Engineering bacteria toward tumor targeting for cancer treatment: current state
and perspectives

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Abstract

One of the primary limitations of cancer therapy is lack of selectivity of therapeutic agents to tumor cells. Current efforts are focused on discovering and developing anticancer agents that selectively target only tumor cells but spare normal cells to improve the therapeutic index. The use of preferentially replicating bacteria as an oncolytic agent is one of the innovative approaches for the treatment of cancer. This is based on the observation that some obligate or facultative anaerobic bacteria are capable of multiplying selectively in tumors and inhibiting their growth. Meanwhile, bacteria have been demonstrated to colonize and destroy tumor, and emerged as biological gene vectors to tumor microenvironment. To improve the efficacy and safety of the bacterial therapy, a further understanding of bacteria between with immune system is required. Furthermore, we want to evaluate how bacterial infection facilitates the “bystander effect” of chemotherapeutic agent and assess that it can be used for additional the antitumor effect when combined with chemotherapy. This study may not only evaluate therapeutic efficacy of bacteria for the treatment of cancer, but also elucidate the mechanisms underlying antitumor activities mediated by bacteria, which involve host immune responses and the cellular molecular responses.

Keywords: Bacteria; oncolytic therapy; tumor-targeting; immunity
Introduction

The problem with cancer therapy is selectivity and specificity. It is relatively easy to induce cell death \textit{in vitro}. However, in order to only induce tumor cell death \textit{in vivo} but spare normal cells, a very selective strategy is necessary. The effective current therapies, such as ionizing radiation, rely on spatial delivery and the increased proliferation rate of tumors in order to differentiate from normal tissue. Some cancer therapeutic strategies rely on the same principle, but other means of selectively targeting tumor cells have also been developed which will add to tumor specificity. Advances in cancer therapy have been made using various methods, but effective and selective delivery of therapeutic agents to tumor sites remains a complex task due to a poor and disorganized blood supply, and high interstitial fluid pressure. Recently, bacteria can be used as tools for treating cancer. Using bacteria as an antitumor agent is not a new idea. The first patient with cancer to be purposefully infected with bacteria was probably treated by German physician W Bush in 1868 (Pawelek et al. 2003). Almost 30 years later, William B Coley, a young surgeon at New York Hospital, encountered a patient with cancer who seemed to be cured by a severe \textit{Streptococcus pyogenes} infection (Pawelek et al. 2003). This observation led Coley to begin treating cancer patients with living bacteria.

Tumor microenvironment

Solid tumors are seldom homogeneous, but instead, they have different
microenvironments that can profoundly influence therapeutic approaches. Perhaps the best-studied component of tumor microenvironment is the presence of low oxygen tension, or hypoxia (Brown and Giaccia 1998). Aggressive tumors often have insufficient blood supply, partly because tumor cells grow faster than the endothelial cells that make up the blood vessels, and partly because the newly formed vascular supply is disorganized (Folkman et al. 1989). This results in acidity and nutrient deprivation, as well as regions with reduced oxygen concentrations. Direct measurements taken in patients showed median oxygen level in normal tissue of 24 to 66 mmHg (3.1 to 8.7% O₂), whereas those in tumors ranged from 10 to 30 mmHg (1.3 to 3.9% O₂). More importantly tumors posse microenvironment with oxygen concentrations of less than 2.5 mmHg (0.3% O₂), levels at which cells are three times more resistant to radiation than aerated cells (Vaupel et al. 1989). The hypoxic regions of tumors are less sensitive to ionizing radiation because its effects depend on oxygen; they are also less sensitive to chemotherapeutic agents because drugs delivered to these regions may be suboptimal (Brown and Giaccia 1998). The hypoxic areas of tumors are poorly vascular supply, and are not treated easily with conventional anticancer agents. Hypoxia is also associated with a more malignant phenotype, such as genomic stability, apoptosis, angiogenesis, and metastasis (Graeber et al. 1996). Hence, hypoxia is considered a major hindrance to therapy. As hypoxia is a common
characteristic of human tumors, which adversely affects the prognosis of patients with cancer, targeting hypoxia may be an effective means of improving cancer treatment. Some anaerobic and facultative anaerobic bacteria have been used experimentally as anticancer agents because of their selective growth in the hypoxic/necrotic regions of solid tumors after systemic administration. Bacteria can actively move away from the vasculature and penetrate into necrotic region of tumors. Bacteria have several advantages for cancer therapy (Fig. 1). We see bacterial therapy as the most innovative and flexible method to modify the tumor microenvironment.

