Biliopancreatic malignancy: Future prospects for progress

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Summary
Several key areas are targeted by novel therapies. Growth factors and their receptors are overexpressed in a high percentage of pancreatic tumors. These factors are critical to tumor cell growth and development. They also promote tumor growth by stimulating angiogenesis. Mutations in other molecules that regulate cell growth, such as the ras protein and the tumor suppressor p53, contribute to a state of continuously stimulated cell proliferation. Other types of molecules such as mucins are also altered or overexpressed in tumors. Mucins are immunosuppressive and are important in tumor cell metastasis.

Identification of risk factors serves two purposes. The first purpose is to allow us to design public education programs that focus on prevention. The second purpose is to pinpoint the population that would most benefit from periodic testing. Because the incidence of pancreatic cancer is so low large scale screenings are not practical with any of growth factor receptors or ras protein. Monoclonal antibodies block interaction between receptor and its ligand. Newly developed drugs prevent tyrosine phosphorylation of growth factor receptors or farnesylation of ras protein. Gene therapy is another approach that is under investigation. Transduction or transfection of genes for wild-type tumor suppressors could correct defects in growth regulation. Vaccines developed against tumor antigens provide hope for the control of not only the primary tumor, but also of the metastatic lesions as well.

Identification of risk factors

Identification of risk factors serves two purposes. The first purpose is to allow us to design public education programs that focus on prevention. The second purpose is to pinpoint the population that would most benefit from periodic testing. Because the incidence of pancreatic cancer is so low large scale screenings are not practical with currently available tests. A screening of 10,162 persons over 40 years of age conducted in Japan found only four cases (0.04%) of the disease [1]. The identification of the risk factors and genetic alterations that are associated with biliopancreatic cancers, and especially those factors that act during the early stages of tumorigenesis, will assist in identifying those who are at greatest risk.

Although a family history of pancreatic adenocarcinoma is not common in patients with pancreatic cancer they are statistically more likely than controls to have a close relative with pancreatic cancer. 5-10% of patients have a first-degree relative with the disease [2]. This suggests that in familial pancreatic cancer the predisposition for the disease is inherited through the germline. A detailed examination of the genetic history of family members may provide information on the process of tumorigenesis. Although the gene(s) associated with familial pancreatic cancer is(are) not yet known there is information about the genes that are probably not involved. The incidence of germline mutations in DPC4 [2] or K-ras [3] in patients with a family history of cancer is not significantly different from that of patients with no family history. Expression of p21WAF-1 and HER-2/neu in these patients was also not significantly different from those with no family history [3]. However, there was a significantly lower incidence of p53 expression [3]. It is known that certain other inherited disorders predispose individuals to pancreatic cancer. One of these is hereditary pancreatitis [4]. Other conditions are reviewed by Lumadue, et al [4].

The rate of pancreatic cancer increases with age. About 80% of the cases occur between ages 60 and 80 [5]. Occurrence in individuals less than 40 yrs of age is rare. This has socioeconomic implications in countries with aging populations. As the proportion of the population over 60 yrs of age grows we may expect to encounter an increase in the incidence of pancreatic cancer. In the United States the incidence is higher in African-Americans than in whites. There is a higher incidence in men than in women, in both the African-American and white populations. However, there has been an increase in the incidence and mortality rate in American women since 1974 [5].

Certain of the risk factors associated with lifestyle are amenable to change. Cigarette smoking is one of the best substantiated behavioral risk factors associated with pancreatic cancer [5]. A recent report done on Swedish women is of interest [6]. It showed an apparent inverse relationship between age at first birth and the risk of...
pancreatic cancer. However, the authors noted that young age at first birth in Sweden may be associated with increased frequency of smoking. The increase in incidence of pancreatic cancer in American women that was noted above may be related to a general increase in the rate of smoking in American women. There is a positive association of pancreatic cancer with consumption of salted food [7] and of red meat [5]. Association with either alcohol or coffee consumption is somewhat ambiguous [5, 8]. On the other hand, consumption of whole grain/high fiber diets [9] and of fruits and vegetables [5, 10] are associated with reduced risk. Recently, there has been considerable interest in the protective components of green tea [11]. Public announcements about the connection between lung cancer and smoking have resulted in a drop in the number of Americans smokers. Reinforcing the public's awareness of the importance of diet and smoking in the etiology of many types of diseases is a cost-effective means of prevention.

