Sparse Initial Entrapment of Systemically Injected *Salmonella typhimurium* Leads to Heterogeneous Accumulation within Tumors

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Abstract

Blood-borne therapeutics, which rely on diffusion and convection for delivery, often do not accumulate in effective concentrations distant from vasculature and are therefore unable to eradicate all cells within a tumor. Motile bacteria have the potential to overcome the diffusion and pressure gradients that prevent passive materials from penetrating into poorly perfused regions of tumors. Here, we test several proposed mechanisms of *Salmonella typhimurium* accumulation in tumors, including: (a) entrapment in the chaotic vasculature of tumors; (b) attraction to specific tumor microenvironments; and (c) preferential replication within specific microenvironments. After systemic injection of *S. typhimurium* into tumor-bearing mice, we used intravital microscopy and histological techniques to quantify their interaction with tumor vasculature. Immediately after injection, few *S. typhimurium* (<4 in 10,000) adhered to tumor vasculature; most remained passively suspended in the blood. Despite this low initial adhesion, ~10,000-fold more *S. typhimurium* accumulated in tumors than any other organ 1 week after the injection, thus demonstrating their specificity. However, within the tumors, we found that most bacteria were located in necrotic tissue as large colonies far (750 μm) from functional vasculature. Together, these results suggest that *S. typhimurium* has limited ability to adhere to tumor vasculature and migrate within tumors and only survives in tissue that becomes necrotic. Although *S. typhimurium* is a promising delivery vehicle because of its tumor specificity, increasing its intra-tumoral motility should improve its therapeutic effectiveness.

Introduction

Bacteria have been investigated as therapeutic agents for tumors for ~150 years (1). A resurgence of interest in nonpathogenic bacteria as drug delivery vehicles (2-5) and tumoricidal agents (6, 7) has been induced by recent advances in molecular biology, including removal of their toxic genes (7-9) and complete sequencing of their genomes (10, 11). These microbes also have the potential to overcome many of the delivery barriers that hinder conventional chemotherapeutics (12).

Recently, Low et al. (8, 13) developed a strain of *Salmonella typhimurium* (VNP20009) that is nonpathogenic in mice, pigs, and humans and accumulates 2000-fold more in tumors than in other organs. However, this strain was not successful at slowing tumor growth in the clinic (13), which prompts the question of how these bacteria are delivered to and specifically replicate within tumors. Obligate anaerobes (e.g., clostridium and bifidobacterium) target the necrotic region of tumors because they can only replicate in the oxygen-free environment found there (2, 7, 14). However, salmonella are facultative anaerobes, able to live in both the presence and absence of oxygen.

Materials and Methods

**Bacterial Culture.** Three strains of *S. typhimurium* were grown in LB\(^4\) broth and on agar plates using standard procedures. SL1344 and the GFP-expressing pSMC21 were a kind gift of Dr. Fred Ausubel, Massachusetts General Hospital and Harvard Medical School; SL7207 was a kind gift of Dr. Bruce Stocker, Stanford Medical School; SL7207 was provided by Vion Corp. (New Haven, CT). SL7207 is an *Aro*\(^{-}\) mutant that is less pathogenic in mice than wild-type SL1344 (16) and is currently being investigated as a tumoricidal agent (5). VNP20009 is a *msb*\(^{B}\) and *pur*\(^{T}\) mutant that was specifically developed as a nonpathogenic, tumoricidal agent (8).

Both SL7207 and VNP 20009 were transfected with pSMC21 by electroporation: 25 μF, 400 Ω, and 2.4 kV, using 0.2 cm cuvettes. Before transfaction, all strains were immediately frozen in LB with 10% glycerol and subsequently maintained in culture with 250 μg/ml kanamycin.

Before injection, all strains were grown overnight in LB from single colonies on fresh agar plates, subcultured 1 in 10, grown to an OD\(_{600}\) of 0.5–0.7, centrifuged at 3700 rpm (3200 \( \times \) g) for 10 min, and resuspended in sterile PBS.

**Mammalian Cell Culture.** LS174T colon carcinoma cells were grown at 37°C, 5% CO\(_2\) in DMEM with 10% fetal bovine serum. Tumor cylinders were formed by constraining spheroids between two parallel horizontal surfaces with a defined spacing of 150 μm (Fig. 1A). Cell aggregates were grown in tissue culture flasks coated with 0.5 mg/cm\(^2\) methacrylate (polyheme) for 1 week to form spheroids. Individual spheroids, 300–400 μm in diameter, were then transferred to 96-well plates, and a polycarbonate lid with protruding cylindrical pins (±25 μm in length and 3 mm in diameter) were lowered onto...
the spheroids (Fig. 1B). Cylindroids were allowed to equilibrate for 24 h before injection of bacteria into the culture medium (final concentration of 1000 CFU/ml) through holes in the polycarbonate lid. Intratumor cylindroid invasion was then monitored microscopically for 24 h.