**Tumor targeting bacteria**

One of strong points of bacterial therapy is the ability to specifically target to tumor sites. In 1964, a series of reports described the use of nonpathogenic *Clostridia* in experimental tumor models (Engelbart and Gericke 1964; Thiele et al. 1964). The rationale for using *Clostridium* is an obligate anaerobic bacterium. Therefore, when injected into a body, the spores replicate and develop only in hypoxic regions. In hosts with advanced cancer, these hypoxic regions can be found and *Clostridium* is presumed to develop and proliferate in these oxygen-poor areas, while being absent from well oxygenated healthy tissues. Moreover, this selective targeting is associated with death of tumor cells. Not all spore-forming bacteria are effective and the spore-forming microorganisms *Bacillus mesentericus* and *Bacillus subtilis* do not
produce oncolysis. These results indicate that although the obligate anaerobic phenotype of *Clostridium* is probably the basis for their ability to specifically target necrotic areas of tumors, other factors may be involved. Although promising, the strategy shows major limitations. First, oncolytic effect is restricted to large, well-established tumors but is undetectable in smaller metastatic nodules, probably because of lacking hypoxic regions in these lesions. Second, *Clostridium*-dependent lysis is found in the center of large tumors, leaving the liquid necrotic center surrounded by a better oxygenated layer of malignant cells that constitute the seed for re-growth of tumor. Finally, some toxicity is observed in preclinical mouse models. Fox *et al.* placed the *Escherichia coli* cytosine deaminase gene into *Clostridium beijerincki* by using a *Clostridium* expression vector, and produced an increased cytosine deaminase activity in extracts of the transformed bacteria (Fox *et al.* 1996). Recent *in vivo* studies of the use of *Clostridia* as tumor vectors have focused on their potential in gene therapy and controlled gene expression by use of radioinducible promoters (Nuyts *et al.* 2001) (Table 1). Another group investigating *Clostridium* in combination with chemotherapy has reported significantly antitumor activity (Dang *et al.* 2001). Many years after the first injection of *Clostridium* spores into tumors, various advances have shown promise for *Clostridium* as tumor-targeting therapeutic vector.
Bifidobacterium is also obligate anaerobic bacterium that has been found to colonize large tumors, because the anaerobic environment is present in parts of large tumors. In contrast to Clostridia, Bifidobacteria are non-pathogenic, non-spore-forming, and find naturally in the digestive tract of humans and some other mammals, and therefore may be safe live bacterial agents to use in the treatment of tumors. In the first of these tumor-targeting studies, Bifidobacterium bifidum was injected into the tail vein and bacteria were assisted by intraperitoneal injections of lactulose. The addition of lactulose, a sugar substrate that is metabolized by bacteria but not by mammalian cells, increased the growth and survival of bacteria within tumors by 1000 times compared with a saline control (Kimura et al. 1980). Subsequent work showed that Bifidobacterium longum also targets carcinogen-induced mammary tumors in rats (Yazawa et al. 2001). Bifidobacterium can also deliver effort genes to tumor sites. Bifidobacterium adolescentis transformed with plasmids encoding endostatin gene, targeted to liver tumor in mouse models (Li et al. 2003). Recently, Cronin et al. also demonstrated that using oral feeding Bifidobacteria resulted in the colonization of tumors (Cronin et al. 2010). Overall, Bifidobacterium can be used to deliver plasmid-encoded antitumor genes and it joins the live bacteria which are potential tumor targeting antitumor vectors (Table 1).

Salmonella are gram-negative, facultative anaerobes that are a common cause of
intestinal infections. Owing to substantial immunostimulation produced by

*Salmonella* lipopolysaccharide (LPS) and other components, systemic infection with

*Salmonella* induce septic shock and high mortality in mammalian. However, the

virulence of *Salmonella* is attenuated in some mutations. It was reported that

attenuated *Salmonella* injected into tumor-bearing mice would preferentially replicate

within tumors, achieving tumor to normal tissue ratios exceeding between 250:1 and

1000:1, depending on the tumor models (Pawelek et al. 1997; Sznol et al. 2000).

