

Immunotherapy for Pancreatic Cancer

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1. Introduction

Patients with pancreatic cancer have an especially poor prognosis, with a 5-year survival rate of <1% and a median survival of 4-6 months (Jemal, Siegel et al., 2010). The management of patients with pancreatic cancer depends on the extent of the disease at diagnosis. However, approximately 80% of patients present with advanced-stage disease that precludes surgical resection (pancreaticoduodenectomy) and long-term survival is poor (Sener, Fremgen et al., 1999). Even after resection, the majority of patients relapse, leading to a median survival of about 18 months after resection (Neoptolemos, Stocken et al., 2004). In this time, gemcitabine-based chemotherapy is typically offered as standard of care. However, most patients treated with gemcitabine alone do not survive longer than 6 months, as the tumor cells are naturally resistant to current chemotherapy (Neoptolemos, Stocken et al., 2004). Importantly, the tumors that develop gemcitabine resistance would still be a suitable target for immunotherapy. Therefore, cancer immunotherapy for pancreatic cancer may be one attractive approach to treatment. This chapter summarizes the effect of immunotherapy for inducing cytotoxic T lymphocytes (CTLs) in patients with pancreatic cancer and discusses recent advances in concept of combination therapy of immunotherapy and chemotherapy.

2. Chemotherapy

Gemcitabine (2',2'-difluorodeoxycytidine) is a synthetic pyrimidine nucleoside analog that has become the standard first-line treatment for patients with advanced pancreatic cancer

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based on clinical benefit and survival improvement compared with 5-fluorouracil (5-FU)-based chemotherapy (Burris, Moore et al., 1997). Gemcitabine is phosphorylated intracellularly to difluorodeoxycytidine triphosphate, which terminates DNA-chain elongation and competitively inhibits DNA polymerase and ribonucleotide reductase, leading the cells into the apoptotic pathway (Storniolo, Allerheilgen et al., 1997). However, most patients treated with gemcitabine alone do not survive longer than 6 months. Moreover, the addition of the cytotoxic agents (platinums, fluoropyrimidines, or topoisomerase inhibitors) or radiation therapy to gemcitabine did not lead to a statistically significant improvement in overall survival (OS) in patients with metastatic pancreatic cancer (Moore, Goldstein et al., 2007; Van Cutsem, Verslype et al., 2007; Philip 2008b; Cascinu, Berardi et al., 2008). Recently, Thierry Conroy and colleagues randomly assigned 342 patients to receive combination chemotherapy regimen of FOLFIRINOX (consisting of oxaliplatin, irinotecan, fluorouracil, and leucovorin) (n=171) or gemcitabine (n=171) (Conroy, Desseigne et al., 2011). In selected patients with good performance status ECOG 0-1, the FOLFIRINOX regimen, when compared with gemcitabine, was associated with significantly increased median survival from 6.8 to 11.1 months. However, as compared with gemcitabine, FOLFIRINOX had increased toxicity. Gemcitabine is still the reference treatment in patients with ECOG performance status 2. Therefore, there is still great need for a novel therapeutic approach with low toxicity for advanced pancreatic cancer. Cancer immunotherapy for pancreatic cancer may be one attractive approach to cancer treatment.

3. Targeted therapy

The era of targeted therapies has generated a lot of interest in discovering better approaches for patients with pancreatic cancer. While traditional cytotoxic drugs also target specific cellular process, the newer generation of agents is set apart by their targeting of a pathway or molecule that derives the growth, speed, survival, or maintenance of tumor cells specially. Overexpression of human epidermal growth factor receptor type 1 (HER1/EGFR) has been suggested to be associated with the malignant transformation of pancreatic cancer (Tobita, Kijima et al., 2003). Therefore, there is a sound rationale for combining HER1/EGFR inhibitor and gemcitabine in pancreatic cancer. Erlotinib (Taraceva, Genentech, South San Francisco) is a small molecule HER1/EGFR tyrosine kinase inhibitor. Pancreatic cancer patients given the combination of erlotinib with gemcitabine showed a statistically significant improved survival compared with those given gemcitabine alone (Moore, Goldstein et al., 2007). The median and 1-year survival rates were better for the combination treatment: 6.24 months versus 5.91 months and 23% versus 17%, respectively. Therefore, the US Food and Drug Administration (FDA) recently approved erlotinib for use in the first-line setting of advanced pancreatic cancer in combination with gemcitabine. However, this survival benefit was small and, therefore, erlotinib has not yet been widely incorporated into standard treatment protocols. On the other hand, cetuximab, a monoclonal antibody, has been shown to significantly suppress the growth of implanted pancreatic cancer cells, and this effect was enhanced by the addition of gemcitabine in mice study (Bruns, Harbison et al., 2000). The study evaluating cetuximab in pancreatic cancer has been completed. In patients with advanced pancreas cancer, cetuximab did not improve the outcome compared with patients treated with gemcitabine alone (Philip, Benedetti et al., 2010). Moreover, the

addition of cetuximab to gemcitabine did not contribute to improvement in the patient-reported health-related quality of life (HRQL) outcomes (Moinpour, Vaught et al., 2010). The next generation of single-target trials is moving toward a focus on antiangiogenic agents, including anti-VEGF and anti-VEGFR strategies combined with gemcitabine. However, the addition of Axitinib that is a potent, selective inhibitor of vascular endothelial growth factor (VEGF) receptors 1, 2, and 3 tyrosine kinase also did not improve overall survival in advanced pancreatic cancer (Kindler, Ioka et al., 2011). These results add to increasing evidence that targeting of EGFR or VEGF signaling is an ineffective strategy in pancreatic cancer. Other chemotherapy, including S-1, ixabepilone, nanoparticle albumin-bound (nab) paclitaxel, FOLFOX (5-FU, leucovorin, oxaliplatin), and XELOX (capecitabine, oxaliplatin) may be better partners with targeted agents (Philip 2008a).

4. Immunotherapy

T cells with the $\alpha\beta$ T-cell receptor (TCR) generally express CD4+ or CD8+ lineage markers and mostly fall into helper or cytotoxic subsets, respectively (Boon, Coulie et al., 1997). On the other hand, T cells expressing the alternate $\gamma\delta$ TCR generally do not express lineage markers. Although CD8+ naive T cells recognize peptides (usually 8-10 amino acids) derived from tumor-associated antigens (TAAs) bound by major histocompatibility complex (MHC) class I molecules on tumor cells, it is not sufficient to initiate a productive generation of antigen-specific CTLs. Induction of CD8+ CTLs need peptides derived from TAAs to be presented on the surface of antigen presenting cells (APCs) in the context of MHC molecules. Moreover, CD4+ T cells recognize peptides (usually 10-30 amino acids) in association with MHC class II molecules on APCs and mediate their helper functions by enhancing the persistence of antigen-specific CD8+ CTLs or through secretion of cytokines such as interleukin (IL)-2 and interferon (IFN)- γ (Steinman and Swanson 1995; Banchereau & Steinman 1998). Therefore, the $\alpha\beta$ TCR interaction with complex of peptides and MHC class I and class II molecules on APCs is a central event in T-cell-mediated antitumor immune responses. Antigen-specific CD8+ CTLs can respond to TAAs derived peptides presented in the context of MHC class I molecules on tumor cells. Therefore, efforts have focused on generating TAAs-specific $\alpha\beta$ CD8+ CTLs (Waldmann 2003).

