

Therapeutic targeting of tumors with imageable GFP-expressing *Salmonella typhimurium* auxotrophic mutants

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ABSTRACT

Tumor targeting *Salmonella typhimurium* has been developed. These bacteria were mutagenized and a strain auxotrophic for leucine and arginine was selected. This strain was also engineered to express GFP. This strain, termed A1, could target prostate tumors in nude mouse models and inhibit their growth. A1 was passaged through a tumor and re-isolated and termed A1-R. A1-R had greater antitumor efficacy and could cure breast, prostate, pancreatic, and lung tumors in nude mouse models.

Keywords: bacteria, auxotrophy, tumor targeting, GFP, imaging, tumor therapy

1. INTRODUCTION

Cancer research has long sought a magic bullet that, in contrast to most current therapeutic protocols, would selectively target and destroy malignant cells. This goal has long remained elusive in large part because of the extreme genetic and phenotypic variability of most tumors. There is, however, an intriguing pathway that recurrently attracts interest, i.e., the restricted colonization of malignancies by certain bacteria (1).

In the 1890s, Coley observed that certain cancer patients with postoperative bacterial infection were cured of their tumors (2). It has been known since the 1940s that anaerobic bacteria can selectively grow in hypoxic and necrotic areas of tumors (3-7). A number of novel approaches to developing tumor-therapeutic bacteria have been described.

Yazawa *et al.* (6) demonstrated that the anaerobic bacterium *Bifidobacterium longum* could selectively germinate and grow in the hypoxic regions of solid tumors after i.v. injection. *B. longum* is a nonpathogenic Gram-positive bacterium found in the lower small intestine and large intestine of humans and other mammals. Yazawa *et al.* showed that *B. longum* selectively grew in 7,12-dimethylbenzanthracene-induced rat mammary tumors after i.v. administration.

Vogelstein *et al.* (8) compared *B. longum* and *Clostridium novyi*, both obligate anaerobes, for their capacity to grow within transplanted tumors. Vogelstein *et al.* created a strain of *C. novyi* depleted of its lethal toxin termed *C. novyi* NT. They showed that i.v.-administered *C. novyi* NT spores germinated within the avascular regions of tumors in mice and destroyed surrounding viable tumor cells (8). *C. novyi* NT grew to a greater extent in the B16 melanoma than *B. longum*. When *C. novyi* NT spores were administered together with conventional chemotherapeutic drugs or radioactivity, extensive hemorrhagic necrosis of tumors often developed within 24 h, resulting in significant and prolonged antitumor effects (8, 9). Vogelstein *et al.* (2, 9) subsequently observed that *C. novyi* NT treatment of tumors elicits an immune response that can, in combination with chemotherapy, result in tumor cure by using syngeneic models with immunocompetent mice.

In another approach, attenuated *Salmonella typhimurium* strains, which are facultative anaerobes, have been described as anticancer agents (10). *Salmonella* strains have been shown to preferentially amplify within tumors and express prodrug converting enzymes such as the herpes simplex thymidine kinase (10). Initially, *S. typhimurium* was attenuated by auxotrophic mutations (11, 12). The internal tumor environment apparently meets the auxotrophic requirements of the mutated *Salmonella*, where they replicated 1,000 times the concentration found in normal tissues (10). Within the tumor,

there may be areas of hypoxia that favor the growth of facultative anaerobes. In addition, the presence of tumor necrosis provides additional nutrients, such as purines, required by the organism. Genetic modification of the *Salmonella* lipid A was done to reduce septic shock (10). Disruption of the *Salmonella msbB* gene reduced TNF α induction and increased the LD₅₀ of *Salmonella* by 10,000-fold. The *msbB* mutant prevents the addition of a terminal myristyl group to the lipid A domain (10). Melanomas in mice treated with this *Salmonella* were 6% the size of tumors in untreated controls (12). Attenuated strains of *Salmonella* have also been modified to secrete immunostimulatory cytokines that may have the potential to stimulate host antitumor responses (13).

The lipid A-attenuated *S. typhimurium* has been evaluated in a phase I clinical trial (14). Twenty-four patients with metastatic melanoma and one patient with metastatic renal cell carcinoma received i.v. bolus infusions containing 10⁶ to 10⁹ cfu/m² of the lipid A-mutant *Salmonella* (VNP20009). To increase safety and reduce toxicity, *S. typhimurium* was attenuated by deletion of both the *purI* and *msbB* genes. The *purI* mutant requires an external source of adenine (14). The resulting VNP20009 strain of *S. typhimurium* could be safely administered to patients. At the highest tolerated dose, tumor colonization was observed. Additional studies are required to reduce doserelated toxicity and improve tumor localization (14) to obtain efficacy.

