THOMSON REUTERS

IDrugs 2010 **13**(10):701-706
 ISSN 2040-3410

FEATURE REVIEW

Bacterial vectors and delivery systems in cancer therapy

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Live bacterial vectors may be useful tools for the development of novel cancer therapies that can be added to the repertoire of existing drugs. Several bacterial strains effectively colonize solid tumors and act as antitumor therapeutics. The naturally occurring tumor-colonizing characteristics of bacterial species such as Salmonella sp, Clostridium sp and Escherichia coli can be further modified by genetic manipulations, making these bacterial systems excellent vehicles for the production and targeted delivery of therapeutic molecules into cancer cells. This feature review summarizes recent research on cancer therapy using genetically modified bacteria. Different approaches – bactofection, DNA vaccination, and bacterially mediated protein and RNAi delivery – in which modified bacteria are used as anticancer therapeutics, are discussed.

Keywords Bacteria, bacterially mediated protein, bacterially mediated RNAi, bactofection, cancer, DNA vaccination, gene therapy, transkingdom RNAi, vector delivery

Introduction

The first observations that bacterial infection may slow the growth of tumors in humans date from the 19th century. The first attempts at using this phenomenon for therapeutic purposes were made more than 40 years ago, following the discovery that bacteria could replicate predominantly in solid tumors. These findings remained generally unexplored until the end of the 20th century, when oncolytic bacteria capable of lysing host cells began to be studied by various research groups.

Most research on gene therapy for cancer has focused on the use of viral vectors that exhibit high gene transfer efficiency. However, certain disadvantages of viral transduction methods have been noted, particularly safety issues, as well as high cost, short bioactivity, size limitations for the DNA payload, and difficulties associated with immunogenicity and cytotoxicity. As a result, alternative delivery vectors are being examined. A framework for the use of bacterial carriers as vectors for the delivery of eukaryotic expression plasmids was introduced in the mid-1990s, following research on a variety of bacteria such as *Shigella* sp, *Salmonella enterica* Typhimurium, *S enterica* Typhi, *Listeria monocytogenes* and *Escherichia coli.*

One of the main reasons for the lack of efficacy of radiotherapy and chemotherapy in many solid tumors is the presence of hypoxic (ie, poorly vascularized) areas that are resistant to these interventions. However, this apparent limitation can be an advantage for alternative approaches, such as with the use of obligate or facultative anaerobic bacteria. Several strains of Clostridia, Bifidobacteria and *Salmonella* sp selectively colonize the hypoxic areas of tumors and destroy tumor cells, thereby providing a more specific tumor-targeted therapy. Despite the selective colonization of hypoxic areas in tumors, several studies indicate that these hypoxia-specific strains are able to destroy various tumor cells, including non-hypoxic cells. Therefore, the off-target tissue toxicity observed for systemic cancer therapies and relapse as a result of residual tumor cell growth, both of which represent two crucial deficiencies of conventional cancer treatments, can potentially be overcome by employing tumor-targeting bacteria.

The concept of bacterial oncolytic therapy has been verified in various experimental studies, as well as a limited number of clinical trials. More recently, a tumor-targeting auxotrophic strain of *Salmonella* Typhimurium was demonstrated to eradicate primary tumors, as well as cancer metastases, almost completely

in various mouse models (*J Cell Biochem* (2009) **106**(6):992-998; *Cell Cycle* (2009) **8**(6):870-875). The use of bacterial systems for therapeutic purposes can be enhanced further by genetic modifications, providing a promising tool for the targeted delivery of genes and their products. Advantages for the use of bacteria in anticancer gene therapy include the natural oncolytic potential of some strains and species, the direct targeting of tumor tissue, and the ease of positive regulation and eradication of the bacteria. Moreover, the naturally occurring anticancer effect of tumor-targeting bacteria can also be achieved after oral administration, circumventing the need for an intravenous route of delivery. Despite notable successes obtained in studies using oncolytic bacteria for the treatment of cancer, bacteriolytic therapy alone has often been insufficient to eradicate tumors completely in experimental models. Therefore, the use of bacteria as carriers of therapeutic molecules has been suggested in order to augment the anticancer efficacy of the treatment.

