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Viral vectors in pancreatic cancer gene therapy

Review Article

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Abbreviations: argine-glycine-aspartate, (RGD); cat endogenous virus, (CEV); conditionally replicating adenoviruses, (CRADs); coxsackie adenoviral receptor, (CAR); cytosine deaminase, (CD); fibroblast growth factor receptors, (FGFRs); gancyclovir, (GCV); gibbon ape leukemia virus, (GALV); herpes simplex virus, (HSV); multiplicity of infection, (MOI); murine leukemia virus, (MLV); rat insulin promoter, (RIP); ribonucleotide reductase M2 subunit, (RRM2); vascular endothelial growth factor, (VEGF)

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Summary

Pancreatic cancer is the fourth leading cause of cancer related deaths, and effective diagnostic and therapeutic strategies are lacking. Molecular research seeking genetic events and signaling pathways that are crucial in pancreatic carcinogenesis holds promise for the development of effective gene therapy strategies. The success of such strategies will depend on efficient, specific, and safe gene delivery to target cells as well as improved target cell-specific gene expression. Adenoviral vectors and retro/lenti-viral vectors have gained significant popularity in cancer gene therapy strategies because of their superior gene transfer efficiency and stable gene expression property, respectively. Many studies have been done to deliver tumor suppressor genes or suicide genes using adenovirus or retro/lenti-virus vectors to treat pancreatic cancer. Another promising therapeutic strategy for pancreatic cancer is the use of conditionally replicating (oncolytic) viruses. These viruses can selectively replicate in cancer cells, and their progeny viruses subsequently spread to surrounding cells, therefore achieving a large scale of viral infection. Several viruses, including adenovirus, herpes simplex virus (HSV), and reovirus have been modified for oncolytic purpose, and incorporation of extra tumoricidal strategies such as enzyme-directed pro-drug and fusogenic viral glycoproteins can further potentiate their anti-tumor capacity. This review provides a brief overview of gene therapy strategies using different viral vectors and anti-tumor activities of oncolytic viruses for pancreatic cancer treatment.

I. Introduction

Pancreatic cancer is the fourth leading cause of cancer deaths in the United States. The mortality rate of pancreatic cancer is the highest among solid tumors, with an overall 5-year survival rate less than 5% (Jemal et al, 2003). Due to its asymptomatic nature in early stages and a lack of sensitive and specific diagnostic tools, pancreatic cancer is usually undetected until metastasis has occurred and curative therapy is no longer possible. There are currently no effective biomarkers available for early detection of the disease, and even the most aggressive monitoring of high-risk patients is inadequate. Furthermore, pancreatic cancer is highly resistant to both chemotherapy and radiation therapy. Recently, many studies have focused on gene therapy as an alternative treatment for pancreatic cancer. The progression of pancreatic cancer is thought to occur through the accumulation of multiple genetic alterations resulting in either gain or loss of gene functionalities. Current molecular research efforts aim to detect the genes that are either over- or under-expressed and the resulting effect on signaling pathways, malignancy potential, chemo- and radio-resistance, and the immune response. Ultimately, this information may lead to the identification of new molecular targets for cancer gene therapy, in which a

specific gene may be delivered for overexpression or for inhibition of abnormally overexpressed genes.

The success of cancer gene therapy strategies largely depends on efficient and safe gene delivery. Among the gene delivery vehicles, viral vectors are the most commonly used to deliver genes of interest and therefore have been widely studied. Adenovirus vectors have been used mainly because of their superior gene transfer efficiency. Retrovirus vectors have been mainly used for stable expression of target genes, which makes them suitable for gene therapy for hereditary and metabolic diseases as they often require large vector doses and long-term gene expression. Furthermore, viral replication and oncolytic properties can be utilized in cancer gene therapy, and the immune response that they elicit may be used to eliminate cancer cells.