To increase the potential of attenuated *Salmonella* as a gene-delivery vector for cancer treatment, Mengesha *et al.* (15) developed a hypoxia-inducible promoter (HIP-1) to limit gene expression specifically to the tumor. Mengesha *et al.* demonstrated that HIP-1 can drive hypoxia-mediated gene expression in bacteria that have colonized human tumor xenografts in mouse models. Expression of both GFP and red fluorescent protein (RFP) under control of HIP-1 demonstrated an \approx 15-fold increase relative to a constitutive promoter when tumors were made hypoxic. Moreover, the use of a constitutive promoter resulted in reporter gene expression in both tumors and normal tissues, whereas reporter gene expression using HIP-1 was confined to the tumor (15).

Yu *et al.* (16, 17) used GFP-labeled bacteria to visualize tumors and metastases in mouse models. Three attenuated pathogens, *Vibrio cholerae*, *S. typhimurium*, and *Listeria monocytogenes*, targeted and replicated in tumors. However, no antitumor efficacy was observed.

Vogelstein's group (18) showed that treatment of mice bearing large, established tumors infected with *C. novyi* NT plus a single dose of liposomal doxorubicin could lead to eradication of the tumors. The bacterial factor responsible for the enhanced drug release was identified as a previously unrecognized protein termed "liposomase." However, *C. novyi* NT was ineffective as monotherapy.

The relative ease and efficacy of externally imaging implanted tumors made fluorescent with GFP or RFP suggested applying the technique to other foreign agents such as infecting bacteria (19). *Escherichia coli* was transfected with a high-expression plasmid containing the GFP gene. The GFP-expressing *E. coli* (*E. coli*-GFP) was administered to mice by various routes, and the fate of the bacteria was readily visualized in real time by whole-body imaging. Real-time images were acquired by using a color CCD video camera by simply illuminating mice at 470 nm (31). We have used the tumor and bacterial imaging technologies to develop therapeutic bacteria capable of targeting tumors as monotherapy (1, 20).

2. MATERIALS AND METHODS

2.1 GFP Gene Transfection of *S. Typhimurium* (1).

S. typhimurium (ATCC 14028) was grown at 37°C to midlogarithmic phase in liquid LB and harvested at 4°C. Bacteria (2.0 \times 10⁸) in 40 μ l of 10% glycerol were mixed with 2 μ l of pGFP (Clontech) vector and placed on ice for 5 min before electroporation with a Gene Pulser apparatus (Bio-Rad) according to the manufacturer's instructions. Electroporation was done at 1.8 kV with the pulse controller at 1,000- Ω parallel resistance.

2.2 Induction of Bacterial Mutations with Nitrosoguanidine (NTG) and Selection for Auxotrophs (1).

Freshly prepared NTG (1 mg/ml in sterile water) was added to the washed culture to a final concentration of 100 μ g/ml in Tris-maleic acid buffer at pH 6.0. The bacteria were incubated with NTG for 30 min. The NTG-treated cells were grown in nutrient broth to express any mutations that were induced. Bacterial colonies were replica-plated in supplemented minimal

agar plates containing specific amino acids to identify the requirements of the auxotrophs. Auxotroph A1, which required leu and arg, was identified.

2.3 Re-isolation of *S. Typhimurium* A1 (20).

S. typhimurium A1 auxotrophs expressing GFP were re-isolated as follows: the A1 bacteria were injected into the tail vein of a HT-29 human colon tumor-bearing nude mouse. Three days after infection, the tumor tissue was removed from the infected mouse. The tumor tissue was then homogenized and diluted with PBS. The resulting supernatant of the tumor tissue was cultured in LB agar plates at 37°C overnight. The bacteria colony with the brightest green fluorescence was picked up and cultured in 5 mL LB medium. This strain was termed A1-R.

2.4 Adherence and invasion assay (20).

RFP-labeled HT-29 human colon cancer cells were grown in 24-well tissue culture plates to a density of approximately 10^4 cells/well. A1-R bacteria were grown to late-log phase in LB broth as previously described. The bacteria were diluted in cell culture medium (1×10^6) and added to the tumor cells and placed in an incubator at 37°C. After 60 minutes, the cells were rinsed five times with 1-2 ml PBS. Adherent bacteria were released by incubation with 0.2 ml 0.1% triton X-100 for 10 min. LB broth (0.8 ml) was then added and each sample was vigorously mixed. Adherent bacteria were quantified by plating to count cfu on LB agar medium. To measure invasion of bacteria, the bacterially-infected cancer cells were rinsed five times with 1-2 ml PBS and cultured in medium containing gentamycin sulfate (20 µg/ml) to kill external but not internal bacteria. After incubation with gentamicin for 12 hours, the cells were washed once with PBS, and the viable intracellular bacteria were evaluated by fluorescence microscopy.