This feature review summarizes recent findings on cancer therapy using genetically modified bacteria as vectors and delivery systems. Different approaches – bactofection, DNA vaccination, and bacterially mediated protein and RNAi delivery – in which bacterial systems are used as anticancer therapeutic vehicles (Figure 1), are discussed.

Bactofection

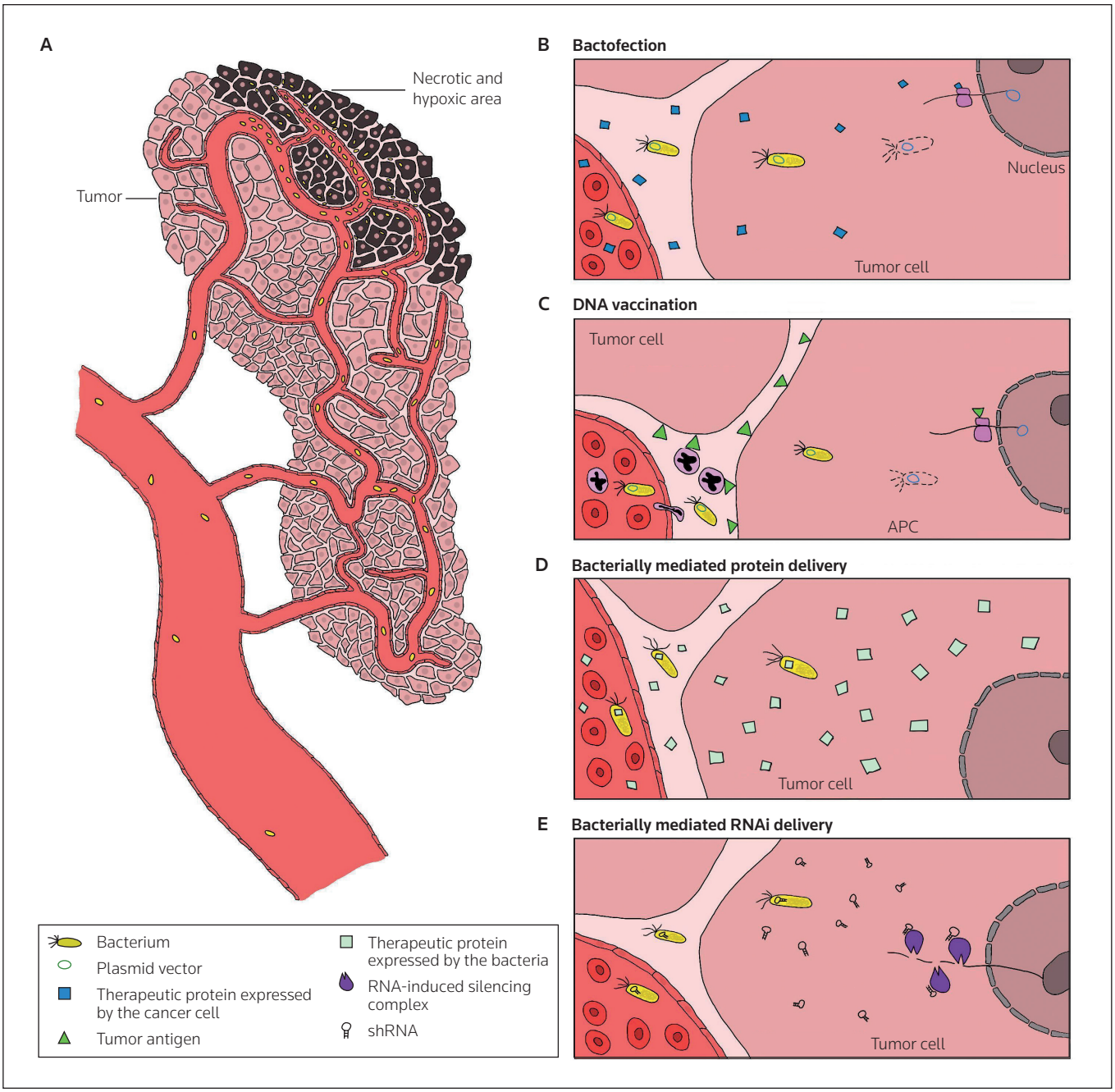
Bactofection is a method in which bacteria are used to transfer genes directly into the target organism, organ or tissue; this basic principle was first described 30 years ago. In the case of cancer therapy, bacteria deliver plasmids encoding the therapeutic (eg, anticancer) gene, under the control of a eukaryotic promoter, into tumor cells. After entering the target cell, the plasmid is released into the cytoplasm and is subsequently transferred into the cell nucleus, where the therapeutic gene is expressed by the host cell's transcription and translation systems. The bacterially mediated transfer of plasmid DNA into mammalian cells thus represents a potent approach for expressing plasmid-encoded heterologous proteins in a large set of different cell types.

