bacterial infection on non-tumor bearing or tumor-bearing mice in vivo, tissue samples were obtained from liver, spleen and tumors after injection as described above. Normal tissues and tumors were excised, weighed and c.f.u. were determined at different time points after homogenizing and plating supernatants on LB agar plates.

For intratumoral (i.t.) injection, E. coli DH5α (sTRAIL) induced by IPTG were harvested, washed and diluted with sterile normal saline. Bacteria were injected directly into the central area of the tumors at a dose of 1 × 10⁸ c.f.u. per 50 μl of sterile normal saline on the first two successive days (total dose of 2 × 10⁹ c.f.u.). For intravenous injection, a total of 5 × 10⁷ c.f.u. of E. coli DH5α (sTRAIL) were injected into the tail vein of tumor-bearing nude mice. One week later, the second injection was performed as described above. Unless otherwise specified, E. coli DH5α (empty vector) was used as a vector control, and sterile normal saline was used as a mock control.

PCR assay and tissue immunohistochemistry
Established H460 tumors were allowed to grow until they were approximately 200 mm³ in size. A total of 5 × 10⁷ c.f.u. per mouse of E. coli DH5α (sTRAIL) was injected i.v. through the tail vein into H460 tumor-bearing nude mice. Four days after injection, animals were killed and tumors, spleens, livers and kidneys were aseptically removed, homogenized, diluted and plated onto LB agar plates supplemented with 50 μg ml⁻¹ penicillin to determine the c.f.u. of E. coli DH5α (sTRAIL). Several clones were randomly picked and PCR was performed using the corresponding sTRAIL primers. At the same time, normal tissues and tumors from nude mice were fixed, paraffin-embedded and sectioned. Tissue immunohistochemistry were performed with an anti-hTRAIL monoclonal antibody (clone 2H12) purchased from R&D Systems (San Francisco, CA) using standard methods.

Western blot analysis
H460 tumor cells were lysed in buffer (25 mM Tris–Cl (pH 7.4), 50 mM NaCl, 0.5% Na-deoxycholate, 2% Nonidet P-40, 0.2% SDS and 10% protease inhibitor cocktail) and cell lysates were subjected to electrophoresis on 15% SDS–PAGE gels and probed with rabbit anti-hDR4/DR5 polyclonal antibody (Santa Cruz Biotechnology, Inc., Santa Cruz, CA).

Tumor size recording and animal survival rate
Mice were imaged along the dorsal view twice a week from day 0 to day 20 after bacteria injection using an IVIS. Bioluminescence was recorded as photons/second. Tumor volume (mm³) was measured by external calipers twice weekly and calculated using the formula V = 0.5LW², where L and W represent length and width of the tumor in millimeters, respectively. All mice were monitored for survival as previously described.

Pathological section and H&E staining
The tumors were removed from nude mice on day 3 after bacteria injection. The tissue was fixed with 10% buffered formalin and processed for paraffin sectioning and hematoxylin–eosin (H&E) staining using standard methods.

Toxicity of sTRAIL in vivo
The toxicity of sTRAIL treatment delivered by E. coli DH5α was examined following intravenous injection of 5 × 10⁷ c.f.u. of E. coli DH5α (sTRAIL) to tumor-bearing mice. Seven days later, a second injection was performed as described above. At 4 and 30 days after infection, serum from all mice was collected and aminotransferase/aspartate aminotransferase/aspartate aminotransferase was analyzed as indicator of liver injury using standard method. Livers, spleens, kidneys and hearts were harvested on day 30 after bacteria injection and fixed in 10% buffered formalin. The tissues were sectioned, stained with H&E and histopathological changes in organs were examined.

Statistical analysis
The in vivo tumor growth data were analyzed by analysis of variance using the Statistica software (StatSoft, Tulsa, OK). The survival rates were analyzed by log-rank test using Statistica. P ≤ 0.05 was considered significant.

