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Review

Facultative or obligate anaerobic bacteria have the potential for multimodality therapy of solid tumours

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ABSTRACT

Recent understanding of the unique pathology of solid tumours has shed light on the difficult and disappointing nature of their clinical treatment. All solid tumours undergo angiogenesis that results in biological changes and adaptive metabolisms, *i.e.* formation of defective vessels, appearance of hypoxic areas, and emergence of an heterogeneous tumour cell population.

This micro-milieu provides a haven for anaerobic bacteria. The strictly anaerobic clostridia have several advantages over other facultative anaerobes such as salmonella or lactic acid-producing, Gram-positive, obligate, anaerobic bifidobacteria. Both pathogenic and non-pathogenic clostridia have been demonstrated to specifically colonise and destroy solid tumours. Early trials of non-pathogenic strains in humans had shown plausible safety. Genetic modifications and adaptation of pathogenic and non-pathogenic strains have further created improved features. However, these manipulations rarely generate strains that resulted in complete tumour control alone. Combined modalities of therapies with chemo and radiation therapies, on the other hand, often perform better, including 'cure' of solid tumours in a high percentage of animals.

Considering that clostridia have unlimited capacities for genetic improvement, we predict that designer clostridia forecast a promising future for the development of potent strains for tumour destruction, incorporating mechanisms such as immunotherapy to overcome immune suppression and to elicit strong anti-tumour responses.

1. Introduction

The word *tumour* originated from Latin *tumor*, meaning 'swelling'. Solid *tumours* are masses of 'swellings' made up of abnormal cells characterised by unrestricted growth in at least three different tissue compartments – the original compartment (primary tumour); the mesenchyme at the primary site (tumour invasion); and distant epiderm, endoderm and mesenchyme (tumour metastasis).[1]

Ninety percent of all human cancers are solid tumours. The initial avascular mass is harmless, but when it grows to and exceeds about 2 mm in diameter, the local vasculatures

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of the surrounding normal tissues become inadequate to support the growing tumour mass.[2] Consequently, some tumour cells are deficient in nutrients and oxygen as well as in accumulating acidic waste products, triggering cellular release of tumour angiogenic factors (TAFs). The diffusion of these TAFs into the surrounding normal tissues stimulates the endothelial cells of the nearby blood vessels to differentiate, divide, and migrate from their original basal lamina, enter the extracellular matrix, and eventually migrate towards the tumour site. Strands of such cells are formed and inter- and intracellular lumina developed to give rise to capillary tubules, which together form a network of new vessels.[2,3] The tumour mass thus becomes vascularised and new blood flow again established although the normal efficient vascular architecture expected is disturbed and is chaotic inside the growing tumour mass leading to areas of tumour hypoxia, acidity, nutrient deficiency and cell death.

2. Angiogenesis and hypoxia – the twin devils of the unique solid tumour pathology

Angiogenesis is thus fundamental for the continuation of local growth, and eventual metastatic spread of solid tumours.[3] These events are coordinated with several TAFs involved. Amongst them, vascular endothelial growth factor (VEGF) is a key stimulator, which exhibits its effect on the vasculature in paracrine and autocrine fashions.[3,4] It not only induces the sprouting, proliferation and outgrowth of capillary endothelial cells, but also increases the permeability of the capillaries and antagonises apoptosis of endothelial cells.[4,5]

In addition to angiogenic events, tumours establish their blood supply via a number of other processes as well. These include vasculogenesis, vascular remodelling, intussusception and possibly vascular mimicry by tumour cells in certain tumours.[6] As a result, the blood vessels become abnormal in structure and function while some are known to have enlarged pore sises, leaky micro-vessels and incomplete ends and yet others become a jumbled mass – thick in some areas and pinched at others.[7] As the tumour mass continues to grow, the diffusion distances between the nutritive microvessels and the number of tumour cells increase and the oxygen transport capacity of the blood is thereby reduced, due to the presence of disease or treatment related anaemia. As a result, this chaotic pathophysiology creates an unbalanced, erratic or hindered blood supply and significantly inefficient perfusion, causing many regions within the tumours to become transiently or chronically oxygen deficient, a phenomenon called hypoxia.[8]