Furthermore, this accumulation is accompanied by delaying the growth of tumor.

*Salmonella* grow under both aerobic and anaerobic conditions so they are able to

colonize small metastatic tumors as well as larger tumors. Attenuated *Salmonella*

hinder tumor growth in a broad range of human and mouse tumor. Tumor growth is

inhibited for long periods, even up to several weeks. These observations, couple with

ease of genetic manipulation, suggested that *Salmonella* are good candidates for

therapeutic anticancer agents, and genetically engineered *Salmonella* have been

developed to express therapeutic genes encoding angiogenic inhibitors (Lee et al.

2004; Lee et al. 2005a), or prodrug-converting enzymes (King I et al. 2002; Pawelek

et al. 1997), aiming to enhance their oncolytic effects. Furthermore, *Salmonella*

accumulated in not only subcutaneous but also orthotopic tumors after systemic

administration (Lee et al. 2008a). VNP20009, an attenuated strain of *S. typhimurium*
(Clairmont et al. 2000), and its derivative, TAPET-CD, which expresses an *E. coli* cytosine deaminase (CD) (Toso et al. 2002), have been evaluated as anticancer agents in clinical trials for cancer patients (Nemunaitis et al. 2003; Toso et al. 2002) (Table 1). VNP20009 is modified from a wild-type strain of *S. typhimurium* by partial deletion of the *msbB* gene, which results in the generation of a lipid A mutant with diminished ability to induce TNF-α-mediated septic shock (Low et al. 1999). It is also a purine auxotroph and cannot metabolize xylose. The targeting and replication of VNP20009 expressing herpes simplex virus thymidine kinase in murine tumors *in vivo* have been noninvasively detected by positron emission tomography (PET) imaging (Soghomonyan et al. 2005). *Salmonella* expressing cytokines exhibited the ability to modulate host immunity and retarded tumor growth (Sorenson et al. 2008). On the contrary, silencing immunosuppressive molecules in tumors can also inhibit tumor growth (Manuel et al. 2011). Previous studies had shown that systemically administered *Salmonella* is present predominantly in the necrotic regions of tumors, but only detected scarcely in the cytoplasm of living tumor cells (Forbes et al. 2003). It appears that *Salmonella* have limited ability to adhere to tumor vasculature and migrate within tumors, and only survives in tissue that becomes necrotic. In this case, either the necrotic microenvironment is advantageous for its growth or tumor microenvironment is an immune privileged site, which provides protection against the
host immune system. Yu et al. demonstrated the real-time visualization of localization, survival, and replication of engineered bacteria in implanted tumors in live animals (Yu et al. 2004). They proposed that a small number of bacteria may enter tumors through leaky vasculature, thereby escaping the host’s immune surveillance and finding sanctuary in the tumor tissues.