Results
E. coli DH5α specifically resides in tumors
A total of 1 × 10⁸ c.f.u. of luminescent E. coli DH5α were i.v. injected into tumor-bearing BALB/c or nude mice using standard methods.
Photon collection (3 min) using the IVIS at 18 h after bacterial injection showed that detectable light emission occurred only at the tumor site, indicating presence of luminescent *E. coli* (Figure 1a). Luminescent *E. coli* DH5α are also present in B16-F10 lung metastases in nude mice (Figure 1a). We performed a dose escalation test of *E. coli* DH5α and found that a dose of $1 \times 10^8$ or $5 \times 10^7$ c.f.u. in BALB/c mice or nude mice, respectively, was optimal for observing bacterial distribution without bacteremia. Unless otherwise specified, these doses were used in all subsequent animal experiments.

To follow the fate of bacteria injected i.v. into the non-tumor-bearing animals, we monitored each animal with the IVIS at different time intervals as indicated in Figure 1b ($n > 10$). Injection of *E. coli* DH5α immediately resulted in light emission localized mainly in the lungs, followed by accumulation of bioluminescent bacteria in the liver, which was present for several hours. Imaging of the same animals at 24 h post-injection revealed that detectable light emission from the earlier time points diminished (Figure 1b). These findings, together with complete absence of bacteria in the blood (data not shown), indicate that the light-emitting bacteria were probably eliminated by the host’s immune system. Bacterial clearance was independently confirmed by absence of light emission in all of the excised organs of the same animal (data not shown).

To further determine the spatial and temporal progression of *E. coli* DH5α infection in animals with implanted tumors, luminescent *E. coli* DH5α were i.v. injected into BALB/c mice ($n > 10$) with melanoma (~200 mm$^3$) that had been growing in the right hind leg. The animals were monitored each day for 15 days with a low-light imager. Bioluminescence distribution patterns determined immediately after injection were similar to distribution patterns in non-tumor-bearing animals. However, at day 1 after injection, no luminescence outside of the tumor region was detected. Continued monitoring of the mice showed that after an initial increase, luminescence decreased until day 4, and then dramatically increased in the tumors (Figure 1b), indicating efficient replication of the bacteria. Furthermore, i.v. injected luminescent *E. coli* DH5α also replicated in the human lung xenografts on nude mice ($n > 10$; Figure 1b). At 30 min and 4 days post-infection, five of the tumor-bearing animals were killed. Luminescence in the excised liver and lung tissue was detected at the 30-min time point as indicated in Figure 1c. In contrast, detectable light emission only existed within the tumor site at day 4 post-infection, indicating that distribution of *E. coli* DH5α into tumors via the bloodstream did not result in significant infection of healthy organs (Figure 1c). The results of the bacterial count using homogenized samples at day 4 post-infection indicate that *E. coli* DH5α preferentially accumulates in tumors rather than in the spleens and livers at a ratio of >10000:1. The numbers of bacteria detected in tumors was maintained as long as 18 days, while those in spleens and livers gradually disappeared (Figure 1d).

**Figure 1** *E. coli* DH5α specifically resides in tumors. (a) Bacteria in tumors can be detected as early as 18 h after injection of $1 \times 10^8$ c.f.u. *E. coli* DH5α through the tail vein (left, B16-F10 murine melanoma in BALB/c mice; middle, H460 human lung carcinoma in nude mice; right, B16-F10 murine lung metastases in nude mice). (b) Tissue distribution of *E. coli* DH5α in immunocompetent BALB/c mice and immunocompromised nude mice using IVIS at different time points. Bars: photons/second (c) Various organs and tumors were removed from the mice and imaged using the IVIS without any processing at 30 min and day 4 after bacteria injection. (d) At different time points after infection, mice were killed. The livers, spleens and tumors were excised, homogenized and assayed for bacterial titers. ND, not detectable. Each value represents mean ± s.d. from 10 mice. Photon collection was for 3 min at different time points after bacteria injection using the Xenogen IVIS 50 imaging system. C.f.u., colony-forming units; IVIS, *In Vitro* Imaging System.
We then constructed a strain of sTRAIL-expressing *E. coli* DH5α, which could specifically target sTRAIL proteins into tumors. To determine whether sTRAIL-expressing *E. coli* DH5α had cytotoxic effects on human tumor cell lines in vitro, H460 lung tumor cells were incubated with *E. coli* DH5α (empty vector) and sTRAIL-expressing *E. coli* DH5α at various MOIs for 12 h. The cells were visualized by light microscopy for signs of apoptosis. Cells incubated with *E. coli* DH5α (empty vector) showed slight toxic effects, whereas cells incubated with sTRAIL-expressing *E. coli* DH5α underwent rapid and massive apoptosis (Figure 2a). A fusion protein having DR4 extracellular domain fused to the immunoglobulin Fc region (DR4: Fc) was added to the cell cultures which reduced, in a concentration-dependent manner, the amount of apoptotic cells and blocked morphological changes associated with apoptosis. As a negative control, 200 ng of TNF receptor:Fc per ml, which binds TNF but not TRAIL, was added to the cells and failed to block DH5α (sTRAIL)-induced apoptosis (Supplementary Figure S1).