II. Overview of gene therapy strategies for pancreatic cancer

In general, optimization of efficient, specific, and safe gene delivery to the cellular target will lead to successful gene therapy strategies for cancer. Current gene therapy strategies for pancreatic cancer can be grouped into two categories: 1) the efficacy of gene therapy does not rely on the rate of transduction of tumor cells; and 2) the efficacy of gene therapy does rely on the efficient transduction of most or all tumor cells. Accordingly, as the efficiency of gene transfer technology continues to improve, strategies in the former group may achieve clinical success earlier than those in the latter group (Tseng and Mulligan 2002). Methods of gene therapy that require only limited transduction include immunotherapy, antiangiogenic strategies, and bone marrow resistance strategies, which all lead to amplified, systemic effects after gene transfer to a relatively small number of cells. Gene therapy strategies that require efficient gene delivery to most or all of the tumor cells *in vivo* include those that aim to revert the cancer phenotype and those that aim to induce selective tumor cell death.

A. Immunotherapy

Current strategies for immunotherapy for pancreatic cancer usually involve one of two approaches. The first involves the modification of tumor cells (primary or allogeneic) to secrete immunostimulatory cytokines (Jaffee et al, 1998, 2001; Sangro et al, 2004). The second approach involves the genetic modification of antigen-presenting cells to express tumor-specific antigens or immunostimulatory gene products (Pecher et al, 2002; Tang et al, 2002). A wide variety of viral and non-viral vectors have been employed in both approaches in pancreatic tumors and other tumor types, and there is currently no consensus as to the optimal method of gene delivery (Tseng and Mulligan 2002).

B. Antiangiogenic therapy

Antiangiogenic gene therapy strategies have been the subject of intense investigation, as angiogenesis is known to be required for solid tumor growth (Folkman, 1971). This strategy may be particularly useful in the treatment of residual or micrometastatic disease commonly occurring after pancreatic resection (Tseng and Mulligan 2002). A number of genes including VEGF receptors and NK4 are being investigated for their ability to interfere with angiogenic signaling pathways (Tseng et al, 2002; Maehara et al, 2002; Saimura et al, 2002). Recently, adenoviral delivery of a soluble form of vascular endothelial growth factor (VEGF) receptor, Flk1, resulted in pancreatic tumor growth inhibition in mice (Tseng et al, 2002). Replication-deficient retroviruses encoding truncated VEGF receptor-2 were found to block VEGF signaling, resulting in significantly reduced subcutaneous tumor growth and inhibition of tumor neoangiogenesis (Tseng et al, 2003). In contrast to conventional therapies, antiangiogenic therapies are believed not to target the tumor cells themselves but their nutritional support via inhibition of blood vessel formation.

C. Reverting the cancer phenotype

Strategies using this approach rely on the premise that either inhibition or restoration of single gene functions may revert the cancer phenotype. Oncogenes that have been targeted in pancreatic cancer gene therapy include *K-ras* and *CaSm* (Kijima and Scanlon, 2000; Brummelkamp et al, 2002; Kelley et al, 2003). Methods used to inhibit oncogene expression include antisense and ribozyme technologies (Kijima and Scanlon, 2000; Kelley et al, 2003). Antisense RNA constructs bind to the complimentary RNA sequence of the targeted gene to block translation, and ribozyme RNAs bind to and cleave the targeted complimentary RNA sequence. During recent years, siRNA technology has been extensively explored for this purpose (Berberat et al, 2005; Taniuchi et al, 2005; Wey et al, 2005). Approaches to restore tumor suppressor gene functions and to revert the cancer phenotype involve *p53* and *p16* genes. Wild-type *p53* gene was delivered to pancreatic cancer cell lines via adenoviral (Bouvet et al, 1998) and retroviral vectors (Hwang et al, 1998), and subsequent suppression of cancer cell proliferation was observed *in vitro* and *in vivo* in an immunocompromised murine model. Replacement of *p16* in pancreatic cancer cell lines has been accomplished using adenoviral vectors and has yielded favorable results (Kobayashi et al, 1999), and concurrent replacement of both *p53* and *p16* has resulted in tumor growth suppression *in vitro* and *in vivo* using adenoviral vectors (Ghaneh et al, 2001).

D. Suicide gene therapy

Gene therapy strategies that rely on efficient transduction of most or all tumor cells in order to cause selective tumor cell death include gene-directed prodrug activation, also referred to as “suicide” gene therapy, selectively delivers genes to tumor cells. When expressed, these genes act to convert systemically administered, non-toxic prodrugs into active chemotherapeutic agents. In this way, the effect of the toxic metabolite is localized to the neoplasm (Yazawa et al, 2002). One of the most commonly used genes in this approach is the herpes simplex virus thymidine kinase (*HSV-tk*) gene, which phosphorylates systemically administered gancyclovir (GCV) to produce gancyclovir monophosphate, that is

then further phosphorylated by the host cells to its toxic form, gancyclovir triphosphate.