**Animal Model.** Dorsal skinfold chambers were surgically implanted into either SCID mice or immuno-competent C3H mice (male, ~30 grams of body weight), under anesthesia (90 mg of ketamine/10 mg of xylazine per kg body weight), as described previously (15). After a 2-day recovery period, small pieces (~1 mm³) of MCaIV murine mammary carcinomas were implanted into the chambers. Tumors were monitored through glass coverslips in the dorsal skin fold chambers (15). Intravital bacterial delivery experiments began once the tumors reached an en face diameter of 4–7 mm. All procedures were carried out following the Public Health Service Policy on Humane Care of Laboratory Animals and approved by the Institutional Animal Care and Use Committee.

**Intravital Microscopy (Measurement of Bacterial Delivery in Vivo).** Five min before bacteria injection, mice were anesthetized (90 mg of ketamine/10 mg of xylazine/kg body weight) and injected with 0.05 ml of 5 mg/ml rhodamine dextran (2 million molecular weight; Molecular Probes, Eugene, OR) to identify functional vasculature as described previously (17).

Anesthetized mice were restrained and placed on an upright microscope (Zeiss, Göttingen) equipped with an automated stage (Burleigh, Fischer, NY). Four to seven vascularized regions were identified in each tumor depending on their size and extent of vascularization. The automated stage was programmed to return to these locations and cycle between them every 20 or 30 s. Images were acquired through a 20X objective, using a FITC filter set at video-rate using a Hamamatsu CCD camera and a Panasonic S-VHS video tape recorder. After one complete cycle was recorded, bacteria were injected i.v. at either 2 or 20 × 10⁶ CFU/mouse suspended in 0.2 ml of sterile PBS.

**Quantification of Bacterial Accumulation.** One week after bacteria injection, the mice were anesthetized and injected with biotinylated Lycopersicon esculentum (tomato) lectin (5 mg/kg; Vector Laboratories, Burlingame, CA) to identify functional vasculature. After 5 min, the mice were euthanized, and the tumors were extracted and cut in half. One-half of the tumor was fixed in 4% paraformaldehyde and embedded in paraffin. The other half of the tumor and five other extracted organs (liver, spleen, lungs, heart, and abdominal skin) were weighed, minced with scissors, and suspended in sterile PBS. The minced organ suspension was serially diluted and plated on LB agar. Bacterial colonies were counted after overnight incubation at 37°C.

The embedded half of the tumor was cut into 5-µm serial sections. Biotinylated lectins bound to the vasculature were conjugated with Avidin (Vectorstain Elite ABC kit) and subsequently visualized with diaminobenzidine according to the manufacturer’s recommendation. Successive sections were Brown-Hopps (Gram’s stain) stained to identify Gram-negative bacteria.

**Statistics.** Statistical significance was determined using ANOVA and Fisher’s post hoc test. The significance in the slope for the bacterial flux to adhesion comparison was determined using the F test for linear regression.

**Results**

**Invasion of S. typhimurium into a Three-dimensional Tumor Model in Vitro.** A three-dimensional tumor model (tumor cylindroids; Fig. 1) was specifically created to observe the invasion of bacteria into tumor tissue. Cylindroids have similar geometric properties to tumor spheroids (18); they contain rapidly proliferating cells in the outer layer, necrotic centers, and a quiescent boundary region (data not shown). However, cylindroids have optically accessible cores and are therefore better suited for microscopically observing the penetration of S. typhimurium deep into tissue (>100 µm). In addition, because the interior of cylindroids can be monitored in real time, bacterial motion can be observed within a growing mass of cancer cells.

Fig. 1, C and D show the extent that S. typhimurium invaded into a cylindroid in 14 h. Rapidly swimming S. typhimuricia were clearly capable of penetrating into tumor cylindroids. During this experiment, bacteria were observed “burrowing” between tumor cells by lining up with the intercellular space and propelling themselves forward (data not shown). These observations demonstrate the ability of S. typhimurium to penetrate into a solid mass of tumor tissue and imply that S. typhimurium is capable of redistributing within tumors in vivo.