In cancer therapy, the therapeutic protein usually acts as a cytotoxic molecule toward the target cell in which it is expressed, as well as toward surrounding cells, by triggering suicide pathways. The bacteria used in such therapy are engineered to undergo lysis after entry into the target cell and to release the plasmid into the cytoplasm of the cell. This lytic mechanism prevents the undesired long-term survival of the bacteria in the host organism. In addition, the products of bacterial lysis may act as potential foreign antigens and adjuvants, inducing cellular immune responses in the host organism and potentially adding to the tumor-ablating effects. However, severe immune reactions (ie, cytokine storms) can cause unwanted toxicities and need to be avoided. Therefore, most bacterial strains used as vectors have been genetically attenuated to avoid serious adverse effects, such as immune system activation and severe infections. However, the use of attenuated strains has not been demonstrated to be effective in clinical trials of such cancer therapy. *Salmonella* strains are the most widely used systems in experiments to develop bactofection-mediated cancer therapy. For example, in one study, a *Salmonella choleraesuis* strain was engineered to carry a eukaryotic expression plasmid encoding the endostatin gene. The expression of endostatin was limited exclusively to tumor tissues colonized by bacteria, and significant inhibition of tumor growth (ie, 40 to 70%) with decreased intratumoral microvessel density was observed (*J Gene Med* (2004) **6**(12):1382-1393). Bactofection using auxotrophic *Salmonella* as a vector to express plasmids carrying genes encoding various cytokines, such as IL-12, GM-CSF, IL-4 and IL-18, as well as other molecules, such as Flt3 ligand, has also been employed effectively in experimental tumor models. In addition, the dual tumoricidal and antiangiogenic effect of *S choleraesuis* carrying an expression plasmid containing the thrombospondin-1 gene under the control of a eukaryotic promoter was observed in a murine model of malignant melanoma (*Cancer Gene Ther* (2005) **12**(2):175-184). The possibility of a treatment for non-solid tumors has also been described using a *Salmonella* Typhimurium-based oral delivery system encoding the CD40 ligand to target B-cell lymphoma. Despite the positive results obtained, only a limited number of studies have been conducted using bactofection for the treatment of cancer. Results of other studies indicate that this approach may also be suitable for targeting cells or tissues involved in other indications, such as the colonic mucosa for colitis and the lungs for acute and chronic lung diseases (eg, acute respiratory distress syndrome and cystic fibrosis). For cancer therapy, a potential limitation of bactofection is that the effector molecule will be expressed exclusively in cells infected by bacteria, leaving a potentially large population of tumor cells untreated. However, if the product of the transgene were secreted from the target cell, then the effector molecule might still have a good therapeutic effect on non-infected tumor cells. However, this proposed mechanism of action has not yet been confirmed experimentally.

