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An Overview on Immunotherapy of Pancreatic Cancer

Fabrizio Romano, Luca Degrate, Mattia Garancini, Fabio Uggeri, Gianmaria Mauri and Franco Uggeri
Department of Surgery, San Gerardo Hospital, University Of Milan Bicocca, Monza, Italy

1. Introduction

Pancreatic cancer is the fourth leading cause of cancer mortality in both men and women. Approximately 32,000 Americans each year will develop and also die from this disease . Despite aggressive surgical and medical management, the mean life expectancy is approximately 15–18 months for patients with local and regional disease, and 3–6 months for patients with metastatic disease 1-2. Even in case of radical surgery it is associated with a poor prognosis and a 5-year survival rate of less than 4%. Early detection methods are under development but do not yet exist in practice for pancreatic cancer. Therefore, most patients present with advanced disease that cannot be cured by surgery (pancreaticoduodenectomy). Clinically, pancreatic cancer is characterized by rapid tumor progression, early metastatization and unresponsiveness to most conventional treatment modalities. In a recent analysis using a database from 1973 to 2003 based on modeled period analysis, 5-year survival of pancreatic cancer patients was 7.1% and 10-year survival was below 5%3. The survival rate is apparently related to the disease stage with a low rate at 1.6–3.3% among patients with distant metastases. Curative resection remains the most important factor determining outcome for resectable tumors. However, the resection rate for pancreatic carcinoma is only 10% and the overall five-year survival rate after resection is still only 10 to 20%. Early diagnosis and effective treatment to control the advanced stages of disease may prolong the survival rate of pancreatic cancer. Otherwise pancreatic cancer remains a disease with high mortality despite numerous efforts that have been made to improve its survival rates.

In developing cancer immunotherapy, the following aims must be considered: (1) detection of immune response to autologous tumor cells, (2) identification of tumor antigens and analysis of the immune responses in patients, (3) analysis of tumor escape mechanisms and development of methods to overcome them, and (4) development of a more efficient immune intervention system by way of animal model experiments and clinical trials. Identification of tumor antigens in the first objective is important because it subsequently allows their use not only as targets for immunotherapy in a more immunogenic form but also enables quantitative and qualitative monitoring of immune responses to tumor cells during immunotherapy. In many animal tumors and in human melanoma, T cells play an

important role in in vivo tumor rejection. Because of their expression of MHC class I, CD8+ T cells are integral in the eradication of most solid tumors. However, CD4+ T cells are also important in the induction and maintenance of final effectors, such as CD8+ T cells and macrophages, as well as for the accumulation of CD8+ T cells in tumor tissues. Thus, we are applying various methods to identify human tumor antigens recognized by T cells.

Immunotherapy has an advantage over radiation therapy and chemotherapy because it can act specifically against the tumor without damaging normal tissue. Immunotherapeutic approaches to PC have included the use of monoclonal antibodies 47, cytokines 8, vaccine 9 and lymphokine activated killer (LAK) cells (10).

2. Immune surveillance and tumour evasion

The extraordinary features of the immune system make it possible to discern self from non-self. However, most human cancers, and pancreatic cancer in particular, are known to be poorly immunogenic, as crucial somatic genetic mutations can generate pancreatic cancer proteins that are essentially altered self proteins. Furthermore, promising immunotherapeutic approaches that have been used for relatively immunogenic cancers such as melanoma have met with variable success[6]. These observations have revealed that for tumours to form and progress, they must develop local and/or systemic mechanisms that subsequently allow them to escape the normal surveillance mechanisms of the intact immune system. Immune-based therapies must therefore incorporate at least one agent against a pancreatic cancer target as well as one or more agents that will modify both local and systemic mechanisms of pancreatic-cancer-induced IMMUNE TOLERANCE.

It is now clear that both local characteristics of the tumour microenvironment as well as systemic factors are important for the immune evasion of tumours. For example, T-cell recognition of pancreatic tumours might be inhibited or suppressed due to the downregulation of human leukocyte antigen (HLA) CLASS I tumour-antigen complexes on tumour cells by a range of intracellular mechanisms[4, 7] — upregulation of immune-inhibition molecules[11, 12, 13, 14, 15, 16, 17], loss of immune-regulation signals[15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30], defects in immune-cell tumour localization[31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, 51] and loss of co-stimulatory molecules[52, 53, 54, 55, 56, 57]. Such alterations within a tumour cell would not be unexpected, as they have unstable genomes. The local inflammatory reaction is also an important triggering event in the recruitment of professional ANTIGEN-PRESENTING CELLS (APCs) and effector cells, such as T cells and NATURAL KILLER (NK) CELLS, to the tumour site. However, pancreatic tumour cells express a range of proteins that inhibit pro-inflammatory cytokines and DENDRITIC CELL (DC) MATURATION[58, 59, 60].

In addition, the numbers of $CD4^+CD25^+$ T regulatory (T_{Reg}) CELLS — a subset of T cells that are known to be important in the suppression of self-reactive T cells (peripheral tolerance) — accumulate in pancreatic tumours[61, 62, 63]. Although these cells are thought to be activated during the immunization process, T_{Reg} cells seem to localize to tumour sites. Tumour production of the chemokine CCL22 probably attracts the T_{Reg} cells by interacting with the CCR4 receptor that is expressed by these cells[64].