2.5 Preparation of Bacteria (20).

The A1-R bacteria were grown overnight on LB medium and then diluted 1:10 in LB medium. Bacteria were harvested at late-log phase, washed with PBS, and then diluted in PBS. Bacteria were then injected into the tail vein of nude mice (5×10^7 CFU/100 µL PBS).

2.6 RFP or GFP Gene Transduction of the PC-3 Human Prostate Cancer Cell Line (1).

For RFP or GFP gene transduction, $\approx 60\%$ confluent PC-3 human prostate cancer cells were incubated with a 1:1 precipitated mixture of retroviral supernatants of PT67 packaging cells and F12K medium containing 7% FBS (Gemini Bioproducts, Calabasas, CA) for 72 h. The packaging cells produced the pLNCX2 vector (Clontech) containing the DsRed2-expressing RFP or the pLEIN vector containing GFP and the neomycin resistance gene. RFP- or GFP-expressing PC-3 cancer cells were selected in medium containing 200–1,000 µg/ml neomycin increased in a stepwise manner. Clones expressing RFP or GFP were isolated with cloning cylinders (Bel-Art Products, Pequannock, NJ) by trypsin/EDTA and were amplified and transferred by conventional culture methods in the absence of selective agent. For RFP gene transduction, 70% confluent human PC-3 cells were used.

2.7 Establishment of Dual Color Cells Expressing GFP and RFP.

For establishing dual-color cells, PC-3-RFP cells were then incubated with a 1:1 precipitated mixture of retroviral supernatants of PT67 packaging cells expressing GFP linked to histone H2B (34) and culture medium. The histone H2B-GFP fusion gene was inserted into the pLHCX vector (Clontech) that also contains the hygromycin resistance gene. To select the double transformants, cells were incubated with hygromycin 72 h after transfection. The level of hygromycin was increased stepwise up to 400 µg/ml. These sublines stably expressed GFP in the nucleus and RFP in cytoplasm.

2.8 Surgical Orthotopic Implantation (SOI) of Prostate Tumors (21).

Tumor fragments (1 mm^3) were prepared from a PC-3-GFP tumor growing subcutaneously in nude mice. Two tumor fragments were implanted by SOI in the lateral lobe of the prostate, which was exposed following a lower midline abdominal incision. After proper exposure of the bladder and prostate, the capsule of the prostate was opened and the two tumor fragments (1 mm^3) were inserted into the capsule. The capsule was then closed with an 8-0 surgical suture. The incision in the abdominal wall was closed with a 6-0 surgical suture in one layer. The animals were kept under isoflurane anesthesia during surgery. All procedures of the operation described above were performed with a 7× magnification microscope (Olympus).

2.9 Surgical orthotopic implantation (SOI) of breast tumors (20).

Tumor fragments (1 mm³) from the MARY-X human breast tumor xenograft (22), grown subcutaneously in nude mice, were implanted by surgical orthotopic implantation (SOI) in the mammary fat pad in nude mice. 8-0 surgical sutures are used to penetrate the tumor pieces to the fat pad. The incision in the skin was closed with a 7-0 surgical suture in one layer. The animals were kept under isoflurane anesthesia during surgery (20).

2.10 Bacterial Infection (1).

When the implanted tumors were externally imaginable, ten mice were administered *S. typhimurium* A1-R (5×10^7) *iv*. Schedules are described below along with the individual experiments.

2.11 Analysis of Antitumor Efficacy (1).

Twenty mice were implanted orthotopically with PC-3 tumors expressing GFP. Ten of the 20 mice served as controls and followed until death without treatment. Ten mice were administered *S. typhimurium* A1 intravenously weekly and survival time was compared to the untreated mice. Progressive tumor enlargement and metastasis were externally imaged by their GFP fluorescence.

2.12 Host Safety (1).

Ten non-tumor-bearing mice served as controls for the possible toxicity of *S. typhimurium* A1-R. The mice were weekly injected *iv* with the bacteria (5×10^7 cfu). Survival time after bacteria infection was monitored and body weight was measured.

