Cancer Gene Therapy - Developments and Future Perspectives

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1. Introduction

Cancer is the second leading cause of death in the Western world. Whilst there are many therapies which can significantly improve patient outcomes, there is still no definitive "cure" for cancer. Over the last decade we have witnessed gene therapy developing at a very fast pace representing a potential new and more effective modality for the treatment of cancer. By the end of 2010, over 1060 gene therapy protocols have been proposed or trialled in the clinical setting for various cancers; this figure represents over 64% of all gene therapy trials in humans in the United States. From many of these trials modest therapeutic responses have been reported, however, unequivocal proof of clinical efficacy is still to be seen. In 2003, the first commercially produced gene based product was manufactured by Shenzhen SiBiono GeneTech. Gendicine™, a replication-incompetent recombinant human Ad5-p53, was approved by the Chinese State Food and Drug Administration to treat head and neck squamous cell carcinoma. Again in 2006, Shanghai Sunway Biotech, commercially released a conditionally replicative adenovirus therapy, Oncorine™[8]. However, it is fair to say that cancer gene therapy has yet to realise its full potential.

2. The gene therapy vector systems

An important feature for any successful gene therapy protocol is the vector system. To date, a number of vectors have been developed, including both viral and non-viral based therapy systems (Table 1). Each of these vectors possesses a number of unique features and each has its own advantages and disadvantages. The most promising existing vectors are the replication-competent oncolytic viral vector based gene therapy systems, particularly the adenoviruses[9, 10].

Vector	Advantages	Disadvantages
Adenovirus	Easy to produce, Can achieve high titre, High efficiency of gene transfer	Large DNA virus, many viral genes, often Immunogenic
Adeno-associated virus	Relatively easy to produce, Has ability for gene integration	Wide type Adenovirus contamination
Retrovirus	Can be made through packaging cells, Has ability for gene integration	Low efficiency of gene transfer, dividing cells only
Herpesvirus	Has Neurotropic affinity, Can be applied to Neurons or Glioma	Large DNA virus, many viral genes, Immunogenic
Viral replicons: Semliki Forest virus	Easy production, less immunogenic	Difficult to purify
Non-viral vectors	Simple, easy to make and easy to use	Low efficiency

Table 1. List of commonly used vectors for cancer gene therapy as well as their advantages and disadvantages

3. Viral vector systems

3.1 Adenoviral vectors

Adenovirus vector (AV) is the most commonly studied and most widely used system in cancer gene therapy. It is of particular use for cancer gene therapy applications, where temporary gene expression is acceptable or even beneficial. There are several serotypes, but the currently employed AVs in clinical trials are mostly based on serotype 5. These vectors can replicate highly and have demonstrated efficient gene transfer into various types of cancer cells[11]. Two AVs related gene therapy products have been approved for clinical use in patients suffering from head and neck cancers in China, replication-incompetent recombinant human Ad5-p53[12], and the other is a conditionally replicative adenovirus therapy.

Several other adenoviruses, based on canine, porcine, bovine, ovine and avian adenoviruses have been developed. The ovine AV is based on serotype 7 and was developed in Australia. Preclinical testing of ovine AV on prostate cancer in animal models has shown therapeutic efficacy[13].

Unfortunately, AVs contain many viral genes which encode for major proteins that elicit a strong host immune response. Of particular concern is the release of cytotoxic T lymphocytes that lyse cells expressing the recombinant genes. Newer generations of AV vector have been designed to overcome some of these problems and initial results are encouraging. New techniques involved in removing the recombinant viral genes and transfecting the non-recombinant plasmid with a helper virus and then separating the helper virus with sedimentation techniques have been developed. Improvements in helper virus have also been trialled that reduces "floxed" helper virus production 1000-fold, but this method still has a 1% wide type (WT) contamination thus still allowing the possibility of *in vivo* recombination. With regard to AV-mediated cancer treatment, high-level tumour transduction remains a key developmental hurdle. To this end, AV vectors possessing infectivity enhancement and targeting capabilities should be evaluated in the most stringent

model systems possible. Advanced AV-based vectors with imaging, targeting and therapeutic capabilities have yet to be fully realized; however, the feasibilities leading to this accomplishment are within close reach[14].