DNA vaccination

The bactofection of plasmids encoding tumor-expressed antigens can lead to the induction of humoral and cellular immune responses in the host, thereby providing a protective defense against tumors. This approach is termed DNA vaccination. Most experimental DNA vaccination studies for anticancer therapy use attenuated-strain *Salmonella* Typhimurium, as the suitability of this vector has been demonstrated in various preclinical investigations. Oral *Salmonella*-based DNA vaccines against VEGFR-2 have exhibited efficacy in suppressing tumor growth in animal models of malignant melanoma, colorectal carcinoma, glioblastoma and lung cancer. Similar antitumor effects have been observed with oral bacterial vaccines against the cytokine IL-18, the apoptosis inhibitor survivin, the tumor endothelial marker 8 and the TGFβ1 coreceptor endoglin. Angiogenesis-related

Figure 1. Antitumor effects of tumor tissue-colonizing bacteria.



(**A**) Auxotrophic bacteria specifically colonize tumors with necrotic and hypoxic areas. The anticancer effect of bacteria can be exerted by different strategies: bactofection, DNA vaccination, and bacterially mediated protein and RNAi delivery. (**B**) Bactofection: after escaping the blood vessel and entering the target cell, bacteria disrupt and release a plasmid vector encoding the therapeutic gene. The plasmid is transferred into the cell nucleus, and the therapeutic protein is expressed by the host cell's expression system. (**C**) DNA vaccination: bacteria deliver therapeutic plasmids into the host cell (eg, APC) in a similar manner to bactofection. The plasmid encodes a tumor cell-expressed antigen to help prime a T-cell response against the tumor antigen that is present on the surface of tumor cells, leading to the induction of humoral and cellular immune responses against the tumor. (**D**) Bacterially mediated protein delivery: bacteria, either within the extracellular environment or within tumor cells, express the therapeutic gene directly and serve as protein delivery vehicles. (**E**) Bacterially mediated RNAi delivery: bacteria deliver plasmid-encoding shRNAs or express the shRNAs to induce RNAi against an oncogene or a tumor-expressed factor. The RNA-induced silencing complex recognizes and cleaves the target mRNA according to homologous shRNAs delivered by the bacteria.
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factors are among the most commonly used target antigens for bacterial anticancer DNA vaccination studies, underlining the relevance of inhibiting tumor blood vessel growth in cancer therapy. In addition to *Salmonella*, other bacterial species have also been used for DNA vaccination against cancer, including *Pseudomonas aeruginosa*.

Most therapeutic cancer vaccines that have been developed induce a protective CD8+ T-cell-mediated immune response that likely reflects a breakdown of peripheral immunological tolerance to self-antigens. Therefore, selecting mainly T-cell epitopes from tumor antigens as targets of vaccination appears to be adequate for cancer therapy. However, the precise molecular mechanism that regulates this phenomenon remains obscure. The need for further research and a better understanding of these observations is highlighted by the fact that similar vector strains are currently used for bactofection-based approaches and for DNA vaccination. In bactofection and bacterial gene therapy approaches, immune activation may be an undesired side effect, whereas in DNA vaccination, the immunogenic properties of the carrier bacteria are a pivotal component of the therapeutic effect.

A main advantage of bacterial DNA vaccination as a cancer treatment strategy is the potential for therapeutic bacterial strains to be administered orally, as demonstrated in animal studies. Current research in the field is focused on the development of clinically relevant oral bacterial vaccines. However, significant knowledge gaps remain, and the mechanism of action for oral DNA vaccines has not been fully elucidated. Prior to proceeding to clinical trials, it is important to have a comprehensive understanding of the underlying processes of bacterially mediated DNA vaccination.

