Journal of Cellular Biochemistry 94:1069–1077 (2005)

PROSPECTS

Immunobiotherapy Directed Against Mutated and Aberrantly Expressed Gene Products in Pancreas Cancer

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Abstract Genetic alterations are responsible for the development of cancer in ductal cells of the pancreas. These genetic changes result in abnormal molecular expression of proteins that are involved in cell proliferation, cell cycle control and adhesion. Some of the genetic mutations result in aberrant proteins that can be recognized as novel or foreign by cells of innate and adaptive immune systems. These are appropriate targets for therapeutic intervention which may involve immunobiologic approaches. These approaches may be less effective because of immune escape mechanisms developed by tumor cells within the microenvironment of the tumor mass. Immunobiotherapy intervention of pancreas cancer must circumvent these obstacles and integrate effective immunotherapy with molecularly targeted approaches to pancreas cancer intervention. J. Cell. Biochem. 94: 1069–1077, 2005.

Key words: mutations; immune surveillance; immune effectors; immune suppression; cancer; pancreas

Adenocarcinomas of the pancreas are aggressively growing cancers that have yet to be successfully controlled with therapy. Despite the dearth of advances in the treatment of pancreas cancer, substantial advances have been made in our understanding of the molecular genetic changes that occur during the progression of a normal ductal cell of the pancreas to pre-neoplastic stages, adenomas in situ, then to malignant and metastatic adenocarcinomas. Like cancers of other tissues, pancreatic ductal cells undergo a series of mutations in genes responsible for driving cell proliferation and controlling the cell cycle (Table I). A complete understanding of the common molecular events that define the evolution of pancreas cancer is vital to the development of specific targeted therapies. This is illustrated by recent advances in the therapy of chronic myelogenous leukemia (CML) and a subset of non-small cell lung cancers. These studies highlight the concept that precise molecular definition of genetic changes that drive cellular proliferation can lead to successful molecular targeting of therapy. In CML, the use of a kinase inhibitor that blocks BCR-Abl, a fusion protein that results from a chromosomal 9/22 translocation and drives "uncontrolled" leukemic proliferation of myelogenous blood cells, can block these events and cause a decrease in leukemia cells in nearly all CML patients [O'Dwyer and Druker, 2000]. Continuous genetic mutations unfortunately often lead to drug resistance as other molecular changes then drive cellular proliferation. In non-small cell lung cancer, the definition of specific mutations in the epidermal growth factor (EGF) receptor has defined a subset of patients whose mutated EGF receptors allow for high binding efficiency of the inhibitor, Iressa (gefitinib) resulting in apoptosis of the cancer cells similar to growth factor withdrawal induced apoptosis [Lynch et al., 2004; Paez et al., 2004]. These examples demonstrate the importance of defining precise genetic changes and the molecular mechanisms that result in driving development of cancers, especially those mutational events that lead to uncontrolled proliferation. The next decade will see further advances in our understanding of the effects of genetic mutations on expressed carcinogenic proteins. This will result in more precise targeting of drugs to control abnormal activities of over expressed and/or mutated proteins, or in the development of genetic constructs to replace proteins' functions in cases

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Received 1 November 2004; Accepted 3 November 2004

DOI 10.1002/jcb.20378

TABLE I. Genetic Change During Pancreas Cancer Genesis

Neoplastic stage[a]	Transitional	PanIN-1A[b]	PanIN-1B[c]	PanIN-2[d]	PanIN-3[f]
Gene mutations	?	HER-2/neu, K-ras	p16(Ink4A)[f]	p53[f]	BRCA2, LOH-6q, DPC4/(SMAD4/MADH4)
Over expression	?	Sonic Hedgehog			[f], Underglycosylated MUC1 Polo-like kinase 1[g]

