Tumour-targeted delivery of TRAIL using Salmonella typhimurium enhances breast cancer survival in mice

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BACKGROUND: An effective cancer therapeutic must selectively target tumours with minimal systemic toxicity. Expression of a cytotoxic protein using Salmonella typhimurium would enable spatial and temporal control of delivery because these bacteria preferentially target tumours over normal tissue.

METHODS: We engineered non-pathogenic S. typhimurium to secrete murine TNF-related apoptosis-inducing ligand (TRAIL) under the control of the prokaryotic radiation-inducible RecA promoter. The response of the RecA promoter to radiation was measured using flow cytometry and immunoblotting. TRAIL toxicity was determined using flow cytometry and by measuring caspase-3 activation. A syngeneic murine tumour model was used to determine bacterial accumulation and the response to expressed TRAIL.

RESULTS: After irradiation, engineered S. typhimurium secreted TRAIL, which caused caspase-3-mediated apoptosis and death in 4T1 mammary carcinoma cells in culture. Systemic injection of Salmonella and induction of TRAIL expression using 2 Gy γ-irradiation caused a significant delay in mammary tumour growth and reduced the risk of death by 76% when compared with irradiated controls. Repeated dosing with TRAIL-bearing Salmonella in conjunction with radiation improved the 30-day survival from 0 to 100%.

CONCLUSION: These results show the pre-clinical utility of S. typhimurium as a TRAIL expression vector that effectively reduces tumour growth and extends host survival.

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Selective targeting of tumours enhances therapeutic delivery by increasing the drug concentration in cancer cells while limiting damage to normal tissue (Jain and Forbes, 2001; Forbes, 2006). Many genera of bacteria have been shown to selectively target tumours (Low et al, 1999; Forbes et al, 2003) and have great promise as adjunctive cancer therapies (Dang et al, 2001; Zhao et al, 2007). Despite this potential, once these bacterial species have been rendered non-toxic to humans, their toxicity is also limited against cancer cells, lowering their clinical significance (Toso et al, 2002; Nemunaitis et al, 2003). The utility of these bacterial therapies can be enhanced by engineering controllable expression of a cytotoxic peptide. Because these bacteria preferentially accumulate in tumour microenvironments (Zhao et al, 2005; Kasinskas and Forbes, 2006), they can also be manipulated to control the location and timing of cytotoxic peptide expression and further reduce host toxicity.

Four main genera of bacteria have been analysed as anticancer agents (Wei et al, 2008) and have been shown to selectively target tumours after intravenous injection: Salmonella (Low et al, 1999), Escherichia (Yu et al, 2004; Stritzker et al, 2007), Clostridium (Lemmon et al, 1997; Dang et al, 2001; Theys et al, 2006), and Bifidobacterium (Yazawa et al, 2001). To improve their efficacy, these bacteria have been engineered to express numerous proteins, including cytosine deaminase (Theys et al, 2001; Dresselaers et al, 2003; Dubois et al, 2007), endostatin (Lee et al, 2004), thrombospondin-1 (Lee et al, 2005), TRAIL (Nuyts et al, 2001b), interleukin-2 (Barbe et al, 2005), and antibodies against HIF-1α (Groot et al, 2007). Among these, only TNF-α is directly toxic to cancer cells. The facultative anaerobes, Salmonella and Escherichia, have an advantage because they can be administered systemically in their active form, which permits them to penetrate and chemotax through tumour tissue to target specific microenvironments (Zhao et al, 2005; Kasinskas and Forbes, 2006). Salmonella has particular promise as a cancer therapeutic because it can be manipulated to target quiescent, diffusion-limited regions in solid tumours (Kasinskas and Forbes, 2007) that are p53 deficient (Yu et al, 2002) and resistant to chemotherapeutics and radiation therapies (Minchinton and Tannock, 2006; Tredan et al, 2007). In addition, Salmonella has been shown to preferentially accumulate in tumours over 1000 times more than other organs after systemic injection, a characteristic that would reduce off-target toxicity from bacterially expressed molecules (Low et al, 1999; Forbes et al, 2003).