Hypoxia is now a well characterised feature and believed to exist in almost every solid tumour.[8] Most of the hypoxic areas have oxygen concentrations of 10 mmHg or less, whereas well oxygenated tissues have oxygen concentrations of 50–60 mmHg.[9,10] To cope with the low oxygen stress, tumour cells respond by converting to anaerobic metabolism, or glycolysis, which in turn produces lactic acids, resulting in lower tissue pH.[11] Prolonged hypoxia can also increase genomic instability and genomic heterogeneity. These adaptive genomic changes allow some tumour cells to overcome nutritive deprivation or to escape from their hostile environment. These survival advantages of tumour cells can be further enhanced by genomic changes leading to loss of apoptotic potential, which may act as a selective pressure for tumour cell variants. These new variants have advantages over less adapted cells in an hypoxic micro-environment and further expand through clonal selection, often becoming the dominant cell types. These variants further intensify hypoxia, establishing a vicious cycle of hypoxia, malignant progression, and treatment resistance.[10]

Hypoxia can also act in an epigenetic fashion, further altering gene expression, such as stimulation of VEGF expression, in which the transcription factor hypoxia-inducible factor 1 (HIF-1) plays a major role. Under hypoxic conditions, HIF-1 upregulates the transcription of VEGF and stabilises its mRNA.[12] Furthermore, hypoxia-induced pathways influence VEGF activity by post-transcriptional regulation of VEGFR-2 and by hypoxia-inducible expression of VEGFR-1.[12] These changes in VEGF upregulation are believed to be associated with increased aggressiveness and metastasis of the solid tumours.

Hypoxia represents one of the most pervasive stresses, eventually resulting in tissue remodelling. Consequently, such a situation arises at the tumour site, *e.g.* the inner mass of the tumour is actually necrotic and this is surrounded by a quiescent cell region while the outer layer is made up of the actively proliferating tumour cells.[7,13,14] All of these abnormalities, *i.e.* the structuring of the primary tumour and the metastatic variants, play a fundamental role in treatment failure with current approaches.[10]

3. Facultative anaerobic and obligate anaerobic bacteria have unique abilities for tumour targeting and tumour lysing

The unique micro-milieu of solid tumours can in fact be turned to advantage as it was noticed that hypoxic regions of tumours fall into three major groups (Table 1):

3.1. *The lactic acid-producing, Gram-positive obligate anaerobic bacteria represented by bifidobacteria*

Three species of the common flora of the human intestine were the foci of past studies, *B. longum*, *B. infantis* and *B. adolescentis*.[15,16] Initial 'proof of concept' testing for the use of bifidobacteria in cancer therapy was by intravenous injection of 5×10^6 colony forming units (CFU) of *B. longum* into mice implanted with Ehrlich ascites tumours. The bacteria were shown to be highly selective and localised primarily within the tumour cells, with virtually no bacteria in other organs after 96 h. At 1 h, 10^2 CFU/g tumour tissues were present, which increased to 10^6 CFU/g by day 7. Unfortunately, no obvious oncolytic effect was observed.[15,16] Later on, *B. adolescentis* was shown to markedly induce tumour apoptosis and prevent occurrence and development of colorectal carcinoma *in vivo* in animal models.[17] The shortcoming of using bifidobacteria for cancer therapy, though, is that they are non-spore formers, and thus are more susceptible to non-permissive conditions and more difficult to store and handle.