**Early diagnosis**

Because of the non-specific and ambiguous nature of the symptoms associated with pancreatic and biliary tract cancers the patient usually does not come into contact with the physician until the disease is well advanced. Serum based immunoassays of mucins (such as CA19-9 and SPan-1) and other tumor antigens showed initial promise for early diagnosis but have now been demonstrated to lack sensitivity and specificity [12, 13]. They retain usefulness in follow-up and assessing prognosis. The lack of specificity of many of the present tests results, in part, from the presence of the antigens in normal pancreas. For example, the CA19-9 and SPan-1 tests identify carbohydrate structures on the MUC1 type of mucin [14], a type of mucin that is prevalent in normal pancreas. Recently, we have shown that another type of mucin, MUC3, is present in very low levels in normal pancreas [15]. Its expression is greatly increased in pancreatic tumors. Part of the MUC3 cDNA sequence was determined with anti-sera directed against deglycosylated pancreatic cancer mucins [16]. The preferential expression of MUC3 in tumors shows promise for a more specific diagnostic test.

Currently there is a large body of literature on the identification of K-ras and p53 gene mutations in body fluids. Up to 95% of exocrine pancreatic tumors contain mutations of the K-ras gene at codon 12 [17]. These types of mutations occur early in pancreatic carcinogenesis in an animal model [18]. Mutations are also present in 75% of small human pancreatic tumors (1.2-3 cm, [19]) and 75% of intraductal lesions, thought to be precursors of adenocarcinomas [20]. K-ras mutations are also detectable in bile of 79% of patients with biliary duct cancer but not in patients with gallbladder cancer or benign biliary diseases [21]. Of those patients who had no apparent malignant cells in bile upon cytological examination 71% had K-ras mutations of DNA in their bile as detected by mutant-allele-specific-amplification [21]. Detection of mutations in K-ras in DNA found in peripheral blood [22] and stool [23] may be the basis of simple non-invasive screening tests. However, caution needs to be taken in the interpretation of a positive test, because of the presence of K-ras mutations in benign pancreatic conditions [24].

**Creation of additional candidates for curative resection**

Curative resection of small localized tumors is still the best hope for survival. Recently, the operative mortality rate has decreased significantly [25]. However, most patients are not good candidates for resection at presentation. Some of these individuals have large invasive tumors. Also surgery does not deal with distant or undetected metastases and residual tumor cells that could lead to relapse. Conventional neoadjuvant treatments have not significantly altered the proportion of patients that will ultimately be able to undergo curative resection. It may be that novel therapeutic approaches will succeed in converting additional patients to resectability. Application of some of the more recent advances in gene therapy, chemotherapy based on chemicals that specifically target signal transduction pathways and immunotherapy may provide new hope in improving mortality rates.

What are some of the key sites that are being targeted with these new therapeutic modalities? The importance of vascularization to the growth of pancreatic (reviewed by Pluda and Parkinson, [26]) and other types of tumors has gained considerable public attention recently. Angiogenesis is also important to tumor cell dissemination [27]. In addition, several steps in the regulation of tumor cell function have now been identified as being aberrant in pancreatic tumor cells. In particular, these include the overexpression of the epidermal growth factor (EGF) receptor family [28] including erbB-2/neu [29]. As noted above mutations in codon 12 of K-ras occur frequently in pancreatic tumors. Mutations in K-ras often lead to the constant activation of the ras protein. Mutations in the tumor suppressor p53, an important factor in cell cycle control, also occur frequently in pancreatic tumors [30]. Mucins are also an important therapeutic target. They inhibit the cells of the immune system and participate in the process of metastasis (reviewed by Ho and Kim, [31]). Their expression on the surface of tumor cells makes them ideal antigens to be targeted by monoclonal antibodies and vaccines.