3.2 Adeno-associated virus (AAV)-based vectors

AAV-based vectors have been shown to be non-toxic and undergo widespread cellular uptake in preclinical evaluation[15]. A recent study has compared five different AAV strains and amongst them, serotype 2 was proven to be the most efficient killers of tumour cells. In another study, serotype 8 AAV vector encoding a soluble vascular endothelial growth factor (VEGF) receptor was able to halt tumour growth in several rodent glioma models. However, difficulties in the development of packaging cell lines for AAV, as well as bulk production and vector purification have been reported as problematic[16]. A new system was developed recently to scale up and bulk production of AAV from insect cells which may solve some of these existing problems[16].

3.3 Retroviral vectors

The earliest system that was developed for gene therapy was the murine Moloney leukaemia virus (MoMLV)-based retroviral vector. More than 23% of all gene therapy trials in patients for various diseases have used a replication-defective or competent MoMLV vector system. This vector's unique ability to transduce dividing cells[17] makes it an ideal choice of vector for continuously dividing and fast growing tumours, such as gliomas. However, poor vector penetration and lack of vector migration away from the injection site is usually seen[18]. To overcome this effect, replication competent MoMLV was developed. A 2005 publication showed complete transduction of human U87 glioma xenografts in nude mice after a single intracranial (*i.c.*) injection of replication-competent MoMLV[19]. In this study, viral envelope was stained positively in glioma cells away from the injection sites. Most importantly, no virus was detected in any non-tumour tissue which is good indication of strict tumour specificity.

Potential limitations of retroviral vector systems are related to their ability to activate cellular oncogenes and inactivate tumour suppressor genes by insertional mutagenesis. One study used MoMLV to transduce bone marrow stem cells for the therapy of severe combined immune deficiency syndrome (SCID), following treatment 4 out of 11 children subsequently developed leukaemia[20]. In addition to improving safety, there are also studies that have shown that dissemination of the vectors in solid tumours still needed to be improved in order to reach clinical efficacy.

Lentiviral vectors are a more recently developed and complicated retroviral vector, based on the Human or Bovine Immunodeficiency Virus. They have all the unique features of MoMLV and have been shown to transduce post mitotic cells *in vitro* and *in vivo*[21]. Studies with the Human Immunodeficiency Virus (HIV)-based vectors have shown efficient gene transfer in tumour models. An understandable reluctance in the use of potentially pathogenic HIV vectors in humans has been seen, although a clinical trial assessing use of lentiviral vector for the therapy of AIDS is underway[22]. To reduce the risk of seroconversion in human patients several bovine based vector systems have been developed, which have the advantage of less or no pathogenicity in humans. We have also developed a bovine lentiviral vector system based on the Jembrana Disease virus (JDV)[23, 24]. JDV only causes disease in a specific species of cattle in the Jembrana district in Bali,

Indonesia, but does not affect humans. Pathological changes in cattle include intense non-follicular lympho-proliferation by reticulum and lymphoblastoid cells in lymphoid organs. Protein and genome sequence studies have confirmed that JDV has a genome of 7732 nucleotides and structure and organisation similar to other members of the lentivirus family. More importantly, JDV possesses several features in common with HIV that are very attractive as a vector, including a high replication rate and the ability to efficiently integrate into chromosomes of non-dividing and terminally differentiated cells.

3.4 Herpesvirus-based vectors

Vectors based on herpesviruses are well-developed and have progressed to clinical trials. As with other viral vectors, replication-defective vectors have not shown much potential. The first replication competent vector was based on a mutant strain, where the vectors are deleted from the main neurovirulence gene r34.5, thus restricting its ability to replicate in adult central nervous system and to form latency. However, later study showed that the mutant strain which had the deletion of the r34.5 gene also had reduced capacity for replication inside tumour cells[25]. New vectors were developed with a deletion of the ICP47 gene which does not appear to impact on efficient replication.

Pre-existing immunity may pose a problem that limits the clinical efficacy of herpesvirus-based vectors. This immunity prevents the transduction of peripheral organs and also can cause liver toxicity. However, a recent mutant strain-secreting cytokine granule macrophage colony stimulatory factor (GM-CSF) or IL-12 was shown to be effective in liver cancer therapy in a murine model which likely involves both direct viral oncolysis and actions of specific immune effector cells[26].