Table 1 – Anaerobic bacteria tested as anticancer agents

Groups	Genus/specie	Features	Advantages	Disadvantages
Group I. Lactic acid-producing bacteria	*Bifidobacterium*	Gram$^+$, obligate anaerobes	Non-toxic – common flora of humans and have been	No obvious oncolytic effect
	B. longum		Used in dairy industry for many years	Non spore former
	B. adolescentis		Have been used as probiotics	More susceptible to non permissive conditions
	B. infantis		Can be used for intravenous or oral administration	More difficult to store and handle
Group II. Intracellular Bacteria	*Salmonella*	Gram$^-$, facultative anaerobes	Attenuated vaccine strain has been proved safe clinically in human	May have difficulty to infect and lyse quiescent cells
	S. typhimurium			
	S. choleraesuis	Agents for intestine infection	Biochemical pathways and genomes are well characterised Auxotrophic isolates for solid tumours have intrinsic anti-tumour activity Can target both large and small tumours Intracellular bacteria can enter target cells or professional antigen presenting cells and induce strong innate immune response	Have a tumour to normal tissue ratio of 1000:1, therefore a significant number of bacteria colonise normal organs *Wild type S. typhimurium* was associated with Gallbladder cancer Virulence factors exist, safety is an issue
	Listeria			
	L. monocytogenes	Gram$^-$, facultative anaerobes		
Group III. Strictly anaerobic bacteria	*Clostridium*	Gram$^+$,	Spore former	Some strains are pathogenic
	Proteolytic	Normal habitat in the soil, aquatic sediments, and intestinal tract of both animals and humans	Spores are easy to produce, stable and economical to use, can be used for intravenous delivery	Strain variation exists
	C. sporogenes		Safety has been shown in early human trials	
	C. oncolyticum		Shown oncolytic ability in animal experiments and human trials	
	C. novyi			
	Saccharolytic			
	C. butyricum			
	C. acetobutyricum			

3.2. *The intracellular, Gram-negative facultative anaerobic bacteria represented by salmonella*

These strains are common causes of intestinal infections and were found to colonise human tumours 50 years ago.[18] However, most salmonellae were pathogenic due to substantial immuno-stimulation produced by salmonella lipopolysaccharide and other components that induced septic shock and high mortality in humans. Salmonella used for anticancer therapy were all attenuated strains. Other bacteria, such as Listeria, have also been tested. These bacteria can infect tumour cells, which could become a target of anti-bacterial specific T-cells.[19]

3.3. *The saccharolytic/proteolytic, Gram-positive, strictly anaerobic, spore forming bacteria represented by clostridia*

The spores are easy to produce, hardy to store, and convenient and economical to use for intravenous delivery. IV injected spores are distributed throughout the body, but germination will only occur when they encounter the requisite anaerobic conditions. Spontaneous colonisation and apparent selective oncolysis were noticed in 1813 when cancer patients seemed to be cured after development of gas gangrene following infection with *C. perfringens*. The first experimental evidence came in 1947 when direct injection of spores of pathogenic *C. histolyticum* into mouse sarcoma causes oncolysis and tumour regression.[20] The first non-pathogenic strain, *C. butyricum* M55, was tested in 1955 and tumour destruction was demonstrated in Ehrlich carcinoma in mice following *i.v.* injection of the spores.[21] The dedicated researchers went on administering spores to themselves and showed that the strain did not cause any pathogenic effects. Follow-up clinical trials in humans also demonstrated safety, but the rate of tumour recurrence remained unchanged.[22] Non-pathogenic strains include proteolytic species, such as *C. sporogenes*, and saccharolytic strains, such as *C. acetobutylicum*.[23,24] In 2001, Vogelstein's group directly compared 26 species of commonly used anaerobic bacteria from the genera of *Bifidobacterium*, *Lactobacillus* and *Clostridium* on colorectal cancer xenografts, and found that

two wild-type strains – *C. novyi* and *C. sordellii* – performed better than the rest because they were highly mobile within tumours, and were particularly sensitive to oxygen. These strains have shown their natural ability as tumour killers (for details see the text below).[14]

4. Designer bacteria – genetic manipulation to improve anticancer potency

Bacteria have an enormous capacity to evolve and advances in recombinant DNA technology have made directional evolution easier, reigniting interest in the use of anaerobic bacteria as anticancer agents. Bifidobacteria have been primarily used as targeted protein delivery vehicles after genetic modification. *B. longum* was first being engineered by Fujimori's group in Japan[15,25,26] who had delivered the suicide gene cytosine deaminase (CD)/5-FC prodrug combination as well as the endostatin gene to tumour models *via i.v.* injection. Xu's group in China has used the oral route.[27] Similarly, *B. adolescentis* and *B. infantis* have also been successfully engineered by two separate Chinese groups.[28,29]