Further analyses demonstrated that the sTRAIL expressed by *E. coli* DH5α induces apoptosis in a dose-dependent fashion (Figures 2b and c). Apoptosis was characterized by FACS analysis in Figure 2d. The sub-G1 cell population increased in the *E. coli* DH5α (sTRAIL)-treated group in a time-dependent manner. These results indicate that *E. coli* DH5α-expressed sTRAIL effectively induces tumor cells apoptosis.

**Antitumor activity of sTRAIL-expressing E. coli DH5α in vivo**

These in vitro results encouraged us to evaluate whether tumor apoptosis by sTRAIL-expressing *E. coli* DH5α could be demonstrated in vivo. Nude mice (n = 20) with *luc*+ labeled H460 lung tumors approximately 200 mm³ in size were intratumorally injected with *E. coli* DH5α (empty vector) or *E. coli* DH5α (sTRAIL). Tumor growth was monitored by two-dimensional caliper measurement and by in vivo tumor imaging after intraperitoneal injection of luciferin. As indicated in Figures 3a and b, H460 tumors in the mock control group grew exponentially, increasing 20-fold in size over the time period examined. Tumor growth was significantly inhibited at 1 week post-injection both in the vector control and in the *E. coli* DH5α (sTRAIL) groups, compared with that in the mock control group (P < 0.0001), and the difference between the vector control and *E. coli* DH5α (sTRAIL) groups was not significant (P = 0.29). In the vector control group, all tumors underwent regrowth during the subsequent 3 weeks, reaching a 17-fold increase as compared with the original tumor size. These results suggest that *E. coli* DH5α alone had little effect on the tumor-targeted delivery of TRAIL protein

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growth of H460 tumors in mice, with less than 10% inhibition on day 30 post-injection. In the E. coli DH5α (sTRAIL)-treated group, tumor growth was substantially suppressed as compared with that in control mice. The difference in tumor volume on day 30 between the sTRAIL-treated group and the mock or vector control groups is statistically significant ($P < 0.0001$; Figure 3b).

We next examined whether in vivo delivery of sTRAIL by E. coli DH5α into tumor tissues induces tumor cell death via apoptosis. To address this question, we injected the mock control, the E. coli DH5α vector control and sTRAIL-expressing E. coli DH5α into established tumors, excised the tumor tissues 3 days later and performed histological analysis on tissue sections. As shown in Figure 3c, H&E staining reveals that tumors injected with sTRAIL-expressing E. coli DH5α showed much more areas of necrosis and numerous cells with condensed nuclei as compared with the control groups. These data clearly indicate that sTRAIL-expressing E. coli DH5α suppresses tumor growth by inducing tumor cell apoptosis.

In general, local therapies such as i.t. injection have limited efficacy in clinical treatments. Therefore, the antitumor efficacy of sTRAIL-expressing E. coli DH5α was tested following systemic delivery. As a result, tail-vein injection of E. coli DH5α had little effect on tumor regression. In contrast, tumor growth was significantly retarded in mice treated with sTRAIL-expressing E. coli DH5α. The mean tumor volumes in mice treated with sTRAIL-expressing E. coli DH5α was dramatically lower than that of tumors treated with sterile normal saline on day 30 ($P < 0.0001$). The difference between the sTRAIL-treated group and the vector control group is also statistically significant ($P = 0.008$; Figure 4a). Moreover, survival of mice treated with sTRAIL-expressing E. coli DH5α was significantly prolonged as compared with that of mock- or vector control-treated mice. The survival of mock- and vector control-treated animals was about 40% at day 30 post-injection, whereas all the mice treated with sTRAIL-expressing E. coli DH5α were still alive at the same time point (Figure 4b).