Pancreatic cancer studies involving HSV-tk transduction by adenoviral and retroviral vectors have yielded success *in vitro* and *in vivo* in pre-clinical models. For example, Makinen et al. demonstrated that rat pancreatic carcinoma cells could be efficiently destroyed by GCV-mediated killing following delivery of HSV-tk by adenovirus and retrovirus vectors, leading to tumor necrosis and shrinkage *in vivo* (in which tumor cells were subcutaneously transplanted after transduction) (Makinen et al, 2000). In a separate study, Rosenfeld et al. demonstrated a similar antitumor effect of GCV with an adenovirus- HSV-tk construct in both *in vitro* and *in vivo* settings in an immunocompromised animal model (Rosenfeld et al, 1997). Block et al, 1997 also demonstrated the efficacy of *in vivo* HSV-tk transduction in producing GCV sensitivity and tumor necrosis. Carrio et al, 1999 has shown enhanced pancreatic tumor regression by a combination of adenovirus and retrovirus-mediated delivery of the HSV-tk gene.

One potential advantage of the HSV-tk/GCV strategy is that nontransduced tumor cells have exhibited cell death in the presence of GCV due to a "bystander effect" (Block et al, 1997; Rosenfeld et al, 1997; Makinen et al, 2000). Potential mechanisms for this effect include: 1) the transfer of gene product and/or activated toxic compound to adjacent nontransduced cells via phagocytosis or gap junctions, and 2) induction of an immune response that leads to nontransduced cell death (Yazawa et al, 2002). Current gene delivery technology does not allow for the expression of HSV-tk in every tumor cell, and the bystander effect may help to compensate for this deficiency. Still, current efforts to improve suicide gene therapy, including the optimization of gene transfer to tumor cells by adenoviral vectors, will likely increase the therapeutic effect of this approach. However, due to the lack of cell specificity of adenoviral vectors, a variety of targeting strategies have been engineered into this vector system. For example, the transcriptional targeting strategy aims to limit transgene expression to the targeted tumor cells and is being employed in suicide gene therapy (discussed in detail in a later section).

III. Adenoviral vectors for gene delivery

Adenoviruses are non-enveloped viruses with double-stranded DNA genome. The virion has icosahedral capsids which are consisted of twelve vertices and seven surface proteins, and is about 70 to 90 nm in size. Adenoviral vectors are well-suited for use as gene delivery vehicles in the treatment of cancer. They provide superior *in vivo* gene transfer efficiency and have the ability to infect a wide variety of dividing and non-dividing cells. Since clinical trials have demonstrated that suboptimal tumor transduction frequencies correlated with limited therapeutic benefit, adenoviral vectors have the advantage of improving levels of transduction efficiency (Yamamoto et al, 2003). In addition to superior transduction efficiency, adenoviral vectors have large cloning capacities and can be produced in high titers (Halloran et al, 2000). Furthermore, their immunogenicity, though poorly understood and perhaps responsible for detrimental toxicity, may actually play a role in the elimination of cancer cells. Adenoviral vectors are particularly attractive vehicles at delivering suicide genes. In addition, a bystander effect seen in suicide gene therapy may compensate for the low transduction efficiency in tumors. This effect may be enhanced through the fusion of the prodrug-activating gene with a secretory gene (Rots et al, 2003). Transduction efficiency and tumor penetration may also be improved by targeting strategies as described below.

The broad tropism of adenoviruses does, however, result in decreased specificity of gene transfer, and the future clinical success of cancer gene therapy strategies will depend on improved targeting specificity of this vector. Improved specificity is particularly crucial in strategies which result in direct cytotoxicity (as in suicide gene therapy) or cytolysis (as in oncolytic viral therapy). Two methods of enhancing therapeutic targeting are currently being investigated for use in several cancer gene therapy strategies, the first one concentrating on transduction and the other one on transcription. In addition, individual strategies may incorporate unique methods of improving specificity.