All salmonella strains tested so far are classified as designer bacteria.[30,31] *S. typhimurium* was the first attenuated and an *S. enterica serovar* Typhi strain was registered as a live oral vaccine against typhoid fever more than two decades ago. This vaccine strain was found to hinder tumour growth in a broad range of human and mouse tumours implanted in mice for long periods, even up to several weeks after untreated control mice had died of tumours.[31] Further genetic modification was focused on the delivery of therapeutic genes, such as those encoding the herpes simplex thymidine kinase (TK),[32] *Escherichia coli* CD,[33] tumour necrosis factor α (TNF-α)[34] and colicin E3.[35] A phase I study of VNP20009, a lipid-A attenuated strain in humans with melanoma, was recently reported. The bacteria were well tolerated. However, there were no anti-tumour responses observed, and bacteria were only cultured from tumour tissue in 12.5% of patients.[36] Change of the kinetics of infusion also failed to improve the response rate or colonisation in a small number of subsequently treated patients.[36] Surprisingly, when the same strain was tested in dogs with spontaneous cancer, tumour colonisation as well as local tumour regression was evident.[37–39]

A recent report detailed a designer salmonella with significant therapeutic efficacy when further mutation and selection for leu/Arg dependent auxotrophy was performed in a prostate cancer model. This approach ensured that the strain invaded tumours regardless of whether they were injected intratumourally or intravenously. The bacteria then replicated intracellularly throughout the prostate cancers, which then stopped growing and regressed by day 20 with no obvious adverse effects.[40] Further adaptation and isolation in a human colon cancer model increased the strain's tumour-targeting capability *in vivo* as well as *in vitro* when compared with the original strain. Treatment with the newly isolated strain resulted in highly effective tumour targeting, including viable tumour tissue and resulted in significant tumour shrinkage in mice with *s.c.* or orthotopic human breast cancer xenografts. Survival of the treated animals was significantly prolonged.[41]

Two metabolically different types of clostridia were used for genetic modification: the first type was the proteolytic clostridia, represented by *C. sporogenes*.[42,43] The techniques needed to genetically modify *C. sporogenes* were considered rather demanding initially. Therefore, the real success was only reported when Brown's group introduced *E. coli* CD into *C. sporogenes* NCIMB10696 by strain specific electroporation.[43] *i.v.* Injection of the spores showed a super capacity of tumour colonisation and at least 10^8 CFU/g of tumour tissue was obtained. This was accompanied by tumour inhibition, which was enhanced by the use of 5-FU. Unfortunately, for reasons unknown, this inhibitory effect did not last.

C. sporogenes (ATCC13732) was considered 'pathogenic' because of its extraordinary capacities in tumour colonisation and liquefaction, so the use of less aggressive clostridia was considered to be more advantageous. The group of Jozef Anné was the driving force behind the use of the saccharolytic clostridium, *C. acetobutylicum*, while the groups of Brown and Minton tested the genetically engineered *C. beijerinckii*.[43,44] These strains have industry values and are 'truly non-pathogenic' and easy to manipulate. Therapeutic genes, encoding CD, nitroreductase or the cytokines TNF-α or IL-12 have been introduced and a considerable amount of heterologous proteins were efficiently expressed and secreted at the tumour site, but no significant tumour inhibition was generated *in vivo* in solid tumour models.[45,46] Several factors may explain the lack of anti-tumour effects: one of them was insufficient recombinant gene expression and secretion at tumour sites, while the other was a low number of colonising bacteria. At this stage, very little work has been published on the use of these groups of non-pathogenic bacteria as successful anticancer agents.