Dendritic cells (DCs) are powerful APCs that play a pivotal role in the initiation, programming, and regulation of tumor-specific immune responses (Steinman 1991). DCs can process endogenously synthesized antigens into antigenic peptides, presented to the cell surface as MHC class I-peptide complexes, and recognized by the $\alpha\beta$ TCR in CD8+ naive T cells (Steinman 1991). DCs are also capable of capturing and processing of exogenous antigens, and presenting antigenic peptide on MHC class I molecules through an endogenous pathway, a process known as antigen cross-presentation (Berard, Blanco et al., 2000). In the case of cancer, cross-presentation after uptake and processing of soluble or particulate matter from apoptotic, necrotic cancer or even live cancer cells is the only important natural mode of presentation (Melief 2003). On the other hand, exogenous antigens from the extracellular environment are captured and delivered to the compartments of the endosome/lysosome, where they are degraded to antigenic peptides by proteases and peptidases, which are complexed with MHC class II and recognized by the $\alpha\beta$ TCR in CD4+ naive T cells (Steinman 1991). Although both immature and mature DCs

are capable of processing and presenting MHC/peptide complexes to TCR, mature DCs are significantly better at CTL induction due to higher expression of MHC class I and class II and costimulatory molecules (Banchereau & Steinman 1998). On the other hand, presentation of antigens by immature DCs, in the absence of proper costimulation, may lead to tolerance induction (Banchereau & Palucka 2005). After antigens uptake and inflammatory stimulation, immature DCs in peripheral tissues undergo a maturation process characterized by the up-regulation of MHC class I and class II and costimulatory molecules, chemokine receptors such as CCR7, and the secretion of cytokines such as IL-12 (Banchereau & Steinman 1998; Forster, Schubel et al., 1999; Steinman 1991). During the process, mature DCs migrate to T-cell areas of secondary lymphoid organs, where they present antigens to CD4+ and CD8+ T cells through MHC class I and class II pathways, respectively (Steinman 1991; Banchereau & Steinman 1998; Banchereau & Palucka 2005). The $\alpha\beta$ TCR in CD8+ CTL can recognize MHC class I-peptide complexes on cancer cells and destroy cancer cells through effector molecules such as granzyme B and perforin (Finn 2008). On the other hand, $\gamma\delta$ T cells generally do not require MHC for antigen presentation, and recognize nonpeptidic antigens. As effective antitumor responses depend on the presence and function of immune cells that are able to recognize and eliminate cancer cells, the aim of immunotherapy is to activate both CD8+ CTLs that recognize TAAs-specific antigens and CD4+ T helper (Th) cells that mediate helper function.

4.1 Immune homeostasis

Now, it is becoming clear that CD4+ Th cells are critical in combating cancer cells and maintaining immune homeostasis. Upon TCR-mediated cell activation, naive CD4+ T cells can differentiate into at least four major polarization patterns including Th1, Th2, regulatory T (Treg), and Th17 cells, all of which participate in different types of immune responses (Zhu & Paul 2010) (Fig. 1). Mainly, immune homeostasis is controlled by two distinct helper T cell subsets, Th1 and Th2 cells. The Th1 cells secrete type I cytokines such as IFN- γ , tumor necrosis factor (TNF)- α , and TNF- β , to activate DCs, which can regulate the survival and persistence of CD8+ CTLs as memory cells (Bachelet, Mariethoz et al., 1998). IL-12 secreted from DCs is a potent inducer of Th1 differentiation. Both CD8+ CTLs and Th1 cells secrete IFN- γ , which can further sensitize tumor cells to CTLs by upregulation of MHC class I molecules on tumor cells and antigen-processing machinery of DCs (Steinman 1991). On the other hand, Th2 cells secrete type II cytokines, such as IL-4 and IL-10 resulted in enhanced generation of a humoral immunity, antibody-based antitumor response (Steinman 1991; Bradley, Yoshimoto et al., 1995; Banchereau & Steinman 1998; Wiethe, Debus et al., 2008). The newly identified Th17 cells secrete IL-17 and IL-22, eliciting tissue inflammation implicated in autoimmunity (Dong 2008). Importantly, cancer cells-derived soluble factors promote the induction of tolerance through the generation of CD4+ α chain of IL-2R (CD25)+ forkhead box P3 (Foxp3)+ natural (n) Treg cell subset (Koido, Homma et al., 2008). Induced (i) Treg cells (CD4+CD25+Foxp3-) secrete transforming growth factor- β (TGF- β) and IL-10 and suppress effector T cells of either Th1 or Th2 phenotype in a cell contact and antigen-specific manner (Shevach 2009; Mougiakakos, Choudhury et al., 2010). Treg cells play a pivotal role in the tumor progression and the suppression of antitumor immunity.

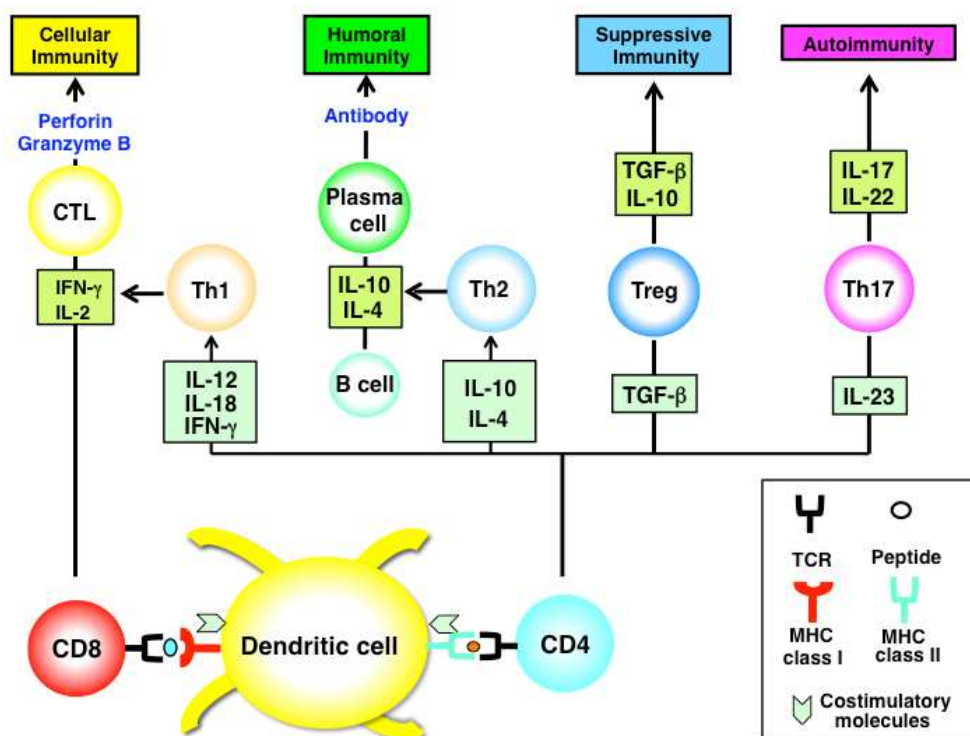


Fig. 1. Immune homeostasis. Upon TCR-mediated cell activation, naive CD4 T cells can differentiate into at least four major lineages, Th1, Th2, Treg, and Th17 cells, all of which participate in different types of immune responses. The Th1 cells produce signature type I cytokines, such as IFN- γ and IL-2 resulting in induction of CD8+ CTLs. Th2 cells secrete type II cytokines, such as IL-4 and IL-10. The Th2 response is associated with the humoral, antibody-based antitumor response. Treg cells that secrete TGF- β and IL-10 suppress Th1 or Th2 cells. Th17 cells secrete IL-17 and IL-22, eliciting tissue inflammation implicated in autoimmunity.