Bacterially mediated protein delivery

Another approach for the use of bacteria in innovative cancer therapies is the bacterially mediated delivery of proteins. This strategy is based on the transfer of bacterially expressed therapeutic proteins to the host organism using genetically modified (ie, transformed) bacteria. Unlike bactofection- and DNA vaccination-based methods, the persistence of bacteria in the target tissue may be a desired property for protein delivery. Persisting bacteria produce the therapeutic polypeptide *in situ*, thus providing the ideal situation of a drug that is distributed locally, but without increasing systemic levels. Compared with bacterially mediated gene delivery (bactofection), the bacterially mediated delivery of protein provides the important possibility of regulating gene expression using low-molecular-weight inducers, depending on the expression system used. Moreover, the effect of bacterial vehicles can possibly be eliminated by using antibiotics.

A widely researched strategy that uses bacterially mediated protein delivery as an anticancer therapy is the enzyme-prodrug approach, in which systemically administered bacteria deliver an enzyme that converts the prodrug into its active compound. Thus, tumor-colonizing bacteria express the enzyme (eg, cytosine deaminase) specifically in tumor tissue and, subsequently, the systemically applied inactive prodrug (eg, 5-fluorocytosine) is converted into the active cytotoxic substance through the activity of the enzyme. This approach limits the toxicity of the active compound to the targeted areas of tumor tissue. Attenuated *Salmonella* Typhimurium, Clostridia and Bifidobacteria have all been used successfully for enzyme-prodrug therapy in preclinical models, and several therapies have advanced into clinical trials. For example, attenuated *Salmonella* Typhimurium has been used for the delivery of cytosine deaminase in several phase I trials. These trials demonstrated that engineered *Salmonella* Typhimurium strains could be administered safely up to a certain dose; however, the delivered protein appeared to lack efficacy in the clinical setting. In preclinical models, the natural oncolytic activity of *Salmonella* Typhimurium has been further enhanced by genetic modification, leading to the production of various antitumor molecules, such as IL-2, IL-18, CCL21 and proapoptotic Fas ligand. In addition, Clostridia appear to be particularly suited to bacterial protein delivery into tumors because of their obligate anaerobic nature, capacity to form spores and ease of transformation. Furthermore, the ability of Clostridia to colonize tumors *in vivo* has been enhanced by the deletion of genes associated with basal oxygen tolerance, such as the gene encoding superoxide dismutase. Conversely, the main advantage of Bifidobacteria is a non-pathogenic nature. Various *Bifidobacterium* strains have been used in preclinical studies as vectors for the expression of endostatin and TRAIL.

A key aspect of the studies investigating *in vivo* bacterially mediated protein delivery is the ability of this system to regulate the bacterial expression of the therapeutic protein specifically. Precise temporal and spatial control is important to optimize the therapeutic effect of the protein, and to minimize any potential adverse effects. One of the key tools in this area is the use of inducible promoters that are activated only in the presence of specific exogenously administered compounds (eg, isopropyl thiogalactoside or arabinose). A system based on an arabinose-inducible bacterial promoter has been used successfully for the specific expression of a therapeutic gene in tumor tissue using the bacterial strains *Salmonella* Typhimurium and *E coli* Nissle 1917. These strains, if administered intravenously, can selectively colonize tumors *in vivo*, but only express the transgene upon the concurrent delivery of L-arabinose. Moreover, the exogenous regulation of therapeutic gene expression can be improved further by the use of bacteria carrying inducible suicide genes.

Another approach to improve regulated gene expression, as well as the targeting of bacteria to the hypoxic regions of tumors, is the use of hypoxia-responsive promoters in tumor-colonizing bacterial strains. A subset of promoters that are activated preferentially inside tumors has also

been identified, suggesting that there are additional functional regulatory mechanisms that could be exploited for targeted expression, in addition to those related to hypoxia. In combination, recent data provide evidence that intravenously administered, non-virulent genetically modified bacteria may be efficiently exploited as vehicles for the local generation of anticancer agents in tumors. Additional research is required to address safety issues and to enhance the efficacy of such bacteria without inducing life-threatening systemic immune responses.