3.5 Viral replicons and transposons

Semliki Forest virus (SFV) subgenomic replicons (i.e. non toxic replication) have been developed that allow stable expression of a required gene e.g. beta-galactosidase (beta-Gal) in mammalian cell lines. Studies showed that expression remained high (approximately 150 pg per cell) throughout cell passages[27].

Since construction of the Sleeping Beauty transposon from defective copies of a Tc1/*mariner* fish element[28], new vertebrate genetic manipulation tools (i.e. transposase enzymes) have become available for gene therapy. This particular transposase in the system binds to the inverted repeats of salmonid transposons that surround the insertion gene and mediate precise 'cut and paste' into fish, mouse and human chromosomes. Potential problems with the use of transposons for gene therapy may arise from having no 'off' switch for the transposase and the relatively low quantities of integrated product, either of which would make retroviral intergrase a more suitable or   alternative enzyme for chromosomal integration.

3.6 Targeted viral vectors

While efforts have been focused on the continuing refinement of various vector systems, several obstacles remain, primarily the low efficiency of gene delivery into target tumour cells. The vascular endothelial wall is a significant physical barrier prohibiting access of systemically administered vectors to the tumour cell. To overcome this obstacle, strategies are currently being developed to take advantage of transcytosis pathways through the endothelium. An AV vector targeted to the transcytosing transferrin receptor pathway,

using the bifunctional adapter molecule has been constructed[29]. The transcytosed AV virions retain the ability to infect cells, establishing the feasibility of this approach. However, efficiency of AV trafficking via this pathway is poor. Other efforts are directed towards exploring other transcytosing pathways such as the melanotransferrin pathway, the poly-IgA receptor pathway, or caveolae-mediated transcytosis pathways. There are hopes to develop mosaic AV vectors incorporating both targeting ligands directed to such transcytosis pathways as well as ligands mediating subsequent targeting and infection of tumour cells present beyond the vascular wall[30].

3.7 Viral vector-associated multifunctional particles (MFPs)

Nanotechnology has recently been incorporated into viral vector systems in the form of multifunctional particles (MFPs). Nanotechnology is defined as the development of devices of 100 nm or smaller, having unique properties due to their scale. The devices that are being developed generally incorporate inorganic or biological material. In this regard, the coupling of inorganic nano-scale materials to targeted AV vectors has much potential for tumour targeting, imaging and amplified tumour killing capacities. For example, magnetic nano-particles have recently received much attention due to their potential application in clinical cancer treatment; targeted drug delivery and magnetic resonance imaging (MRI) contrast agents[31]. However, despite the useful functionalities that might derive from metal nanoparticle systems, the lack of targeting strategies has limited their application to locoregional disease. Thus, tumour-selective delivery is the key to improve therapeutic applications of this technology.

AAV has been developed with MFP, by virtue of genetic capsid modifications, to incorporate additional functionalities, such as modified fibres, combined with imaging motifs on the pIX protein, to simultaneously target tumour cells while monitoring viral replication and spread. Herpes simplex virus thymidine kinase (HSV TK) has been incorporated at pIX site of the AAV capsid. This enzyme is compatible with available PET imaging ligands such as ^{18}F-penciclovir, providing an imaging system for viral replication that can directly be translated for clinical applications. Interestingly, HSV TK is an enzyme that has utility in so-called suicide gene therapy, in which the expressed enzyme converts a substrate such as ganciclovir to its phosphorylated metabolite, which can then be further phosphorylated by cellular kinases to a toxic metabolite, causing cell death[32]. Also, tumour cells expressing this gene product induce the death of adjacent cells via the so-called 'bystander effect', thus representing an 'amplifying strategy' as mentioned above.

3.8 Oncolytic virus

An oncolytic virus is a virus that has the ability to infect cancer cells and cause oncolysis. It is with obvious reasons that these types of viruses have received much attention in the field of cancer therapy as they can result in direct destruction of the tumour cells. Initial research into the anticancer potential of oncolytic viruses examined naturally occurring oncolytic viruses including adenovirus, poliovirus and Coxsackie virus[33-36]. However, these studies highlighted a number of limitations with naturally occurring oncolytic viruses including uncontrolled infection, incomplete oncolysis and the development of an immune response[33, 35]. The stimulation of the immune system prevented the virus from destroying the cancer and therefore reduced the efficacy of the treatment. Due to these limitations many researchers discontinued their research into naturally occurring oncolytic viruses.

