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GENE THERAPY AND PANCREATIC CANCER

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1. ABSTRACT

Adenocarcinoma of the pancreas is associated with a short survival due to frequent delays in diagnosis and the lack of effective systemic therapies. Advances in understanding the molecular basis of pancreatic cancer have allowed identification of molecular targets which are amenable to therapeutic intervention. Such targets include p53, K-ras, p16, and DPC-4. Gene therapy involves the transfer of genetic constructs which alter the neoplastic potential of the cancer cell. Vectors used in gene transfer include viral and non-viral methods. Presently, gene therapy of pancreatic cancer is limited to pre-clinical studies using *in vitro* and *in vivo* models. However, the initial results from these pre-clinical studies have been encouraging and will form the basis for clinical studies of gene transfer in patients with pancreatic cancer.

2. INTRODUCTION

Given the aggressive biologic nature of pancreatic cancer and the need for additional therapeutic strategies, attention has turned to the use of novel therapeutic approaches which target this disease at the molecular level. Gene therapy is one such approach which utilizes the transfer of genetic constructs into the cancer cell. Once genetic transfer has taken place, expression of the gene product may alter the biologic behavior of the tumor. This alteration can occur due to blocking transformation of known oncogenes or by restoring tumor suppressor function. Other gene therapy approaches focus on augmenting the immunologic attack against cancer cells. These gene transfer strategies and others will be discussed in this chapter. First, however, gene therapy of pancreatic cancer is based on three important principles which will be reviewed: 1) genetic alterations responsible for neoplastic transformation of the pancreatic duct cell and hence possible interventional targets in pancreatic cancer, 2) delivery systems which allow transfer of genetic constructs into the pancreatic cancer cells, and 3) objective measurement of the cellular response to these novel therapeutic agents.

3. MOLECULAR BIOLOGIC TARGETS IN PANCREATIC CANCER

3.1. p53

As a therapeutic target in oncology, the tumor suppressor gene, p53, has received much interest due to its high incidence of mutation in human cancers (1-3). The p53

gene codes for a gene product that is a nuclear protein capable of arresting the cell cycle or inducing apoptosis (programmed cell death). Loss of both p53 alleles through point mutation, deletion, or rearrangement results in the transformation of normal cells to malignant cells. Restoration of wild-type p53 function can induce both cell cycle arrest and apoptotic cell death. This forms the basis of ongoing clinical trials using retroviral p53 as a method of gene therapy in patients with advanced lung cancer (4). Mutations in p53 occur in as high as 70% of pancreatic adenocarcinomas (5-8). In addition, some investigators have identified an association between the presence of a p53 mutation in the resected specimen and decreased survival in patients with pancreatic cancer (9). Given these observations, p53 represents a reasonable target for novel therapeutic approaches in pancreatic cancer which include gene therapy techniques.

3.2. K-ras

K-ras is a protooncogene located on chromosome 12 which encodes the p21-ras protein. Ras proteins serve in signal transduction pathways. Mutations in the ras protein result in an activated state which contributes to cellular transformation. These mutations occur in 75-90% of pancreatic cancers and can be detected in human bile, blood, stool, and FNA samples by DNA amplification via the polymerase chain reaction (10-12). Extensive characterization of ras signaling pathways has allowed formulation of farnesyl transferase inhibitors which block a crucial step in the ras metabolism. These agents are currently being evaluated in clinical studies. Gene transfer techniques now allow further opportunity to inhibit function of this oncogene.

3.3. DPC-4, p16, and Rb

A unique genetic alteration in pancreatic cancer has been recently discovered which functions in a tumor suppressor fashion. This mutation, termed Deleted in Pancreatic Cancer 4 (DPC4), occurs in one-half of pancreatic adenocarcinomas (13). Investigators at The M.D. Anderson Cancer Center have demonstrated that DPC4 is involved in the signal transduction pathway of transforming growth factor-beta (TGF-beta) (14). By providing normal copies of DPC4, gene transfer provides a potential avenue to restore appropriate signal transduction within pancreatic cancer cells. Similarly, a cyclin-dependent kinase (CDK) inhibitor called p16 is altered in up to 85% of pancreatic carcinomas (15). If p16 is mutated or deleted, then the Rb (retinoblastoma) protein is phosphorylated resulting in cell cycle progression. Both p16 and Rb represent additional targets in pancreatic carcinogenesis.

