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Review

Salmonella-based Anticancer Vaccines and their Efficacy

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ABSTRACT

Surgery, chemotherapy, and radiotherapy are successfully used to treat patients with tumors or cancers. However, the innovation of more potent therapeutic modalities is essential for the efficient treatment of patients with advanced cancers. More than two centuries ago, bacteria have been observed to have beneficial effects in some cancer patients. Virulence factors of some bacteria and their infectious behavior in the body suggest their effectiveness in tumor suppression. At present, bacillus calmette-guérin (BCG), a live attenuated strain of *Mycobacterium bovis*, is currently used to treat bladder cancer. Some other bacteria have also been found to have antitumor activities. Anaerobic bacteria can colonize solid tumors and exert an intrinsic antitumor effect. *Salmonella* is the most studied bacterium in the field of bacterial anticancer therapy in preclinical studies. In this article, we discuss progress in the development of bacterial anticancer vaccines, especially *Salmonella*-based vaccines, their antitumor efficacy, and mechanisms involved in vaccine-mediated cancer cell death.

Keywords
Cancer; Vaccine; Bacteria; *Salmonella*; Apoptosis; Immune responses; Cancer therapy efficacy.

Abbreviations
MHC: Major Histocompatibility Complex; TGF-β: Transforming Growth Factor-Beta; VEGF: Vascular Endothelial Growth Factor; IL: Interleukin; PD: Programmed Cell Death; APCs: Antigen Presenting Cells; PD-L1: PD-Ligand 1; TLRs: Toll-Like Receptors; BCG: Bacillus Calmette-Guérin; CFU: Colony-Forming Units; TNF-α: Tumor Necrosis Factor-Alpha; TRAIL: Tumor Necrosis Factor-Related Apoptosis-Inducing Ligand; PSA: Prostate-Specific Antigen; CTLA-4: Cytotoxic T Lymphocyte Associated Antigen-4, IFN-γ: Interferon-Gamma; Th1: T Helper Type 1; Omp A: Outer Membrane Protein A.

INTRODUCTION

Hundreds of years ago, variolation has been used to protect humans against smallpox. At the end of the 18th century, the cowpox virus was introduced by Edward Jenner as a safer alternative to the variola virus, even though the virus was not identified at that time. During the 19th century after the discovery of bacteria as causative agents of infectious diseases, attenuated or killed microorganisms or a component of the whole microorganism were widely used to induce immunity against infectious diseases. Use of these vaccines led to a dramatic decrease in death induced by viral and bacterial agents. In recent decades, researchers attempted to develop therapeutic vaccines against cancer with the aim of recruiting lymphocytes and other immune cells to destroy cancerous cells. These vaccines were used as a monotherapy or in combination with conventional therapeutic modalities such as surgery, chemotherapy, and radiotherapy to prevent metastases and recurrence of cancer.[1]

T-cells are an important part of protective adaptive immune responses. Immunogenic cancer cell growth can result in the generation of antitumor immune responses, especially T-cell responses.[2] Intravenous infusion of tumor-sensitized T-cells from immune donors has resulted in regression of large established tumors in T-cell-deficient recipients.[3,4] High number of intraepithelial $CD8^+$ tumor-infiltrating T-cells was associated with the

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absence of lymph node metastases in patients with human papillomavirus-positive cervical cancer.[5] High $CD8^+/CD4^+$ T-cell ratio of tumor-infiltrating lymphocytes was associated with better clinical outcome in colorectal cancer patients.[6] In contrast, it has been shown that T-cell-mediated suppression of antitumor immunity occurs in progressive growth of an immunogenic tumor.[3] Indeed, immunogenic tumor cells can induce $CD8^+$ T-cell responses, however, tumors can avoid immune cell-mediated destruction by induction of T-cell tolerance as well.[7,8]