A. Transductional targeting

Transductional targeting produces cell-specific infection, thereby allowing systemic administration of the vector yield localized infection at sites of interest. Such improved specificity results in more specific transgene expression, reducing the required therapeutic dose and avoiding the involvement of normal tissues. In addition, inflammatory and immune responses against the vector may be reduced (Rots et al, 2003). Finally, infectivity and transduction efficiency may be enhanced in tumors that do not express the primary adenoviral cell surface receptors.

Adenovirus infection begins with the interaction between the host cell primary coxsackie adenoviral receptor (CAR) and the adenoviral fiber protein, termed "knob". Internalization of the virus particle involves the interaction between host cell integrins and the integrin-binding motif argine-glycine-aspartate (RGD) sequence of the viral penton base protein (Rots et al, 2003). One method of transductional targeting utilizes recombinant fusion proteins which bind to the adenoviral fiber and to a non-CAR tumor cell surface receptor. This retargeting towards non-CAR receptors is particularly important in order to increase the infectivity and transduction efficiency in tumors such as pancreatic cancer, as these tumors do not express adequate levels of CAR. This approach was used to redirect adenoviral vectors to epidermal growth factor receptors (EGFRs) on pancreatic carcinoma cells, leading to enhanced gene transduction efficiency and specificity (Wesseling et al, 2001). Redirection with specific fusion proteins that bind the adenoviral knob and fibroblast growth factor receptors (FGFRs) on pancreatic carcinoma cells resulted in increased efficiency of suicide gene delivery (Kleeff et al, 2002). A second method of transductional targeting genetically alters the adenoviral knob by deleting CAR-binding sequences and replacing it with foreign sequences that recognize specific receptors

such as integrins or other proteins that are selectively expressed on the surface of tumor cells. Such genetic alteration of the adenoviral fiber protein may, as in the first strategy, result in enhanced infectivity in tumor types which do not express adequate levels of CARs.

B. Transcriptional targeting

Transcriptional targeting utilizes tumor-specific promoters for gene expression. This targeting strategy is particularly important in suicide gene therapy and oncolytic viral therapy, in which cytoxicity or cytolysis of the involved cells takes place. One recent study of suicide gene therapy utilized the rat insulin promoter (RIP) to drive the expression of the 5-fluorcytosine-activating enzyme, cytosine deaminase (CD), demonstrating pancreatic cancer-specific cytotoxicity *in vitro* (Wang et al, 2004).

In oncolytic viral therapy strategies, tumor-specific promoters may be used to drive the expression of an essential gene of the virus. The efficacy of transcriptionally targeted adenovirus vectors has been studied in a number of tumors, including hepatocellular carcinomas, breast carcinomas, colon cancer, melanoma, neuroblastomas, and prostate cancer (Post et al, 2003). A recent phase I trial in prostate cancer patients documented the safety of intraprostatic delivery of CV706, a PSA-selective, replication competent adenovirus (DeWeese et al, 2001). In one preclinical study for pancreatic carcinoma, the cycloxygenase (COX)-2 promoter was incorporated into infectivity-enhanced conditionally replicating adenoviruses (CRADs) to drive the expression of the adenovirus E1 gene, resulting in strong and selective antitumor effects *in vitro* and *in vivo* (Yamamoto et al, 2003).

C. Toxicity and the immune response

Efforts to improve cell-specific gene delivery and expression will likely decrease the toxicity of the viral vector, by preventing transduction and expression of the gene in normal cells. Targeted delivery will also reduce the required therapeutic dose, minimizing the inflammatory and immune responses to the vector (Rots et al, 2003). Indeed, the humoral and cellular immune responses towards the vector, transgene, and infected cells are known to be substantial and may result in severe toxicity (Hemminki and Alvarez, 2002). The mechanisms underlying these responses are extremely complex and poorly understood, as they include both the innate and acquired immune responses to both viral and therapeutic genes.

Efforts to fully understand the complex interactions between the vector and host became particularly important after a large-dose infusion of the first generation adenovirus resulted in the death of a young patient with ornithine decarboxylase deficiency (Marshall, 1999). This unfortunate event revealed the danger of large-dose adenoviral vector administration, especially in the treatment of hereditary and metabolic disease in which large doses are required and readministration is often necessary. However, adenoviral vectors remain in favor for use in cancer gene therapy strategies. Clinical trials involving intravascular adenoviral delivery in cancer patients have shown acceptable toxicity profiles to date (Reid et al, 2002), and the immune response generated by adenoviral vectors may actually help to eliminate noninfected cancer cells.