4.2 Immunosuppression in tumor microenvironment

Pancreatic cancer cells express TAAs such as Wilms' Tumor gene 1 (WT1) (Sugiyama 2005), mucin 1(MUC1) (Mukherjee, Ginardi et al., 2000), human telomerase reverse transcriptase (hTERT) (Seki, Suda et al., 2001), mutated K-RAS (Gjertsen, Bakka et al., 1995), survivin (Wobser, Keikavoussi et al., 2006), carcinoembryonic antigen (CEA) (Nair, Hull et al., 1999), HER-2/neu (Larbouret, Robert et al., 2007), or p53 (Hoffmann, Nakano et al., 2000) as potential targets for immunotherapy. Therefore, immunotherapy targeted such a TAA may be an approach in patients with advanced pancreatic cancer. However, the microenvironment in pancreatic cancer is consisted not only cancer cells but also stroma cells such as cancer-associated fibroblasts (CAFs), tolerogenic DCs, myeloid-derived suppressor cells (MDSCs), immunosuppressive tumor-associated macrophages (TAMs), and

Treg cells (Fig. 2). These immune suppressive cells secrete vascular endothelial growth factor (VEGF), IL-6, IL-10, TGF- β , soluble Fas ligand (Fas-L), and indolamine-2,3-dioxygenase (IDO) (Koido, Homma et al., 2010c). As a result, immunosuppressive cells inhibit antitumor immunity by various mechanisms, including depletion of arginine and elaboration of reactive oxygen species (ROS) and nitrogen oxide (NO). The tumor microenvironment also promotes the accumulation of Treg cells that suppress CD8⁺ CTL function through secretion of IL-10 or TGF- β from Treg cells and tumors. Therefore, immunotherapies that struggle against pancreatic cancer cells with CTLs as well as inhibition of Treg cells may tip the balance in favor of immunostimulation. Currently, the field of cancer immunotherapy using peptide- or cell (DC or whole tumor cell)-based approaches is in an active state of preclinical and clinical investigations.

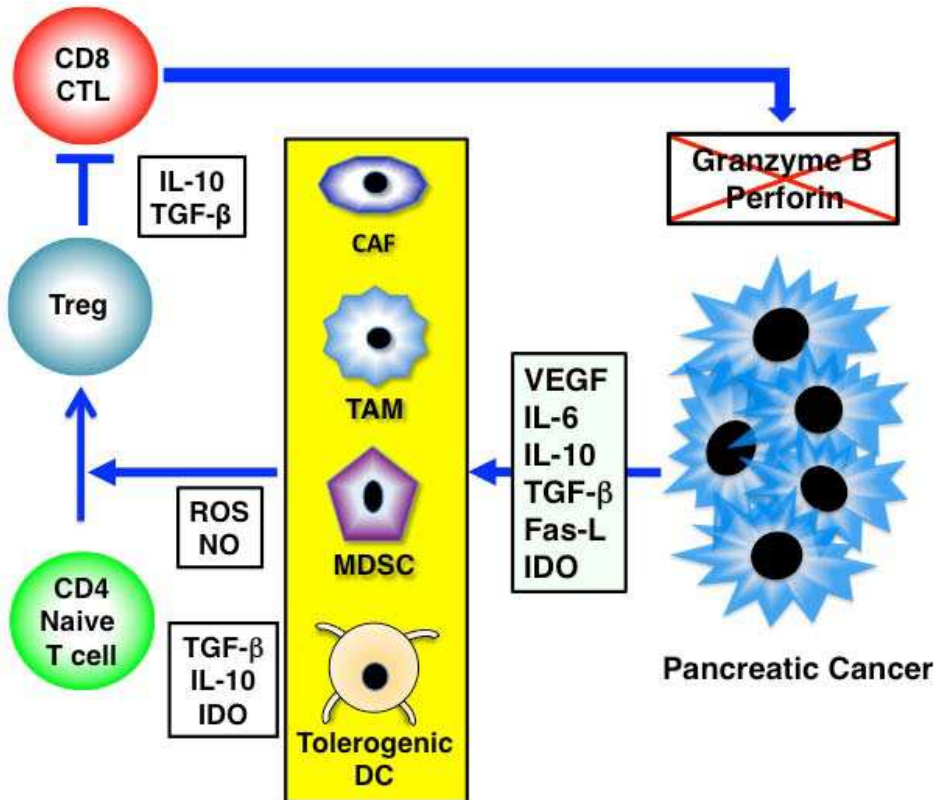


Fig. 2. Immunosuppression in tumor microenvironment. Pancreatic cancer cells secrete various factors such as VEGF, IL-6, IL-10, TGF- β , Fas-L, and IDO, all of which promote the accumulation of heterogeneous populations of CAFs, TAMs, MDSCs, or tolerogenic DCs. These immunosuppressive cells in tumor microenvironment inhibit antitumor immunity by various mechanisms, including depletion of arginine and elaboration of ROS and NO. The tumor microenvironment also promotes the accumulation of Treg cells that suppress CD8⁺ CTL function.