Temporal control of gene expression has been previously shown in Clostridium (Nuyts et al, 2001a,b) and viral vectors (Senzer et al, 2004; Mezirir et al, 2006). Both of these systems...
use radiation to trigger gene expression because of its ability to penetrate through tissue and trigger promoters that respond to DNA damage (Anderson and Kowalczykowski, 1998). In Clostridium, the prokaryotic promoter to RecA has been shown to control TNF-α production after irradiation (Nuys et al., 2001a,b). Similarly, temporal control of TNF-α expression has been shown using an adenoviral vector that encodes the eukaryotic Egr1 promoter upstream of TNF-α (Mehzir et al., 2006). In phase I clinical trials, this system was well tolerated (Senzer et al., 2004) and has shown to regress tumours (McLoughlin et al., 2003); however, it requires intratumoural injection to ensure its local effect, and is thus limited by access.

TNF-related apoptosis-inducing ligand (TRAIL) is a promising cancer therapeutic because it has been shown to selectively induce apoptosis in cancer cells when compared with normal tissue (Yagita et al., 2004). TRAIL is effective in many cancer cells, including colon, breast, lung, prostate, renal, ovarian, bladder, glioma, and pancreatic tumours (Walczak et al., 1999; Hylander et al., 2005; Shanker et al., 2008). Similar to TNF-α, TRAIL exerts an effect by the death receptor pathway of apoptosis, which activates caspase-8 and leads to activation of caspase-3, an important apoptotic mediator (LeBlanc and Ashkenazi, 2003). As an expressible peptide, TRAIL has great potential because it is non-toxic to most normal tissues, unlike TNF-α (Ashkenazi et al., 1999; Cretney et al., 2006), and because bacterially produced TRAIL has been shown to be cytotoxic to cancer cells (Kim et al., 2004). However, TRAIL has a limited role as a blood-borne therapy because of its rapid renal clearance and short half-life (Kelley et al., 2001). Despite its specificity towards tumours over most tissues, TRAIL may also induce hepatic cell death (Zheng et al., 2004), which suggests that an approach that allows for its selective delivery into tumours would avoid potential hepatotoxicity.

We have designed a targeted cancer therapy that provides spatiotemporal control of cytotoxic protein delivery by using Salmonella typhimurium as a vector to deliver TRAIL into tumours. To create a radiation-inducible system, the TRAIL gene was coupled to the promoter sequence for RecA, a gene involved in the prokaryotic SOS response to DNA damage (Anderson and Kowalczykowski, 1998). RecA promoter stimulation was shown using fluorometry, and TRAIL expression, secretion, and function were shown using immunoblotting and mammalian cell culture. A syngeneic murine breast cancer model was used to show the ability of radiation-inducible TRAIL secretion using S. typhimurium to suppress tumour growth and enhance host survival. Because of the ability to control the spatial and temporal delivery, TRAIL delivery using S. typhimurium has a great potential to be an adjunctive treatment strategy for solid tumours.

**MATERIALS AND METHODS**

**Development of plasmid constructs**

A series of prokaryotic-expression plasmids were created that contained either murine TRAIL or the green fluorescent protein, ZsGreen, under the control of the endogenous S. typhimurium RecA promoter (Figure 1). Plasmid cloning was performed using Escherichia coli DHS-3 (Invitrogen, Carlsbad, CA, USA) and all restriction endonucleases were obtained from New England Biolabs (Ipswich, MA, USA). To create a prokaryotic expression construct specific for S. typhimurium, the RecA promoter sequence (−91 to +84) was PCR amplified from the S. typhimurium genome (McClelland et al., 2001) using the forward primer 5’-CAC CAGCGAAAGGGACAGGCGACGACATA-3’ (Sapi restriction site underlined) and the reverse primer 5’-AGAACCCTGGACGC