Nevertheless, a better understanding of the immune response to these vectors is necessary in order to ensure safety and to preserve readministration efficacy in cases of recurrent cancer. Indeed, modulation or evasion of the immune response is particularly important during readministration of the vector, when the neutralizing antibody response to a previous exposure may be significant. Preclinical attempts to achieve this have included adenoviral serotype switching, masking the vectors with polymers such as peglation during both initial treatment and readministration, plasmapharesis (removing circulating neutralizing antibodies), coadministration of anti-inflammatories, and incorporation of immuno-modulatory genes into the vectors.

IV. Retrovirus mediated gene delivery

Retrovirus is another widely used vector for cancer gene therapy. Unlike adenovirus, retroviruses (except for lentiviruses) only infect dividing cells. The retrovirus genome can be stably integrated into the host chromosome DNA. Although the transduction efficiency of a retrovirus is usually less than 10%, the inserted gene can be stably expressed in target cells, which makes the retrovirus vector an attractive strategy for cancer gene therapy.

Duxbury et al. used retrovirus mediated siRNA to block the ribonucleotide reductase M2 subunit (RRM2), and found that RRM2 gene silencing decreased pancreatic adenocarcinoma cell invasiveness and gemcitabine chemoresistance (Duxbury et al, 2004). Brummelkamp et al. constructed a retroviral vector to specifically and stably inhibit the expression of the oncogenic *K-rasV12* allele, but not the wild type K-ras, in human tumor cells. Loss of expression of *K-rasV12* led to the loss of anchorage-independent growth and tumorigenicity. This study demonstrated that viral delivery of siRNAs can be used for tumor-specific gene therapy to reverse the oncogenic phenotype of cancer cells (Brummelkamp et al, 2002). Many studies have also used retrovirus vectors to deliver suicide genes into tumor cells (Carrio et al, 2001; Greco et al, 2002). One report indicated that significant inhibition of pancreatic tumor growth could be achieved by a combined delivery of HSV-tk by adenovirus and retrovirus vectors (Carrio et al, 1999).

Modifying the retrovirus vector to make it more effective in transducing tumor cells is another important aspect in tumor gene therapy. Howard et al. constructed a pseudotyped retroviral vector containing either the amphotropic murine leukemia virus (MLV-4070A) envelope, the cat endogenous virus (CEV) envelope RD114, or the rhabdovirus vesicular stomatitis virus glycoprotein (VSV-G), and used these pseudotyped vectors to transduce three pancreatic cancer cell lines. They found that retroviral vectors pseudotyped with VSV-G provided the best transduction efficiency for human pancreatic tumor cells when compared to either MLV-4070A- or CEV RD114- pseudotyped retroviral vectors.

Their results suggest that the use of VSV-G glycoprotein for pseudotyping recombinant retroviruses enhances the delivery and expression of the therapeutic gene in human pancreatic tumor cell lines and may be important for designing modified retroviral vectors for better transduction efficiency in pancreatic cancer gene therapy (Howard et al, 1999).

V. Oncolytic viral therapy

Human viruses have the natural ability to efficiently infect and kill target cells. Therefore genetically engineering human viruses to kill tumor cells, while sparing normal cells, represents an attractive approach to antitumor therapy. Oncolytic viruses have two principal advantages. First, unlike conventional chemotherapy and radiotherapy, they specifically target cancer cells because of their restricted ability to replicate in normal cells. Second, unlike replication-incompetent vectors, oncolytic viruses produced from initially infected tumor cells can spread to surrounding tumor cells, thereby achieving greater distribution of virus and enhanced antitumor effects.

Oncolytic viruses are most commonly constructed by deleting viral genes necessary for efficient replication in normal cells but not tumor cells. They can also be constructed by regulating the transcription of viral replication proteins through the use of exogenous, tissue-specific promoters (i.e. transcriptional targeting), or by retargeting viral infection specifically to tumor cells (i.e. transductional targeting) (Post et al, 2003). Several of these conditionally-replicating viruses are under investigation in clinical trials. Additionally, other viruses such as reovirus and Newcastle Disease virus are being investigated for their inherent tumor-selective properties in oncolytic viral therapy (Norman and Lee, 2000; Sinkovics and Horvath, 2000).