Other important elements in regulating the T-cell recognition of pancreatic tumours are the inhibitory pathways, known as 'immunological checkpoints'. Immunological checkpoints

serve two purposes. One is to help generate and maintain self-tolerance, by eliminating T cells that are specific for self-antigens. The other is to restrain the amplitude of normal T-cell responses so that they do not 'overshoot' in their natural response to foreign pathogens. The prototypical immunological checkpoint is mediated by the cytotoxic-T-lymphocyte-associated protein 4 (CTLA4) counter-regulatory receptor that is expressed by T cells when they become activated[15, 23]. CTLA4 binds two B7-FAMILY members on the surface APCs — B7.1 (also known as CD80) and B7.2 (also known as CD86) — with roughly 20-fold higher affinity than the T-cell surface protein CD28 binds these molecules. CD28 is a co-stimulatory receptor that is constitutively expressed on naive T cells. Because of its higher affinity, CTLA4 out-competes CD28 for B7.1/B7.2 binding, resulting in the downmodulation of T-cell responses[20].

A range of B7-family members interact with co-stimulatory and counter-regulatory inhibitory receptors on T cells. Two recently discovered B7-family members, B7-H1 (also known as PD-L1) and B7-DC (also known as PD-L2) also seem to interact with T-cell co-stimulatory and counter-regulatory inhibitory receptors[18, 29, 30]. PD-L1, which is upregulated on T cells when they become activated, seems to control a counter-regulatory immunological checkpoint when it binds PD-1 26,28,29. Activating receptors for B7-DC and B7-H1 have not yet been definitively identified. B7-DC is expressed on DCs, and is likely to have a co-stimulatory role in increasing activation of naive or resting T cells. In contrast to B7.1, B7.2 and B7-DC, B7-H1 is also expressed on several peripheral tissues and on many tumours, including pancreatic tumours[30].

Another new B7-family member, B7-H4, seems to mediate a predominantly inhibitory function in the immune system[14]. Recent data indicate that pancreatic tumours also express B7-H4 (D.L. and E.M.J., manuscript in preparation), and both B7-H1 and B7-H4 probably protect tumours from immune-system attack. Preclinical studies have already demonstrated that it is possible to downregulate B7-H1 signalling in mice, improving the antitumour response to vaccination[18]. Monoclonal antibodies that downregulate B7-H1 and B7-H4 are currently in clinical development. These antibodies will probably begin clinical testing in patients with pancreatic cancer within 2 to 3 years.

3. Cancer immunotherapy protocols

Clinical trials using various immunotherapies, active immunization with tumor antigens, or tumor cell–derived products, and adoptive immunotherapy using antitumor immune cells were conducted in various cancers, most extensively in melanoma, and tumor regression was observed in some patients. Active Immunization Immunizations with synthetic peptides, particularly MHC class I–binding epitopes, were performed in various trials. Since native epitopes have relatively low immunogenicity, various immunoaugmenting methods, including coadministration of adjuvants and cytokines [incomplete Freund adjuvant (IFA), IL-2, IL-12, or GM-CSF], were applied to achieve efficient immunization. Tumor regression in melanoma patients was observed in various clinical trials using melanocytespecific antigens such as MART-1 and gp100 and, in particular, the HLA high-binding modified peptide. Since CD4+ T cells appear to be directly and indirectly important in tumor rejection, combined immunization with both Th and CTL antigens is being attempted. Immunization with proteins containing multiple Th and CTL epitopes may be effective,

although production of recombinant GMP-grade proteins is costly, and modifications such as particle formation may be required for effective presentation of MHC class I–restricted epitopes. To facilitate peptide immunization in melanoma, coadministration of the anti-CTLA4 antibody, which blocks regulatory T cells and negative feedback regulation of T-cell activation, was carried out. Although tumor regression along with autoimmune reactions was observed, augmentation of the immune response to the administered peptides was not observed in peripheral blood.[24] In pancreatic cancer, intradermal immunization with the mutated K-*ras* peptides and GM-CSF resulted in the induction of a memory CD4+ T-cell response and prolonged survival, compared with nonresponders.[15] Immunization with the MUC1 peptide and BCG resulted in augmented immune responses without tumor regression.[22] Immunization with recombinant viruses or plasmids containing tumor antigen cDNA (DNA immunization) rather than peptide/proteins may be applied. In melanoma clinical trials, a generation of neutralizing antibodies against viral proteins appeared to interfere with the induction of immune response to tumor antigens following immunization with recombinant adenovirus and vaccinia virus.[25] However, recent protocols using a recombinant fowlpox virus containing the modified gp100 cDNA or the ER signal sequence–conjugated gp100-epitope minimal gene demonstrated frequent induction of tumor reactive T cells.[26] Interestingly, tumor regression was observed in patients after subsequent administration of IL-2.

Intramuscular immunization with the recombinant gp100 plasmids appeared to be insufficient to induce an antitumor T-cell response.27 DC are the most potent professional APC that can process antigens for both MHC class I and II pathways and activate both naive CD4+ T cells and CD8+T cells in vivo. In murine studies, immunization with DC pulsed with tumor antigens resulted in better antitumor effects than direct peptide administration. In immunization trials using DC pulsed with tumor lysates or synthetic peptides, tumor regression was observed in patients with various cancers, including melanoma, prostate cancer, colon cancer, and B-cell lymphoma.[28] Although most clinical trials have used monocyte-derived DC, peripheral blood DC as well as CD34+ cell–derived DC have been used in some protocols.[29] Antigen loading on DC using various antigens including RNA, cDNA, recombinant virusand cell-penetrating peptide conjugated proteins has also been exploited. DC fused with tumor cells and leukemia clone– derived DC have also been used in clinical trials. K-ras– specific T cells were detected in pancreatic cancer patients following multiple intravenous infusions of peptide-pulsed antigen presenting mononuclear cells obtained by leukapheresis, although no therapeutic effect in patients was observed. In addition, no tumor regression was observed following immunization with DC transfected with MUC1 cDNA. A decrease in tumor marker was observed in a patient with a pancreatic neuroendocrine tumor, following immunization with DC pulsed with autologous tumor lysates. Intratumoral administration of immature DC following intraoperative irradiation is currently being conducted in Japan. Thus far, any antitumor effects observed in these DC-based clinical trials for pancreatic cancer are weak. Protocols for the optimal use of DC in immunotherapy, including the source of DC, kinds of tumor antigens, methods for maturation and antigen loading, site and schedule for administration, remain to be determined. Based on murine experiments, immunization with more immunogenic tumor cells that are modified using various techniques, including hapten conjugation, foreign antigen introduction, and transfection with various genes such as cytokines (eg, GM-CSF, IL-2, TNF-_, IFN-_, IL-4) have been employed in melanoma, prostate cancer, and lung