5. Peptide vaccines

Peptide-based cancer vaccines are preparations made from antigenic protein fragments that represent the minimal immunogenic region of TAA (Purcell and McCluskey 2007; Bijker, Melief et al., 2007). As peptide vaccines are simple, safe, stable, and economical, multiple MHC class I-binding peptides have been identified and vaccination with synthetic peptides has been examined for their immunogenicity in clinical trials for pancreatic cancer (Dummer 2001; Jaffee, Hruban et al., 2001; Yanagimoto, Mine et al., 2007; Miyazawa, Ohsawa et al., 2010). In early phase clinical trials, vaccination of mutant K-ras (Gjertsen, Bakka et al., 1995; Gjertsen, Buanes et al., 2001; Abou-Alfa, Chapman et al., 2011), MUC1 (Yamamoto, Ueno et al., 2005b; Ramanathan, Lee et al., 2005), or telomerase (Bernhardt, Gjertsen et al., 2006) peptide to patients with advanced pancreatic cancer are significantly associated with antitumor responses. As almost all pancreatic cancers involve mutations in the K-ras oncogene, it is believed that activating K-ras mutations are critical for initiation of pancreatic cancer (Gjertsen, Bakka et al., 1995; Gjertsen, Buanes et al., 2001; Abou-Alfa, Chapman et al., 2011). In a clinical phase I/II trial involving 48 patients with pancreatic cancer (10 surgically resected and 38 with advanced disease), vaccination of synthetic mutant K-ras peptides in combination with granulocyte-macrophage colony-stimulating factor (GM-CSF) produced immune responses to mutant K-ras and showed prolonged survival from the start of treatment compared to non-responders (Gjertsen, Buanes et al., 2001). Abou-Alfa et al. (Abou-Alfa, Chapman et al., 2011) also vaccinated 24 patients with resected pancreatic cancer with mutant K-ras peptide in combination of GM-CSF and found that the vaccination proved to be safe and tolerable with no elicitable immunogenicity and unproven efficacy. On the other hand, almost all pancreatic cancer cells also express MUC1 that is high molecular weight glycoproteins (Chhieng, Benson et al., 2003). The MUC1 peptide derived from tandem repeat core was also recognized by CD8+ T cells in an MHC-restricted and -unrestricted manner. Therefore, MUC1 peptide vaccine was subsequently used in the immunization of patients with pancreatic cancer (Finn, Jerome et al., 1995). Ramanathan et al. (Ramanathan, Lee et al., 2005) used 100 mer MUC1 peptide with SB-AS2 adjuvant in 16 patients with resected or locally advanced pancreatic cancer. They found that 100 mer MUC1 peptide with SB-AS2 adjuvant induced low but detectable MUC1-specific humoral and T-cell responses in some patients. Yamamoto et al. (Yamamoto, Ueno et al., 2005b) also, in a clinical phase I trial involving 6 patients with advanced pancreatic cancer, reported that vaccination with 100 mer MUC1 peptide and incomplete Freund's adjuvant resulted in increased circulating anti MUC1 IgG antibody in some patients. Moreover, human telomerase reverse transcriptase (hTERT) is the catalytic subunit of telomerase and a prototype for a novel class of universal tumor antigens due to its expression in the vast majority of human tumors (Beatty & Vonderheide, 2008). Therefore, it is one of widely applicable target antigen recognized by CTLs in pancreatic cancer. Bernhardt et al. (Bernhardt, Gjertsen et al., 2006) reported the results of a phase I trial of telomerase peptide in combination with GM-CSF for non-resectable pancreatic cancer patients (n=48). The immunotherapy was safe and induction of an immune response was correlated with prolonged survival. Recently, Itoh et al. (Itoh, Yamada et al., 2009) have developed personalized peptide vaccines. In this regimen, pre-vaccination peripheral blood mononuclear cells (PBMCs) were screened for their reactivity in vitro to each peptide in

patients, and only the reactive peptides were used as vaccines to 11 patients with advanced pancreatic cancer. In the personalized peptide vaccine, increased cellular and humoral immune responses to at least one of peptides used for vaccination were observed in the post-vaccination PBMCs (Yamamoto, Mine et al., 2005a). In all of these peptide vaccines, only a limited success has occurred in clinical trials. Generally, the drawback of this strategy comes from numerous factors: (i) only a limited number of known synthesized antigenic peptides can be available (Mocellin, Pilati et al., 2009), (ii) CD8⁺ CTLs may be ineffective in reacting with pancreatic cancer cells due to down regulation of certain antigens and MHC class I molecules, (iii) impaired function of DCs in patients with advanced pancreatic cancer (Yanagimoto, Takai et al., 2005; Koido, Hara et al., 2010a), and (iv) tumor microenvironment where immune suppressive cells such as Treg cells, CAFs, MDSCs, or TAMs exist (Finn 2008). The more attractive peptide-based vaccines may be synthetic long peptides. As synthetic long peptides are not able to bind directly on MHC class I and class II molecules on DCs, they need to be taken up, processed and presented by DCs. Therefore, the long peptide vaccines can be presented on MHC class I and class II molecules long time resulted in induction of antigen-specific polyclonal CD4⁺ and CD8⁺ T cells (Melief & van der Burg 2008; Bijker, van den Eeden et al., 2008). Peptide vaccines for the treatment of established pancreatic cancer may require long-lived presentation of epitopes by MHC class I and class II molecules on appropriately activated DCs. Such presentation is essential for induction of robust therapeutic CD4⁺ and CD8⁺ T-cell responses. Recently, Weden et al. (Weden, Klemp et al., 2011) treated 23 patients who were vaccinated after surgical resection for pancreatic cancer with long synthetic mutant K-ras peptides designed mainly to elicit T-helper responses. Surprisingly, 10-year survival was 20% (four patients out of 20 evaluable) versus zero (0/87) in a cohort of nonvaccinated patient treated in the same period. The key elements for the development of therapeutic peptide vaccines for pancreatic cancer may be the combination with chemotherapy to overcome robust cancers. Indeed, Wobser et al. (Wobser, Keikavoussi et al., 2006) reported a case of complete remission (CR) of liver metastasis of pancreatic cancer refractory to gemcitabine chemotherapy under vaccination with a survivin peptide. Peptide vaccines alone should be tested in cancer patients in remission to prevent recurrence and metastasis after surgical resection.

6. DC-based vaccines

For T-cell activation, three signals are required: (i) effective presentation of multiple TAAs in MHC class I and class II molecules; (ii) costimulation by membrane-bound receptor-ligand pairs; and (iii) soluble factors to direct polarization of the ensuing efficient antitumor immune responses. DCs derive their potency from constitutive and inducible expression of essential costimulatory ligands on the cell surface including B7, ICAM-1, LFA-1, LFA-3, and CD40 (Inaba, Witmer-Pack et al., 1994). These proteins function in concert to generate a network of secondary signals essential for reinforcing the primary antigen-specific signal in T-cell activation (Inaba, Pack et al., 1997). Therefore, now it is clear that DCs have the ability to provide all three signals essential for induction of antitumor immunity (Banchereau & Palucka 2005). These findings have provided the rationale for ex vivo antigen loading of DC as vaccines. More than 200 clinical trials have been performed using DC as cellular adjuvants in cancer. Several strategies to deliver TAAs into DCs have been developed to generate potent antitumor immune responses (Fig. 3).