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**Figure 1** Radiation-inducible prokaryotic expression constructs for ZsGreen and TRAIL. (A) The plasmid construct, pRA-ZsG, has the S. typhimurium promoter for RecA upstream of the green fluorescent protein, ZsGreen. The plasmid construct, pRA-TR, substitutes ZsGreen with the apoptosis-inducing peptide, mTRAIL. (B) Fluorescence imaging shows green fluorescence from bacteria electroporated with pZsGreen and pRA-ZsG plasmid constructs. (C) Fluorometry showing relative induction of fluorescence by the RecA promoter. S. typhimurium VNP20009 electroporated with pZsGreen and pRA-ZsG were induced with 2 Gy γ-irradiation or 5 J m⁻² UV irradiation, and then grown at 37 °C, 250 r.p.m. for 4 h. Data were normalised to the bacterial absorbance at 600 nm, and are reported as relative fluorescence of VNP pRA-ZsG compared with VNP pZsGreen. Gene expression is significantly increased with genotoxic damage in comparison with the un-induced control (∗∗P<0.05). (D) Immunoblot showing TRAIL secretion. 15% SDS–PAGE was performed on 20 μg protein obtained from cytosolic (c) and supernatant (s) fractions of overnight bacterial culture transfected with plasmid constructs. After transfer to PVDF, an immunoblot was performed using 1 : 200 Rabbit anti-TRAIL polyclonal antibody.
plasmid pZsGreen (Clontech, Mountain View, CA, USA) by subcloning the RecA promoter into the SapI and NcoI sites to substitute the lac promoter. Two plasmids for expression of murine TRAIL were developed, pTRAIL and pRA-TR (Figure 1A), by excising the murine TRAIL fragment from pORF5-mTRAIL (InvivoGen, San Diego, CA, USA) using NcoI and Nhel, and subcloning into the Ncol and Spel sites on pRA-ZsG.

The construct pQCXIN mCh was developed by subcloning the BamHI/EcoRI digested fragment from pSET_B mCherry into the retroviral vector, pQGXIN (Clontech). Appropriate frame and sequence of inserts was confirmed using an ABI Prism 3730xl DNA Sequencer (Genewiz, South Plainfield, NJ, USA).

S. typhimurium transformation using electroporation

The mshB purI xyl Salmonella typhimurium strain, VNP20009 (Vion Pharmaceuticals, New Haven, CT, USA), was grown in Luria-Bertani (LB) media at 37 °C until mid-log phase, and then harvested at 4 °C. Cells were made electrocompetent after serial washes in ice-cold 10% glycerol, followed by resuspension in GYT medium (10% glycerol, 0.125% yeast extract, 0.25% tryptone) at 2.5 × 10^6 cells ml^-1. Electroporation was performed in 0.2 cm cuvettes after mixing 40 μl competent cells with 25 ng plasmid DNA using a Bio-Rad Micro Pulse with settings of 2.5 kV, 25 μF, and 200Ω. Bacteria were selected and maintained in LB media with 50 μg ml^-1 ampicillin.

Confirmation of RecA promoter function using imaging and fluorometry

Bacteria electroporated with ZsGreen plasmid constructs were grown until an OD600 of 0.5, and then exposed to 1.0 mM IPTG, 2 Gy γ-irradiation, or 5 J m^-2 UV irradiation. Bacteria were incubated at 37 °C, 250 r.p.m. for 4 h. Fluorescence was observed using a Maestro In-Vivo Imaging System (Cambridge Research & Instrumentation, Inc., Woburn, MA, USA). Fluorescence intensity was quantified using a microplate reader with 485 nm excitation and 535 nm emission filters.