Recent advances in genetic modification techniques of oncolytic viruses have again awoken researchers' interest into these agents for cancer therapy. There are now many different oncolytic viruses currently being trailed as potential therapeutic agents (Table 2)(reviewed in [37]). The first to be produced for the clinic was ONYX-015 which has been shown to be a safe anticancer agent[38, 39].

Name	Oncolytic virus strain	Type of tumour
ONYX-015	Adenovirus	Head & neck
		Ovarian
		Primary & secondary liver tumours
		Pancreatic
CV706	Adenovirus	Prostate
CV787	Adenovirus	Prostate
G207	HSV	Glioma
NV1020	HSV	Colorectal
		Liver metastases
Vaccinia-GM-CSF	Vaccinia	Melanoma
PV701	NDV	Advanced solid tumour
1716	HSV	Glioma
		Melanoma

Table 2. List of oncolytic viruses used in clinical trials (*adapted from* [37]).

4. Limitation of viral vectors

Although both viral vectors and oncolytic viruses are widely used in cancer gene therapy research, these vectors suffer from several limitations. The first of these is the virus's inability to specifically seek out and target the tumour. The majority of therapies that utilize these vectors currently require intratumoural injection to elicit an effect. Whilst this approach may be beneficial in some cases, its uses are limited as many tumours are inaccessible and may have already spread to other areas of the body at the time of diagnosis and treatment, making them difficult to locate and treat. Another deficiency of viral vectors is their lack of capacity to efficiently penetrate and kill every cell within the tumour mass. This results in non-tumour stromal cells being unaffected thus having the ability to regrow into another tumour. Finally, the tumours hypoxic microenvironment also reduces the effectiveness of the viral vector.

Hypoxia is a prevalent feature found within most solid tumours. When a tumour grows to and exceeds about 2mm in diameter, the local vasculatures of the surrounding normal tissues become inadequate to support the growing mass[40]. This results in a increase in angiogenesis within the tumour resulting in the tumour becoming more vascularised. However, the normal efficient vascular architecture is disturbed and chaotic inside the growing tumour mass which leads to areas of tumour hypoxia, acidity, nutrient deficiency and cell death. These hypoxic regions have the ability to reduce the effectiveness of viral vectors thereby decreasing gene expression and proliferation leaving a proportion of the tumour mass unaffected, which may result in tumour regrowth.

4.1 Oncolytic clostridia

Solid tumours account for approximately 90% of all diagnosed cancer. Our current understanding of the unique microenvironment of solid tumours has highlighted the deficiencies of both traditional therapies and new viral gene therapies and now requires a rethink in the design of vectors. As mentioned, this hypoxic core provides a barrier for current gene therapy vectors; however, it is also the perfect environment for anaerobic bacteria.

Class	Species	Features	Advantages	Disadvantages
Class I:	*B. longum*	Gram+ non-motile	Non-pathogenic present in common intestinalflora,	No obvious oncolytic effect
Bifido-bacteria	*B. adolescentis*	obligate anaerobes	Have been used in human for many years	Non-spore former
	B. infantis		Probiotic bacteria	More susceptible to non-
			Can be used for intravenous or oral administration	permissive conditions
			Expression of recombinant protein	More difficult to store and handle
Class II:	*Salmonella*	Gram– facultative	Attenuated vaccine strain has been proved safe	Intracellular bacteria, thus may have difficulty to
Facultative	*S. typhimurium*	anaerobes	clinically in human,	infect and lyse quiescent
intracellular	*S. choleraesuis*	Agent for	Biochemistrypathways and	cell
bacteria		intestine infection	genomes are well characterized	Have a tumour to normal tissue ratio of 1000:1,
			Auxotrophic isolates for	therefore a significant
			solid tumours have intrinsic antitumour activity	number of bacteria colonize normal organs
				Cell wall components are immunogenic
	Listeria	Gram+, facultative	Grow under aerobic and anaerobic conditions, thus	Virulence factors exist, especially LPS in the
	L. monocytogenes	anaerobes	can target both large and	bacterial cell wall, thus
	E. coli	Gram–,	small tumours, enter	safety is an issue