3.4. Bcl-2

The cytotoxicity of chemotherapeutic agents and radiation is often promoted through a process called apoptosis, or programmed cell death. Apoptosis is regulated by a balance of proapoptotic and antiapoptotic mediators. The bcl-2 protein has been well-characterized in its ability to inhibit apoptosis. Bcl-2 overexpression occurs in approximately 50% of pancreatic carcinomas (16). Increased levels of Bcl-2 could provide pancreatic carcinoma cells with a protective effect against naturally occurring or cytotoxic-induced apoptosis. Potential gene transfer approaches to augment apoptosis in cancer cells include blocking Bcl-2 function and overexpressing proapoptotic mediators.

3.5. Others

Recently, another putative tumor suppressor gene has been identified in pancreatic carcinoma cell lines. The FHIT (fragile histidine triad) gene, which encodes for a hydrolase, is found on chromosome 3 and is mutated in 70% of pancreatic carcinoma cell lines (17). Presently, the role of FHIT inactivation in pancreatic tumorigenesis is unknown. Furthermore, overexpression of epidermal growth factor receptor and fibroblast growth factor receptor in pancreatic cancer appears to be characteristic of aggressive tumors and may represent additional strategic targets (18-19).

4. METHODS FOR GENE TRANSFER

The two major *in vitro* methods for the introduction of foreign genes into tumor cells include viral- and physically mediated gene transfer. Although physical methods, e.g., liposomal transfer, are applicable to human gene therapy, viral transfer has been more widely used due to its high transduction efficiency.

4.1. Viruses

4.1.1. Retroviruses

Retroviruses are composed of an RNA genome encapsulated into a complex virus particle containing viral and cellular components. The RNA genome consists of the long terminal repeats (LTRs), *gag*, *pol* , and *env* regions, and a packaging signal, psi. The *gag* and *pol* regions encode core proteins, a protease, reverse transcriptase, and an integrase. The *env* region encodes the envelope proteins and is responsible for binding and uptake of the virus into the host cell. The LTRs are essential for viral integration. The psi sequence is necessary for packaging of RNA molecules into virions prior to budding from the host cell membrane. Thus, the retrovirus is able to accomplish two of the most important functions necessary for gene transfer: entry into cells and integration of genes into the host DNA in a stable and heritable fashion.

However, there are several potential limitations of recombinant retroviruses (20-21). Replication of the target cells is necessary for proviral integration to occur, making successful gene transfer dependent on the ability of the retrovirus to induce proliferation of the target cells. The target cells must have the appropriate retroviral receptor for successful transfection. In addition, the retroviral capacity is limited to about 8 kilobases, making packaging of larger genes a problem. The currently achievable retroviral titers are 1×10^7 particles/ml; five to six logs lower than that of adenoviral titers. Such titers are not sufficient for the treatment of large tumors. Lastly, retroviruses are relatively labile compared with other viruses and, in general, cannot be purified without significant loss of infectivity.

4.1.2. Adenoviruses

Adenoviruses are large, complex structures which contain a linear, double-stranded DNA genome approximately 36 kb pairs long. Adenoviruses are internalized by receptor-mediated endocytosis and transported to the nucleus where the immediate early genes, E1a and E1b, are expressed. The products of these genes regulate the expression of a variety of host genes and activate the expression of the early delayed genes, which include E2, E3, and E4. The concerted activities of these

early genes contribute to the initiation of the late phase of viral replication and activation of the major late promoter (MLP). A large mRNA transcript produced from this MLP undergoes extensive posttranscriptional processing, leading to expression of five sets of late proteins (L1 through L5) that are structural components of the virion.