Measurement of TRAIL expression using immunoblotting

Bacteria electroporated with plasmid constructs were grown overnight in modified M9 Media (0.4% glucose, 1% tryptone, 200 μM purine base, and 50 μg ml^-1 ampicillin). Bacteria were centrifuged at 15 000 g, with aspiration of the supernatant fraction. Secreted proteins from media supernatants were precipitated with 0.1% sodium deoxycholate and 7% trichloroacetic acid, followed by washes in acetone and resuspension in phosphate-buffered saline (PBS). The cell pellet was incubated in bacterial lysis buffer (50 mM potassium phosphate, 400 mM NaCl, 100 mM KCl, 10% glycerol, 0.5% Triton X-100, and 10 mM imidazole, pH 7.8) at 4 °C to extract cytosolic proteins. Protein concentration was determined using a BCA assay kit (Pierce, Rockford, IL, USA). With 20 μg of protein per well, 15% SDS–PAGE was performed, followed by transfer onto PVDF membrane. Membranes were blocked in 5% non-fat milk in Tris-buffered saline with 0.1% Tween-20 (TBST), and then incubated overnight at 4 °C in 1:200 Rabbit anti-TRAIL polyclonal antibody (Abcam, Cambridge, MA, USA). After serial washes in TBST, membranes were incubated in 1:5000 HRP-conjugated goat anti-rabbit polyclonal antibody, and then washed again and visualised using Supersignal chemiluminescent substrate (Pierce).

Mammary tumour cell lines

Mammary carcinoma 4T1 cells (American Tissue Type Collection, Manassas, VA, USA) were maintained at 37 °C, 5% CO2 in RPMI-1640 with 10% fetal bovine serum. Using tumours composed of syngeneic 4T1 cells in BALB/c mice was one of the few appropriate cancer models. It was necessary to use immunocompetent mice.
because attenuated bacteria have deleterious effects on immuno-compromised mice (Forbes et al, 2003). Because of rejection by the immune system, only syngeneic tumours will form in immuno-competent mice.

An attenuated mammary carcinoma cell line, 4T1/red, was developed because implanted 4T1 cells invaded the peritoneum and caused diffuse metastatic disease within 3 to 4 weeks. The new 4T1/red cell line was developed by G418 selection after retroviral infection of 4T1 with pQCXIN-mCh viral supernatants, obtained after transformation of the EcoPack 2–293 cell line (Clontech). Tumours formed from 4T1/red cells had an increased doubling time and no longer created pulmonary or hepatic metastases, with decreased intraperitoneal invasion. Using 4T1/ red cells allowed for observation of growth of the primary tumour without mice succumbing to bowel obstruction or diffuse metastatic disease. Prevention of metastatic disease enabled a more accurate test of the hypothesis that bacterial treatment influence growth of the primary tumours. Immediately before in vivo use, trypsinised 4T1/red cells were resuspended in PBS at 5 × 10⁶ cells ml⁻¹.

Toxicity of bacterial secreted protein on mammalian cell culture

Filtered supernatants were obtained from bacterial strains (VNP pRA-ZsG and VNP pRA-TR) grown in modified M9 Medium (0.4% glucose, 1% tryptone, 200 μM purine base, and 50 μg ml⁻¹ ampicillin) and resuspended in 4T1 media at a concentration of 50 ng ml⁻¹. The control treatment used sterile modified M9 Medium. Recombinant mouse TNF-α (Sigma, St Louis, MO, USA) was also suspended in 4T1 media at a concentration of 50 ng ml⁻¹.

Caspase activity assays

ApoAlert caspase activity assays (Clontech) were conducted on 4T1 cells after application of experimental supernatants or recombinant mouse TNF-α (Sigma) at 50 ng ml⁻¹ for 24 h. Cell lysates were obtained from 2 × 10⁶ cells and incubated in caspase-3 substrate (DEVD-pNA) or caspase-8 substrate (IEDT-pNA) according to the manufacturer’s directions. Validation of the assays was performed by incubating cell lysates from the TNF-α treatment group with DEVD-fmk, a caspase-3 inhibitor.