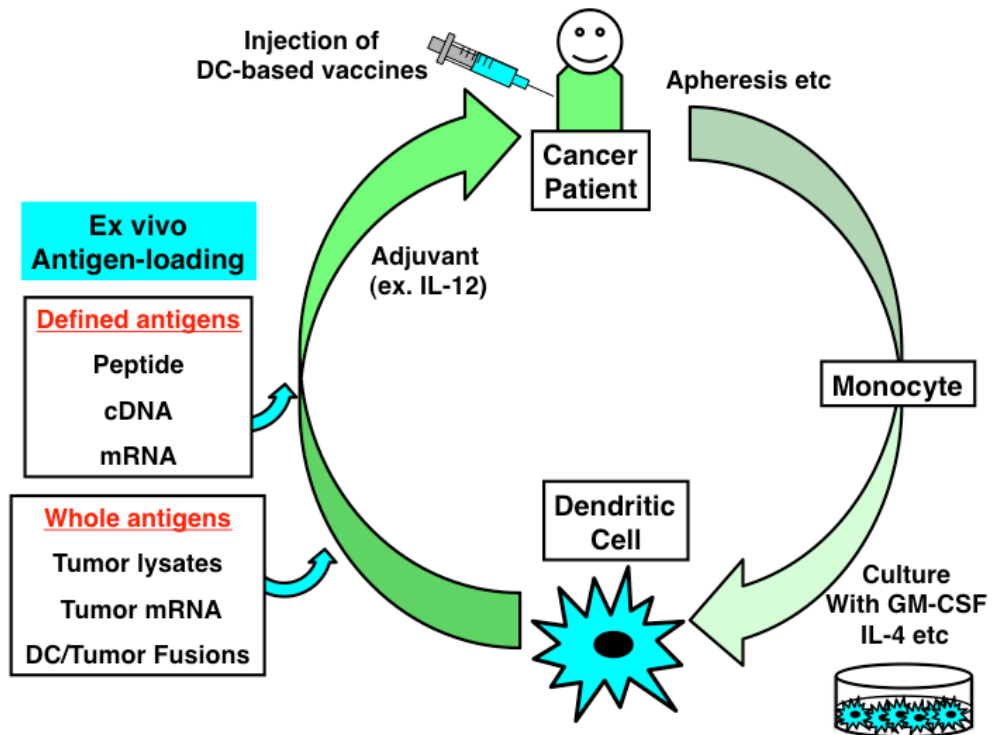


Fig. 3. Strategies to deliver defined or whole antigens to DCs. DCs used for cancer vaccines have been generated from the peripheral blood monocytes of the patients using cytokines including GM-CSF and IL-4 etc. To generate antigen-specific CTL responses against tumor cells, DCs loaded with synthetic peptide, antigenic cDNA, or mRNA have been used. Moreover, whole tumor associated antigens including defined and unidentified have been also loaded to DCs.

DCs have been loaded with tumor antigens in the form of peptides (Nestle, Alijagic et al., 1998), tumor lysates (Mackensen, Herbst et al., 2000), apoptotic tumor cells (Palucka, Ueno et al., 2006), or mRNA (Nair, Boczkowski et al., 1998; Koido, Kashiwaba et al., 2000). Alternatively, whole tumor cells have been fused with DCs to facilitate the entry of TAAs, including both known and unidentified, into the endogenous antigen-processing pathway in the DCs (Fig.4). The strategy for DC/tumor fusion vaccine is based on the fact that DCs are the most potent antigen-presenting cells in the body, whereas tumor cells express abundant tumor antigens. In animal studies, DC/tumor fusion vaccines have been shown to possess the elements essential for processing and presenting tumor antigens to host immune cells, for inducing effective immune response, and for breaking T-cell tolerance to TAAs (Gong, Chen et al., 1997; Koido, Hara et al., 2007; Gong, Koido et al., 2008; Koido, Hara et al., 2009; Koido, Hara et al., 2010a; Koido, Homma et al., 2010b). Recently, we have reported that fusions of human pancreatic cancer cells and DCs induce CTL responses against pancreatic cancer cells in vitro (Koido, Hara et al., 2010a). Although DC/tumor fusion

vaccines have proven clinically safe and efficient to induce tumor-specific immune responses, only a limited number of objective clinical responses have been reported in cancer patients (Avigan, Vasir et al., 2004; Kikuchi, Akasaki et al., 2004; Homma, Kikuchi et al., 2005; Homma, Sagawa et al., 2006).

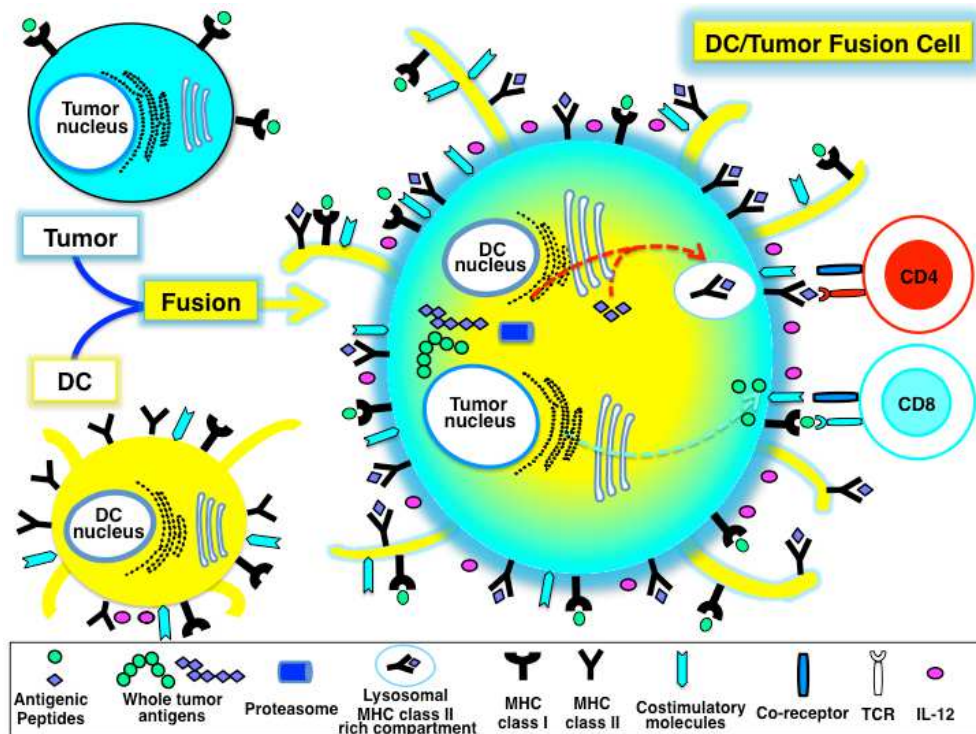


Fig. 4. Fusions of DC and tumor cell. DC/tumor fusions express MHC class I and class II, costimulatory molecules (CD80 and CD86), and multiple tumor-associated antigens. The DC/tumor fusions are able to process multiple TAAs derived from tumor. They form MHC class I-peptide complexes, in the endoplasmic reticulum, which are transported to the cell surface of DC/tumor fusions and presented to CD8⁺ T cells. The DC/tumor fusions can also synthesize antigenic peptides in the endoplasmic reticulum, which are transported to the cytoplasm, where MHC class II-peptide complexes are assembled with multiple tumor-derived peptides. These complexes are presented to CD4⁺ T cells, which are essential for induction of efficient antigen-specific polyclonal CTLs.

Clinical trials of antigen-pulsed DCs have been conducted in patients with various types of tumors including pancreatic cancer. In a phase I/II clinical trial of a MUC1 peptide-loaded DC vaccines in pancreatic and biliary cancer patients following resection of their primary tumors, 4 of the 12 patients followed for over four years were alive, all without evidence of recurrence (Lepisto, Moser et al., 2008). Moreover, MUC1 specific immune responses were observed even in patients with pretreated and advanced disease, following immunization with DC transfected with MUC1 cDNA (Pecher, Haring et al., 2002). Findings from initial

clinical trials demonstrate that hTERT-specific immune responses can be safely induced in cancer patients. Suso et al. (Suso, Dueland et al., 2011) recently reported that vaccination with DC transfected with hTERT mRNA (DC/hTERT mRNA) had the potential to induce strong immune responses to multiple hTERT epitopes. In this therapy, a patient who could not continue chemotherapy due to severe neutropenia had been treated with DC/hTERT mRNA alone for 3 years and resulted in no evidence of active disease. Moreover the CR was associated with induction of hTERT-specific immune responses against several hTERT-derived Th and CTL epitopes. Therefore, DC/hTERT mRNA may be an attractive approach to induce potent antitumor immunity. On the other hand, combined injection of unloaded DCs and activated lymphocytes resulted in prolonged survival of refractory pancreatic cancer patients (Nakamura, Wada et al., 2009). To improve the clinical efficacy of DC-based cancer vaccines, we need to design novel and improved strategies that can boost adaptive antitumor immunity to break overcome the immunosuppressive tumor microenvironment.