Because of its ability to transduce both quiescent and dividing cells, the adenovirus has been engineered to deliver genes to a wide variety of mammalian cells (22). First generation of adenoviral vectors have been rendered replication incompetent by deletion of the E1 region and can be propagated *in vitro* in 293 cells, a human embryonic kidney cell that stably expresses E1a and E1b (23). The maximum size of the foreign insert is limited to 7.5 kb in such vectors. The advantages of adenoviral vectors include transduction of a variety of cell types, stability, easy production of high titers (10^{12} pfu/ml), little to no integration of the adenoviral genome in the host cell genome, accommodation of large gene constructs, and the ability to transduce non-dividing cells (24).

The major hurdle to the use of adenoviral vectors has been the host immune response against the vector. Both humoral and cellular immune responses have been demonstrated (25). More recent modifications to adenoviral vectors have included deletion of other regions of the genome in an attempt to make the vector less immunogenic. In addition, several researchers have proposed using immunosuppressive agents such as cyclophosphamide and etoposide to limit the immune response in patients previously exposed to the adenovirus (26-27).

4.1.3. Adeno-associated viruses

Adeno-associated viruses (AAV) are small, linear single-stranded DNA viruses which belong to the parvovirus family. AAV requires the presence of a helper virus, usually adenovirus, to initiate productive infection. The wild-type (wt) virus has the ability to integrate at a specific location in chromosome 19; however, this activity seems to be lost in the recombinant virus (28). Because most of the viral genome has been deleted, the vector has the advantage of generating less of an immune response. The inability to develop a high-titer-producing packaging cell line continues to be the limiting factor for the efficient use of this system.

4.2. Liposomes

Liposomes, when combined with DNA of any size, form a lipid-DNA complex that is safe, useful in a variety of cell types, and of relatively low cost (29). These vectors are designed to deliver therapeutic genes to cells without the aid of a virus. Liposomes are completely synthetic and there is no limitation on the size and type of nucleic acid which can be incorporated. The disadvantages of liposomes include a lack of specific cell targeting and a relative inefficiency of gene transfer.

Liposomal gene transfer may be useful for pancreatic diseases. In one study, the LacZ marker gene was complexed to cationic liposomes and introduced into the rat pancreas either by intraductal or intraarterial injection (30). Up to 28 days after *in vivo* gene transfer, beta-galactosidase activity could be demonstrated in the pancreas. Intraductal injection induced expression in lining duct cells preferentially, whereas intraarterial injection resulted in transduction of endothelial cells of intrapancreatic arteries, as well as in the spleen, lymph nodes and liver. Only occasionally were acinar cells positive for blue staining indicating gene transfer by either type of treatment.

4.3. Naked DNA technology

The simplest system for gene therapy is the delivery of DNA without the use of a virus or synthetic vector. This has been accomplished by using mechanical methods, such as direct injection of DNA into tissue or by high-velocity bombardment of tissues with DNA attached to gold particles (the "gene gun") (31). Such injection of naked DNA into muscle has led to *in vivo* gene expression sufficient to generate immune responses against the proteins encoded by the delivered DNA; a strategy which has been applied in clinical trials to melanoma and colon cancer (22).

5. MANIPULATION OF GENETIC TARGETS

5.1. Restoration of Tumor Suppressor Genes and Anti-Oncogene Strategies

5.1.1. Gene Overexpression

The inactivation of certain genes may lead to tumor growth. Such genes have been termed "tumor suppressor genes". Replacement of the mutated tumor suppressor gene with the wild-type construct can result in restoration of the tumor suppressor activity. This can occur through overexpression of the gene product as is typical of adenoviral-based gene transfer. An adenovirus expression vector has been developed for the delivery of wild type human p53 cDNA to cells. By overexpressing p53, apoptosis was induced in cancer cells with mutated or deleted p53 but only minimally affected growth of cells containing wt-p53 (32). The p53-adenovirus vector construct has been demonstrated to inhibit the growth of lung and colon carcinomas in experimental mouse models (33-34). To determine the transduction efficiency of epithelial cells of the pancreatic duct, Vickers *et al* perfused the pancreatic duct of viable cadaveric pancreas with recombinant adenoviral vectors containing the beta-galactosidase (LacZ) gene (35). Intense blue staining of the adenoviral vector-transduced cells was indicative of gene transfer into the pancreatic duct cell. However, in a commentary, Parekh raises several questions regarding this study (36). First, there appeared to be an overall low transduction efficiency achieved. Second, only superficial lining cells were transduced. If this technique is to be applicable to the treatment of pancreatic cancer, penetration of the vector must involve deeper tissue and should be targeted to tumor cells to limit transduction of normal pancreatic parenchyma.