[Brat et al., 1998; Westra et al., 1998; Iacobuzio-Donahue et al., 2000; Hruban et al., 2001; Rosty et al., 2003] [Day et al., 1996; Hahn et al., 1996; Moskaluk et al., 1997; Villanueva et al., 1998; Wilentz et al., 1998; Goggins et al., 2000; Wilentz et al., 2000; Fukushima et al., 2002; Cowgill and Muscarella, 2003; Real, 2003; Thayer et al., 2003; Arvanitakis et al., 2004; Gray et al., 2004; Iacobuzio-Donahue et al., 2004].
[a]Progression of histological changes and associated genetic changes that accumulate with neoplastic development.
[b]Pancreatic Intraepithelial Neoplasia 1-A (Flat).
[c]Pancreatic Intraepithelial Neoplasia 1-B (Papillary).
[d]Pancreatic Intraepithelial Neoplasia 2 (Atypical papillary).
[e]Pancreatic Intraepithelial Neoplasia 3 (Adenocarcinomas in situ).
[f]Aberrant methylation is observed with increasing frequency at progressing stages.
[g]Neoplastic stage of inital expression unknown.

of gene deletions or inactivations and subsequent loss of functions. While targeted therapy of once deadly cancers will tame these diseases, their eradication can only occur by destroying cancer stem cells in a given patient or by prevention throughout the population. Defining aberrantly expressed and mutated gene products will aid in this goal by providing specific targets that designer drugs can inhibit and by identifying aberrantly expressed peptides that can serve as antigens for vaccines to boost immune responses to mutated, malignant cells. Depletion of carcinogens from our environment and development of tumor antigen vaccines will serve someday as major cancer preventive measures. To date, vaccines to established tumors have met with limited success. While the use of vaccines for tumors is a plausible future scenario, the efficiency of immune effector cells may be limited to prevention, or destruction of minor sites of malignant cells or of cancer stem cells rather than destruction of large tumor masses. Vaccines may be most effective during early stages in tumor development, before tumors in their microenvironment evolve protective mechanisms to evade immune cells that could kill them. This is particularly true in pancreas cancer where evidence for immune cell reactivity against these tumors has been gathered for more than 25 years [reviewed in Plate and Harris, 2000]. Yet, tumors of the pancreas persist because, in part, the tumors protect themselves against invading immune cells. Mechanisms of tumor evasion from immune attack must be better understood, and blocked in order for immune cells to effectively reach the tumor cells and kill them. This manuscript will examine potential targets for immunobiotherapy of pancreas cancer, mechanisms through which tumors evade destruction by immune effector cells, and possible strategies for overcoming immune escape by tumor cells so that immune self defenses against these mutated "foreign" cells will effectively rid them from the host.

MOLECULAR TARGETING OF THERAPY

Many clinical trials of pharmacological agents that may down-regulate particular signaling pathways or specific steps in a given pathway are proposed or underway for pancreas cancer (http://www.clinicaltrials.gov). The rationale for most appears to justify the clinical trial yet there are many target pathways or signals that are up regulated in pancreas cancer but are not directly responsible for ductal cell transformation [reviewed in Pino et al., 2004; Xiong, 2004]. Drug therapy, especially in clinical trials, should be specifically targeted to molecules functional in initiating transforming events. Mutational changes that are not critical to neoplastic transformation may not serve as effective targets for drug therapy, but could be appropriate for vaccine development if the proteins affected by those mutations are expressed in cancer cells in each stage of carcinogenesis. Broadly acting drugs, such as inhibitors of DNA synthesis may deplete many malignant cancer cells, but they may not be effective against cancer stem cells which may remain quiescent for sufficient periods of time to escape destruction by such therapy. The concept of cancer stem cells, although relative new, is supported with increasing evidence in a variety of tumors [Reya et al., 2001; Singh et al., 2003;

Sell, 2004]. Pancreas stem cells likely undergo an initial mutation, such as a single codon change in the K-ras proto-oncogene as observed in >90% of pancreas tumors [Hruban et al., 1993], and as a result of this mutation become pancreas cancer stem cells, cells already capable of self renewal but additionally are driven to more frequent turnover and with increased risk of further mutations. The K-ras mutation in pancreas cancer cells results in the constitutive activation of a key signal transduction pathway, yet it is insufficient to drive pancreatic ductal cells into malignant transformation. Additional mutations in key cell regulatory proteins are required (Table I). Mutations in K-ras, however, are most likely a significant driving force in the process of pancreas cancer genesis.