Immunity and bacteria

The immune response plays a special role in tumor microenvironment: it can not only promote tumor progression but also select tumor cells that are more aggressive. Tumors develop several immune evasion strategies that allow the generation of more aggressive tumor. The colonization of bacteria in tumor sites may destroy the immunosuppressive phenotype of tumor microenvironment. Bacteria are potent adjuvants that induce antitumor immunity by activating immune cells in host. The host immune responsiveness is highly complex after administration bacteria. Previously, it was detected strong IFN-γ production in the tumors derived from mice after bacterial treatment (Lee et al. 2008b). Bacteria within tumors may induce inflammatory responses, leading to the recruitment of immune cells, such as macrophages, neutrophils, and lymphocytes to the tumor site. Bacterial replication and lysis of tumor cells may induce cell-mediated immune responses to tumor cells, higher oncolysis could account, in part, for an increased infiltrate of CD8+ T cells in
bacteria-treated tumors. The cytotoxic T cell response against tumor cells may
enhance the antitumor efficacy of bacteria (Lee et al. 2011). Antitumor effects of
neutrophils, in particular, after being activated by substances derived from
microorganisms have also been demonstrated in tumors (Westphal et al. 2008).
Previously, we demonstrated that TLR4 signaling is involved in the antitumor effector
of Salmonella (Lee et al. 2008a). Furthermore, Salmonella induced IFN-γ production
and polarized the T cell response to a Th1-dominant state in wild-type mice, but not in
CD4⁺ T cell deficient mice. Thus, bacteria-activated CD4⁺ T cell infiltrating tumor
may be a relevant source of IFN-γ in the tumor microenvironment and may contribute,
a least in part, to the host antitumor immunity induced by Salmonella. Meanwhile, the
accumulation of Salmonella in tumor sites provoked a potent inflammatory response,
which recruited large numbers of immune cells. IFN-dependent chemokines, such as
IFN-inducible chemokines CXCL9 (MIG), and CXCL10 (IP-10) (Lee et al. 2008a),
induced by Salmonella are expected to recruit effector cells to tumor. Actuality, it was
found a large number of infiltrating immune cells such as macrophages, neutrophils
within tumor microenvironment. Recently, the CD8⁺ T cell immune response induced
by Salmonella reported by Saccheri observed antimicrobial response present in
tumors to activate cytotoxic CD8⁺ T cells via gap junction protein (Saccheri et al.
2010). Bacteria act both locally, by recruiting T cells that inhibit tumor growth, and
systemically, where bacteria provide the development of immune response via the cross-presentation of tumor antigen (Saccheri et al. 2010). Bacteria can also be modified to express cytokines that have antitumor activity. Oral administration of *Salmonella* expressing interleukin-2 had been shown to increase the systemic natural killer cell populations (Sorenson et al. 2008).

**Administration route**

Some studies have been demonstrated several factors significantly influenced the tumor colonization of bacteria. It was noticed that different bacterial administration routes could affect bacterial colonization. Mei *et al.* showed that intraperitoneal injection of bacteria resulted in less tumor colonization compared to intravenous injection (Mei *et al.* 2002). Previously, we also examined whether bacteria could target the untreated tumor when injected intratumorally into one of the bilaterally implanted tumors. These results indicated that bacteria, administered via either intratumoral or systemic route, were able to accumulate in the tumors at distant sites, and as a consequence, conferred contralateral antitumor effect (Lee *et al.* 2005b). Thus, this tumor-targeting property of bacteria provides impetus to explore its use in inhibiting tumor growth at distant sites. Meanwhile, In addition, oral administration of *Salmonella* still showed antitumor effects and reduced toxicity (Chen *et al.* 2009).

**Bacteria induce the death of tumor cell**
We and other groups demonstrated that the induction of apoptosis in the tumor sites was correlated with bacterial accumulation (Lee et al. 2005b; Ganai et al. 2011). Bacteria in tumor induced apoptosis by multiple mechanisms including competition for nutrients, stimulation of immune response. Moreover, LPS from *Salmonella* may induce the apoptosis of tumor and endothelial cells. As bacterial replication in tumors and subsequent lysis of tumor cells may induce cell-mediated immune responses to tumor cells, higher oncolysis could account, in part, for an increased infiltrate of immune cells in tumors (Figure 2). The cells undergoing bacteria-induced cell death exhibit heterogeneous morphological features (Chen et al. 1996; Boise and Collins 2001). It is clear that more than one mechanism is involved in the bacteria-induced killing of cells (Hernandez et al. 2003). Autophagy is a cellular process that mediates the degradation of long-lived proteins and unwanted organelles in the cytosol. Autophagy pathway interacts with intracellular bacteria in a variety of ways (Kirkegaard et al. 2004). Malignant cells frequently display lower levels of basal autophagic activity than their normal counterparts and fail to increase autophagic activity in response to stresses. Autophagy is involved in the cell defense elimination of bacteria. The signaling pathways leading to activation of bacteria-induced autophagy in tumor cells remain to be elucidated. To date, a possible interaction of bacteria with tumor cells has not been examined. The bacteria in
controlling tumor growth may induce autophagy signal pathway.