C. novyi and *C. sordellii* have a super ability of inside the tumour colonisation and spreading, but have significant toxicity, with 40% of animals dying of toxicity after spore injection. Innovative design enabled removal by heating of the lethal toxin (α), known to be located on a bacterial phage in *C. novyi*, thus creating a genetically modified, attenuated derivative, *C. novyi-NT*.[14]

5. The anaerobic bacteria – 'platform technology' for combined modalities of cancer therapy

Thirty years ago during the hey-days of *C. oncolyticum* M55(which is the same strain as *C. butyricum* M55 and *C. sporogenes* ATCC13732 after twice being reclassified!), the newly emerged oncolytic therapy was combined with an existing beamed radiofrequency therapy to further facilitate effective tumour killing. The hypothesis was that the short-termed radiofrequency could warm up the tumour, damage tumour cells, and thus increase the necrotic areas, which in turn should create a favourable environment for *C. oncolyticum* M55 to germinate and multiply. Radiofrequency therapy was used to warm the tumours up to 42–44 °C and was followed by bacterial delivery in 861 mice bearing three types of tumours on the neck, *i.e.* Ehrlich solid carcinoma, Harding-Pasey-Melanoma, and fibrosarcoma induced by methylcholanthrene. In all the tumours tested, radiofrequency significantly enhanced oncolysis by *C. oncolyticum* M55.[47]

Further experiments were performed to optimise the time needed between applications of each therapy. Both Ehrlich

adenocarcinoma and Harding-Passey-Melanoma were implanted in the neck of mice. Pre-treatment with radiofrequency therapy was carried out first, followed by *i.v.* administration of *C. oncolyticum M55* spores. A clear dependence on the time interval between the two therapies was evident. Tumour oncolysis was most significant when spores were administered 12 h after initial radiofrequency therapy in rapidly growing Ehrlich adenocarcinoma.[48] For slowly growing melanoma, a three-therapy combination, including radiofrequency therapy, *i.v.* administration of *C. oncolyticum M55* spores and local X-irradiation was tested, resulting in a cure rate of approximately 20% of the animals. However, although the survival time was significantly longer, relapses at the sites of the primary tumours finally killed the rest of the animals. A second and third round of repeats of the three modalities combination increased the 'cure' rate, but only slightly.[49]

Another strategy to increase the oncolytic effect of *C. oncolyticum M55* was by decreasing the oxygen concentration to 11–12% in the air that the tumour-bearing animals breathe in. This combination significantly increased the bacterial oncolytic effect, resulting in microscopically complete oncolysis (Ehrlich-Solid-Tumour increased by 62% and Harding-Passey-Melanoma by 64%). With this approach, 30% of animals were cured completely of their tumours.[50]

Unfortunately, clinical use of these strategies did not generate the expected effects, and thus, there were few publications in the 80s. However, in a summarised review of 34 years of work with 1316 gliomas (508 glioblastomas = 39% patients), Heppner reported in 1986 that in addition to the operative removal and postoperative X-ray therapy, various attempts had been undertaken to prevent recurrences. These included intracerebral application of Cobalt60 ($n = 6$); locally applied antimitotic agents ($n = 76$); intracarotid administration of *C. oncolyticum M55* spores (Oncolysis, $n = 67$); circumscribed heating of the extirpation cavity with metal and high-frequency electromagnetic field ($n = 85$); and vaporisation of the tumour bed with the defocused CO2- or Neodymium-YAG-Laser beam ($n = 177$). Permanent cure was only obtained in a single case. Therefore, it was fair to say that none of the procedures were working. However, by comparison, he concluded that bacterial oncolytic therapy with *C. oncolyticum M55* in combination with surgery and periodic postoperative radiofrequency therapy locally, in the excision cavity of the tumour, might justify cautious optimism about future developments of effective therapy for glioblastomas.[51]