Induction of T-cell responses is the major aim of vaccines. Vaccines can produce T-cell responses to a large number of antigens. It is preferable to include more than one antigen in vaccines to decrease the likelihood of immune escape. But, immunological tolerance to cancer cell antigens is a usual phenomenon. Furthermore, tumor cell growth and proliferation can lead to the establishment of tumor tissue with an immunosuppressive tumor microenvironment.[9] Therefore, breaking immunological tolerance and immunosuppression is necessary for therapeutic cancer vaccines. Tumors employ numerous mechanisms limiting natural or vaccine-induced antitumor immune responses. Some of these mechanisms include down-regulation of major histocompatibility complex (MHC) class I molecules, lack of expression of costimulatory molecules CD80 and CD86, expression of Fas ligand, and secretion of immunosuppressive cytokines such as transforming growth factor beta (TGF-β), vascular endothelial growth factor (VEGF), and Interleukin 10 (IL-10).[10,11] Tumor infiltrating dysfunctional $CD8^+$ T-cells expressing the immune checkpoint molecule programmed cell death 1 (PD-1) have been detected in various types of cancer,[12-14] which was associated with poor clinical outcome for patients.[12] Tumor cells and antigen-presenting cells (APCs) expressing PD-L1 can impair proliferation and effector function of $PD\text{-}1^+$ T-cells in the tumor microenvironment.[15] In clinical trials, blocking PD-1/PD-L1 interactions with anti-PD-1 antibodies led to improved antitumor immunity.[16] In addition, increased frequency of immunosuppressive cells such as regulatory T-cells has been reported in the peripheral blood and tumor microenvironment of cancer patients.[17] Depleting these cells or blocking their immunosuppression functions by monoclonal antibodies has resulted in improved antitumor immunity in some types of cancer.[18] However, more effective therapeutic approaches should be developed to produce potent antitumor immune responses in patients with advanced cancer.

APCs have a crucial role in antitumor immunity as they trigger adaptive immune responses by processing and presenting antigens for recognition by T-cells.[19] Bacterial products can strongly activate APCs through pattern recognition receptors such as toll-like receptors (TLRs). Some bacterial products have antitumor properties by apoptosis induction in cancer cells. For instance, the bacterial protein azurin, a cupredoxin type of electron transfer and purified redox protein from *Pseudomonas aeruginosa*, selectively induced apoptosis in human breast cancer cells[20] and oral squamous carcinoma cells.[21] This bacterial protein effectively enters human cancer cells, but not normal cells,[22] and it has been shown to induce apoptosis through stabilization of the tumor suppressor protein p53.[23] In some studies, bacterial products have been used as an adjuvant for anticancer vaccines.[24] In addition, live bacteria expressing tumor antigen have been used for tumor antigen delivery in *vivo*.[25, 26] These therapeutic vaccination approaches did not lead to the eradication of established experimental tumors or human tumors. Nonetheless, some live bacteria have been successfully used for cancer treatment.[27]

APPLICATION OF BACTERIA IN ANTICANCER THERAPY

More than two centuries ago, cancer remission was reported in cancer patients after recovering from bacterial infections. In the late 19th century, William Coley used live and heat-killed bacteria such as *Streptococcus pyogenes* and *Serratia marcescens* to treat cancer patients. The Coley's heat killed bacteria, known as Coley's toxin, has been used for sarcoma patients until 1963. In the mid-1980s, Bacillus Calmette-Guérin (BCG), a live attenuated strain of *Mycobacterium bovis*, was used to treat superficial bladder cancer.[27] At present, BCG is a common treatment for bladder cancer and it is the only bacterial agent approved by the US Food and Drug Administration (FDA) for primary therapy of carcinoma *in situ* of the bladder. BCG is successfully used to treat some non-invasive bladder cancers two weeks after surgery.[28] Some other bacteria have also been found to have antitumor activities.