cancer. Strong antitumor effects, however, were not observed in the reported clinical trials. In pancreatic cancer, vaccination with GM-CSF transduced allogeneic pancreatic cancer cell lines along with adjuvant radiation and chemotherapy following surgical excision demonstrated possible benefit in disease-free survival, which appeared to be associated with the increase of postvaccination DTH responses against autologous tumor cells.

4. Adoptive Immunotherapy with antitumor

4.1 Immune cells

Passive immunotherapy with large doses of activated antitumor lymphocytes was also employed since there was a possibility that active immunization would be insufficient to induce enough of an immune response to cause tumor regression in the immunosuppressed patient with a large tumor burden. Adoptive transfer of tumor-reactive T cells cultured from tumor-infiltrating lymphocytes, along with IL-2, resulted in a clinical response in melanoma patients.[65] Adoptive transfer of EBV-specific T cells resulted in regression of EBV-associated lymphoma. Intraportal infusion of in vitro MUC1-stimulated T cells was performed in pancreatic cancer, yielding preliminary results that indicate inhibition of liver metastasis. Although the clinical use of tumor-reactive T cells was previously limited due to the difficulty in generating tumor-reactive T cells for most cancers, it is now possible to generate these cells from the PBMC of cancer patients by in vitro stimulation, using the identified tumor antigens.[66] Tumor-reactive T cells from patients preimmunized with tumor antigens were generated more efficiently, which suggests that combined use of active and passive immunotherapies is ideal. One of the problems that arises from adoptive transfer of cultured T cells is the low efficiency of administered T cells in in vivo maintenance and accumulation in tumor tissues. However, it was recently reported that nonmyeloablative, lymphodepletive pre-treatment with cyclophosphamide and fludarabine resulted in extended persistence of administered tumor-reactive T cells in peripheral blood and tumor tissues and increased tumor regression, which may be due to suppression of patient immune responses or the need to make room for homeostatic proliferation of transferred lymphocytes.[67] Adoptive immunotherapy with IL- 2–activated PBMC, LAK (lymphokine activated killer) cells displayed some antitumor effects when locally administered (ie, by intrapleural or intraarterial infusion) for lung or liver cancer. Intraportal administration following intraoperative irradiation in pancreatic cancer patients is reported to result in possible prolongation of survival[68].

Adoptive immunotherapy involves harvesting the patient's peripheral blood T-lymphocytes, stimulating and expanding the autologous tumour-reactive T-cells using IL-2 and CD3-specific antibody, before subsequently transferring them back into the patient. Twelve patients with advanced pancreatic cancer who underwent resection, intraoperative radiotherapy and intraportal infusion of LAK cells with recombinant IL-2 had lower incidence of liver metastasis compared to controls (three of 12 vs ten of 15; $p<0.05$)69. There was no significant difference in overall survival, but more patients were alive three years later (36% vs none).

Telomerase— Telomerase is a reverse transcriptase that contains a RNA template used to synthesise telomeric repeats onto chromosomal ends. Activation of telomerase and its maintenance of telomeres play a role in immortalisation of human cancer cells, as telomeres

shrink after each cell division [70]. Telomerase activity is found in 92-95% of pancreatic cancers [71-72], and is associated with increased potential of invasion and metastasis and poor prognosis [73-74]. Upregulation of telomerase may also be responsible for the development of chemotherapy resistance [75]. Adenovirus-mediated transduction of p53 gene inhibited telomerase activity in MIAPaCa-2, SUIT-2 and AsPC-1 cells, independent of its effect on apoptosis, cell growth and cycle arrest [76]. Antisense to the RNA component of telomerase seemed to increase susceptibility of Panc-1 cells to cisplatin [77]. Telomerase reverse transcriptase antisense oligonucleotide (hTERT-ASO) was found to inhibit the proliferation of BxPC-3 cells *in vitro* by decreasing telomerase activity and increasing apoptosis [78]. Adoptive transfer of telomerase-specific T-cells was studied in a syngeneic pancreatic tumour mouse model [79]. T-cells were produced *in vitro* by coculturing human lymphocytes with telomerase peptide-pulsed dendritic cells (DCs) or *in vivo* by injection of peptide with adjuvant into C57BL/6 mice. Animals treated with these T-cells showed significantly delayed disease progression.

MUC1— Adoptive transfer of MUC1-specific cytotoxic T-lymphocytes (CTLs) was able to completely eradicate MUC1-expressing tumours in mice [80]. Intraportal infusion of *In vitro* MUC1-stimulated T-cells was performed in patients with pancreatic cancer, with subsequent inhibition of liver metastasis [81]. In a study of eleven patients with lung metastases (from colorectal, pancreatic, breast, lung, or melanoma primaries), effector cells were generated *in vitro* using cultured DCs, synthetic peptide, peripheral blood lymphocytes, IL-2 and anti-CD3 antibody [82]. A partial response of the lung metastases was observed in a patient with pancreatic cancer who received these cells stimulated with MUC1.