Class	Species	Features	Advantages	Disadvantages
		facultative	professional antigen	when large amount of bacteria
		anaerobes	presenting cells and induce strong innate immune response	are delivered
			Have the potential as a vaccine vector for tumour therapy	
			Biology is well studied and known	
Class III:	*Clostridium*	Gram+, strictly	Spore former	Some strains are
Strictly	Proteolytic	anaerobes	Spores are stable, easy to	pathogenic
Anaerobic	*C. sporogenes*	normal habitat in the soil,	produce and economic to use	Some strains are difficult to manipulate genetically
bacteria	Saccharolytic	aquatic sediments,	Clostridialspores can be delivered non-invasively	Only colonize in large tumours with area of
	C. novyi	and intestinal tract of both	and systemically, i.e. intravenous injection	hypoxia/necrosis
	C. butyricum	animals and humans	Have shown extensive oncolytic ability	Oncolysis interrupted at the rim causing incomplete
	C. acetobutylicum		Spores are non-immunogenic and can be	tumour lysis
	C. oncolyticum		repeatedly delivered	
	C. beijerinckii		Oncolysis occurs irrespective of tumour cells' heterogeneity or growth status	

Table 3. Types of aerobic and facultative anaerobes used in cancer gene therapy (*adapted from* [41]).

Both facultative and obligate anaerobic bacteria have several advantages over traditional viral vector systems as they have been shown to selectively target, colonise and regerminate into vegetative cells in the hypoxic microenvironment of solid tumours when delivered systemically (Table 2)[41].   One such bacterium, the strictly anaerobic *Clostridia*, have also

been shown to cause tumour lysis and destruction even without any genetic modification. A number of non-pathogenic strains of *Clostridia* have also shown plausible safety when used in humans. However, these vectors also have limitations. As these bacterium require an anaerobic environment to survive, oncolysis is almost always stalled at the proliferative outer rim of the tumour mass. More recently, combinational approaches have to be implemented to deal with these limitations and a number of trials have commenced to test these protocols.

The intrinsic property of *Clostridia* to specifically target and colonise only within the hypoxic core of the tumour enables them to serve as perfect vectors for the delivery of genes for cancer therapy. Already, clostridial spores have been genetically manipulated to deliver genes for cancerstatic factors, prodrug converting enzymes, or cytokines with the aim of improving their innate oncolytic activity. Furthermore, when used in combination with conventional chemotherapy or radiation therapies these vectors often perform better then *Clostridia* alone. Another advantage of clostridial spores is their seemingly unlimited capacity to carry exogenous genes. This remarkable characteristic will allow for the development of novel ways to equip *Clostridia* with gene combinations that may have the ability to break immune suppression, overcome the limitation of tumour lysis in the outer rim or cause a strong anti-tumour response with the ability to eliminate tumour metastases, the ultimate cause of cancer death.

It is obvious that a major step towards the development of an effective cancer therapy will be to construct a vector that targets the tumour alone, and is capable of spreading to and throughout the tumour found in tissues. Clostridial spores fit into this equation very well. Clostridia are strictly anaerobic. They are gram-positive, rod-shaped, and form spores under unfavourable conditions. There are about 80 species and several of these have been tested in solid tumours. All known species require anaerobic conditions to grow but do vary in their oxygen tolerance and their biochemical profile. Clostridial spores have been administered intravenously and showed a distinct advantage for use in cancer therapy as they are easy to produce and store. Germination of spores will only occur when they encounter the requisite anaerobic conditions. Spontaneous colonization of tumours in cancer patients and the apparent selectivity of Clostridia for tumours were noticed more than 50 years ago. The first experiment in 1947 showed that direct injection of spores of *C. histolyticum* into mouse sarcoma caused oncolysis (liquefaction) and tumour regression[42]. Later experiments proved this selectivity by injecting mice *i.v.* with spores of C. tetani, the causative agent of tetanus. Injected non-tumour bearing animals remained healthy. However, tumour bearing mice died within 48 h because of *C. tetani* colonisation and tetanus production. This provided evidence that the *C. tetani* were able to germinate/replicate selectively in the anaerobic environment found within tumours, and released their toxins systemically[6]. Obviously, it would not be appropriate to use pathogenic strains of Clostridia for clinical therapy in humans. A non-pathogenic strain of *C. butyricum* M-55 has been isolated[43]. M-55 was later reclassified as *C. oncolyticum* and taxonomic studies have now clearly established that it is a *C. sporogenes* strain (ATCC13732). This is a proteolytic species causing liquefaction of colonised tumours. This was later verified by testing more isolates.