Almost 70-years ago, it has been shown that anaerobic bacteria can selectively grow in tumors.[29,30] Several species of anaerobic bacteria have the ability to colonize solid tumors and induce tumor shrinkage. Colonization of anaerobic bacteria in solid tumors is largely due to impaired blood flow and extensive necrosis in the central part of tumor tissue. Selective localization of bacteria in chemically-induced rat mammary tumors was observed after systemic injection of *Bifidobacterium longum*, a nonpathogenic, anaerobic bacterium.[31] It has also been shown that obligate anaerobic bacteria *Clostridium novyi* and *Clostridium sordelli* localize solid tumors, especially in the hypoxic parts of tumor tissues.[32] However, small tumors, as well as metastatic tumors, may not be targeted by obligate anaerobic bacteria as these regions have sufficient blood circulation and are not hypoxic. In experimental syngeneic and allogeneic murine tumor models using light-emitting bacteria, several facultative anaerobic bacterial species such as *Escherichia coli*, *Salmonella enterica serovar Typhimurium (S. typhimurium), Vibrio cholerae*, and Listeria monocytogenesis were able to selectively enter solid tumors and metastases and replicate within tumors.[33,34] Intravenously injected facultative anaerobic bacteria can enter and replicate within tumors with different efficacy. It has been shown that *Escherichia coli* robustly replicates in tumor while *Streptococcus pyogenes* shows a much lower level of replication in the tumor. Marine bacteria *Photobacterium phosphoreum* and *Vibrio fischeri* did not show tumor-specific colonization after intravenous injection. The colonization process was independent of the tumor type but largely dependent on the stage of tumor development.[35] The majority of injected bacteria have been found in the central part of the tumors. This part of large tumors is usually necrotic. Moreover, the bacterial dose can affect the colonization of tumor,[35] as a small number of bacteria may be cleared by the body's immune system. Thus, an appropriate number of bacteria with antitumor properties are required to overcome the immune clearance mechanisms in order

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to survive during tumor entry.

SALMONELLA-BASED ANTICANCER VACCINES, THEIR THERAPEUTIC EFFICACY, AND MECHANISMS INVOLVED IN CANCER CELL DEATH

Salmonella is a gram-negative, facultative anaerobe, and a pathogen to human and animals. This bacterium is extensively studied in the field of bacterial therapy of cancer. Some *Salmonella* strains preferentially colonize solid tumors and show an intrinsic antitumor effect. *S. typhimurium* strains exhibited high tumor colonization following systemic administration into tumor-bearing mice, resulting in more than 1×108 CFU per gram tumor tissue.[36] After intravenous administration of *S. typhimurium*, bacteria were found in blood, spleen, and liver. Low numbers of bacteria were detected in tumors associated with blood vessels. A rapid increase of the proinflammatory cytokine TNF-α was detected in the blood which was linked to a tremendous influx of blood into tumors by vascular disruption, resulting in bacteria flushing into the tumor. Blood influx was followed by necrosis formation, bacterial growth and infiltration of neutrophils.[37] Bacterial motility or chemotactic responsiveness have not been required for tumor invasion and colonization of *Salmonella*.[38] Both intravenous and intraperitoneal injection of *S. typhimurium* led to complete tumor clearance. In contrast, after oral administration, tumor colonization was transient, inefficient, and delayed with no therapeutic effect observed.[38] Furthermore, oral infection with *Salmonella* may increase the risk of gall-bladder cancer[39] and colon cancer.[40] This pathogen also has the potential for causing sepsis. However, tumor-colonizing *Salmonella* can be readily controlled by systemic administration of antibiotics.[41]