**Validation and toxicity**

To further confirm that sTRAIL-expressing E. coli DH5α localizes preferentially in tumors, we performed PCR on bacterial colonies obtained from tumor tissues. H460 tumors were allowed to grow in nude mice until they were approximately 200 mm$^3$ in size, and then $5 \times 10^7$ c.f.u. of sTRAIL-expressing E. coli DH5α were injected through the tail vein. Four days after injection the animals were killed and tumors, spleens, livers and kidneys were aseptically removed, homogenized, diluted and plated onto LB agar plates supplemented with 50 μg/ml penicillin to determine the c.f.u. of sTRAIL-expressing E. coli DH5α. As shown in Figure 5a, sTRAIL-expressing E. coli DH5α was found preferentially within the tumor tissue. PCR analysis using sTRAIL primers of randomly picked bacterial colonies showed that the bacteria cultured from the tumors contained the sTRAIL plasmids. Immunohistochemical analysis of tumor tissues
also indicated positive sTRAIL staining (Figure 5b). In addition, sTRAIL protein was undetectable by a TRAIL-specific ELISA in mice serum on day 1, 4 and 7 after 5 × 10^7 c.f.u. of DH5x(sTRAIL) were injected i.v. (data not shown).

To determine whether E. coli DH5x (sTRAIL) caused hepatocellular toxicity in vivo, we measured the serum levels of two indicators of hepatocellular damage, alanine aminotransferase and aspartate aminotransferase, at 4 and 30 days after infection. As shown in Figure 5c, the values for serum aminotransferase and aspartate aminotransferase were within normal ranges, and no significant difference was found among the groups. Furthermore, histological analysis of liver sections performed on day 30 showed that expression of sTRAIL did not cause any pathological changes as compared with those in
mock- and vector control-treated mice (Figure 5d). Histological analysis of other tissues produced similar results to those obtained from liver (data not shown). Notably, mice injected with $5 \times 10^7$ c.f.u. of sTRAIL-expressing *E. coli* DH5α once per week for two consecutive weeks tolerated the infection and survived longer than mock- and vector control-treated mice (Figure 4b). We also examined the effect of DH5α (sTRAIL) on the TRAIL sensitivity of tumor cells and demonstrated that H460 cells aseptically separated from the tumor-bearing mice treated with DH5α (sTRAIL) are still susceptible to TRAIL-induced apoptosis (Supplementary Figure S2a). Consistently, expression of TRAIL receptor DR4/DR5 was not obviously affected by DH5α or DH5α (sTRAIL) administration (Supplementary Figure S2b). All these results suggest that *E. coli* DH5α-mediated sTRAIL expression kills tumor cells without detectable toxicity on normal cells.

**Discussion**

Specifically inducing tumor cells to undergo apoptosis is a promising therapeutic approach for cancer. In this study, we showed that non-pathogenic *E. coli* carrying a soluble TRAIL protein administered systemically, selectively localized in tumors and resulted in persistent tumor growth inhibition in mice. This treatment combines the advantages of tumor-targeting *E. coli* and the potent antitumor effects of sTRAIL.

The sTRAIL protein in various forms has been demonstrated to induce apoptosis of many cancer cells *in vitro* and inhibit growth of many tumors in rodent models. The sTRAIL protein acts by activating the cell-surface DR4 and DR5.\(^\text{35,36}\) In our study, inhibition of tumor growth was mediated by sTRAIL protein expressed in *E. coli*, since *E. coli* carrying the empty vector did not have tumor inhibition activity and a DR4:Fc fusion molecule could block the sTRAIL-harboring *E. coli* from inducing H460 cell apoptosis *in vitro*.