Saccharolytic clostridia, such as *C. beijerinckii* NCIMB8052 spores administered intravenously to EMT6 tumour-bearing mice germinated in the necrotic tumour regions while the oxygenated tumour areas remained free of spores[44]. Equally, intravenous injection of rhabdomyosarcoma-bearing rats with at least 107 spores of *C. beijerinckii* ATCC17778, C. acetobutylicum DSM792 (= ATCC824) or *C. acetobutylicum* NI-4082

(reclassified as *C. saccharoperbutylacetonicum*) showed tumour colonisation without complete tumour lysis[45].

C. sporogenes was the first *Clostridium* to be gene modified and this was performed with the E. coli Colicin E3 gene. Colicin E3 encodes a bacteriocin shown to have canceriostatic properties[46]. However, the overall anti-tumour efficacy of this bacteriocin was limited. This may have resulted from poor gene modification methodologies which were improved with the application of electroporation. In 2002 Prof. Brown's group introduced *E. coli* cytosine deaminase (CD) into *C. sporogenes* NCIMB10696 by electroporation[47]. Intravenous injection of the recombinant spores followed by the systemic administration of the prodrug 5-FC inhibited tumour growth which was more pronounced than the use of prodrug alone. Unfortunately, for reasons unknown this inhibition in tumour growth did not persist. However, it was clear that *C. sporogenes* has a great capacity to colonise the tumour. At least 10e8 CFU/g of tumour was obtained following the intravenous injection of the spores.

Saccharolytic Clostridia strains including *C. beijerinckii* ATCC17778, *C. acetobutylicum* DSM792 (ATCC824) or *C. acetobutylicum* NI4082 (reclassified as *C. saccharoperbutylacetonicum*) and *C. butyricum* are non-pathogenic and their development has been industry funded. Therapeutic genes, encoding the cytokine tumour necrosis factor *alpha* (TNF-α), CD or nitroreductase (NTR) have been introduced into these strains[48, 49]. Following transformation of *C. acetobutylicum* using strain-specific electroporation protocols, CD expression was monitored in lysates and supernatants of early logarithmic growth phase cultures of recombinant *C. acetobutylicum* (pKNT19closcodA)[50]. A considerable amount of heterologous protein was expressed and efficiently secreted. Also, *C. acetobutylicum* strains NI4082 and DSM792 engineered to produce cytosine deaminase were able to express and secrete this enzyme at the tumour site[48, 49]. Functional CD enzyme was detected in the tumour of rhabdomyosarcoma-bearing WAG/Rij rats that were injected with the recombinant *C. acetobutylicum*, but not in control animals. Animals, concomitantly treated with antivascular chemical agent, CombreAp, showed higher incidence of CD-positive tumours (100 versus 58%). Moreover, the level of active CD in these tumour specimens was considerably higher (mean conversion efficiency of 5-FC to 5-FU ~11%) as compared to tumours not treated with the vascular targeting drug (mean conversion efficiency of 5-FC to 5-FU ~11%) when compared to untreated tumours (mean conversion efficiency of 5-FC to 5-FU ~3%)[49]. However, when these recombinant strains were used in solid tumour models *in vivo*, there was a consistent lack of significant tumour regression observed. Factors that may have contributed to this lack of efficacy include a low level of bacterial colonisation of the tumour or insufficient recombinant gene expression and secretion at the tumour site[51]. Recent studies have reported the development of vectors utilising super tumour coloniser *Clostridial* strains *C. sporogenes* or *C. novyi-NT*. Recombinant *C. sporogenes* and *C. novyi-NT* overexpressing NTR showed significant *in vivo* anti-tumour effects[52] when used with prodrug demonstrating the clinical potential of these vectors.

5. Advantages of clostridial spores for cancer gene therapy

At present, there are various gene therapy vector systems under development against cancer. However, due to the complexity of the solid tumours involving angiogenesis, hypoxia, stromal cell, tumour cell heterogeneity and the emergence of de-differentiated stem cells, none of the existing vectors are holding any real promises. The clostridial spore-based vector system is not infectious, and has gained renewed interest, because of the following true advantages.