The key to developing therapy for eradication of cancer is to define those initiating mutational events that give rise to self-renewing cancer stem cells and target the molecular consequences of those mutations for therapy. When this key transitional event gives rise to altered proteins, such proteins could serve as targets for vaccines. Immune effector cells then would target the cancer stem cells as well as their further mutated, malignant, and metastatic tumor cells. While drug therapy for nonresectable tumor mass must be targeted at molecular pathways controlled by key transforming events in order to induce death specifically of malignant pancreas tumor cells, most of these therapies may not effectively remove cancer stem cells. Biotherapies, and particularly immunobiotherapy, directed against aberrantly expressed proteins involved in initiating events in pancreas cancer stem cells, events that allow these cells to self renew and provide for opportunities for more frequent mutations leading eventually to neoplastic development, may accomplish this goal and may play a prominent role in the future arsenal of therapies for treatment of cancer.

IMMUNE RESPONSES TO PANCREAS CANCER

While an accumulation of mutations in genes that drive and control cell proliferation are essential elements in the development of cancers, immune surveillance mechanisms continuously recognize mutated gene products and effectively rid the body of mutated cells, some of which may have had the potential to become tumor cells. In pancreas cancer patients, despite the tenacity of their disease, significant levels of immune cell activity have been detected. Molecular changes observed have resulted in the identification of a number of antigenic proteins including K-ras, MUC1, HER-2/neu, and mesothelin [Barnd et al., 1989; Peiper et al., 1997; Iacobuzio-Donahue et al., 2003a]. Expression of the mutated K-ras oncogene results in a novel peptide which can be recognized by immune cells. Investigators took advantage of this novel peptide and created a vaccine for treatment of pancreas cancer patients. The K-ras mutant-peptide based vaccine, in combination with the myeloid specific growth factor GM-CSF, induced peptide specific immune responses in over half of the patients tested. The authors concluded that subjects whose T-cells responded experienced a doubling of their survival time over those patients who did not respond (148 vs. 61 days) suggesting that vaccine driven immune augmentation could have a beneficial effect. [Gjertsen et al., 1995, 2001]. Another aberrantly expressed protein in epithelial cell tumors is MUCIN 1 (MUC1). MUC1 is normally richly glycosylated and expressed on the apical surface of ductal cells. In tumor cells, MUC1 is often over expressed, underglycosylated, and redistributed around the entire cell surface. The underglycosylation of MUC1 thereby exposes its protein backbone to the immune system as new antigens. Immune responses to MUC1 had been demonstrated in pancreas cancer patients, hence a MUC1 peptide vaccine was generated to boost these responses [Gendler et al., 1988; Barnd et al., 1989; Finn et al., 1995]. MUC1 vaccines studied in phase I clinical trials resulted in the generation of T-cell responses in most of the subjects vaccinated. It remains to be determined whether the responses triggered by this vaccine are effective against pancreas tumor cells in vivo [Goydos et al., 1996; Ramanathan et al., 2004]. While under glycosylated MUC1, over expressed HER-2/neu and mesothelin, and mutated K-ras have been defined as a targets of immune cells in pancreas cancer patients, any mutated protein that is processed into peptides in endocytic vesicles or via proteosomes can serve as antigens for T-cell recognition if (1) they can properly "fit" into the MHC groove and if (2) appropriate T-cell receptors (TCR) have evolved to recognize that antigenic peptide. It is expected that more

antigenic determinants on pancreas tumors will be defined and that vaccines using whole cells, cell lysates, or RNA isolated from tumor cells could present these, as yet undefined antigens, to patients' immune systems. Vaccines have been created, therefore to boost immune responses to these potential unknown antigens. In one study pancreas tumor cells were transfected with the growth factor gene, GM-CSF, with the aim of activating dendritic cells of the host to process allogeneic tumor cells and facilitate presentation of tumor antigens [Jaffee et al., 2001, 2002; Iacobuzio-Donahue et al., 2003b]. In clinical trials some beneficial effect of these growth factor transfected, allogeneic tumor cell vaccines was reported, albeit in a minority of the subjects vaccinated. Overall, early clinical trials of various vaccines in pancreas cancer patients show promise in that activation of immune responses are demonstrated, however the achievement of significant effects on tumor growth and patient survival have not been forthcoming. Whether the failures of these vaccines to achieve significant control of tumor growth in most subjects is due to ineffective antigenic targets or to an inability of activated immune cells to reach tumor sites and induce their effector functions is unclear and requires further evaluation.