5.1.2. Antisense, Ribozymes, and Drug Susceptibility Genes

Antisense technology involves introduction of a gene construct into the cell that has a base sequence complementary to the RNA sequence targeted for inhibition. By binding this RNA sequence, oncogenic protein expression is inhibited. This strategy has been used to study the effects of eliminating expression of a mutant K-ras oncogene in human lung cancer cells (37). Another approach involves the introduction of ribozymes directed against oncogene mRNA. Ribozymes are RNA molecules

designed to bind and cleave specific mRNA sequences, thus destroying the template for oncogene expression. Directed enzyme pro-drug therapy involves the delivery of a drug susceptibility gene to a cancer cell that will confer sensitivity to a therapeutic agent. The herpes simplex virus thymidine kinase (HSV-tk) phosphorylates ganciclovir to a potent DNA synthesis inhibitor. The gene for the bacterial enzyme cytosine deaminase has also been used in gene transfer. Its mechanism of action is through conversion of 5-fluorocytosine to the active form 5-fluorouracil.

5.2. Immunotherapy

An immune response against syngeneic tumors can be generated in animal models using a variety of tumors induced by chemical carcinogens and viruses. Tumor regression can result from manipulating the human immune response with interleukin 2 (IL-2). The response rates of cancer patients to these immune manipulations is low and primarily confined to patients with melanoma and renal cell cancer. Other approaches utilize cytotoxic T-lymphocytes (CTLs) which are specific for tumor cells. Enhancing immunologic defense against tumor cells through gene transfer is being increasingly explored.

5.3. Bystander Effect

A significant bystander effect (killing or growth arrest of nontransduced tumor cells mediated by transduced tumor cells) has been noted with p53-mediated tumor suppressor gene therapy. Although the exact mechanism of this effect has not been fully established, it is possible that p53 downregulation of vascular endothelial growth factor (VEGF) and the resultant inhibition of angiogenesis may contribute to the bystander effect (38). The bystander effect has also been noted during herpes simplex thymidine kinase/ganciclovir-based gene therapy of human gastrointestinal tumor cells and may be related to the uptake of toxic metabolites by non-transduced cells via intercellular communication (39).

6. MEASURING RESPONSE TO GENE THERAPY

6.1. Gene Expression

Gene therapy relies on efficient transgene expression of the genetic construct upon delivery to the cell. Prior to evaluating the response to gene therapy, confirmation of transgene expression is required. Reverse-transcriptase-PCR (RT-PCR) allows determination of mRNA expression from the cDNA of the gene transfer element. By developing primers which overlap portions of the adenovirus or promoter element, the RT-PCR technique can be vector-specific and only amplify the construct of gene delivery and not endogenous genes within the cell. In addition, overexpression of protein can be determined by Western blot analysis. This is important in gene therapy methods which restore normal (wild-type) gene function to cells with a mutant oncogene or tumor suppressor gene. If transgene expression is not observed, there should be no expectation of response to gene therapy.

6.2. Apoptosis and Cell Cycle Arrest

Apoptosis is a measurable endpoint of gene therapy. The cytotoxity of several gene therapy agents, such as p53 alone and in combination with chemotherapy or irradiation, is through apoptotic cell death. There are several laboratory techniques to objectively measure induction of apoptosis resulting from gene transfer both *in vitro* and *in vivo*. These include flow cytometry with propidium iodide exclusion, terminal deoxynucleotidyl transferase-mediated dUTP nick end-labeling (TUNEL) staining, and DNA fragmentation assays. Inhibition of cell proliferation by cell cycle arrest can result in therapeutic efficacy in solid tumors. An example of this is G1 arrest through induction of p21 by p53. Cells treated with gene therapy agents can also be evaluated for cell cycle arrest by flow cytometric methods.