5.1 Preferentially growing within the unique tumour microenvironment

The biological properties of virus-based vectors, in particular the ability to enter and replicate (in the case of replication-competent viral vectors) within a tumour cell and then spread from cell to cell are highly relevant for effective cancer therapy. However, recent understanding of tumour pathology has revealed that several features of the tumour environment may not be conducive for viral replication[40, 53]. Hypoxia is an important feature of solid tumours and the ability of viruses to enter and replicate in hypoxic cells may be a critical determinant for the success or failure of viral vector-mediated cancer gene therapy. Turning off protein translation is a central process in the cellular adaptation to many types of stress, including viral infection and hypoxia. The hypoxic cells, the apoptotic cells, the quiescent cells are all refractory to viral entry and replication[54]. This is a major problem for virus-based vectors because if the vector can't reach a tumour cell, it can't act or deliver a therapeutic gene. On the contrary, clostridial spores are able to home in on these niche environments because of their own unique metabolic need, which enable them to utilise the tumour micro milieu and respective tissues for their own proliferation. Both wild-type and genetically modified Clostridia have been demonstrated to specifically colonise and destroy solid tumours. "Trojan horse" vectors have further created improved features that enable them to kill tumour cells through multimodality mechanisms.

5.2 Easy production

All of the viral vector systems need sophisticated cell culture systems, expensive culture media, rounds of filtrations and purifications and dedicated centrifugation and storages. On the contrary, clostridial spores can be easily and inexpensively produced from anaerobic bacterial culture. There are only a few steps involved and the spores, once produced can be stored at room temperature for at least 3–6 months.

5.3 Easy administration

While most viral vectors have to be intratumourally injected, intravenous injection of resuspended clostridial spores are possible and sufficient as they will be leaked out of the incomplete vessels in the solid tumour, thus specifically targeting to and colonising the hypoxic regions of the tumours.

5.4 Safety

Safety is always a concern when live vector systems are used for human gene therapy. Some of the hurdles of using viral vectors include: (1) whether the vector is sufficiently targeted to tumour alone; (2) whether the vector expresses low levels of viral genes that may lead to increased toxicity and immunogenicity[55]; (3) possible immunogenicity of the transgene that may be reduced with a reduction in the duration of gene expression[56]; and (4) whether viral particles are sequestered within the target cells or secreted into body fluid such as urine and subsequently spread into environment. We postulate that the use of clostridial spore based vectors may be a safer option to using viral vectors. Clostridia are strictly anaerobic, are tumour targeted and would be unable to live in non-hypoxic environments. A recent experiment with *C. novyi-NT* has demonstrated that the strain was unable to colonise artificially created infarcted heart where the level of hypoxia was inadequate to support the replication of the Clostridia[7]. Early trials of non-pathogenic Clostridia strains in patients have demonstrated safety. In the unlikely event of an adverse effect, clostridia can be eliminated from the blood stream with the use of readily available antibiotics such as

metronidazole which showed total spore clearance from the blood stream after 9 days of treatment[4].

6. Indiscriminant destruction of all cell types within the tumour

Solid tumours comprise not only malignant cells, but also extracellular matrix and many other non-malignant cell types, including stromal cells such as fibroblasts, endothelial cells and inflammatory cells. The mechanisms of clostridial vector-mediated tumour killing consist of several aspects: one is from its transgene that encodes prodrug converting enzymes for suicide-gene therapy or cytokines for immuno-gene therapy. These are essentially the same as the viral vectors. However, there is another side of the tumour killing effect that is resulting from the consequences of an innate antitumour effect of the clostridial strain due to production of hydrolytic enzymes including proteases, lipases, and nuclease. Furthermore, there is also a nutrients competition between the clostridia and cells surrounding them (including tumour cells, stromal cells and stem cells), where the clostridia multiplied much faster than the mammalian cells. The cumulative multiplications and the combined events of energy and substance metabolism effectively depleted the limited nutrient source and deprive the tumour cells, causing starvation and death. More recently, there were observations that indicate the germination of the clostridial spores, the transformation from spores to vegetative rods, and the continue multiplications of the vegetative rods inside the tumour activated the immune system, assisting the antitumour effects[57]. These tumour killing mechanisms destroy not only tumour cells, but also any other cells in their vicinity. These are characteristics that viral vectors are not so well equipped, nor any existing convectional cancer therapies.