Results from studies, many of which have been performed on animal models, suggest that inhibition of tumor-associated angiogenesis will result in tumor regression. Vascular endothelial growth factor (VEGF) is one of the major angiogenic inducers [32]. One strategy to negate the influence of VEGF has been to transfect anti-sense VEGF cDNA. Melanoma cells transfected with the anti-sense cDNA formed small tumors with little vascularization and extensive necrosis in nude/SCID mice [33]. Moreover, anti-sense transfected cells formed few lung colonies. On the other hand, melanoma cells transfected with sense VEGF formed well vascularized tumors and numerous tumor colonies in the lung. Other forms of gene therapy that target angiogenesis are reviewed by Kong and Crystal [34]. Another means of inactivating VEGF is through the use of a neutralizing antibody. Such an antibody inhibited both primary and metastatic tumor growth of fibrosarcoma cells in a nude mouse system [35]. Endostatin and angiostatin are two antiangiogenic peptides that are effective in controlling tumor growth. Endostatin is a 20 kilodalton portion of the C-terminus of collagen XVIII. E. coli derived endostatin caused primary tumors to regress to dormant microscopic lesions [36]. Angiostatin is an internal fragment of
plasminogen. Recombinant angiostatin suppressed growth of primary and metastatic Lewis lung carcinoma tumors in an animal system [37].

Several approaches have been used against the EGF family of receptors. Transfection of a ribozyme to specifically cleave receptor mRNA [38] reduced the level of the receptor. The receptor tyrosine kinase can be inactivated by specific inhibitors [39]. Application of anti-growth factor receptor monoclonal antibodies has been reviewed by Fan [40]. Recently, the antibody, Herceptin [41], which is directed against ErbB-2/neu (HER-2), has recently been approved by the FDA for the treatment of advanced breast cancer. An antibody against the EGF receptor induced antibody-dependent cellular cytotoxicity (ADCC) in pancreatic cancer patients [42]. Cells expressing a particular receptor can also be destroyed by targeting that receptor with a recombinant form of the ligand that had been modified by inclusion of a protein toxin [43]. Inhibition of the activity of the EGF receptor tyrosine kinase not only slows growth of colonic tumor cells but it can also induce apoptosis of the cells [44]. Neutralizing antibodies that block interaction between EGF or erbB-2/neu ligand and their receptors also down-regulated expression of VEGF in human epidermoid carcinoma cells [45]. Treatment with a monoclonal antibody can also improve the effectiveness of conventional chemotherapy. For example, treatment with Herceptin enhanced the anti-tumor activity of paclitaxel and doxorubicin in human breast cancer xenografts [41]. It is attractive to theorize that the replacement of a defective tumor suppressor genes or the blocking of a constantly active gene product will be sufficient to reverse the malignant characteristics of a tumor cell, if not kill it. However, it must be remembered that cells that can be identified as being malignant already have a number of genetic mutations [46]. For example, in one study all cases of pancreatic cancers that contained p53 mutations also had codon 12 K-ras gene mutations [30]. On the other hand, gene replacement may also have a bystander effects. Replacement with wild-type p53 by transduction with a viral vector into colonic cancer cells decreased expression of RNA for VEGF [47]. The reduction in VEGF expression was dose dependent. Several approaches have been tested to alter the effects of K-ras and p53 mutations: transfection of anti-sense K-ras gene fragment into pancreatic cancer cells [48]; administration of a viral vector bearing the wild-type p53 gene into the peritoneum of nude mice to inhibit growth of primary pancreatic xenograft tumors and to reduce peritoneal tumor deposits [49]; and transduction of pancreatic cells with the p21WAFl gene, the product of which is a downstream effector of p53 function [50]. Farnesylation of ras is required for its activity. Inhibitors specific for farnesyltransferase are another means of preventing ras activity [51]. Pancreatic cancer patients have been vaccinated with mutant ras peptides [52]. Vaccinated patients exhibited a transient T-cell response. p53 is also a target for vaccine development [53].