7. CURRENT STATUS OF PRE-CLINICAL GENE THERAPY STUDIES IN PANCREATIC CANCER

A spectrum of gene transfer techniques and genetic constructs have been evaluated in the laboratory using pancreatic cancer models. These models include both *in vitro* and *in vivo* studies. A spectrum of established pancreatic cancer cell lines with various gene mutation status are available for these studies. This section includes current information on laboratory studies using gene therapy in pancreatic cancer. Studies utilizing viral vector-based gene therapy are summarized in table 1.

Due to its frequent mutation in pancreatic cancer, K-ras represents an attractive gene therapy target. Aoki and colleagues have utilized antisense constructs to K-ras to inhibit *in vitro* proliferation of pancreatic cancer cell lines containing K-ras mutations (40). This inhibitory effect was not observed in cells with wild-type K-ras. They demonstrated decreased peritoneal dissemination of pancreatic cancer in an *in vivo* model. Shichinohe and colleagues utilized an H-ras mutant, N116Y, which has dominant negative activity to ras function (41). Growth inhibition was demonstrated in eight human pancreatic cell lines when introduced by lipofection. Furthermore, the N116Y-expressing clones were determined to be nontumorigenic *in vivo*. Ribozymes represent an additional, potential strategy specific for K-ras mutations. Ribozymes function to initiate degradation of K-ras mRNA, thus preventing oncogenic protein expression (42).

Replacement of mutated p53 with wild-type p53 by retroviral and adenoviral gene therapy has been safely used in phase 1 clinical trials for patients with non-small cell lung cancer (4). A recent study in p53-null pancreatic carcinoma cells undergoing replacement with wild-type p53 did not show alteration of tumorigenicity (43). However, recent observations from our institution revealed a significant decrease in cell proliferation of pancreatic cell lines after undergoing adenoviral mediated p53 gene replacement and was a direct result of apoptotic cell death (44).

The retinoblastoma (Rb) tumor suppressor gene, involved in cell cycle regulation, has been shown to be mutated in pancreatic cancer although with a lower incidence as compared to p53 and K-ras (45). To evaluate the effect of gene transfer, investigators incorporated a constitutively active form of the Rb gene into an adenoviral vector. The result was growth inhibition of human pancreatic cancer cells *in vitro* which was not due to apoptotic cell death (46). Other investigators have utilized the adenoviral vector to overexpress the p21 tumor suppressor gene to inhibit the proliferation of pancreatic cancer *in vitro* (47).

Table 1. Experimental models of viral vector-based gene therapy for pancreatic cancer

Institution (Reference number)	Journal (Year)	Vector	Gene
Duke (57)	Surgery (1994)	Retrovirus	HSV-tk
USC (54)	Ann Surg (1996)	Retrovirus	HSV-tk
Baylor (55)	Pancreas (1997)	Adenovirus	HSV-tk
Univ Michigan (46)	Surgery (1997)	Adenovirus	Rb
Wayne State (47)	Pancreas (1998)	Adenovirus	p21
MD Anderson (44)	Ann Surg Oncol (1998)	Adenovirus	p53

Fas ligand is a member of the tumor necrosis factor (TNF) family that is capable of inducing an apoptotic signal when bound to its receptor, Fas.

Researchers have demonstrated that a fusion protein between Fas and the ligand-binding domain of the estrogen receptor (MfasER) induces apoptosis (48). When MfasER cDNA was expressed in pancreatic cell lines in the presence of estrogen, DNA fragmentation occurred (49). However, a recent report suggests that even though Fas and Fas ligand are expressed by human pancreatic adenocarcinomas, pancreatic tumor cells are resistant to Fas-mediated apoptosis (50).