6.1 Extracellular agent
While viral vectors need access to viable target cells and their cellular machinery to achieve transgene delivery and expression, this goal is often difficult to fulfil as some tumour cells are not viable at the time of gene delivery. Furthermore, none of the existing vector systems efficiently transfer genes to every tumour cell which subsequently allows for tumour regrowth. On the other hand clostridial spore replication is not tumour cell dependent and occurs via rod multiplication extra-cellularlly. Furthermore, the tumour killing mechanism of clostridial spores may operate independently of the requirement for gene transfer. Without the requirement for gene integration into the host cell genome removes the possibility of insertional mutagenesis when using Clostridia. Therefore, Clostridia may show tumour killing irrespective of the tumour cell heterogeneity found within the tumour environment.

6.2 No restrictions on accommodating therapeutic genes
One of the primary limitations of most viral vectors has been the small size of the virion, which only permits the packaging of very limited sizes (usually a few kilobases) of exogenous DNA that includes the promoter, the polyadenylation signal and any other enhancer elements that might be desired. However, for clostridia size limitations are far less restricted, not only because the plasmids used can harbour much larger DNA fragments, but in case the foreign gene is integrated in the host chromosome there is in fact unlimited capacity for insertion of therapeutic genes, forecasting the promising future for the development of ever powerful vectors.

7. Other developments for cancer gene therapy

Another potential direction for cancer gene therapy has come from the discovery of small non-coding RNAs that can significantly interfere with gene expression levels at the post-transcriptional level. RNA interference (RNAi) is an evolutionary conserved mechanism for specific gene silencing that can result in the degradation or inhibition of homologous mRNA. The most widely studied types of RNAi include both micro-RNA (miRNA) and short interfering RNA (siRNA).

Post-transcriptional gene silencing by RNAi represents an essential part of endogenous gene regulation, with much evidence to suggest that RNAi regulates more than one third of all cellular mRNAs, and that each RNAi can control hundreds of gene targets in both normal and diseased conditions. More recently mutations or aberrant expression patterns in RNAi have been shown to correlate with various diseases, including cancer, and indicate that some RNAi may have a tumour suppressor function or operate as oncogenes. It is now believed that RNAi may prove to be a beneficial factor in the treatment of cancer by either altering the endogenous levels of these RNAi or by the introduction of new RNAi to alter the expression of cancer causing genes.

RNAi therapy has already been trialled in a number of different models, however, several critical limitations must first be overcome. These limitations include non-specific effects in non-tumour tissue, potential toxicity due to interactions with endogenous RNAi, half-life of silencing, and inability to specifically target only the tumour mass. One possible approach, to overcome many of these limitations, is to use RNAi in combination with *Clostridia* as a gene delivery system to the hypoxic core of the tumour.

8. Conclusion

The unique pathophysiology of solid tumours presents a huge problem for the conventional therapies. Thus, the outcomes of current therapies are so far disappointing. Several new approaches aiming at developing effective treatments are on the horizon. These include a variety of virus-based therapy systems[2, 58, 59]. Amongst all these, replication-competent viral vector-mediated cancer therapy is most promising[60, 61]. 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 vector does not efficiently enter and spread to sufficient numbers of tumour cells. Third, hypoxia, a prevalent characteristic feature of most solid tumours, reduces the ability of the viral vector to function and decrease viral gene expression and production. Consequently, a proportion of the tumour mass is left unaffected and capable of re-growing. Fourth, pre-existing immunity pose a problem for the efficacy of viral vectors. Therefore, there have rarely been any cures with the use of the system.

The strictly anaerobic clostridia, on the other hand, have been shown to selectively colonise in solid tumours when delivered systemically and has resulted in high percentage of "cures" of experimental tumours. A phase I clinical trial combining spores of a non toxic strain (*C. novyi-NT*) with an antimicrotubuli agent has been initiated[62]. Genetic manipulation of clostridia to make them into "Trojan horse" vectors will provide further tumour killing mechanisms and amplifying antitumour effects. Clearly, it is just a matter of time that a "Trojan horse" type of clostridium will become a clinical reality, especially if we can further improve upon the system by providing additional features, ideally including (i) targeting tumours only and not anywhere else, (ii) able to effective kill primary tumours as well as metastases. Current

technologies are in place to achieve these goals. Newer and effective therapies for solid tumours based on the "Trojan horse" will be a reality in a very near future.

9. References

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