Annexin V-FITC/propidium iodide flow cytometry

4T1 cells were exposed to experimental treatments for 48 h. Cells were prepared using an Annexin V-FITC apoptosis detection kit II (BD Biosciences, San Jose, CA, USA), according to the manufacturer’s directions. Flow cytometry was conducted on 10,000 cells per treatment using a FACSCalibur flow cytometer (BD Biosciences). Normalisation and compensation were performed on unstained controls.

Syngeneic murine tumour model

Animal care was conducted in accordance with the National Institute of Health guidelines for care and use of laboratory animals. Previous approval from the institutional animal care and use committee of the Baystate Medical Center was obtained. At 8 weeks of age, Balb/c mice received a subcutaneous injection of 50,000 4T1/red cells using a 10 μl Hamilton syringe at the level of the right third mammary fat pad.

Biodistribution

To determine bacterial biodistribution, 4T1 tumours were grown for 21 days. At this time, mice received systemic injection through tail vein with 100,000 cfu g⁻¹ VNP20009. Mice were killed after 48 h. The tumour and liver samples were weighed and then minced in a known volume of PBS until homogenous, followed by plating of serial dilutions on LB agar. After 24- to 48-h incubation at 37 °C, colony-forming units (cfu) were counted to determine bacterial concentration (cfu g⁻¹).

Salmonella immunohistochemistry

Tumours were fixed in 10% neutral buffered formalin and embedded in paraffin. Antigen retrieval was performed on rehydrated equatorial tissue sections using an EZ Retriever System with CitraPlus Antigen Retrieval Solution (BioGenex, San Ramon, CA, USA). A DakoCytomation Autostainer (Carpinteria, CA, USA) was programmed according to the manufacturer’s settings using an Envision G2/2 AP System. Sections were incubated in a 1:200 dilution of Rabbit Polyclonal Antibody to Salmonella (Abcam) in 0.2% BSA for 30 min. An alkaline phosphatase polymer was applied for 30 min, followed by staining with Permanent Red. Sections were counterstained with hematoxylin. Positive controls were performed on a tumour with intratumoural injection of VNP20009.

Image acquisition

An Olympus IX71 Inverted Epi-fluorescence Microscope (Center Valley, PA, USA) equipped with a Ludd Motorised Z-Stage (Hawthorne, NY, USA), a monochromatic Hamamatsu cooled-CCD Digital Camera (Hamamatsu City, Japan), and a CRI MicroColor trichromatic filter (Woburn, MA, USA) was used to acquire images at ≥ 10 objective lens magnification from immunohistochemically-labeled slides. A script in IPLab (version 3.71, BD Biosciences) was used to automate image acquisition and assemble a tiled montage of individual images comprising three RGB channels, creating macroscopic composite colour images of entire tumour sections.

Figure 3 Spatial distribution of S. typhimurium after systemic administration. (A) Bacterial concentration (cfu g⁻¹) in 4T1 mammary tumours and livers of BALB/c mice at 48 h after systemic injection of 100,000 cfu g⁻¹ VNP20009. (B) Band of bacteria in a 4T1 tumour (stained red with white arrows) identified by anti-Salmonella immunohistochemistry. Scale bar is 100 μm. (C) Composite image of 4T1 tumour stained using Salmonella immunohistochemistry to visualise bacteria (red with white arrows). Scale bar is 5 mm.
Tumour response to bacterial treatment

After 21 days of tumour growth, Balb/c mice received systemic injection through tail vein with 100 000 cfu g⁻¹ VNP pRA-ZsG, VNP pRA-TR, or PBS (control). After 2 days, subgroups of mice received a single dose of 2 Gy whole-body γ-irradiation by exposure to a Gammator-50 137Cs Source (Radiation Machinery Co., Parsippany, NJ, USA). This time point was determined from observations in 4T1 tumours showing that bacteria were furthest away from perfused vasculature at 48 h. Tumour volume was determined every 2 days with measurements obtained from an electronic vernier caliper using the equation (length)(width)(height)π/6. Mice with tumours ≤75 mm³ at 21 days of tumour growth were excluded from analysis. Time of killing was determined when mice were moribund or tumour volume exceeded 1000 mm³. Follow-up was limited to 30 days.