4.2 Cytokines and immunomodulators

TNFerade— TNF-α is a multifunctional cytokine that has shown antitumour potency [83-85]. TNFerade Biologic (TNFerade) is a replication-deficient adenovirus carrying the gene for human TNF-α, regulated by a radiation-inducible promoter Early Growth Response (Egr-1). The latter would ensure maximal gene expression when infected tissue is irradiated 86. TNFerade was effective in combination with radiation in a number of human xenograft models, including glioma [87], prostate [88], oesophageal [89] and radiation-resistant laryngeal cancers [90]. The multicentre phase II/III Pancreatic Cancer Clinical Trial with TNFerade (PACT) is currently ongoing and involved patients with locally advanced pancreatic cancer. Patients were given radiotherapy, 5-FU with or without CT-guided transabdominal injection of TNFerade. Preliminary data of 51 patients revealed that the one-year survival increased from 28% to 70.5% with the addition of TNFerade, with MS of 335 and 515 days respectively[91].

Virulizin— Virulizin (Lorus Therapeutics Inc.) is a biological response modifier obtained from bovine bile [92]. It stimulates the expression of TNF-α and activates macrophages, which subsequently activates natural killer cells via IL-12 [93-94]. Evidence exists to show that it also induces the production of IL-17E with resulting eosinophilia [95].

In vivo studies showed that Virulizin significantly inhibited the growth of human pancreatic cancer xenografts (BxPC-3, SU [86.86] and MIAPaCa-2) in nude mice, as well as potentiated the antitumour effect of gemcitabine and 5-FU [96-97]. A phase III trial was conducted to study the

effect of gemcitabine with or without Virulizin in 434 chemotherapy-naïve patients with advanced pancreatic cancer [341]. MS was not significantly better for the gemcitabine and Virulizin group compared to gemcitabine with placebo (6.3 vs 6 months). However for stage 3 patients who received Virulizin in a salvage setting, a significant difference in survival was demonstrated (10.9 vs 7.4 months, p=0.017).

4.3 IL-2

Pancreatic cancer could thus constitute a paradigmatic example of neoplasia where tumor-related variables and host immunosuppressive status have the same importance in determining an unfavourable prognosis. The severe suppression of anticancer immunity, which characterizes patients suffering from pancreatic cancer, is further aggravated by surgical treatment [98]. In fact, it is known that surgery may inhibit anticancer immunity by provoking a postoperative decline in the absolute number of circulating lymphocytes [99-101], which play a fundamental role in generating an effective anticancer immune reaction; this is fundamentally an IL-2-dependent phenomenon [102].

Surgery-induced immunosuppression could represent one of the main factors responsible for relapse in cancer patients treated by radical surgery, by possibly promoting the growth of micro-metastases, already existing at the time of the surgical removal of the tumor . Previous clinical studies have shown that the immunosuppressive status occurring during the postoperative period is particularly severe in patients with pancreatic cancer and this evidence could explain, at least in part, the high percentage of recurrences occurring in patients radically operated for cancer of the pancreas [103]. At present, the only molecule which has been proven to correct the lymphocytopenia is IL-2, representing the main growth factor for lymphocytes, including T lymphocytes and natural killer (NK) cells [104] and the stimulation of lymphocyte proliferation would constitute the main mechanism responsible for the antitumor activity of IL-2 in the immunotherapy of cancer [105]. Moreover, the preoperative administration of IL-2 for only few days prior to surgery was effective in preventing surgery-induced lymphocytopenia [106]. In addition, the abrogation of surgery-induced lymphocyte decline has been shown to improve the prognosis of patients with colorectal cancer in whether treated by radical or palliative surgery [107]. The therapeutic impact of IL-2 presurgical administration remains to be better defined in gastric cancer [108], despite its efficacy in preventing the postoperative lymphocytopenia. Finally, the prevention of postoperative lymphocyte decline by IL-2 presurgical immunotherapy was associated with clear lymphocyte and eosinophil intratumoral infiltration in colorectal cancer patients, which, in contrast, was less evident in patients with gastric carcinoma. Preliminary clinical studies have suggested that preoperative injection of IL-2 may also prevent surgery-induced lymphocytopenia in patients with pancreatic cancer [109]. According to previous investigations, IL-2 presurgical immunotherapy may also completely abrogate surgery-induced lymphocytopenia also patients with pancreatic carcinoma, as well as previously described for both colorectal and gastric carcinomas. Moreover, in agreement with the clinical results previously reported for colorectal cancer patients and in contrast to those more controversially reported in gastric cancer, this study would suggest that a preoperative immunotherapy with IL-2 may improve the clinical course of the pancreatic cancer in terms of both FFPP and OS. Therefore, particularly because of its unfavourable prognosis, presurgical immunotherapy with IL-2 could represent a simple but effective

clinical strategy to improve the prognosis of pancreatic cancer patients undergoing macroscopical radical surgery.

4.4 Allogeneic antigen-specific immunotherapy

Allogeneic antigen-specific immunotherapies, nonmyeloablative SCT (minitransplant) and DLI (donor leukocyte infusion), are reported to have some antitumor effect [graft versus tumor (GVT)] on solid tumors, including RCC, breast cancer, and pancreatic cancer, in addition to haematological malignancies.[110]GVT effects were also observed in pancreatic cancer patients in minitransplant protocols conducted in Japan. Although the mechanisms of the antitumor effects, such as allogeneic responses to minor histocompatibility antigens (mHa), on hematological malignancies are well studied, they remain unclear with regard to solid tumors. One of the major problems in allogeneic treatment of the solid tumor is severe GVHD. Several strategies for the separation of GVT and GVHD have been developed for hematological malignancies. Whether this separation is possible for solid tumors, however, is unclear.

Was reported on the efficacy of adoptive immunotherapy (AIT) with cytotoxic T lymphocytes (CTLs), induced from autologous pancreatic tumors but not from AIT with LAK cells. Although these immunotherapies have a potential as alternative treatments for PC, the effects have been limited.