Bacteria, including *S. typhimurium*, have recently gained attention as tumor-targeting vectors.\(^\text{37,38}\) These bacteria selectively localize in tumor tissues when administered systemically, while the widely used adenoviral vector and adeno-associated virus vector do not have inherent tumor tropism and their tumor specificity is usually achieved via i.t. vector delivery.\(^\text{39}\) Otherwise, side effects associated with systemic administration of these viral vectors and the transgene products expressed would be a significant concern.\(^\text{40}\) The tumor targeting in mice by *E. coli*, as observed in this study, is impressively specific and efficient. *E. coli* DH5α administered i.v. localized specifically in several types of solid tumors in mice (murine B16 melanoma in BALB/c mice, human H460 lung carcinoma in nude mice) and also in multiple tiny metastatic sites of murine B16 melanoma in nude mice. This specific tumor targeting is possibly a mechanism related to the unique leaking tumor vasculature, the immune-suppression environment or the rich nutrient environment of tumor tissues.\(^\text{41}\) The tumor-targeting property of *S. typhimurium* has been confirmed in dogs and humans in addition to mice, although with varied efficiency.\(^\text{27,42-44}\) Nevertheless, these findings offer cautious optimism for using bacteria as tumor gene therapy vectors.

Another prominent feature of bacterial gene delivery vectors is that they are fully replication-competent while adenovirus or adeno-associated virus gene delivery vectors used for *in vivo* therapy purpose are usually replication-incompetent. This feature provides a basis for the *in vivo* persistence of the bacterial vectors and the therapeutic gene they carry. In our experiments, *E. coli* could be detected from the tumor tissue up to 1 month after administration (data not shown). The recombinant sTRAIL protein has a short half-life *in vivo*: from 3 to 5 min in mouse and 30 min in cynomolgus monkeys or chimpanzees.\(^\text{12}\) But the *E. coli*-expressed sTRAIL protein could be detected in tumor tissue at 4 days after administration. sTRAIL expression at later time points had not been determined, but the flat tumor growth curve at 3 weeks after the last administration suggests that sTRAIL expression in tumor might have lasted much longer. Moreover, *E. coli* DH5α delivery of sTRAIL protein results in diffusion throughout tumor tissues through the bloodstream. This may lead to increased extensive tumor killing, transcending the limitations of current gene therapies by affecting infected cells and adjacent areas.\(^\text{45}\)

Compared with other bacterial vectors with potential for tumor gene delivery, the *E. coli* vector employed here has certain advantages. First, *E. coli* is an extracellular bacterium while *S. typhimurium* is mainly intracellular. Since the sTRAIL molecule acts by interacting with the extracellular portion of its receptor, it would be advantageous and more efficient to have sTRAIL released by an extracellular bacterium. Secondly, as a facultative anaerobic bacterium, *E. coli* can grow in both small and large tumors, whereas anaerobic bacterium can only grow in large tumors with a necrotic center.

Bacterial vectors administered systematically would inevitably result in some complications, including certain level of bacteremia and induction of circulating proinflammatory cytokines due to their endotoxin. In clinical trials of the attenuated *S. typhimurium* strain VNP20009, tumor localization was only found in some patients receiving the bacteria at high doses where dose-limiting toxicity was found.\(^\text{27}\) *E. coli* will probably share some of these complications. However, many *E. coli* species are harbored by humans and other mammalian species in their digestive tract in a symbiotic relationship; thus non-pathogenic *E. coli* bacteria are likely to evoke a lower immune response and toxicity response in animals as compared with that by attenuated *S. typhimurium*. In this study, the endotoxin levels in mice plasma on day 0.5, 1, 3 and 7 after intravenous injection of $5 \times 10^7$ c.f.u. DH5α were found to differ non-significantly between the bacteria-injected mice and normal saline-injected mice (data not shown). Thus, in terms of safety profile and
efficiency in tumor localization in humans, *E. coli* might hold more potential than other bacteria. Furthermore, *E. coli* are the most thoroughly studied and known bacteria; numerous well-characterized strains and tools are available for their manipulation, which will certainly facilitate related studies.

To our knowledge, this is the first report of tumor suppression *in vivo* achieved by using *E. coli* as the protein delivery vector. This *E. coli* DH5α (sTRAIL) therapy could serve as a prototype for further development of a feasible and effective form of treatment for solid tumors. In addition, our study has general implications for the application of non-pathogenic *E. coli* as human gene therapy vectors.

**Conflict of interest**

The authors declare no conflict of interest.

**Abbreviations**

TRAIL, tumor necrosis factor-related apoptosis-inducing ligand; *E. coli*, *Escherichia coli*; IVIS, *In Vivo* Imaging System; c.f.u., colony-forming units; i.v., intravenously; i.t., intratumoral; MOI, multiplicity of infection.

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