A. Oncolytic adenovirus

The first engineered virus to enter clinical trials was dl1520 (ONYX-015), a conditionally-replicating adenovirus (CRAD). This particular CRAD has a deletion of *E1B*-55kD, a gene that inhibits the function of the tumor suppressor gene, p53. It was hypothesized that ONYX-015 replication would be limited in normal cells (i.e. cells containing wild type p53 gene) but that replication could occur in tumor cells (i.e. cells containing mutant p53 gene) (Kasuya et al, 2005).

Preclinical data suggest that the modulation of ONYX-015 replication by p53 expression may vary according to the origin of tumors and that the virus may not possess clear tumor selectivity; some preclinical studies have demonstrated a clear association (Bischoff et al, 1996; Rogulski et al, 2000), while others have not (Rothmann et al, 1998; Harada and Berk, 1999). Furthermore, clinical trials involving ONYX-015 as a single agent have demonstrated low efficacy. Recent phase II trials of ONYX-015 administered to head and neck cancer patients via intratumoral injection did, however, demonstrate selective tumor destruction, transient viral replication, and mutant p53-associated necrosis (Nemunaitis et al, 2000, 2001). A recent phase I trial in pancreatic cancer patients demonstrated no objective tumor response and did not document viral replication. However, this clinical trial did show that CT-guided local injection of ONYX-015 was well-tolerated (Mulvihill et al, 2001).

As in suicide gene therapy, improved transductional and transcriptional targeting is needed for more efficient and specific gene delivery and expression. In the case of oncolytic viral therapy, transcriptional targeting may be achieved by utilizing tumor-specific promoters to direct transcription of essential viral genes such as E1a.

Although preclinical and clinical studies of oncolytic viral therapy have shown limited single-agent efficacy, this strategy may be enhanced by combining traditional chemotherapy and radiotherapy (Chen et al, 2001), or by the addition of immunostimulatory genes such as IL-12, IL-24 and GMCSF to maximize the anti-tumor effect (Zhao et al, 2005; Qian et al, 2002). The administration of ONYX-015 in combination with cisplatin and 5-fluorouracil has yielded significant phase II clinical success in head and neck cancer patients, including a high proportion (27%) of complete responses (Khuri et al, 2000). A recent phase I/II trial in pancreatic cancer patients demonstrated the feasibility and safety of administering the virus under endoscopic ultrasound-guided injection in combination with gemcitabine administration (Hecht et al, 2003). Furthermore, this study demonstrated that increased tumor responses could be achieved by the combination therapy.

Conditionally-replicating adenoviruses may also function as gene delivery vehicles. Prodrug activation genes ("suicide" genes) are the most studied therapeutic genes delivered by this strategy. However, the incorporation of *HSV-tk* into the genomes of oncolytic adenoviruses has yielded conflicting results: the findings of Nanda et al, 2001 demonstrated enhanced antitumor activity over oncolytic viral therapy alone, while the findings of Lambright et al, 2001 demonstrated no augmentation of antitumor activity, possibly due to the inhibition of viral replication by GCV activation. The combination of oncolytic adenoviral therapy and double suicide gene therapy has also been explored. The incorporation of *HSV-tk* and *cytosine deaminase* (which leads to the activation of the prodrug, 5-fluorocytosine) yielded antitumor activity in prostate cancer patients (Freytag et al, 2002). Preclinical evidence suggests that this last strategy of combining oncolytic viral therapy with double suicide gene therapy may be able to potentiate the effectiveness of radiotherapy in a clinical setting (Rogulski et al, 2000).