Pancreatic cancer cells present an enormous challenge, as they are naturally resistant to current chemotherapy and radiation therapy. In addition, known pancreatic cancer antigens have generated relatively weak immune responses. This is probably due to a combination of mutations in oncogenes such as *KRAS* and tumour-suppressor genes such as *TP53*, *CDKN2A*, *DPC4* (deleted in pancreas cancer 4), *BRCA2* and *ERBB2* (also known as HER2/neu), as well as overexpression of growth factors such as transforming growth factor-α (TGFα), interleukin-1 (IL-1), IL-6 and IL-8, tumour-necrosis factor-a (TNFα), or vascular endothelial growth factor (VEGF), their receptors, or constitutive expression of multidrug-resistant genes[2, 3, 4, 5]. Alternative therapeutic approaches are therefore urgently needed for this disease.

Immune-based therapies aim to recruit and activate T cells that recognize tumour-specific antigens. In addition, recombinant monoclonal antibodies are being designed to target tumour-specific antigens — these would kill tumour cells either by direct lysis or through delivery of a conjugated cytotoxic agent. Both approaches are attractive for the treatment of pancreatic cancer for several reasons. First, these immune-based therapies act through a mechanism that is distinct from chemotherapy or radiation therapy, and represent a non-cross-resistant treatment with an entirely different spectrum of toxicities. Second, through the genetic recombination of their respective receptors, the B cells and T cells of the immune system are capable of recognizing a diverse array of potential tumour antigens. In addition, both T and B cells can distinguish small antigenic differences between normal and transformed cells, providing specificity while minimizing toxicity. New insights into the mechanisms by which T cells are successfully activated and by which tumours evade immune recognition are driving the development of new combinatorial immunotherapy approaches. In addition, recent advances in gene-expression analysis have allowed for the identification of new pancreatic targets, including candidate tumour antigens that might

serve as T-cell and antibody targets. These advances now make it possible to exploit the immune system in the fight against pancreatic cancer.

4.5 Targeting signalling molecules

By the time that patients are diagnosed with pancreatic cancer, the tumour has typically progressed and invaded adjacent structures. Perineural invasion, metastasis to lymph nodes and liver, and an intense DESMOPLASTIC STROMAL REACTION are commonly observed. A range of signalling pathways, including epidermal growth factor receptor (EGFR) and the PI3K–AKT–mTOR–S6K cascades, are known to mediate pancreatic tumour growth and progression[111]n addition, new blood-vessel formation (angiogenesis) is required for the growth of primary pancreatic tumours and is essential for metastasis. In pancreatic tumours, this process is probably regulated by fibroblast growth factor, platelet-derived endothelial-cell growth factor and VEGF family members. In fact, several pancreatic-cancer-associated genes have been linked to angiogenesis. DPC4 upregulates VEGF expression, and mutated KRAS expression is associated with increased micro-vessel density[112].

Monoclonal antibodies that target a range of these pathways have demonstrated efficacy in preclinical models[113-115]dition, monoclonal antibodies that target EGFR and VEGF receptor have been tested in patients with a range of cancers, including pancreatic cance[115,117]hough these antibodies have demonstrated only modest results as single agents, the pathways they affect are also candidate targets for immune intervention.

Preclinical evidence has also shown that specific inhibitors of these signalling pathways can also increase immune activation. For example, VEGF is a key inhibitor of pro-inflammatory cytokines as well as dendritic-cell maturation, and it can also directly inhibit T-cell development. So antibodies that block signalling by this growth factor can promote antitumour immune responses. Furthermore, downregulation of the ERBB-receptor-family members with drugs such as herceptin promotes tumour-antigen presentation by HLA class I molecules, improving the potential for T-cell recognition and lysis[118]onoclonal antibodies that target these signalling pathways are now being developed for clinical trials as agents that potentially synergize with other immune-based approaches, including vaccines.

4.6 Vaccines against pancreatic tumour antigens

To develop the ideal vaccine for pancreatic cancer, the following wish list would probably need to be fulfilled. First, specific cell-surface proteins must be identified that are that are crucial in the cancer growth or progression pathway and are unique to pancreatic cancer tumours. Second, these tumour-exclusive proteins should be shown to elicit a vigorous tumour-protein-specific immune response. Third, the best carrier to deliver the appropriate immunogenic tumour proteins should be identified. Fourth, molecules that are immune stimulatory as well as molecules that can abrogate the natural immune-inhibition signalling that is seen in pancreatic cancer should be identified to enhance the immune response. Fifth, additional synergistic immune help should be identified (for example, antibodies or *ex vivo* tumour-reactive T cells). Several proteins, such as carcinoembryonic antigen (CEA), mutated KRAS, mucin-1 (MUC1) and gastrin, have in fact been identified to be specifically overexpressed in most pancreatic cancers[119-125] antigens were identified over 10 years ago using various methods to analyse gene expression in cancer cells. Vaccines and antibodies

designed to target these antigens have been tested in early-phase clinical trials[126-131]hese antigens are known to have weak inherent immune potential, various immune-modulating agents were co-administered, including granulocyte–macrophage colony-stimulating factor (GM-CSF), and interleukin-2 (IL-2). So far, a few studies have demonstrated post-vaccination immune responses to the relevant peptides or whole proteins. Significant clinical responses have not yet been observed. This might be due to the lack of pooling of the right antigens, to the existence of host mechanisms of immune tolerance, the inability of the relevant immune cells to effectively localize to the sites of disease, or a combination of these factors.

Vaccination involves administering an antigen that is unique for a particular type of tumour with the aim of stimulating tumour-specific immunity. Antigens could be delivered in the form of DNA or peptide, as well as tumour cells or antigen-pulsed DCs. Additional synergistic help is added to elicit a more vigorous and effective immune response, such as cytokines and immunostimulating adjuvants.