The use of *C. novyi-NT* spores for *i.v.* or intratumoural injection did not result in significant tumour control, but had toxicity.[14] Therefore, a combined bacterial therapy was proposed and conventional chemo agents, dolastatin-10, mitomycin C, vinorelbine and docetaxel were used in addition to the administration of *C. novyi-NT* spores, resulted in significant anti-tumour properties. Ironically, the tumour lytic effects were too good as sometimes severe systemic toxicities were evident and animals died of toxaemia or tumour lysis syndrome. *C. novyi-NT* was also combined with radiation. Three modes of radiotherapy were combined with *C. novyi-NT* and all showed an additive effect on tumour regression, resulting in long-term remission in several mouse models.[52] Radiation-enhanced effects of *C. novyi-NT* were believed to be the consequence of bacterial attacks on tumour cells. This was because tumour cells were least sensitive to radiation as they were not dividing. Radiation was only effective in rapidly proliferating cells. In solid tumours, only 3–5% of cells are in the growth fraction and 95% of tumour cells are hypoxic or prenecrobiotic.[53] Furthermore, the use of bacterial oncolysis could reduce the effective radiation dose when compared with that used in conventional radiotherapy.[54] The results clearly point out the importance and potential of combined multi-modality approaches, including newer genetic manipulations for the development of effective cancer therapies.

6. Conclusions

The unique pathophysiology of solid tumours causes huge problems for conventional therapies, meanwhile presenting opportunities for the development of new and innovative strategies. Several new approaches are on the horizon, including a variety of viral and non-viral based gene therapy systems.[54–56] Amongst these, replication-competent, viral vector-mediated cancer therapy is most promising.[57,58] However, even this system suffers from several deficiencies: first, the vectors currently have to be injected intratumourally to elicit an effect. This is far from ideal as many tumours are inaccessible and spread to other areas of the body making them difficult to detect and treat. Second, because of the heterogeneity within a tumour, the vectors do not efficiently enter and spread to every tumour cell. Third, hypoxia, a prevalent characteristic feature of most solid tumours, reduced the ability of the viral vector to function and decreased viral gene expression and production. Consequently, a proportion of the tumour mass is left unaffected and capable of re-growing. Therefore, there have rarely been any protocols that have gone on to phase III trials.

However, anaerobic bacteria have been shown to selectively colonise and regerminate in solid tumours when delivered systemically. The bacteria cause oncolysis irrespective of the tumour heterogeneity and the hypoxic environment which has turned out to be a favourable attraction to the bacteria, rather than a deterrent. With innovative genetic modifi-

Table 2 – Bacterial vectors versus replication competent viral vectors

Advantages	Oncolytic viral vectors	Modified bacteria
Safety	+	+++
Easy and economic production	+	++
Convenient to store	0/+	+++
Easy administration	+	+++
Systemic delivery	0/+	+++
Extracellular growth	0	+++
Transduce tumour cells to kill	+++	0
Effect on stromal cells	0	+++
Gene integration	+/++	0
Mutagenesis	+/++	0
Clear out of the circulation	0	+++
Potential long-term side effect	++/+++	+
'Cure' tumours	0/+	++

0, no effect; +, small effect; ++, medium effect; +++, significant effect.

cations or smart combined therapies, bacterial oncolysis has resulted in 'cures' of solid tumours in several animal models. A direct comparison of replication-competent, viral vector-mediated cancer therapy with oncolytic anaerobic bacterial therapy has shown several distinct differences (Table 2).

Recently, a number of immunosuppressive mechanisms at the solid tumour site have been recognised, including 'tolerance to self antigens' and the presence of regulatory immune cells that shut-down effector T-cell function.[59,60] Considering that the delivery of *C. novyi-NT* generated a potent immune response against syngeneic tumours in immuno-competent animals with 30% of the animals having had a 'cure', we believe that new antigens from the destroyed tumour cells or 'molecules' secreted by or associated with *C. novyi-NT* may have broken up tumour 'self tolerance'.[61] Identification of these mechanisms will provide significant opportunities for designing new clostridia that can achieve higher 'cure' rates, such as generating clostridial strains that can combine with antibody-mediated therapies, and other forms of immunotherapies. Current recombinant DNA technologies are well in place to achieve these goals. Newer and effective therapies for solid tumours based on designer bacteria in combination with novel clinically applicable strategies will be a reality in a very near future.

Conflict of interest statement

None declared.

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