2.13 Imaging in Live Mice (1).

The Olympus OV100 Small Animal Imaging System (Olympus Corp., Tokyo, Japan), containing an MT-20 light source (Olympus Biosystems, Planegg, Germany) and DP70 CCD camera (Olympus), was used for imaging in live mice. The optics of the OV100 fluorescence imaging system have been specially developed for macroimaging as well as microimaging with high light-gathering capacity. The instrument incorporates a unique combination of high numerical aperture and long working distance. Five individually optimized objective lenses, parcentered and parfocal, provide a 10⁵-fold magnification range for seamless imaging of the entire body down to the subcellular level without disturbing the animal. The OV100 has the lenses mounted on an automated turret with a high magnification range of 1.6× to 16× and a field of view ranging from 6.9 to 0.69 mm. The optics and anti-reflective coatings ensure optimal imaging of multiplexed fluorescent reporters in small animals. High-resolution images were captured directly on a PC (Fujitsu Siemens, Munich, Germany). Images were processed for contrast and brightness and analyzed with the use of Paint Shop Pro 8 and Cell^R (Olympus Biosystems) (23).

3. RESULTS AND DISCUSSION

Salmonella typhimurium A1 grows in tumor xenografts. In sharp contrast, normal tissue is cleared of these bacteria even in immunodeficient athymic mice. *S. typhimurium* A1 is auxotrophic (Leu/Arg-dependent) but apparently receives sufficient support from the neoplastic tissue to grow locally. Whether additional genetic lesions are present is not known. In *in vitro* infection, the GFP-expressing bacteria grew in the cytoplasm of PC-3 human prostate cancer cells and caused nuclear destruction. These effects were visualized in cells labeled with GFP in the nucleus and red fluorescent protein in the cytoplasm. *In vivo*, the bacteria caused tumor inhibition and regression of xenografts visualized by whole-body imaging. The bacteria, introduced *i.v.* or intratumorally, invaded and replicated intracellularly in PC-3 prostate cancer cells labeled with red fluorescent protein grafted into nude mice. By day 15, *S. typhimurium* A1 was undetectable in the liver, lung, spleen, and kidney, but it continued to proliferate in the PC-3 tumor, which stopped growing. When the bacteria were injected intratumorally, the tumor completely regressed by day 20. There were no obvious adverse effects on the host when the bacteria were injected by either route. The *S. typhimurium* A1 strain grew throughout the tumor, including viable malignant tissue. This result is in marked contrast to bacteria previously tried for cancer therapy that were confined to necrotic areas of the tumor, which may account, in part, for the strain's unique antitumor efficacy (1).

In order to increase tumor-targeting capability of A1, the strain was reisolated after infection of a human colon tumor

growing in nude mice. The tumor-isolated strain, termed A1-R, had increased targeting for tumor cells *in vivo* as well as *in vitro* compared with A1. Treatment with A1-R resulted in highly effective tumor targeting, including viable tumor tissue and significant tumor shrinkage in mice with s.c. or orthotopic human breast cancer xenografts. Survival of the treated animals was significantly prolonged. Forty percent of treated mice were cured completely and survived as long as non-tumor-bearing mice. These results suggest that amino acid auxotrophic virulent bacteria, which selectively infect and attack viable tumor tissue, are a promising approach to cancer therapy (20).

A1-R was used to treat metastatic PC-3 human prostate tumors that had been orthotopically implanted in nude mice. GFP was used to image tumor and metastatic growth. Of the 10 mice with the PC-3 tumors that were injected weekly with *S. typhimurium* A1-R, 7 were alive and well at the time the last untreated mouse died. Four A1-R-treated mice remain alive and well 6 months after implantation. Ten additional nontumor-bearing mice were injected weekly to determine the toxicity of *S. typhimurium* A1-R. No toxic effects were observed. The approach described here, where bacterial monotherapy effectively treats metastatic prostate tumors, is a significant improvement over previous bacterial tumor-therapy strategies that require combination with toxic chemotherapy (24).

S. typhimurium A1-R-GFP was administered to both axillary lymph and popliteal lymph node metastasis of human pancreatic cancer and fibrosarcoma, respectively, as well as lung metastasis of the fibrosarcoma in nude mice. The bacteria were delivered via a lymphatic channel to target the lymph-node metastases and systemically via the tail vein to target the lung metastasis. The cancer cells expressed red fluorescent protein (RFP) in the cytoplasm and GFP in the nucleus linked to histone H2B, enabling color-coded real-time imaging of the bacteria targeting the metastatic tumors. After 7-21 days of treatment, the metastases were cured without the need of chemotherapy or any other treatment. No adverse effects were observed. This new strategy demonstrates the clinical potential of targeting and curing cancer metastasis with engineered bacteria without the need of toxic chemotherapy (Hayashi, K., Zhao, M., Hoffman, R.M., unpublished data).

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