MECHANISMS OF ESCAPE FROM IMMUNE SURVEILLANCE

Unfortunately, the Darwinian theory of "natural selection or survival of the fittest" applies to tumor cells as well as to intact organisms. Survival of mutated cells that give rise to malignant tumors is affected by the entire host and the microenvironment surrounding the tumor, particularly the natural and adaptive immune defenses. Thus, as tumor cells continue to evolve, the "fittest" often survive because they have developed means to thwart the immune system (Table II). While the immune system normally functions well in controlling "mutated" cells that could become tumors, additional mutations result in a down regulation of expression of MHC molecules on tumor cell surfaces such that even if mutated peptides could fit into their antigen binding grooves, insufficient numbers of HLA molecules reach the outer membranes to be able to trigger immune responses [Torres et al., 1996]. Tumor cells also release byproducts that suppress phagocytic cell function. Without reprocessing of tumor antigens by dendritic cells, no co-stimulator molecules from antigen presenting cells such as B7.1 or B7.2 (CD80 and CD86) are available to co-signal T-cells of impending danger from the mutated cells. Tumor cell presentation of antigenic peptides to potentially responsive T-cells therefore leads to the induction of an anergic state, tolerization, or even elimination of immune T-cells capable of recognizing those peptides.

The fact that tumors often contain an accumulation of lymphocytes that are reactive against antigens expressed on tumors when assayed in vitro, attests to the ability of the immune system to recognize tumors as altered self, or foreign. Even with the activation of specific immune cells, however, tumors can still evade being killed by down regulating functional activities of the immune cells. One characteristic often described of T-cells isolated from tumors is the down-regulated expression of the CD3 zeta chain [reviewed in Whiteside, 2004]. CD3 zeta chain is the signal transducing protein associated with the T-cell receptor. The mechanism behind CD3 zeta chain down-regulation is under investigation but it likely results from signals within the tumor microenvironment; i.e., a down-regulation signal for T-cells triggered by tumor cell products. Again, these are mechanisms evolved by tumor cells to

TABLE II. Tumor Induced Mechanisms of Escape From Immune urveillance

Means of immune escape	Tumor by-products
Decreased MHC expression	
Decreased antigen presentation	Soluble MUC1
Induction of T-cell anergy, tolerance, or deletion	TGF-β, TGF-α
Induction of regulatory/suppressor T-cells	Prostaglandins
Inhibition of T- and NK-cell activity	Soluble MICA—Major histocompatibility complex class I-related Chain A/B
Decreased CD3 zeta function; i.e., decreased signal transduction in immune effector cells	ROS—Reactive oxygen species
Fas ligand expression	
Increased ligand expression for PD-1 (B-7) family	Soluble PD-1 family ligands (PD-H1/L1, others??)

evade immune attack. Tumors also express and release proteins and a variety of other by-products capable of down-regulating immune responses (Table II). The soluble form of the tumor antigen, MUC1 for example, can suppress T cell proliferation and cytolytic functions, and can block antigen processing by dendritic cells [Hiltbold et al., 2000; Plate and Harris, 2000]. Soluble MUC1 hence could potentially suppress T-cell killing of tumors, in vivo. TGFβ, which can suppress T-cell activation and proliferation, and prostaglandins, that can inhibit macrophage functions, are additional tumor cell byproducts that can inhibit immune cell activity. Additionally, membrane associated major histocompatibility complex class I-related chain A/B molecules (MICA/B) which normally enhance immune cytolytic cell and NK activity, are also released in soluble forms from tumors, including those of the pancreas, and can block immune cytotoxic activity [Groh et al., 2002]; (Xu et.al., personal communication). Other tumor products such as reactive oxygen species (ROS) have also been demonstrated to down regulate immune cell activity. Another tumor defense mechanism may utilize molecules that the immune system uses to regulate itself. Tumor cells may take advantage of systems that have evolved to regulate immune responses such that once an invading organism or foreign antigen is cleared from the body, and the specific immune responding cells are no longer needed, they are down-regulated or eliminated and only memory cells prevail [reviewed in Leibson, 2004]. Fas, Programmed Death-1 (PD-1), and B- and T-lymphocyte attenuator (BTLA) are some known inhibitory receptors expressed on activated lymphocytes. Ligands for these receptors, namely Fas ligand, PD-H1 (PD-L1) or PD-DC (PD-L2), and BT3, are frequently expressed on tumors, hence tumor cells can trigger Fas, or PD-1, CD28-related, receptors expressed on activated T-lymphocytes and natural killer cells (NK) [Satoh et al., 1999; von Bernstorff et al., 1999; Tamura et al., 2003; Compte et al., 2004]. Inhibitory motifs or death domains are subsequently activated to induce cellular apoptosis, or at least a down-regulation of T-cell functional activities.