B. Oncolytic herpes virus

HSV has been modified for oncolytic purposes, most commonly by deleting either one or both of the virally encoded *r34.5* or *ICP6* genes. Deletion of the viral *r34.5* gene, which functions as a neurovirulence factor during HSV infection (Chou et al, 1990), blocks viral replication in nondividing cells (Chou and Roizman, 1992; Bolovan et al, 1994; McKie et al, 1996). The viral *ICP6* gene encodes the large subunit of ribonucleotide reductase, which

generates sufficient dNTP pools for efficient viral DNA replication (Boviatsis et al, 1994; Mineta et al, 1994; Chase et al, 1998), and is abundantly expressed in tumor cells but not in non-dividing cells. Consequently, viruses with a mutation in this gene can preferentially replicate in and kill tumor cells. The oncolytic HSV G207, which has been extensively tested in animal studies and is currently being tested in clinical trials, has a deletion of both copies of the *r34.5* locus and an insertion mutation in the *ICP6* gene with the *E. coli lacZ* gene (Mineta et al, 1995; Todo et al, 1999; Walker et al, 1999). Alternatively, an oncolytic HSV can be constructed by using a tumor-specific promoter to drive *r34.5* or other genes essential for HSV replication (Chung et al, 1999).

As compared with other viruses that have been investigated for oncolytic purposes, HSV possess several unique features that enhance their potential as antitumor agents. First, antiherpetic medications such as acyclovir and gancyclovir are available as safety measures in the event of undesired infection or toxicity from the HSV. Second, productive infection with HSV usually kills target cells much more rapidly than infection with other viruses. For example, HSV can form visible plaques in cultured cells in only 2 days, in contrast to 7 to 9 days for adenovirus. In vitro studies have also shown that at a multiplicity of infection (MOI) of 0.01, an HSV can kill almost 100% of cultured cancer cells in 2 days (Fu and Zhang, 2002), while a much higher dose or a longer infection time is required to achieve equivalent cell killing with adenovirus (Yu et al, 1999). Rapid replication and spreading among target cells may be important for a virus to execute its full oncolytic potential *in vivo*, as the body's immune mechanism may be more likely to restrict the spread of slower growing viruses. Third, HSV seems to be able to replicate and spread even in the presence of anti-HSV immunity. This feature has been most clearly demonstrated during recurrent HSV infection, in which the virus can still grow and spread extensively in local skin, despite obvious antiviral immunity. Moreover, pre-existence of anti-HSV immunity in experimental animals has no significant effect on the therapeutic potency of oncolytic HSVs administered either intratumorally or systemically (Chahlavi et al, 1999; Lambright et al, 2000; Yoon et al, 2000). Fourth, HSVs have wide cell tropism, infecting almost every type of human cells that have been tested so far. Thus, oncolytic viruses derived from HSV would likely have wide applicability among cancer patients. Finally, the risk of introducing an insertion mutation during HSV oncolytic therapy appears minimal because HSVs generally do not integrate into cellular DNA.

Oncolytic HSVs were initially designed and constructed for the treatment of brain tumors, especially glioblastomas (Martuza et al, 1991; Mineta et al, 1995). Subsequently, they have proved to be effective in treating a variety of other human solid tumors, including breast cancer (Toda et al, 1998; Fu and Zhang, 2002). The safety of the oncolytic virus G207 has been extensively tested in mice (Sundaresan et al, 2000) and in a primate species (*Aotus*) that is extremely sensitive to HSV infection (Hunter et al, 1999; Todo et al, 2000). These studies have confirmed that oncolytic HSVs are safe for *in vivo* administration. These encouraging results in animals have prompted clinical trials of these viruses in patients with malignant gliomas (Markert et al, 2000; Rampling et al, 2000).