Statistical analysis

Data are reported as means with their 95% confidence intervals in parentheses. Hypothesis testing was performed using Student’s t-test with significance determined by *P < 0.05. Regression analysis was performed according to logarithmic functions for tumour growth. Survival analysis was performed using Kaplan–Meier curves, with comparisons between groups made using the log-rank test, *P < 0.05, †P < 0.05 compared with PBS.
Results

TRAIL expressed using S. typhimurium induces apoptosis and cell death

Gene expression from the created plasmids was confirmed using fluorescence imaging and immunoblotting (Figures 1B-D). Figure 1B shows the presence of green fluorescence from VNP pZsGreen and VNP pRA-ZsG. To assess the ability of the RecA promoter to be triggered by genotoxic damage, the fluorescence of VNP pRA-ZsG was compared with VNP pZsGreen. The ratio of fluorescence induction by the RecA promoter to the lac promoter significantly increased by 22% at 4 h after 2 Gy γ-irradiation in comparison with non-induced controls (P < 0.05; Figure 1C). Fluorescence present in the non-irradiated control is because of the low-level constitutive expression of the RecA promoter (Nuyts et al, 2001a). In bacterial cultures, immunoblots for TRAIL protein showed that engineered S. typhimurium produced TRAIL that was predominantly secreted into the media supernatant and only minimally retained in the bacterial cytoplasm (Figure 1D). Although not quantitative, this immunoblot shows that pTRAIL and pRA-TR produce considerable amounts of secreted mTRAIL.

Supernatant fractions from the bacterial vectors for TRAIL delivery induced apoptosis and promoted cell death in 4T1 murine mammary carcinoma cells (Figure 2). Supernatants derived from VNP pRA-TR activated both caspase-8 and caspase-3, with responses similar to those observed for TNF-α, as compared with liver (Figure 3A). The average tumour density was almost 1000-fold greater than the liver density, which is significantly reduced the activity of both caspases, confirming that the positive control, TNF-α, did activate caspase-3. Supernatants from the negative control, VNP pRA-ZsG, did not activate any of the two caspases (Figure 2A). Because the only difference between VNP pRA-TR and pRA-ZsG was the mTRAIL gene, the different cellular responses to the bacterial supernatants indicate that activation of the death receptor pathway was specifically dependent on mTRAIL expression. Annexin-V/propidium iodide flow cytometry showed that bacterial supernatants from VNP pRA-TR promote 4T1 cell death (annexin-V positive, propidium iodide positive) and early apoptosis (annexin-V positive, propidium iodide negative; Figures 2B and C). The proportion of cells undergoing cell death was increased in comparison with the control group (P < 0.05), with more robust results than TNF-α. In addition, VNP pRA-TR supernatants significantly increased the annexin-V-positive fraction of cells (P < 0.05; Figure 2C), indicating the early apoptotic stages of phosphatidylserine membrane translocation (Corsten et al, 2006).

Different modes of action confer a functional benefit of TRAIL over TNF-α. Although both act through the death receptor pathway, TRAIL stimulates both pro-apoptotic signals (through caspase-8) and mitochondrial-stabilising anti-apoptotic signals (through NF-κB), leading to cells with resistance to TNF-α-mediated apoptosis (Beg and Baltimore, 1996). TRAIL has attenuated the induction of NF-κB, leading to a pronounced death signal through p53-independent mechanisms (LeBlanc and Ashkenazi, 2003). This may explain the increase in early apoptosis, but overall lack of change in 4T1 cell death observed with TNF-α in Figure 2C.