**Tumor Specificity and Dose Dependence of S. Typhimurium Accumulation in Vivo.** Fig. 2 shows the accumulation of S. typhimurium (strain VNP20009) in the organs of mice bearing MCaIV tumors, 1 week after injection. Significantly (>10,000-fold) more bacteria (P < 0.0002) accumulated in the tumor than any other organ for C3H mice injected 2 million CFU (Fig. 2, A and B). The spleen...
The ratio of bacterial accumulation in the tumor to accumulation in the spleen (generally the organ with the greatest accumulation) was strongly dependent on dose (Fig. 2B). Surprisingly, mice injected with 20 million CFU accumulated similar numbers of bacteria in tumors compared with the mice injected with 2 million CFU. On the other hand, bacterial accumulation in the other organs increased with dose escalation (Fig. 2A). This phenomenon was observed in both immunocompetent C3H mice and immunodeficient SCID mice, indicating that it is not dependent on a fully functional immune system. Either a maximum bacterial growth rate is achieved in tumors or a threshold for clearance is crossed when bacteria can no longer be cleared from normal organs. Low et al. (8) also observed that only a narrow dose range could produce significantly large accumulation ratios.

The higher bacteria dose affected the health of the SCID mice; four of five SCID mice did not survive injections of 20 million CFU. The liver of the surviving mouse (Fig. 2C) had obvious infarcts, possibly caused by ischemia as a result of bacterial occlusion or aggregates of leukocytes and platelets activated in the presence of the bacteria. The higher dose induced splenomegaly (Fig. 2D) in both strains of mice. However, the higher dose did not appear to affect the health of the C3H immunocompetent mice; all survived and were normally active 1 week after the injection.

The accumulation of S. typhimurium strain SL7207, which is also currently being investigated as a tumoricidal agent (5), was greater in normal organs than the tumors (Fig. 2E). However, SL7207 detrimentally affected the health of the mice. Every mouse had splenomegaly, infarcted livers (similar to Fig. 2C), and pungent ascites 1 week after injection with 2 million CFU.

**Sparse Adhesion of Bacteria to Tumor Vasculature.** The delivery of S. typhimurium into McAv tumors was observed using intravital microscopy (Fig. 3). After an injection of 20 million CFU, bacteria could clearly be seen flowing in the blood stream in each observed tumor location (Fig. 3A). Numerous bacteria were observed (>3.6 bacteria/sec, maximum 9.2 bacteria/sec) in each 0.59 × 0.44 mm field of view. After an injection of 2 million CFU, 10 times fewer bacteria were observed in each location. To determine adhesion events, 20 million CFU bacteria were injected.

No bacteria adhered to the walls of vessels with high flow rates (defined as >~1.5 mm/sec) in any of the 14 mice observed. In vessels with lower flow rates, occasional interactions occurred. Fig. 3, B–E show a bacterium adhering; it enters via a small vessel on top of the larger vessels that are more clearly visible in these figures. During 1 h of observation, no qualitative drop in bacterial flux into each field of view was detected. However, no bacteria were detectable in the blood 24 h after injection.

The appearance and disappearance of 106 adherent bacteria were tracked in 23 locations of four mice (Fig. 3F). Only bacteria that adhered for >2 min were tracked. Each either dislodged, slowly disappeared, or persisted until the end of observation. Fluorescence of the bacteria may slowly disappear because of photobleaching, bacterial migration, endocytosis, reduced GFP expression, or GFP diffusion through bacterial membranes with increased permeability. Total flux of bacteria into a location was determined by counting all bacteria that entered the field of view over a given period of time. This overall flux represents the sum of all bacterial fluxes through each of the vessels visible in the field. Only 0.035 ± 0.015% (or <4 in 10,000) of the bacteria that flowed into the tumors permanently adhered (defined as disappearing or persistent bacteria observed for >12 min).

On a per field basis, the number of adherent bacteria per time (expressed as rate of adhesion) increased with an increasing overall bacterial flux (Fig. 3G; P < 0.002). In other words, the more bacteria that flowed into a location, the more that adhered. The dependence in Fig. 3G coupled with the observation that bacteria do not adhere to

accumulated more bacteria than the other organs for most of the mice (two had slightly more in the liver). This supports the findings of Low et al. (8), who report a tumor accumulation ratio >2000 for this strain of S. typhimurium in mice.
rapidly flowing vessels suggests that bacteria will preferentially adhere (in terms of bacteria per volume) in tissue that contains numerous slow and tortuous vessels, i.e., tumors. Note that the average residence time of dislodged bacteria was 7.6 min.