However, recent studies indicate that current oncolytic viruses, although safe, may have only limited anti-tumor activity on their own. Pro-drug converting enzymes, such as HSV tk, as mentioned above, have been combined with oncolytic viruses in an attempt to improve the anti-tumor potency. This has not been successful most likely because the antitumor effect of pro-drug activation is offset by its inhibitory action on viral replication (Gustin et al, 2002; Tseng and Mulligan 2002). Recently, it was suggested that the syncytia-forming property of fusogenic membrane glycoproteins might be useful in cancer therapy (Kasuya et al, 2001; Gilliam and Watson, 2002; Fu and Zhang, 2002 Nakamori et al, 2003; Nakamori et al, 2004a, b). Since these viruses kill their target cells through formation of multinucleated syncytia, involving membrane fusion between infected and uninfected cells, they have the theoretical advantage affecting adjacent cells that have not been directly infected. For example, it has been shown that a C-terminal truncation of the gibbon ape leukemia virus (GALV) envelope glycoprotein leads to a constitutive and hyperfusogenic version of the GALV envelope glycoprotein (GALV.fus) (Lieberman et al, 2001; Fu et al, 2003). Transduction of this gene into a range of human tumor cells results in efficient cell destruction through syncytia formation (Sakorafas and Tsiotos, 2001; Fu and Zhang, 2002; Fu et al, 2003). Furthermore, the bystander killing effect from this hyperfusogenic glycoprotein is at least ten times higher than the effect from the suicide genes HSV-TK or cytosine deaminase (Gunzburg and Salmons, 2001). We have demonstrated that incorporation of cell-membrane fusion properties into an oncolytic HSV can dramatically enhance the antitumor activity of the virus (Fu et al, 2003). This new class of oncolytic viruses, called fusogenic oncolytic HSVs, kill tumor cells by two efficient and complementary mechanisms: direct cytolysis (through virus replication) and cell membrane fusion. The combination of these tumor-killing mechanisms may even yield a synergistic antitumor effect, as syncytia formation in the tumor tissue can facilitate the spread of the virus, leading in turn to widespread syncytia formation. Our studies have demonstrated that the fusogenic oncolytic HSV virus potently infects and kills human pancreatic cancer cells *in vitro* (unpublished data). It has also recently been discovered that the insulin promoter is active in human pancreatic adenocarcinoma and could thus be used as a pancreatic cancer specific promoter (Halloran et al, 2000). It has been reported that the insulin promoter-tk with gancyclovir selectively ablates human pancreas cancer both *in vitro* and *in vivo*. Therefore, the combination of the pancreatic cancer specific promoter, and the potent fusogenic oncolytic virus might be an attractive strategy in pancreatic cancer gene therapy. The genetically engineered tumor-specific fusogenic oncolytic virus using the insulin promoter will have two major advantages. First, the tumor-specific expression of the fusogenic protein will allow systemic delivery resulting in

efficient tumor cell killing in primary and metastatic lesions while sparing the host from toxicity. Second, unlike the replication-incompetent vectors, this oncolytic virus will spread from initially infected tumor cells to surrounding tumor cells. The use of this knowledge to create a tumor specific fusogenic, oncolytic HSV virus is promising as a potential adjunct to surgical resection. This strategy could also potentially be applied to other cancers.

C. Reovirus therapy

Reovirus is a unique oncolytic virus because it infects cells with an activated Ras signaling pathway (Wilcox et al, 2001; Ring, 2002; Etoh et al, 2003). About 80% of pancreatic cancer cells have Ras mutations, which makes the reovirus an attractive candidate in pancreatic cancer therapy. Etoh et al. used oncolytic reovirus to infect five different pancreatic cancer cells (Panc-1, MIA PaCa-2, PK1, PK9, and BxPC-3). They found that all five cell lines were infected by reovirus and the susceptibility to reovirus infection correlated with elevated Ras activity in these cell lines. After intratumor injection of reovirus, decreased tumor growth was observed in a unilateral murine xenograft model using Panc-1 and BxPC-3 cells. Moreover, reovirus replication was observed only within the tumor and not in surrounding normal tissues. These results indicate that reovirus might be a novel oncolytic viral therapy against pancreatic cancer (Etoh et al, 2003).

VI. Conclusion

Adenovirus and retrovirus vectors have been widely used in cancer gene therapy because of their superior gene transfer efficiency and stability, respectively. Tumor suppressor genes, suicide genes, or immunomodulatory genes have been delivered to tumor cells using adenovirus or retrovirus vectors to treat pancreatic cancer, and the results are promising. Numerous efforts have also been made to enhance the therapeutic benefits by improving transduction efficiency and tumor specificity. Oncolytic viral therapy offers another great promise for pancreatic cancer therapy because the virus targets cancer cells specifically, and spreads within the tumor mass even if it only infects a small number of cells initially. Several viruses, such as adenovirus, herpes simplex virus (HSV), and reovirus have been used as oncolytic viruses to treat cancers. Modified oncolytic HSV that contains a fusogenic viral protein provides more potent anti-tumor capacity in several cancers. A comprehensive therapy using combined oncolytic virus and tumor suppressor gene or suicide gene therapy, along with conventional radiation and chemotherapy, might provide superior treatment for pancreatic cancer.

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