Whole-Cell—GM-CSF is one of a few cytokines that has shown significant antitumour effect *in vivo* [342]. It is an important growth factor for granulocytes and monocytes, and has a crucial role in the growth and differentiation of DCs, the most potent antigen-presenting cells (APCs) for triggering immune response. *In vivo* growth of AsPC-1 cells, retrovirally transduced with the GM-CSF gene, was inhibited and associated with increased survival of the nude mice, even in the mature T-cell-deficient condition 132. Jaffee et al. conducted a phase I study using allogeneic GM-CSF-secreting whole-cell tumour vaccine for pancreatic cancer 133. This is based on the concept that the localisation of GMCSF in the implanted tumour environment together with the shared tumour antigen expressed by the primary cancer would effectively induce an antitumour immune response. In this study two pancreatic cancer cell lines (PANC 10.05 and PANC 6.03) were used as the vaccine, both genetically modified to express GM-CSF. 14 pancreatic cancer patients who had undergone pancreaticoduodenectomy eight weeks prior were given variable doses of the vaccine intradermally. Three of the eight patients who received ≥10 × 107 vaccine cells developed postvaccination delayed-type hypersensitivity (DTH) responses associated with increased disease free survival time, and remained disease-free for longer than 25 months after diagnosis. Side effects were mainly limited to local skin reactions at the site of vaccination. In a recently completed phase II study of 60 patients with resected pancreatic adenocarcinoma, patients received five treatments of 2.5 × 108 vaccine cells, together with 5-FU and radiotherapy134. The reported MS was 26 months, with a one- and two-year survival of 88% and 76% respectively.

4.7 Peptide and DNA

- **Ras:** As described earlier, mutated ras is highly prevalent in pancreatic cancer. A phase II study was done using mutant ras peptide-based subcutaneous vaccine in 12 cancer patients (five with fully resected pancreatic and seven with colorectal cancers). Five out of 11 patients showed showed ≥1.5 fold increase in interferon-γ (IFN-γ) mRNA copies in peripheral blood mononuclear cells. The pancreatic cancer patients showed a disease-free survival of >35.2 months and post-vaccination survival of >44.4 months 135. Gjertsen et al tested an intradermal vaccine of APCs loaded *ex vivo* with synthetic ras peptide corresponding to the ras mutation found in the patient's tumour 136. In this

phase I/II study of five patients with advanced pancreatic cancer, two of them showed induced immune response. They also studied ras peptide in combination with GM-CSF in a phase I/II trial involving 48 patients with pancreatic adenocarcinoma of variable stage 137. Peptide-specific immunity was induced in 58% of patients. Of patients with advanced disease, those who responded to treatment showed increased survival compared to non-responders (148 and 61 days respectively; p=0.0002).

As IL-2 is involved in T-cell-mediated immune response, a vaccine consisting of mutant ras peptide in combination with GM-CSF and IL-2 was tested in a phase II trial of 17 patients with advanced cancers (14 colorectal, one non-small cell lung and two pancreatic cancers) 138. Of the six patients with positive immune response (by means of IFN-γ mRNA copies), the MS and the median PFS were 39.9 and 17.9 months compared to 18.5 and 15.6 months for nonresponders, respectively. Grade III toxicities led to IL-2 dose reduction in three of the patients.

- **CEA and MUC1:** Carcinoembryonic antigen (CEA) glycoprotein is expressed at a low level in normal colonic epithelium but is overexpressed in many malignant diseases, including those of the colon, rectum, stomach and pancreas (85-90%) 139. Its serum level is sometimes used as a marker for the diagnosis of pancreatic cancer, with a sensitivity of 25-40% and a specificity of 70-90% 140-141.

To boost MUC1-specific immune response, a vaccine composed of MUC1 peptide and SBAS2 adjuvant was tested in a phase I study 142. There was an increase in the percentage of CD8+ T-cells and MUC1-specific antibody (some developed IgG). Hope for the CEA or MUC1 vaccine was nevertheless crushed when a phase III trial of 255 patients using PANVAC-VF (vaccine consisted of recombinant vaccinia and fowlpox viruses coexpressing CEA, MUC-1 and TRICOM) failed to improve overall survival compared to palliative chemotherapy or best supportive care.

- **Gastrin:** G17DT (Gastrimmune or Insegia) is an immunoconjugate of the amino-terminal sequence of gastrin-17 (G-17) linked by means of a spacer peptide to diphtheria toxoid. Given intramuscularly it induces the formation of antibodies that can neutralise both amidated-G-17 and the precursor glycine-extended G17 143. In a phase II study of 30 patients, 67% mounted an antibody response. A significantly higher response (82%) was achieved in those given the highest dose of 250μg compared to 46% in the 100μg group. MS was significantly higher (217 days) for the antibody responders compared to non-responders (121 days; p=0.0023).

When used as a monotherapy for patients with advanced pancreatic cancer unwilling or unsuitable to take chemotherapy, MS was 151 compared to 82 days in the placebo group (p=0.03) [360]. G17DT was subsequently tested in a phase III trial with or without gemcitabine in 383 untreated patients with locally advanced, recurrent or metastatic pancreatic adenocarcinoma. This unfortunately showed that the addition of G17DT did not improve overall survival or secondary endpoints Increasing -17 antibody titre levels in a subset of patients, however, were associated with increased survival.