Attenuation of *Salmonella* strains has been performed by genetic alterations to prevent bacteria-induced septic shock, especially by lipopolysaccharide deletion.[42,43] Several genetically modified *Salmonella* strains have been developed such as VNP20009, LT2, CRC1674, A1-R, and CRC2631.[43-46] Intravenous administration of attenuated *S. typhimurium*, VNP20009, has been evaluated in phase I clinical trial for the treatment of 24-patients with nonresponsive metastatic melanoma and one patient with renal cell carcinoma. Dose-limiting toxicity, including thrombocytopenia, anemia, persistent bacteremia, hyperbilirubinemia, diarrhea, vomiting, nausea, elevated alkaline phosphatase, and hypophosphatemia, was observed in patients receiving 1×109 CFU/m^2. VPN2009 induced a dose-related increase in the circulation of proinflammatory cytokines IL-1 beta, TNF-α, IL-6, and IL-12. Focal tumor colonization was observed in two patients receiving 1×109 CFU/m^2 and in one patient receiving 3×108 CFU/m^2. Objective tumor regression was not observed in the patients, including those patients with colonized tumors.[47] The genetically modified, amino acid auxotrophs, *S. typhimurium* A1 strain grew throughout the tumor, including viable malignant tissue. The bacteria invaded and replicated intracellularly in PC-3 prostate cancer cells grafted into nude mice and caused tumor inhibition and regression. Normal tissue was cleared of these bacteria even in immunodeficient athymic mice.[45] *S. typhimurium* A1 was also able to inhibit PC-3 human prostate cancer experimental bone metastasis and significantly improved the overall survival of mice with multiple bone metastases.[48]

In a mouse infection model, *S. typhimurium* mutants from which Braun lipoprotein genes (lppA and B) and the multicopy repressor of high-temperature requirement (msbB) gene were deleted a minimally induced proinflammatory cytokine production was observed. Immunization with these mutants followed by challenge with the wild-type *S. typhimurium* significantly inhibited tumor growth, as 88% regression in tumor size was observed in ippB/msbB mutant immunized mice. The tumor size regression was correlated to downregulation of CD44 high and $CD4^+CD25^s$ regulatory T-cells.[49] Tumors are a rich source of purines with adenosine triphosphate concentrations. A *Salmonella* strain deficient in synthesizing purines by a mutation in the purl gene which encodes for purine biosynthetic enzyme has been used with tumor-specific localization.[50] Live attenuated *S. typhimurium* genetically modified at purl and msbB genes (VNP20009 strain) when administrated intravenously to tumor-bearing mice inhibited the growth of tumors, which did not depend on the presence of T and B-cells.[51] Increased number of bacteria was detected in the tumor. The bacterial number reached levels 10,000 times higher than in the normal liver reservoir. *Salmonella* growth was observed in areas of the tumor which partially inhibit tumor growth. However, a rim of tumor survived and resulted in progressive tumor growth.[52]

Orally administration of attenuated *S. typhimurium* carrying a eukaryotic expression vector that contains the second-derived activator of caspases (Smac) and TRAIL genes under the control of the human telomerase reverse transcriptase promoter inhibited tumor growth by 70-90% and prolonged the survival of mice.[53] Immunization of mice with recombinant *S. typhimurium* expressing C-Raf significantly reduced tumor growth in transgenic mouse models of Rafoncogen-induced lung adenomas.[54] Vaccination with recombinant attenuated *S. typhimurium* aroA strain secreting prostate-specific antigen (PSA) and cholera toxin subunit B induced cytotoxic $CD8^+$ T-cell responses and efficient prevention of tumor growth in mice.[55] Intravenous injection of attenuated *S. typhimurium* strains reduced immunosuppression in the tumor and tumor-draining lymph node.[56,57] However, injection of unmodified *S. typhimurium* or genetically-modified *S. typhimurium* expressing recombinant tumor proteins did not result in eradication of long-established tumors in mice.

Immunogenic melanoma tumors can grow progressively even when the tumor is infiltrated by $CD8^+$ T-cells. Long-established immunogenic tumors have been shown to contain a high percentage of PD-1^+ tumor-specific $CD8^+$ T-cells. Treatment with PD-L1 and CTLA-4 blocking antibodies was ineffective in preventing the growth of progressive tumors. Exogenous tumor-specific antigen delivery into tumors using *S. typhimurium* expressing a recombinant antigen resulted in induction of proliferation of tumor-specific $CD8^+$ T-cells in the lymphoid organs and recovered effector function of tumor-specific $CD8^+$ T-cells in the tumor. Immunization with this vaccine led to improved mice survival and rejection of 32% of long-established immunogenic melanoma tumors. However, following the treatment, the majority of tumor-specific $CD8^+$ T-cells expressed a high-level of PD-1 in the tumor. Combination of injection of *S. typhimurium* expressing the recombinant antigen with programmed cell death-ligand 1 (PD-