7. Whole tumor cell-based vaccines

Although cancer vaccines with defined TAAs are commonly used, the advantage of using autologous whole tumor cells is that tumor cells express a whole array of TAAs that are both characterized and uncharacterized. Moreover, this rich source of antigens contains epitopes of both CD8+CTLs and CD4+ T helper cells (Koido, Hara et al., 2005; Koido, Hara et al., 2009; Koido, Homma et al., 2010c). Thus, whole tumor cells could greatly diminish the chance of tumor escape compared to using single epitope peptide vaccines. In clinical trials, autologous tumor cells have been used as cancer vaccines to induce polyclonal CTL induction against colorectal (Harris, Ryan et al., 2000), renal cell cancer (Jocham, Richter et al., 2004), or melanoma (Berd, Sato et al., 2004), and several trials have shown clinical responses in the initial clinical studies. However, in many cases, even though a tumor-specific immune response has been observed, none has shown significant efficacy in the randomized phase III trials. To improve immunogenicity of vaccines, autologous whole tumor cells have been genetically modified to secrete GM-CSF and have shown promising results in patients with prostate (Simons, Jaffee et al., 1997) renal cell (Simons, Mikhak et al., 1999), metastatic non-small-cell lung carcinoma (Salgia, Lynch et al., 2003), and melanoma (Soiffer, Hodi et al., 2003). This approach is based on the concept that GM-CSF is required at the site of the tumor to prime TAAs-specific immunity effectively (Nemunaitis 2005).

Autologous cancer cells would be the best source of immunizing proteins, however, only 10-15% of pancreatic cancer patients diagnosed are eligible for surgical treatment. Therefore, autologous tumor cells may not be provided in almost of the patients with pancreatic cancer. Moreover, even if the patients are treated by surgical resection, it is difficult to prepare sufficient amounts of autologous tumor cells due to the length of culture time and potential contamination of bacteria and fungus (Koido, Hara et al., 2005a). To circumvent this problem, allogeneic pancreatic tumor cell lines with shared TAAs have been used instead of autologous tumor cells to deliver shared TAAs into autologous DCs (Jaffee, Hruban et al., 2001; Lutz, Yeo et al., 2011). The whole allogeneic tumor cell line-based vaccines have numerous advantages. (i) Allogeneic tumor cell lines that share one or even several of the TAAs as autologous tumor cells. (ii) Allogeneic tumor cell lines can be propagated in large quantities in cell factories. (iii) It is not necessary to determine HLA typing of patients and allogeneic tumor cells, because autologous DCs can process and

present multiple TAAs from allogeneic tumor cells owing to cross-presentation in the context of autologous MHC class I and class II alleles. (iv) Both antigens-specific polyclonal CD4+ and CD8+ T cells can be induced simultaneously. While currently explored allogeneic approaches as whole tumor cell-based vaccines represent an improvement in terms of standardization over their autologous counterparts, they nevertheless entail the culture of large batches of cells under good manufacturing practice (GMP) grade conditions. Further optimization of these *in vitro* culture methodologies may be required. Moreover, the quality must be easily assessed and monitored in GMP facilities. One of the challenges that face the generation of whole allogeneic tumor-based vaccines for clinical use may be to overcome the potential hazards of fetal calf serum (FCS) (Koido, Hara et al., 2010a).

In a phase I trial, cancer vaccines using irradiated allogeneic pancreatic cancer cells secreting GM-CSF were safe and induced systemic antitumor immunity in patients with surgically resected pancreatic cancer (Jaffee, Hruban et al. 2001). From the same group, GM-CSF secreting allogeneic pancreatic cancer cells alone or in sequence with cyclophosphamide in patients with advanced pancreatic cancer showed minimal treatment-related toxicity and induction of mesothelin-specific T-cell responses (Laheru, Lutz et al., 2008). In addition, cyclophosphamide-modulated immunotherapy resulted in prolonged overall survival in a gemcitabine-resistant population. Recently, a single institution phase II study of 60 patients with resected pancreatic adenocarcinoma was performed (Lutz, Yeo et al., 2011). This approach integrated with chemoradiation was safe and demonstrated prolonged overall survival in resected pancreas cancer. While this approach for pancreatic cancer is a safe and promising therapy, their clinical efficacy remains to be established. Further clinical evaluation of the approach in patients with pancreatic cancer is warranted.

8. Combined therapy of immunotherapy and chemotherapy

In established pancreatic cancer patients, the effect of immunotherapy alone is limited by the number of CTLs able to penetrate tumor and by the number of tumor cells expressing specific antigens. Even if large numbers of CTLs generated *ex vivo* were injected into the patients, CTLs cannot penetrate into tumor site because of tumor stroma. Moreover, in tumor site, Treg cells or MDSCs produce immunosuppressive cytokines such as IL-10 and TGF- β . As a result, antitumor clinical responses may not induce in patients with advanced pancreatic cancer treated with immunotherapy alone.

Cytotoxic chemotherapy is well known to blunt immune responses, because of its toxicity for dividing cells in peripheral lymphoid tissue as well as the bone marrow. Indeed, several of the cancer chemotherapeutic agents such as cyclophosphamide (Weiner and Cohen 2002) and methotrexate (Weinblatt, Coblyn et al., 1985) are also used as immunosuppressants for the treatment of severe systemic autoimmune diseases. Therefore, the chemotherapeutic approach was considered to be inappropriate based on a widely held belief that the immunosuppressive effects of the chemotherapy would negate the efficacy of cancer vaccines (Zitvogel, Apetoh et al., 2008). However, increasing evidences have been mounting to suggest that immunotherapy has the possibility of achieving better success when used in combination with conventional chemotherapy (Gabilovich 2007; Smith, Kasamon et al., 2010). Gemcitabine that is a standard cytotoxic agent for pancreatic cancer has been also generally considered immunosuppressive due to neutropenia and lymphopenia being common adverse side effects. There is increasing evidence, however,

that gemcitabine plays important roles in the induction of antitumor immune responses. Gemcitabine inhibited B cells (Nowak, Robinson et al., 2002) and CD11b+GR1+ MDSCs (Suzuki, Kapoor et al., 2005), the phenomenon that may skew antitumor immunity towards beneficial T-cell responses (Qin, Richter et al., 1998). Moreover, gemcitabine treatment in patients with pancreatic cancer induced the proliferation of CD14+ monocytes and CD11c+ DCs (Soeda, Morita-Hoshi et al., 2009). To induce efficient therapeutic CTL responses, cross-presentation of TAAs by DCs is essential. Treatment of pancreatic cancer cells and DCs with gemcitabine results in enhanced cross-presentation of TAAs by DCs, CTL expansion, and infiltration of the tumor, all of which are associated with augmented CTL (Nowak, Lake et al., 2003a; Nowak, Robinson et al., 2003b; Dauer, Herten et al., 2005; Correale, Cusi et al., 2005). A recent report that chemotherapeutic agents caused up-regulation of cation-independent mannose 6-phosphate receptor (CI-MPR) expression on cancer cells and a concurrent increase in the uptake of granzyme B by activated CTLs also strongly suggests chemotherapy can function in synergy with induction of CTL responses to cure established pancreatic cancer (Ramakrishnan, Assudani et al., 2010).