S. typhimurium VNP20009 preferentially colonises tumours

In a syngeneic subcutaneous mammary carcinoma model, systemic inoculation of 100 000 cfug⁻¹ VNP20009 resulted in the preferential colonisation of bacteria within 4T1 mammary tumours as compared with liver (Figure 3A). The average tumour density was almost 1000-fold greater than the liver density, which is similar to the numbers obtained in previous studies (Forbes et al, 2003). At both macro- and microscopic length scales, Salmonella were present in all tumours that were analysed (Figures 3B and C). At 48 h after administration, VNP20009 preferentially accumulated in necrotic regions of 4T1 tumours (white arrows, Figure 3C).
S. typhimurium expressing TRAIL retard tumour growth in mice

Treatment with bacterial vectors and γ-irradiation synergistically delayed tumour growth in 4T1/red tumour-bearing BALB/c mice (Figure 4; Table 1). Mice were systemically injected with PBS controls, control bacteria (VNP pRA-ZsG), or TRAIL-expressing bacteria (VNP pRA-TR), both with and without activation by 2 Gy γ-irradiation at 48 h after infection. All treatments significantly increased tumour doubling time compared with PBS controls (P < 0.05; Figure 4B; Table 1). At all time points, except the day of injection, the tumour volumes of VNP pRA-TR with 2 Gy radiation were significantly less than PBS controls (P < 0.05; Figure 4A). The combination of VNP pRA-TR with 2 Gy radiation increased the tumour doubling time from 6.6 days (95% CI 6.4 – 6.9 days) to 12.2 days (95% CI 11.7 – 12.8 days; P < 0.05; Figure 4B) and caused a significant delay of 19.0 days (95% CI 15.0 – 23.1 days) for tumour growth to 1000 mm³ compared with the PBS controls (P < 0.05; Figure 4C).

The delay in tumour growth for VNP pRA-TR with 2 Gy radiation was significantly greater than the control treatments, VNP pRA-ZsG with 2 Gy irradiation and VNP pRA-TR alone (Figure 4C; P < 0.05), indicating that both TRAIL expression and induction by radiation were necessary for maximum growth delay. The significant difference in growth delay between irradiated VNP pRA-TR and VNP pRA-ZsG (P < 0.05; Figure 4C) indicates that TRAIL is produced in tumours. Although this evidence is indirect, the only difference between the plasmids on these two strains is the TRAIL gene. If TRAIL was not produced, these two strains would have induced the same response. Compared with VNP pRA-TR with 2 Gy irradiation, radiation alone only delayed tumour growth by 3.5 days (95% CI 0.1 – 7 days), whereas VNP pRA-TR alone delayed growth by 9.1 days (95% CI 6.7 – 11.7 days), suggesting that the combined response was synergistic rather than additive. This synergy shows that there is activation of TRAIL expression by the RecA promoter as well as stimulation of bystander effects by the combination of radiation and TRAIL.

In addition to retarding growth, treatment with VNP pRA-TR and radiation improved mouse survival (Figure 4D; Table 2). To maintain animals according to humane principles, death was not used as a primary end point. For survival analysis, mice were killed when moribund or when tumours measured > 1000 mm³. Although there was no difference in median survival between PBS with and without irradiation, median survival was nearly doubled by VNP pRA-TR with radiation (26 days) compared with PBS controls (14 days), with a significant increase in the 30-day survival from 0% to 37.5% of mice (log-rank test, P < 0.05; Figure 5A). The 30-day survival was 100% after redosing with VNP pRA-TR and 2 Gy radiation reduced the risk of death by 76% when controlled against radiation treatment alone (hazard ratio 0.24; 95% CI 0.08 – 0.75; P < 0.05).