**Combination therapy**

The primary limitation of cancer therapy is lack of selectivity of therapeutic agents to tumor cells. Current efforts are focused on discovering and developing anticancer agents that selectively target only tumor cells but spare normal cells to improve the therapeutic index. The use of preferentially replicating bacteria as an oncolytic agent is one of the innovative approaches for the treatment of cancer. *Salmonella*, a facultative anaerobe, has been employed as an antitumor agent that is capable of preferentially amplifying within tumors and inhibiting their growth. Furthermore, *S. typhimurium* has been demonstrated to grow in the necrotic and relatively hypoxic foci within tumors, but not in well-oxygenated tumors at the rim of the growing nodules (Rosenberg et al. 2002). The limited ability of *S. typhimurium* to disperse throughout the tumor may be the most important shortcoming in its use as an anticancer agent. Regarding the importance of the dispersal capability of bacteria for oncolytic activity, Dang *et al.* showed that *Clostridium novyi*, an obligate anaerobe, is able to disperse evenly and eradicate tumors in mice when combined with anti-vascular agent (Dang *et al.* 2001). Therefore, a genetically modified *Salmonella* strain capable of dispersing more homogeneously throughout tumors would be a more desirable tumoricidal agents or transgene vectors. Our previous data suggested that
the capability of *Salmonella* to disperse within tumors and hence to delay tumor growth was augmented when combined with low-dose cisplatin (Lee et al. 2005b).

Meanwhile, the combination of *Salmonella* with low-dose radiotherapy dampened tumor immune escape mechanism (Avogadri et al. 2008). Additional strategy to enhance antitumor efficacy would involve inhibiting the viable rim of tumor growth or modifying the tumor matrix that would facilitate the motility and penetration of tumor-targeting bacteria.

**Further perspectives**

Bacteria are a powerful antitumor agent due to its tumor-targeting potential, antitumor capability, and ability to deliver therapeutic gene. Host factors including innate and adaptive immune responses play roles in bacteria-induced antitumor activity. In phase I study, patients received VNP20009 that rapidly cleared from blood, and most tumor were not detectable the colonization of *Salmonella*. Patient had pre-existing anti-*Salmonella* antibodies and was not accompanied the colonization of *Salmonella* in the tumor sites after systemic administration (Toso et al. 2002). In agreement with clinical study, the higher anti-bacteria antibody titers in the host cause fewer amounts of bacteria in the tumor sites. Anti-bacteria antibody results in a noticeably lower total number of bacteria in the tumor sites and decreases the antitumor effect of bacteria (Lee et al. 2009). Therefore, avoiding neutralizing
antibody emerges as a key issue in the development of bacterial therapy approaches. Moreover, preexisting *Salmonella* neutralizing antibodies are common in the human population due to the ubiquitous nature of *Salmonella* serotypes and therefore, may interfere with antitumor activity of *Salmonella* even upon the first inoculation of *Salmonella*. Masking of the immunogenicity of *Salmonella* by linking polymer may be the approach to curtail antibody-mediated *Salmonella* neutralization. Recently, we found that natural antibodies produced by B cells result in a slightly lower total number of bacteria in the tumor sites, but decrease the inflammation and cytokine production after systemically *Salmonella* treatment (Lee et al. 2011). These results will develop the new opinion that gives thought to the complex interactions between bacteria and host immunity to maximize the chances of therapeutic success (Figure 2).

The development of tumor-targeting bacteria is an exciting area of research. Avoiding side effect emerges as a key issue in the development of safety bacteria therapy approaches.
Acknowledgements

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Conflict of interest

The author declares that he has no conflict of interests.
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### Table 1.
**Bacteria as carriers**

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CD, cytosine deaminase; TNF-α, tumor necrosis factor-α; TK, thymidine kinase; IL-2, interleukin-2; STAT3, signal transducer and activator of transcription 3; shRNA, short hairpin RNA.
Figure legends

**Fig. 1.** Schematic representing the advantage offered by bacteria as an antitumor agent. Bacteria offer several advantages like (i) tumor-targeting potential, (ii) mobility, (iii) transgene capacity, and (iv) cheap.

**Fig. 2.** The accumulation of bacteria within tumor microenvironment. Bacteria inhibit tumor growth by inducing the death of tumor cells, increasing infiltrating cells or increasing antiangiogenic cytokine expression. Host nature antibodies or neutralizing antibodies block the ability of tumor-targeting bacteria.