- **Mesothelin:** Thomas and colleagues provided the first direct evidence, by using mesothelin epitopes, that pancreatic cancer-specific CD8+ T-cell response can be generated via crosspresentation by an approach that recruits APCs to the vaccination

site 144. Gaffney et al studied the mesothelin DNA vaccine in combination with the anti-glucocorticoid-induced TNF receptor antibody (anti-GITR) in mice with syngeneic mesothelin-expressing pancreatic cancer 145. 50% of animals treated with mesothelin were tumour-free 25 days after tumour injection compared to 0% of non-treated mice. This increased to 94% with the addition of anti-GITR. The agonist anti-GITR served to enhance T-cell-mediated response of the vaccine 146-147.

- **Telomerase:** The telomerase peptide vaccine GV1001 was tested in a phase I/II study of 48 patients with unresectable pancreatic cancer 148. They received intradermal injection in combination with GM-CSF. Immune responses, as measured by DTH skin reaction and T-cell proliferation *in vitro*, were demonstrated in 24 of 38 evaluable patients, with the highest percentage (75%) in the intermediate dose group. MS for this group was significantly longer at 8.6 months, and one-year survival was 25%. GV1001 was given to patients in a phase I trial using imiquimod as an adjuvant149. Imiquimod acts by binding to Toll-like receptor 7 on immune cells, resulting in the production of cytokines such as IFN-α, IFN-β and IL-12. Immune response was found in up to six (46%) of 13 evaluable patients.
- **Survivin:** Survivin-specific CTLs were isolated from pancreatic cancer patients and these could lyse pancreatic carcinoma cell lines *in vitro* 150. Vaccination with survivin DNA prolonged survival in murine pancreatic and lymphoma tumour models, associated with slower tumour growth and increased lymphocyte infiltration . Survivin peptide was tested in a patient with gemcitabine refractory pancreatic cancer . Whilst on treatment he had complete remission of liver metastases after six months. However when he was weaned from the vaccination he developed recurrent disease. Vaccine-induced immune activity was detected by IFN-γ enzyme-linked immunospot (ELISPOT) assay.

Antigen-pulsed DCs— Antigen-specific T-cell responses are initiated by DCs. They capture antigens secreted or shed by tumour cells and present peptides in association with the MHC class I and II molecules. This results in the expression and upregulation of cytokines and costimulatory molecules which in turn stimulate CD4+ and CD8+ T-cells to mount an antitumour response. As such DCs that carry the tumour antigen of interest is an ideal adjuvant in cancer immunotherapy.

- **MUC1:** In a phase I/II trial, human autologous DCs transfected with MUC1 cDNA were used as a vaccine for ten patients with advanced breast, pancreatic or papillary cancer 151. Four patients showed a two- to ten-fold increase in the frequency of mucin-specific IFN-γ-secreting CD8+ T-cells, suggesting an immune response. In a phase 1b study, eight patients with pancreatic or biliary tumours were vaccinated with DCs pulsed with MUC1 152.

As discussed previously, monoclonal antibodies have so far been the most successful form of immunotherapy clinically. They are being used as diagnostic tools, prognostic indicators, and for the treatment of many cancers. Advantages include their specific targeting of tumour cells while sparing normal tissue, their relative ease of administration, and their low toxicity profile. The major disadvantages include the absence of T-cell activation, which therefore precludes T-cell-mediated cytotoxic killing and the generation of memory immune responses. In addition, a potential limiting factor in its use involves tumour heterogeneity. Specifically, all tumour cells within a proliferating mass might not express the antigen that is

being targeted. Inhibitors to EGFR and to VEGF have been tested in combination with gemcitabine and are currently in Phase III trials either with other approaches have used dendritic cells as the carrier of the antigen of interest. To date, CEA and MUC1 antigens have been among the initial antigens tested, with mixed results153-154 se of adoptively transferred pancreatic-cancer-specific T cells has been proposed to be another opportunity to augment the immune response. Although this strategy has been promising preclinically, and has been used with some success in melanoma, there have not been any clinical trials in pancreatic cancer so far.

A current limitation to the development of vaccines for pancreatic cancer has been the inability to correlate *in vitro* measures of antitumour immunity with *in vivo* responses. Post-vaccination DTH responses to autologous tumour are a potential useful surrogate, but this approach is not ideal. At present, it is technically challenging to produce sufficient quantity and purity of autologous tumour material for testing, as tumours vary in their composition of tumour cells versus other cell types between patients. Although other biological end points, such as an antibody response or *in vitro* CYTOLYTIC T LYMPHOCYTE (CTL) ASSAY against a vaccine-delivered tumour antigen (or antigens), have been measured and provide important 'proof of concept' data, these end points have also not been demonstrated to be predictors of traditional clinical end points, including tumour response and survival benefit.

It is difficult to assess whether the lack of improved survival after immunotherapy is due to inefficient antigen delivery, which could result in ineffective immunization, inappropriate selection of antigen targets, or both. As discussed above, there are formidable barriers to inducing an antitumour immune response, even when the vaccine itself is potent enough to reduce significant cancer burdens in more immunogenic tumour systems. Effective immunization will therefore require the targeting of relevant pancreatic tumour antigens using optimized antigen-delivery systems with immune-stimulating cytokines, in sequence with other therapeutic interventions that alter immune checkpoints in the tumour microenvironment, such as inhibitors to regulatory molecules on T cells (for example, antibody to CD152/CTLA4).

5. New immunotherapy targets

The inability of previously tested antigens (including CEA, KRAS, MUC1 and gastrin) to induce immune-specific responses underscores the challenge to identify more relevant immunogenic targets. Indeed, these antigens were chosen only because they were overexpressed or had altered expression in pancreatic tumours, and not because they had been shown to be immunogenic. Therefore, there might be additional as-yet-unidentified antigens that might be more immunogenic for inducing effective immunity against pancreatic cancers. How will such new candidate pancreatic cancer antigens be discovered? Two methods are routinely used in an attempt to identify new targets. The first method, serological analysis of recombinant tumour cDNA expression libraries (SEREX), uses serum to screen phage-display libraries prepared from tumour cells to identify candidate antigen targets that have elicited both humoral and cell-mediated immune responses in cancer patients. This method has identified coactosin-like protein (an actin-filament-binding protein that interacts directly with 5-liopoxygenase and has an important role in cellular leukotriene synthesis) as a potential pancreatic cancer target antigen. This protein seems to be recognized by antibody and T-cell responses in patients with pancreatic cancer[155].