Colonization of S. Typhimurium in the Necrotic Regions of Tumors. The location of bacteria that had accumulated in the tumors was determined by Brown-Hopps staining of transverse sections, cut orthogonally from the coverslip in the dorsal-skin-fold chamber (Fig. 4). The same mice were used for intravital microscopy, quantification of bacterial accumulation, and histological sectioning. For each of the sections, ×100 magnification was used to locate bacteria and identify whether they were in colonies or spread sparsely (Fig. 4, A–C). Biotinylated lectin perfusion stained only vessels with active blood flow, allowing identification of functional vasculature. (Fig. 4E).

No bacteria were found in the living tumor tissue of any of the mice; they were only present in the necrotic regions (Fig. 4, A–C). Bacteria could not be found in smaller tumors that lacked necrosis. Most of the bacteria were found in large colonies (Fig. 4D) that occupied a small percentage of the total tumor volume (the sections shown in Fig. 4, A–C contained more bacteria than the sections from the other mice). The average distance between the colonies and the functional vasculature was 750 ± 40 μm with a minimum distance of 100 μm.

Discussion

S. Typhimurium Localization to Tumor Necrosis. In our experiments, S. typhimurium were injected into tumor-bearing mice systemically. These bacteria were observed flowing through tumor vasculature and adhering sparsely (0.035%; Fig. 3). After 1 week, the bacteria had specifically accumulated within the necrotic regions of the tumors, significantly distant from functional vasculature (Figs. 2 and 4).

There are three possible mechanisms to explain this localization to tumor necrosis. Either (a) S. typhimurium are specifically attracted to necrotic tissue and migrate there from the vasculature through living tumor tissue; (b) S. typhimurium adhere sparsely on tumor vasculature, migrate a short distance outside the vessels but only successfully colonize tissue that becomes necrotic; in this case, either the necrotic microenvironment is advantageous for growth or these regions provide shelter from the immune system; or (c) S. typhimurium occlude tumor vasculature in a similar fashion to the liver infarcts in Fig. 2C. Thus, the bacteria never need to leave the blood vessel but survive and colonize in these occluded/dying vessels in tissue soon to become necrotic. This would explain the large distance from functional vasculature and is the subject of future experimentation.

Entropically, it seems implausible that bacteria would migrate to necrotic tissue and congregate in colonies such as those in Fig. 4. If they were attracted to components in necrotic tissue and freely motile, they would most likely spread out to more uniformly consume these components. The stochastic nature of bacterial chemotaxis implies that bacteria migrate toward and consume chemo-attractant, moving on to new sources once consumed, thereby resulting in a more homogeneous distribution than observed. The fact that this did not appear to happen suggests that S. typhimurium have limited motility within the tumor, instead surviving only in tissue that becomes necrotic after injection (mechanisms II and III).

S. Typhimurium as a Future Therapeutic. Previously, we suggested the properties of an ideal anticancer bacterium (1). An ideal bacterium would be: (a) nontoxic to the host; (b) only able to replicate within the tumor; (c) motile and able to disperse evenly throughout a tumor; (d) slowly and completely eliminated from the host; (e) non-immunogenic; and (f) able to cause lysis of tumor cells. Our results suggest that the biggest shortcoming of S. typhimurium is its inability to disperse throughout the tumor. Dang et al. (7) dramatically dem-
onstrated the importance of this capability by selecting a strain of clostridium (C. novyi) able to disperse evenly and eradicating tumors in mice by combining its administration with chemotherapy. An additional strategy would involve modifying the tumor matrix that would facilitate the motility and penetration of the bacteria (19, 20).

Regardless, S. typhimurium has a couple of advantages over the obligate anaerobe C. novyi. Being a facultative anaerobe, it can be injected active and motile, potentially increasing its tumor specificity. Being a Gram-negative enteric bacterium, similar to Escherichia coli, it is far easier to genetically manipulate than any obligate anaerobe.

S. typhimurium was found to aggressively penetrate into three-dimensional cultured tumor tissue in vitro. Intravital microscopy demonstrated that <0.04% of S. typhimurium that flow into tumors are able to adhere to tumor vasculature. Subsequently, S. typhimurium accumulates only in necrotic regions of tumors that are distant from tumor vasculature. These results may explain why tumors were not eradicated in mouse models treated with S. typhimurium and the limited success of preliminary clinical trials. Additionally, these results help define the obstacles necessary to overcome for a successful bacteriolytic therapy. An engineered strain of S. typhimurium able to disperse more homogeneously throughout a tumor would be a more successful drug delivery or tumoricidal agent. This could potentially be accomplished by genetically inducing matrix-degrading enzymes and/or enhancement of intrinsic motility machinery (19).

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References

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