Redosing bacteria and irradiation accentuated the therapeutic effect

Because a single dose of VNP pRA-TR activated by 2 Gy radiation showed decreased growth for approximately 1 week, we reasoned that additional redosing at 1 week would potentiate the therapeutic effect (Figure 5). Additional mice received a series of two treatments, with intravenous injection of VNP pRA-TR or PBS on days 0 and 6, followed by 2 Gy radiation on days 2 and 8 (Figure 5A). The effects of redosing VNP pRA-TR with radiation were considerable, delaying the expected time to tumour volume of 1000 mm³ by 30.3 days (95% CI 26.6 – 34.1 days; P < 0.05; Figure 5B; Table 3). The 30-day survival was 100% after redosing with VNP pRA-TR and radiation, compared with 25% after redosing with PBS and radiation (log-rank test, P < 0.05; Figure 5C).

Figure 5 Suppression of tumour growth and enhanced survival by repeated dosing of VNP pRA-TR and γ-irradiation. (A) Regression curves for tumour growth after treatment with two doses of VNP pRA-TR and radiation (n = 4), two doses of PBS and radiation (n = 4), and PBS alone (n = 11). Experimental groups received intravenous injection with PBS or VNP pRA-TR at days 0 and 6 (small arrows), followed by 2 Gy irradiation 2 days after experimental treatments (days 2 and 8; large arrows). Significant differences in tumour volume after redosing VNP pRA-TR and radiation were observed at all time points except days 0 and 2 in comparison with the PBS control group (P < 0.05). (B) Estimates of time to tumour volume of 1000 mm³ show a delay of 30 days after two doses of VNP pRA-TR with irradiation in comparison with the PBS control (*P < 0.05; redosed VNP pRA-TR + 2 Gy, in comparison with all other groups). (C) Kaplan–Meier survival curves for the treatment groups in (A). At 30 days of follow-up, 100% of mice survived with two treatments of VNP pRA-TR with 2 Gy irradiation, compared with 25% after repeated dosing with PBS and 2 Gy, and no survival from PBS controls (log-rank test, P < 0.05).
DISCUSSION

These results show that S. typhimurium can express and secrete a functional TRAIL protein, with control of expression by genotoxic damage from a small dose of radiation. Secreted TRAIL, by activating the death receptor pathway, can stimulate apoptosis in carcinoma cells. Furthermore, S. typhimurium bearing pRA-TR significantly decreases tumour growth and increases survival when activated by radiation; an effect amplified by repeated dosing.

Complementary to these direct results, there are several added benefits in our approach. Numerous tactics have been developed with the intention of purposefully targeting cancers, including viral gene therapy, liposomal delivery systems, monoclonal antibodies, small-molecule protein inhibitors, and nanoparticle technologies; however, the ability to achieve spatial and temporal control while minimising toxicity has been limited. The first benefit of our approach is the ability of S. typhimurium to effectively target tumour microenvironments, with the ability to induce apoptosis within regions of quiescence bordering tumour necrosis (Kasinskas and Forbes, 2007). S. typhimurium has both tumour tropism and microenvironment specificity, with patterns of distribution within tumour microenvironments that are time dependent, offering opportunity for both spatial and temporal control of peptide delivery. The second benefit is the ability of TRAIL to target cancer cells with specificity, allowing for induction of apoptosis by p53-independent mechanisms while avoiding toxicity to the host observed with TNF-α (Takeda et al., 2007).

The third benefit is the ability of the RecA promoter to act as a switch, turned on by genotoxic damage from radiation, allowing for temporal and spatial control of gene expression (Mitchell and Gu, 2004). Fourth, radiation therapy can potentiate responses to TRAIL delivery and influence the tumour microenvironment through bystander effects (Belka et al., 2004; Mothersill et al., 2004). Finally, repeated dosing in the setting of limited toxicity may provide a method to limit exponential growth in tumours. Once fully developed, we envision that this bacterial cancer therapeutic with spatial and temporal control of delivery will provide considerable therapeutic benefit by enhancing efficacy while limiting host toxicity.

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