These findings open a novel field of investigations for future clinical trial design, taking into account the immunostimulatory capacity of chemotherapeutic agents such as gemcitabine, and using them in combined chemoimmunotherapy strategies in patients with pancreatic cancer (Correale, Aquino et al., 2003; Nowak, Lake et al., 2003a; Correale, Cusi et al., 2005; Dauer, Herten et al., 2005; Correale, Del Vecchio et al., 2008). Now the immunostimulatory effects of gemcitabine have been confirmed in patients with cancer. In patients with pancreatic (Plate, Plate et al., 2005), nonsmall-cell lung (Levitt, Kassem et al., 2004) or colon (Galletto, Buttiglieri et al., 2003) cancer, standard cytotoxic agent, gemcitabine combined with recombinant cytokines or cancer vaccines could synergistically enhanced the frequency of tumor-specific CTL precursors. Therefore, patients with advanced pancreatic cancer have been treated by combination therapy of gemcitabine with peptide vaccine. For instance, both clinical and immune responses to personalized peptide vaccination combined with gemcitabine were evaluated in 21 patients with non-resectable pancreatic cancer (Yanagimoto, Mine et al., 2007; Yanagimoto, Shiomi et al., 2010). In this report, the reactive personalized peptides (maximum of 4 kinds of peptides) were administered with gemcitabine. Median overall survival time of all 21 patients was 9.0 months with a one-year survival rate of 38%. Immune boosting in both cellular and humoral responses was well correlated with overall survival. Combination therapy of a epitope peptide from vascular endothelial growth factor receptor 2 (VEGFR2) with gemcitabine was also conducted in 18 patients with metastatic and unresectable pancreatic cancer (Miyazawa, Ohsawa et al., 2010). The median overall survival time of all 18 patients who completed at least one course of the treatment was 8.7 months. Moreover, VEGFR2-specific CTL responses could be induced by the combination therapy. Similar findings were observed in 5 patients with inoperable locally advanced pancreatic cancer using gemcitabine, OK-432 stimulated DCs injected into the tumor sites, and intravenous infusion of lymphokine-activated killer (LAK) cells stimulated with anti-CD3 monoclonal antibody (Hirooka, Itoh et al., 2009). In this regimen, one patient had partial remission (PR) and 2 had long stable disease (SD) more than 6 months. Recently, we also reported that combination therapy of DC-based immunotherapies with gemcitabine/S-1 was effective in patients with advanced pancreatic cancer refractory to standard chemotherapy (Kimura, Imai et al., 2011). As WT1 is one of the excellent TAAs for the target of immunotherapy and is frequently expressed in pancreatic cancer cells (Oka, Tsuboi et al., 2004; Cheever, Allison et al., 2009), 38 out of 49 patients had

received vaccination with WT1 peptide pulsed DCs with or without combination of other peptides such as MUC1, CEA and CA125 in this report. Prior to this combination therapy, 46 out of 49 patients had been treated with chemotherapy, radiotherapy, heavy particle radiotherapy, or hyperthermia, but elicited no significant effects. In spite of these handicapped conditions, surprisingly, of 49 patients, 2 patients showed CR, 5 PR, and 10 SD, and median survival time was 360 days. We recently reported that gemcitabine sensitized the pancreatic cancer cells with WT1 specific T cell-mediated antitumor responses in vitro (Takahara, Koido et al., 2011), also supporting the significance of the combination therapy (Fig.5). In this study, gemcitabine treatment of human pancreatic cancer cells increased WT1 mRNA, and this increase was associated with nuclear factor kappa B (NF- κ B) activation. Gemcitabine treatment also shifted WT1 protein from the nucleus to the cytoplasm, which may promote proteasomal processing of WT1 protein and generation of antigenic WT1 peptide. Moreover, presentation of HLA-A*2402-restricted WT1 peptide increased in gemcitabine-treated pancreatic cancer cells. Indeed, we observed clinical response in a phase I clinical trial of combination therapy of WT1 peptide vaccine and gemcitabine (manuscript in preparation). Pancreatic cancer cells already, which have acquired gemcitabine resistance by the activation of NF- κ B might be killed by WT1-specific CTLs. Assessment of the clinical response to the combined therapy of WT1 peptide vaccine and gemcitabine is presently underway.

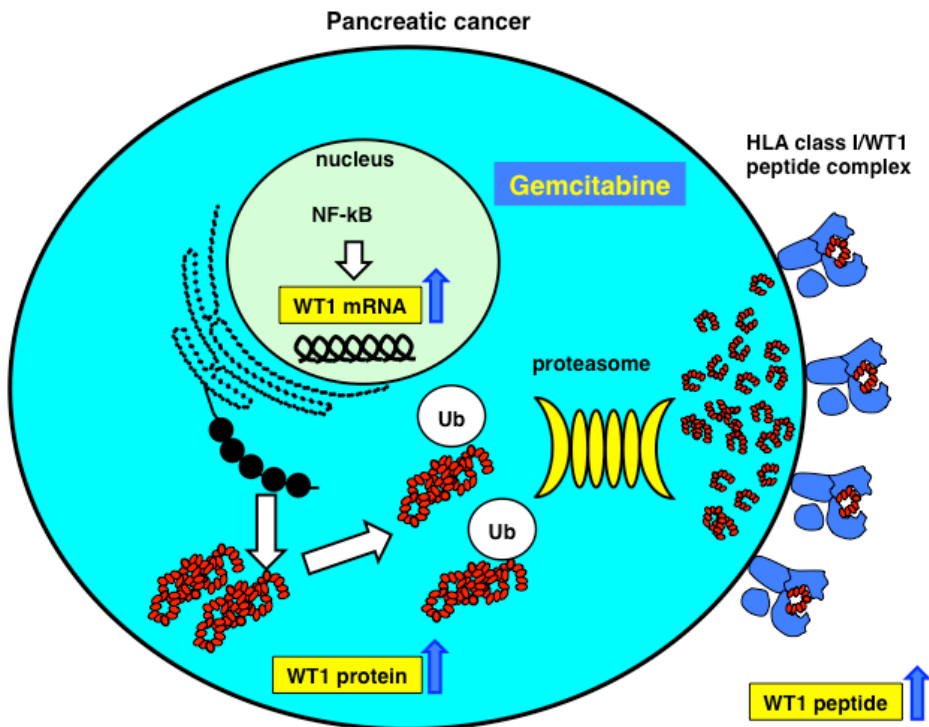


Fig. 5. Synergistic therapeutic antitumor effects of gemcitabine and WT1-specific CTLs. Gemcitabine enhanced WT1 expression in human pancreatic cancer cells and sensitized pancreatic cancer cells with WT1-specific T-cell-mediated antitumor immune responses.