Immunotherapy has always had appeal because immune recognition is highly specific. Antibodies and immune cells will target only those cells presenting a particular antigen, which ideally are only the tumor cells. Immune responses are effective against the metastases (known and unknow) as well as against the primary tumor. There has been a recent study using $^{131}$I labeled monoclonal antibodies for the treatment of patients with non-resectable liver metastases of colon cancer cells [54]. Two main obstacles to the effectiveness of antibodies in the past have been the delivery of a sufficient effective dose and, paradoxically, the extreme specificity of the antibodies. In the first case, delivery of a large number of macromolecules such as antibodies to tumor cells is limited by barriers imposed by the blood supply, vessel wall, and interstitium [55]. A growing understanding of these barriers will help in the design of new delivery methods. A recent detailed analysis of the killing effectiveness of immunoconjugates to toxins showed that as few as 1000 molecules of immunotoxin per cell were necessary to kill tumor cells [56]. Thus immunotoxins can be effective even at relatively low doses.

Monoclonal antibodies can also be too specific. Because of tumor cell heterogeneity not all cells in a tumor will express the targeted antigen. One means of circumventing this problem is with the use of immunoconjugates to high energy radioisotopes such as $^{131}$I [54,57]. These radioisotopes will affect neighboring cells as well as the targeted cells. Antibody-directed enzyme prodrug therapy (ADEPT), where a prodrug is administered systemically to be activated by enzymes that have been pre-targeted by antibodies [58], results in a local production of active drug. As concentrations build in the interstitium the drug will also affect neighboring cells not targeted by antibody/enzyme. We are in the process of determining if increasing the level of a targeted antigen (Nd2 mucin-associated antigen) and the number of cells expressing that antigen [59] will enhance antibody binding and uptake. Up-regulation of EGF receptor enhanced antibody dependent cell mediated cytotoxicity (ADCC) in one recent clinical study of pancreatic cancer patients [42].

There is considerable interest in involving the patient's own immune system in the process of tumor control. On the one level there are bispecific antibodies that react with both a tumor antigen and an antigen present on cells of the patient's immune system. One study examined bispecific antibodies that target MUC1 mucin and a surface antigen of killer cells [60]. These antibodies effectively mediated interaction between bile duct carcinoma cells and lymphokine activated killer cells. Another means of targeting lymphokine activated killer cells to bile duct cells was to chemically conjugate staphylococcal enterotoxin A to a MUC1 specific antibody [61]. It has also been observed that human/murine chimeras of monoclonal antibodies directed against mucins are much more effective as mediators of ADCC than the native murine antibody [62, 63].