Listeria sp are being actively developed as therapeutic agents using the anticancer vaccination strategy. In this approach, the bacteria are engineered to produce an antigen that will elicit an antitumor response by the immune system. This strategy has been demonstrated to be successful for the treatment of experimental mesothelioma and various other cancers *in vivo*, and is being evaluated in clinical trials by several biotechnology companies, including Aduro BioTech Inc (formerly Anza Therapeutics Inc) and Advaxis Inc. ADXS11-001 (Advaxis), a live attenuated *L monocytogenes* vector that secretes the *Listeria* protein listeriolysin O (LLO) fused to the HPV16 E7 antigen, was demonstrated to be safe in a phase I trial in patients with cervical cancer (*Vaccine* (2009) **27**(30):3975-3983). Phase II trials of the agent in patients with cervical cancer and cervical intraepithelial neoplasia are ongoing.

Bacterially mediated delivery of RNAi

A promising new approach for bacterially mediated anticancer therapy is the combination of two distinct methodologies: bacteriotherapy and RNAi. As noted, bacteria have been employed as a versatile gene delivery vector, and have been demonstrated to be an effective, safe and inexpensive strategy for delivering RNAi to mammalian cells. Two systems that have been used to exploit bacteria for the delivery of RNAi are transkingdom RNAi (*tk*RNAi) and bacterially mediated RNAi (*bm*RNAi). *tk*RNAi, developed at the Beth Israel Deaconess Medical Center, uses genetically modified bacteria to produce and deliver shRNA against oncogenes or tumor-expressed factors into tumor cells. Thus, the invasive bacteria enter the target tumor cell, release shRNA into the cytoplasm and activate the RNAi pathway to induce gene silencing. In terms of the production of therapeutic molecules by bacterial carriers, *tk*RNAi resembles bacterially mediated protein delivery, but may have the advantage of not requiring host/target cell-mediated transcription of a transgene. The delivery of shRNA into the cytoplasm of the host cell is sufficient to induce RNAi. The invasive *E coli* has been used successfully as a vector for the delivery of shRNA against the human colon cancer oncogene catenin β-1 and the multidrug-resistance gene MDR1. Such experiments have demonstrated the efficacy of orally administered or systemically injected *tk*RNAi bacteria in mouse models of human cancer. *tk*RNAi is being developed as a novel method for the delivery of RNAi-based therapeutics by Cequent Pharmaceuticals and ViThera Laboratories, with various human and veterinary applications ranging from the treatment of colon cancer to gastrointestinal inflammatory conditions.

Unlike the *tk*RNAi delivery system, carrier bacteria in the *bm*RNAi system do not produce shRNA, but instead transfer an shRNA expression plasmid to the host cell. The transcriptional machinery of the host cell is then used to produce shRNA. Thus, *bm*RNAi acts analogously to bactofection. Attenuated *Salmonella* Typhimurium has been used for the *in vivo* delivery of eukaryotic expression plasmids encoding shRNA against STAT3, MDR1 and the anti-apoptotic protein Bcl-2 to various mouse models of cancer, such as mouse prostate carcinoma, human tongue squamous cell carcinoma and murine melanoma, respectively. While the bacterially mediated delivery of RNAi clearly provides a new area of cancer therapeutic research, additional studies are required to uncover its potential applications.