Adoptive immune therapy has been tested in a variety of cancers, and in such trials the relationship of the expression of immune regulatory ligands on tumor cells to the effectiveness of adoptive T-cell immunity may also be an important clue to regulatory events. Recent reports have raised an intriguing issue of T-cell selection in adoptive immunity which may result from the ability of tumors to act as "immune regulators." In adoptive immune therapy of cancer patients with cloned T-cells, little benefit has been realized. The adoptive transfer of large mixtures of T-cell lines, however resulted in objective responses to melanoma tumors in the majority of subjects [Rosenberg and Dudley, 2004]. Monitoring of donor T-cell receptors in these recipients demonstrated the survival of dominant clones, suggesting that the other clones may have been down-regulated or eliminated. Selection of immune clones may be determined by molecules aberrantly expressed on tumor cells. Tumor cells may accomplish selection against immune cells by expression of "down-regulator" molecules such as those defined as members of the PD-H1/B-7 family of ligand molecules [Freeman et al., 2000]. Therefore, as activated T-cells migrate into tumors, they are confronted by a potential multitude of inhibitors aimed at preventing destruction of the tumor cells. Even if functional immune cells successfully reach a tumor cell, then PD-1 ligand surface regulators, BT3, or apoptosis inducing proteins such as FasL expressed on tumor cells might prevent immune effector cell function by "turning" them off, down-regulating cytokine production, or inducing their apoptosis (activated T-cell death). Only lymphocytes that have alternative receptors for these ligands may survive to kill tumor cells. While these clinical trials of adoptively transferred immune cells significantly enhance our understanding of lymphocyte-tumor interactions, it is difficult to envision that adoptive therapy for the treatment of 30,000 new pancreas cancer patients each year with autologous T-cell lines will be feasible, especially with respect to financial considerations, time and technological requirements.

PERSPECTIVES FOR THE FUTURE

The design of tumor vaccines has evolved to take many of the problems associated with tumor cell antigens and activation of T-cells into consideration. It was quickly realized that the use of "purified," naked antigenic proteins or peptides to vaccinate subjects is insufficient. Instead tumor antigens need be directed specifically to antigen presenting cells, usually den-