The second method uses tumour-specific T cells that have been isolated from patients with pancreatic cancer to screen cDNA libraries prepared from autologous tumour cells. This method requires the isolation and culture of tumour-specific T cells, along with tumour cells, from patients with pancreatic cancer and is a technically challenging approach. This approach has been most successful in identifying melanoma-associated antigens[156].

A relatively newer, more promising method of tumour-antigen identification is the use of the patient's lymphocytes to evaluate proteins that are found to be differentially expressed by pancreatic cancer[157-158] approach has several advantages. First, it allows for a rapid screen of a large number of candidate antigens but requires the isolation from patients of only a few lymphocytes, which are limited in availability. Second, this approach is not dependent on the availability of autologous tumour cells, which are difficult to isolate in large enough numbers for generating cDNA libraries. Third, this approach can be used to identify tumour antigens that are expressed by any HLA type, allowing for the generalization of this approach to most patients. Finally, this approach has the potential to rapidly identify 'immune relevant' antigens, as it uses immunized lymphocytes from patients vaccinated with a whole-tumour-cell vaccine approach who ideally have demonstrated clinical evidence of immune activation following vaccination. So this method provides the best insurance that the antigens identified are ones that the patient's immune system is reacting to after immunization.

As additional 'immune relevant' pancreatic tumour antigens are identified, the next significant challenge lies in developing strategies to improve the *in vivo* delivery of these antigens to APCs and thereby allow effective antigen processing and presentation, and subsequent activation of a potent antitumour immune response. DCs are now accepted as the most efficient APCs in B- and T-cell activation. Several clinical trials have tested *ex vivo* expanded and primed DCs as a vaccine approach. However, these studies have revealed the difficulty in reliably producing phenotypically mature DCs for clinical testing, as only mature DCs are capable of efficiently presenting antigens to T cells. If an antigen is not presented in the proper context by mature DCs, immune downregulation or tolerance can occur. It has been shown in animal models that immature DCs induce T-cell tolerance. As an alternative to DC-based delivery, recombinant viral- and bacterial-vector delivery systems are currently under development or are already undergoing clinical testing. The use of modified viral particles or targeted bacteria to deliver tumour antigens to the immune system is based on the innate ability of the agent to efficiently infect APCs *in vivo*. Early approaches have included viruses such as vaccinia [159,160] er, the use of immunogenic vectors in cancer patients who have been previously exposed to a similar vector often induces vigorous immune responses against the vector before effective priming against the tumour antigen can occur. As such, other viral particles and bacterial delivery systems are currently nearing or are already undergoing clinical development for the treatment of pancreatic cancer.

6. Future directions

The limitations of currently available therapy for pancreatic cancer are more clearly exposed as we begin to appreciate the molecular changes behind the complex transformation of normal pancreatic ductal cells into frank pancreatic cancers, and the mechanisms of pancreatic cancer resistance to traditional anticancer modalities. It is clear that the most

effective therapy will require a combined approach incorporating the best targeted interventions taken from each respective modality. Preclinical models have already revealed the synergy between immunotherapy and other targeted therapeutics, such as inhibitors of VEGF and EGF signalling. These combinations are about to be tested in patients with pancreatic cancer.

Pancreatic cancer remains one of the most resistant cancers to traditional forms of therapy. Until techniques for early detection can be developed, most patients will continue to present with incurable disease. The pancreatic cancer research community is committed to developing new therapies for this disease. Pancreatic cancer patients and their families, through a number of national pancreatic cancer non-profit organizations such as Pancreas Cancer Action Network have organized to support this effort. It is crucial that we move forward with scientifically driven innovative therapies, as the empirical approaches have failed. Recent developments in the design of mouse models that recapitulate early pre-invasive genetic changes in *KRAS* activation, inactivation of *CDKN2A*, *TP53* and *SMAD4* tumour-suppressor genes should provide the opportunity to test such approaches in a timely manner[161,162].

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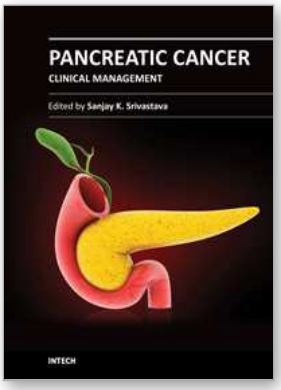

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This book covers pancreatic cancer risk factors, treatment and clinical procedures. It provides an outline of pancreatic cancer genetic risk factors, biomarkers and systems biology for the better understanding of disease. As pancreatic cancer suffers from lack of early diagnosis or prognosis markers, this book encompasses stem cell and genetic makers to identify the disease in early stages. The book uncovers the rationale and effectiveness of monotherapy and combination therapy in combating the devastating disease. As immunotherapy is emerging as an attractive approach to cease pancreatic cancer progression, the present book covers various aspects of immunotherapy including innate, adaptive, active, passive and bacterial approaches. Management of anesthesia during surgery and pain after surgery has been discussed. Book also takes the reader through the role of endoscopy and fine needle guided biopsies in diagnosing and observing the disease progression.

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University Campus STeP Ri
Slavka Krautzeka 83/A
51000 Rijeka, Croatia
Phone: +385 (51) 770 447
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中国上海市延安西路65号上海国际贵都大饭店办公楼405单元
Phone: +86-21-62489820
Fax: +86-21-62489821