Another means of involving the patient's immune system is by vaccines. Vaccine targets include mutated oncopgenes [52, 53] and mucins. Mucin carbohydrates [64], protein [65] and DNA [66] have been used. Recently, the dendritic cell was demonstrated to be a highly effective antigen-presenting cell (reviewed by Pardoll, [67]). In addition, they also express high levels of molecules that activate T-cells. Dendritic cells pulsed in vitro with a variety of antigens have proved effective in producing specific anti-tumor effects in vivo, including the inhibition or regression of metastatic tumors [68,69]. Use of whole tumor cells or lysates, as opposed to specific tumor antigens, circumvents the problem of tumor cell heterogeneity. Tumor cells secreting cytokines such as
pancreatic cancer cells [77]. This enzyme makes tumor cells
mice with established peritoneal metastases of human
retroviral vector was injected into the peritoneum of nude
deposits of Bxpc3 pancreatic cancer cells [49]. In another
resulted in a significant inhibition of peritoneal tumor
greatly improved 3-year patient survival and reduced the rate
fluorouracil was continuously infused through the hepatic
principal
expressed atypically in primary pancreatic tumors [15] they
MUC1 is also an applicable antigen for the pancreas and bile
for vaccine development.
Identification, prevention, and treatment of metastases
As more becomes known about what distinguishes a
pancreatic metastasis new diagnostics tests can be designed.
One study reports that the immunophenotype of metastases
of epithelial malignancies closely resembles that of the
primary tumor [71]. In an animal model differences were
rare in lymph node and liver metastases of pancreatic tumor
cells, although they occurred more frequently in peritoneal
metastases [72]. Detection of pancreatic metastases in
regional lymph nodes by polyclonal chain reaction that
targets K-ras mutations in codon 12 has been reported [73].
Detection of breast cancer micrometastases in axillary lymph
nodes by reverse transcriptase-polymerase chain reaction
targeted MUC1 mucin mRNA [74]. As discussed above
MUC1 is also an applicable antigen for the pancreas and bile
duct. Because other types of mucins such as MUC3 are
expressed atypically in primary pancreatic tumors [15] they
may also be appropriate target antigens to identify pancreatic
metastases.
In breast cancer the number of microvessels is an
independent predictor of metastatic disease [27]. Angiogenesis forms highly permeable new vessels that can
be easily penetrated by tumor cells. Novel anti-angiogenic
treatment discussed in an earlier section may also be
effective in reducing the likelihood of metastases and also
their growth if established. Hepatic metastases is a common
cause of treatment failure after curative resection in
pancreatic cancer. One major challenge is to prevent their
establishment. Carbohydrate structures of mucins on the
surface of tumor cells act as ligands in the metastasis of
pancreatic tumor cells (reviewed in ref. [31]). The principal
ligand is the sialylated Lewis\(^a\) structure. Antibodies that
recognize this epitope, such as 19-9 and Span-1 [14], may be
useful in reducing liver metastases. Such an antibody has
been shown to be effective in reducing the development of
liver metastases of pancreatic tumor cells in an animal model
[75]. Regional delivery of cytotoxic drugs is another strategy
to prevent establishment and growth in new sites such as the
liver and peritoneal cavity. In one clinical study 5-
fluorouracil was continuously infused through the hepatic
artery and portal vein for 28-35 days [76]. This treatment
greatly improved 3-year patient survival and reduced the rate
of hepatic metastases. In an animal study a retroviral p53
vector was injected into the peritoneum of nude mice. This
resulted in a significant inhibition of peritoneal tumor
deposits of Bxpc3 pancreatic cancer cells [49]. In another
example of regional gene therapy a thymidine kinase
retroviral vector was injected into the peritoneum of nude
mice with established peritoneal metastases of human
pancreatic cancer cells [77]. This enzyme makes tumor cells
susceptible to ganciclovir. Subsequent administration of
ganciclovir resulted in a reduction of the mass of tumor deposits.
In an animal model of liver metastases mice were vaccinated
with dendritic cells prior to the intrasplenic injection of tumor cells [67]. The dendritic cells impeded the
establishment and growth of liver metastases. This occurred
even when the dendritic cells were not pulsed with tumor
cell lysate. However, the effect was greater in mice receiving
dendritic cells that had been pulsed with tumor cell lysates. A recent clinical study of melanoma patients with metastatic
disease patients were vaccinated with irradiated autologous
melanoma cells engineered to secrete human GM-CSF [68].
Metastatic lesions resected after vaccination were infiltrated
with T lymphocytes and plasma cells, which was not the case
prior to vaccination. In addition, there was extensive tumor
destruction and fibrosis.
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