Conclusion

The application of new approaches using bacteria for the transfer of therapeutic genes or for the production of therapeutic proteins or nucleic acids has the potential to advance cancer gene therapy significantly. Recent studies indicate that treatments targeting a single molecule or pathway, even if such molecules or pathways have pleiotropic effects, are unlikely to eradicate tumors completely; however, if the naturally occurring antitumor activity inherent to some anaerobic bacteria strains can be combined successfully with their ability to deliver agents targeting tumor angiogenesis, apoptosis or the immune system, then this approach represents a significant step toward achieving tumor eradication. Future directions that may progress the field include improvements in the spatial and temporal regulation of bacterial therapy, improved specificity (ie, tumor and gene targeting), and the inclusion of other novel therapeutic payloads (eg, microRNA agonists or antagonists). All of these improvements could be combined with bacterial delivery systems to achieve synergistic anticancer effects for combination therapy. Nevertheless, safety issues and public attitude will have a significant impact on the adoption of bacterially mediated anticancer therapy in the clinical setting. Therefore, these considerations will need to be addressed to progress bacterial-based therapies from the bench to bedside. The careful selection of therapeutic indications for bacterial therapies, combined with the ability to minimize toxicities and side effects, will be crucial to the success of this approach. The use of bacterial therapeutics that are specific for organs in which a local bacterial flora is present, and for which strains derived from this flora may be used to construct the therapeutic agent, appears to be the optimal approach. For example, such therapeutic bacteria could be used in the gastrointestinal tract and the skin, as well as in the genitourinary tract. Various opportunities exist with bacterial agents, including the prevention of colon cancer, the treatment of inflammatory conditions of the gastrointestinal tract, luminal enzyme replacement

treatment, and the prevention of sexually transmitted diseases, mucosal cancers and dysplasia caused by viral infections. Focusing the development of bacterial therapeutics to these areas is expected to facilitate clinical success and to prevent challenges caused by the occurrence of major adverse events. Thus, the strategic development of bacterial therapeutics should enable bacteria to become more acceptable both as carriers of therapeutic molecules and as therapeutic 'factories' for use in innovative therapies for cancer and other debilitating diseases.

Further reading

1. Agorio C, Schreiber F, Sheppard M, Mastroeni P, Fernandez M, Martinez MA, Chabalgoity JA: **Live attenuated *Salmonella* as a vector for oral cytokine gene therapy in melanoma.** *J Gene Med* (2007) **9**(5):416-423.

2. Buttaro C, Fruehauf JH: **Engineered *E coli* as vehicles for targeted therapeutics.** *Curr Gene Ther* (2010) **10**(1):27-33.

3. Hoffman RM: **Tumor-targeting amino acid auxotrophic *Salmonella* Typhimurium.** *Amino Acids* (2009) **37**(3):509-521.

4. Loeffler M, Le'Negrate G, Krajewska M, Reed JC: **Inhibition of tumor growth using *Salmonella* expressing Fas ligand.** *J Natl Cancer Inst* (2008) **100**(15):1113-1116.

5. Nemunaitis J, Cunningham C, Senzer N, Kuhn J, Cramm J, Litz C, Cavagnolo R, Cahill A, Clairmont C, Sznol M: **Pilot trial of genetically modified attenuated *Salmonella* expressing the *E coli* cytosine deaminase gene in refractory cancer patients.** *Cancer Gene Ther* (2003) **10**(10):737-744.

6. Niethammer AG, Xiang R, Becker JC, Wodrich H, Pertl U, Karsten G, Eliceiri BP, Reisfeld RA: **A DNA vaccine against VEGF receptor 2 prevents effective angiogenesis and inhibits tumor growth.** *Nat Med* (2002) **8**(12):1369-1375.

7. Pálffy R, Gardlík R, Hodosy J, Behuliak M, Resko P, Radvánský J, Celec P: **Bacteria in gene therapy: Bactofection versus alternative gene therapy.** *Gene Ther* (2006) **13**(2):101-105.

8. Stritzker J, Weibel S, Hill PJ, Oelschlaeger TA, Goebel W, Szalay AA: **Tumor-specific colonization, tissue distribution, and gene induction by probiotic *Escherichia coli* Nissle 1917 in live mice.** *Int J Med Microbiol* (2007) **297**(3):151-162.

9. Xiang R, Luo Y, Niethammer AG, Reisfeld RA: **Oral DNA vaccines target the tumor vasculature and microenvironment and suppress tumor growth and metastasis.** *Immunol Rev* (2008) **222**(1):117-128.

10. Xiang S, Fruehauf J, Li CJ: **Short hairpin RNA-expressing bacteria elicit RNA interference in mammals.** *Nat Biotechnol* (2006) **24**(6):697-702.