Although the concept is still far from being firmly established, these reports may be sufficient to provide a platform for the combination of immunotherapy with chemotherapy. A combined approach of conventional therapies such as radiation or chemotherapy kill the bulk of tumor cells and CTLs that target TAAs may represent a promising approach for the treatment of patients with advanced pancreatic cancer. Evaluation is warranted to examine the effect of the combined approach on disease-free survival and overall survival.

9. Immunotherapy targeting cancer stem cells

It has been well known that the majority of patients with advanced pancreatic cancer that respond initially to standard chemotherapies ultimately undergo relapse due to the survival of small populations of cells with cancer-initiating/cancer stem cell (CSC) fraction (Wang, Li et al., 2011). These CSCs are a subpopulation of the tumor more capable than other cancer cells (CC) to self-propagate, initiate new tumors differentiate into bulk tumor, and therefore sustain tumor growth. It has been reported that pancreatic cancer cells resistant to chemoradiotherapy are rich in CSC fraction (Du, Qin et al., 2011). Moreover, CSCs could be

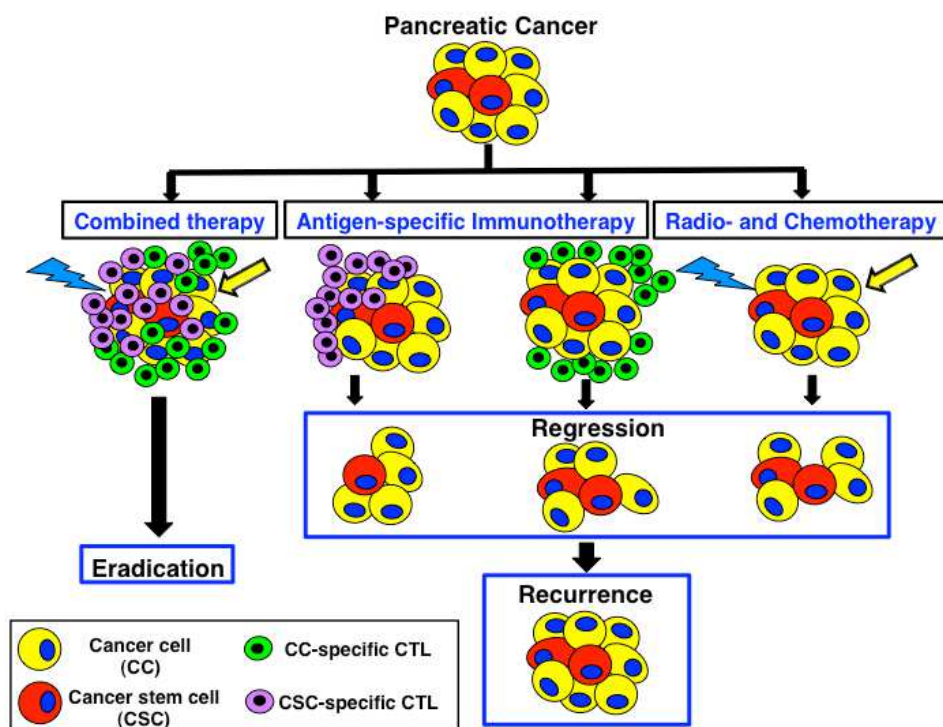


Fig. 6. Combination therapies of immunotherapy and standard radio- and chemotherapy. Currently applied standard therapies such as radio- and chemotherapy target bulk CCs that are less resistant than CSCs. This leads to initial regression of the tumor mass but eventually regrowth from residual CSCs. Combined therapies of standard therapies and immunotherapeutic approach targeting CSCs would cut off the rejuvenating supply of CSCs and resulted in tumor eradication.

expanded during the acquisition of gemcitabine resistance (Hong, Wen et al., 2009). Thus, targeted therapy against CSC fraction that is resistant to chemotherapy could be applied to overcome drug resistance in the treatment of pancreatic cancer (Fig. 6). Importantly, the tumors that develop chemotherapeutic drug resistance would still be a candidate target for immunotherapy. TAAs can be classified into two categories: i) CSC-specific antigens, such as SOX2 (Hong, Wen et al., 2009) or ALDH1A1 (Inoda, Hirohashi et al., 2011) and ii) shared antigens, such as CEP55 (Hirohashi, Torigoe et al., 2010), MUC1 (Engelmann, Shen et al., 2008; Weng, Song et al., 2010), or WT1 (Cheever, Allison et al., 2009; Sugiyama 2010) between CSCs and more differentiated subpopulations. Therefore, the development of strategies that target the CSC population by immunotherapy may be highly desirable. For example, DC-based cancer vaccine, $\gamma\delta$ T cells, or natural killer (NK) cells killed human cancer stem cells (Pellegatta, Poliani et al., 2006; Todaro, D'Asaro et al., 2009; Pietra, Manzini et al., 2009; Weng, Song et al., 2010), in vitro. Success of these potential therapies will depend on how well immunological responses to CSCs can be modulated for example by vaccines upregulating antigen-processing and -presentation in DCs. Recently, we used fusions of DC and CSC to activate potent CSC-specific CTL responses and resulted in expression CTLs with elevated levels of IFN- γ and enhanced killing of CSCs in vitro (Weng, Song et al., 2010). Moreover, the classification of conclusive CSC markers followed by the identification of defined T cell-recognized CSC epitopes in the future may also lead to the clinical application of anti-CSC vaccination strategies.

10. Conclusion

The prognosis of patients with pancreatic cancer remains grim, and current thinking toward the development of curative therapy is likely to require eradication of the CSC population. A combined approach of conventional therapies such as radiation or chemotherapy kill the bulk of pancreatic cancer and CTLs that target CSC and CC fraction may represent a more promising approach for the treatment of patients with advanced pancreatic cancer. Clinical evaluation is warranted to examine the effect of the combined approach earlier in the disease course and in patients with less aggressive disease.

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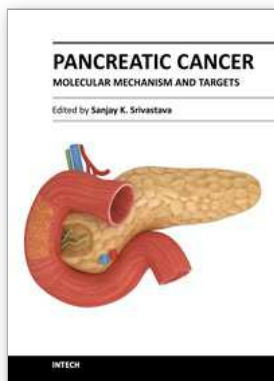
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This book provides the reader with an overall understanding of the biology of pancreatic cancer, hereditary, complex signaling pathways and alternative therapies. The book explains nutrigenomics and epigenetics mechanisms such as DNA methylation, which may explain the etiology or progression of pancreatic cancer. Book also summarizes the molecular control of oncogenic pathways such as K-Ras and KLF4. Since pancreatic cancer metastasizes to vital organs resulting in poor prognosis, special emphasis is given to the mechanism of tumor cell invasion and metastasis. Role of nitric oxide and Syk kinase in tumor metastasis is discussed in detail. Prevention strategies for pancreatic cancer are also described. The molecular mechanisms of the anti-cancer effects of curcumin, benzyl isothiocyanate and vitamin D are discussed in detail. Furthermore, this book covers the basic mechanisms of resistance of pancreatic cancer to chemotherapy drugs such as gemcitabine and 5-fluorouracil.

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