An immunologic approach to genetic therapy of tumors utilizes cytotoxic T-lymphocytes (CTL). CTL clones specific to mucin, which is expressed on the surface of tumor cells, have been generated. A plasmid expression vector was used to introduce mucin cDNA into immortalized B-cells. The mucin-specific CTL clones were able to recognize and lyse these mucin-bearing tumor cells (51). The investigators concluded that this represents a possible mechanism of immunizing patients with pancreatic cancer.

A new approach utilizes gene transfer to enhance the sensitivity of pancreatic tumor cells to recombinant human TNF. By transfection of a gene for the TNF receptor, in combination with high affinity mutein TNF, both pancreatic cancer cell lines and tumor models demonstrated increased susceptibility to TNF administration (52).

There are several reports evaluating the transfer of drug susceptibility genes in experimental models of pancreatic cancer. These genes, once expressed by the cancer cell, render it susceptible to therapeutic agents by a process called directed enzyme pro-drug therapy. The two genes most commonly used are the herpes simplex virus thymidine kinase (HSV-TK) gene which increases sensitivity to ganciclovir and the bacterial enzyme, cytosine deaminase, This enzyme converts 5-fluorocytosine (5-FC) to the active form 5-fluorouracil (5-FU). The HSV-TK gene, in a recombinant adenoviral vector, has been demonstrated to be effective in reducing tumor burden in mice that were injected intraperitoneally with pancreatic tumor cells. A significant bystander effect was demonstrated in an *in vitro* model by Rosenfeld and colleagues (53). A similar approach using a retroviral vector was evaluated in an intraperitoneal murine model by Yang and colleagues (54). After injecting pancreatic cancer cells into the tail of the pancreas, reduction of metastatic deposits were found in the HSV-TK group treated with ganciclovir. Evaluating the treatment of hepatic metastases from pancreatic cancer, researchers combined direct, intraperitoneal, and intratumoral injection of the adenoviral HSV-TK gene, and intraperitoneal administration of ganciclovir to demonstrate growth inhibition of this metastatic form of pancreatic cancer (55). However, other investigators found that repeated administration of ganciclovir resulted in loss of the integrated HSV-TK gene and therefore, resistance to ganciclovir (56).

Another strategy utilizes promoter elements that are driven by overexpressed proteins within cancer cells. To allow greater tumor specific gene expression, the promoter for the carcinoembryonic antigen (CEA) was coupled to the HSV-TK gene in a retroviral vector, and resulted in tumor size reduction *in vivo*. This suggests utility against CEA-expressing pancreatic cancer cells (57).

A similar efficacy using enzyme prodrug systems was shown for an adenoviral vector containing the cytosine deaminase gene. By conversion of 5-FC to active 5-FU, this strategy resulted in anti-neoplastic activity *in vitro* and *in vivo* (58). A different approach utilizes the nitroreductase gene which activates a weak alkylating agent, CB1954. Inserted into a retroviral vector, nitroreductase clones of pancreatic tumor lines were up to 500-fold more susceptible to CB1954 than parental cells (59).

8. PERSPECTIVE

Pancreatic cancer remains one of the most difficult malignancies to treat. This is often due to delayed diagnosis and aggressive metastatic potential. New diagnostic and therapeutic strategies continue to be developed. Increasingly, these new strategies are enhanced by utilizing information from the molecular biology of pancreatic cancer. Gene therapy is an exiting, new approach in the treatment of malignant disease. However, a realistic view of gene therapy for pancreatic cancer is necessary for both physicians and their patients. First, with the exception of one or two early clinical trials, the present status of gene therapy for pancreatic cancer remains in the laboratory. Success in treating human pancreatic cancer xenografts in mice does not necessarily predict success in treating naturally occurring human pancreatic cancer. Hurdles in gene therapy for this disease include more efficient gene delivery, targeting tumor cells versus normal pancreatic parenchyma, and development of systemic delivery strategies to complement local gene delivery. Gene therapy represents a challenge to the basic science and clinical investigators who use these molecular targets in the development of more effective treatment of patients with pancreatic cancer.

9. ACKNOWLEDGEMENTS

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