L1) blocking antibody enhanced the expansion of tumor-specific $CD8^+$ T-cells and resulted in 80% tumor rejection.[58] Genetically modified *S. typhimurium* harboring short hairpin RNA against inhibin alpha subunit caused remarkable cytotoxicity in cancer cells compared with unmodified *S. typhimurium*. This tumor-targeted therapy also significantly inhibited the growth of colon cancers and melanomas and prolonged the survival of mice bearing syngeneic tumors.[59]

Genetically modified *S. typhimurium* A1 has been shown to grow in the cytoplasm of PC-3 human prostate cancer cells and caused nuclear destruction *in vitro*. The bacteria, introduced intravenously or intratumorally, caused tumor inhibition and regression of xenografts in nude mice. *S. typhimurium* A1 was undetectable in the liver, lung, spleen, and kidney, but it continued to proliferate in the PC-3 tumor. Intratumoral injection of the bacteria resulted in complete tumor regression by day 20.[45] Preferential destruction of mitochondria has been observed 8 h after inoculation of genetically modified *Salmonella* in PC-3M human prostate cancer cells, but, the nucleus was not apparently affected by *Salmonella* within 8 h.[46] *Salmonella* invasions can induce apoptosis in infected cells as apoptosis has been observed in *Salmonella*-infected macrophages[60,61] and intestinal epithelial cells.[62] Furthermore, apoptosis of *Salmonella*-infected cancer cells can result in antitumor immune responses through triggering cross-presentation of tumor antigens on MHC class I molecules of professional antigen presenting cells to cytotoxic T-cells.[63]

Infection with *Salmonella* results in activation of both innate and adaptive immune responses. This bacterium induces production of numerous cytokines such as IL-1β, IL-6, TNF-α, IFN-γ, and IL-12 and recruits and activates APCs such as dendritic cells. Importantly, *Salmonella* can trigger Th1 polarization which is favorable to antitumor immune responses. *S. typhimurium* outer membrane protein A (OmpA) induces the maturation of tumor antigen-pulsed dendritic cells resulting in IL-12 production and generation of Th1 immune responses. Bone marrow-derived dendritic cells stimulated with OmpA of *S. typhimurium* generated effective antitumor immunity in a mouse tumor model.[64] *Salmonella*-based anticancer vaccines reducing the frequencies or functions of immunosuppressor cells, such as regulatory T-cells and myeloid-derived suppressor cells, may show improved efficacy in cancer patients. Intratumoral injection of attenuated *S. typhimurium* significantly inhibited Her-2/neu-expressing tumor growth which was associated with increased levels of TNF-α-secreting neutrophils ($CD11b^+Gr1^+$ myeloid cells) and reduced levels of $CD4^+CD25^+Foxp3^+$ regulatory T-cells in vaccinated mice.[65]

CONCLUSION

Salmonella is the most studied bacterium for developing a bacterial anticancer therapeutic vaccine. This bacterium is an intracellular microorganism and can induce Th1 immune responses and other antitumor immune cells. Furthermore, *Salmonella* preferentially colonizes solid tumors and exhibits an intrinsic antitumor effect. Vaccination with *S. typhimurium* strains resulted in therapeutic outcomes in several preclinical studies. However, enhancing their therapeutic efficacy is essential for clinical application in cancer patients. Elucidation of *Salmonella*-mediated mechanisms in cancer cell destruction can lead to improved bacterial anticancer therapy by utilization of bacterial strains with potent cytotoxic activities against cancer cells. Increasing our understanding of the tumor cell-specific metabolisms may also be beneficial to identify tumor cell metabolic demands and metabolic final products in the tumor microenvironment to find more appropriate bacterial strains with desired metabolic pathways for tumor-selective colonization and destruction in cancer patients.

CONFLICTS OF INTEREST

The authors declare that they have no conflicts of interest.

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