dritic cells. Further, vaccines now often include an association with a growth factor so that the dendritic cells that take up the tumor antigen vaccine will be driven to mature into antigen presenting cells capable of triggering both T-cell receptors and co-stimulatory molecules so that T-cells are driven to mature into effectors rather than to cause their anergy or tolerance. Another strategy that has met with some success in generating immune responses, is to load dendritic cells with mRNA isolated from tumors [Kalady et al., 2004]. Loading of dendritic cells with RNA might be advantageous not only for the potential expression of novel tumor antigens that can be presented by the dendritic cells, but certain RNA sequences may serve as "an adjuvant" or danger signal that may induce dendritic cells to produce appropriate cytokines for immune activation. These RNA sequences, like CpG sequence repeats of DNA, may trigger dendritic cells, or other cells of the innate immune system through toll-like receptors. Toll-like receptors (TLR) are one mechanism through which the innate immune system can be activated. TLR recognize a variety of environmentally encountered ligands and activate first-line defenses of inflammation and immunity [Janeway and Medzhitov, 2002]. Successful vaccines, then may do well to incorporate into their composition, a mixture with activators of toll-like receptors so that the activation and selection of immune cells is bolstered. While these additional features may successfully direct the activation of immune T-cells capable of killing tumor cells, particularly when assayed in antigen binding or cytotoxicity assays in vitro, immune cells may be unable to function at the tumor site due to the suppressive/inhibitory forces of the tumor by-products or regulatory surface products. Tumor cell defenses are generally not considered in the development of vaccines designed to activate or augment immune responses to tumors. A major question then is how can we overcome these "down-regulator" factors? One possibility is to target specific therapy to tumor byproducts. Preliminary studies in animal models suggest that one route to successful therapy may be by blocking the function of suppressive ligands expressed on tumor cells. Anti-PD-H1 antibody treatment, for example, resulted in specific activation of immune effector cells directed against tumors [Iwai et al., 2002]. Monoclonal antibody therapy in some cancers has met with significant effects. The objectives of those therapies, however has not been to induce specific immunity and to depend upon the development of immune effector cells to eradicate the tumors as would be expected from antibodies directed against down-regulatory ligands. Clearly, vigorous autologous responses to tumors would be an ideal mechanism to destroy metastatic sites of tumor cells as well as "hidden" sites of cancer stem cells.

It is our opinion that molecular targeting of drugs to aberrantly expressed pathways is the best method to treat and eradicate pancreas tumors. In patients where surgical resection or destruction of the entire tumor mass is not possible or where cancer stem cells could give rise to other metastatic sites of tumors, tumor antigen vaccines or "pre-activated" T-cell lines specific for tumor antigens should be applied. Vaccines that are presented through dendritic cells will trigger development of both innate and adaptive immune effector cells, while other protein-based vaccines must include an "adjuvant" such as a toll-like receptor agonist to prevent the induction of suppressor cells, anergy or tolerance to tumor antigens. Also, reagents to interfere with the tumors' ability to suppress immune effector cells must be included in the therapy to ensure that immune effector cells can perform their functions within the tumor microenvironment.

In conclusion, the future of pancreas cancer therapy will be with a combination of therapies that are always evolving in their design to hone in on tumors and salvage normal tissue. Surgical resection of large masses, with laser treatments of nonresectable areas, combined with radiotherapy techniques will result in reduction of the major tumor masses. A combination of targeted biotherapy with immunotherapy, however will be required to get rid of nonresectable metastatic sites or beds of pancreas cancer stem cells (Table III). Targeted biotherapies will require precise definition of the molecular changes resulting from genetic mutations in pancreas cancer. While immunotherapy with activated cytolytic T-cell lines shows some promise, it is unlikely to serve as a therapy available to the general public due to financial and technological requirements. Vaccines derived from peptides, mRNA or DNA of mutated gene products in combination with antibodies or other reagents directed against molecules that enable tumors to escape immune

TABLE III. Bio-Immunotherapeutic Targets for the Treatment of Pancreas Cancer

Tumor targeted inhibitors	Immune agonist or activators	Tumor specific vaccines
Mimetics for mutated proteins targeted to enzyme active sites, protein–DNA or protein–protein, protein–lipid binding motifs	Toll-Like Receptor agonists	Antigen loaded or genetically transduced dendritic cells
Interference of over expressed proteins using anti-sense, siRNA, or inhibitors of down-stream substrates	Inhibitors of down-regulatory ligands; e.g., PD-H1/PD-L1 ligands, BTS ligands, Fas ligand, and others	Mutant protein or recombinant peptides
Target mutated proteins with antibodies, immunotoxins	Activate expression of, or co-transfect co-stimulatory molecules	Tumor derived RNA
Tumor cell transfections with functional gene replacements, or death inducing factors; e.g., TNFα	Activate genes coding alternate adhesion molecules to prevent tumor cell migration	DNA based
Identify pancreas cancer stem cells and target mutant gene(s)	Targeted delivery of cytokines	Mutated peptides from unique stem cell proteins

effector cells may serve to destroy residual malignant cells and cancer stem cells.

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