Review

Gene therapy for pancreatic and biliary malignancies

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Summary

Advances in our understanding of the molecular genetics of pancreatic and biliary cancers have given us new targets for therapy using molecular and genetic approaches. Replacement of tumour suppressor gene function using adenoviruses to transfer wild-type p53 and p16 genes can produce dramatic anti-tumour effects, both in vitro and in vivo. Blockade of dominant oncogene function using dominant negative technology may have a particular application for mutated K-ras which occurs almost ubiquitously in pancreatic adenocarcinoma. Genetic prodrug activation therapy using tumour-selective gene promoters to drive the expression of so-called suicide genes is showing remarkable promise. Targeted delivery of such therapeutic constructs may also be possible through knowledge of the expression of surface receptors by particular tumour cell types. Genetic immunomodulation using cytokine genes as well as specific vaccines against tumour-associated antigens are now being brought into clinical trials.

Key words: Cholangiocarcinoma, DNA vaccination, gene therapy, immunotherapy, pancreatic cancer, prodrug activation, ras.

Introduction

Cancers of the pancreas and biliary tree have long carried bleak prognoses. Recent advances in our knowledge of tumour biology have begun to offer the potential of developing novel therapeutic strategies for these diseases. We discuss the possible roles of these genetic-based therapies as applied to ductal adenocarcinoma of the pancreas and cholangiocarcinoma.

Molecular genetics

The molecular genetic profile of pancreatic adenocarcinoma has been extensively studied and the common genetic alterations described [1,2]. In particular, activating mutations of the K-ras oncogene are seen in over 90% of cases, although the magnitude of this association has recently been challenged [3]. Defects in one or more of the three tumour suppressor genes CDKN2, p53 and DPC4 occur in 80%, 60% and 50% of cases respectively (see Table 1). Individual tumours display varying combinations of these abnormalities. Less common alterations have been found in other genes such as BRCA2 [4], more rarely the APC (adenomatous polyposis coli) gene [5] and a possible 'mutator phenotype' with microsatellite instability [reviewed in reference 2]. Pancreatic adenocarcinomas also demonstrate abnormally high expression of polypeptide growth factors and their receptors, particularly of the epidermal growth factor (EGF) family [6], which may contribute to the neoplasm's growth advantage by autocrine and paracrine effects.

Given the close embryological origins of the liver, biliary tree and pancreas, together with their connection by a continuous epithelium, it is not surprising that proliferative lesions of these organs can share important similarities as well as regional differences [7]. For example, there have been numerous reports of pancreatic differentiation occurring in hepatic oval cell proliferation [8] and conversely of hepatocellular differentiation in some pancreatic tumours [9]. Cholangiocarcinomas do share some important clinical and molecular characteristics with pancreatic cancer. Not only is the prognosis correspondingly poor, with less than 10% of cases being suitable for curative surgery [10], but there is also a high rate of K-ras mutations in the same codon 12 position found in pancreatic cancers. The reported incidence of K-ras mutations in cholangiocarcinoma has varied widely,

Table 1. Common genetic alterations in pancreatic adenocarcinoma.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Locus</th>
<th>Frequency of alteration</th>
</tr>
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<tbody>
<tr>
<td>K-ras</td>
<td>12p12</td>
<td>90%</td>
</tr>
<tr>
<td>CDKN2*</td>
<td>9p21</td>
<td>80%</td>
</tr>
<tr>
<td>p53</td>
<td>17p13</td>
<td>60%</td>
</tr>
<tr>
<td>DPC4*</td>
<td>18q21.1</td>
<td>50%</td>
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</tbody>
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* Also known as p16 or MTS1
* Also known as SMAD4

Table 2. Examples of gene-therapy based techniques applicable to cancer treatment.

<table>
<thead>
<tr>
<th>Procedures</th>
<th>Examples</th>
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<tbody>
<tr>
<td>Tumour suppressor gene replacement and/or induction of apoptosis</td>
<td>Wild-type p53, p21, p16, Retinoblastoma gene</td>
</tr>
<tr>
<td>Genetic prodrug activation therapy</td>
<td>HSV thymidine kinase, E.Coli cytosine deaminase</td>
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<tr>
<td>Immunotherapy</td>
<td>Cytokine gene therapy, Costimulatory molecule gene therapy</td>
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Table 2. Examples of gene-therapy based techniques applicable to cancer treatment.
from as high as 100% in one United Kingdom study [11] down to just 8% in Thailand [12]. These differences may reflect the geographical variations in the prevalence of aetiological agents such as Opistorchis Viverrini infestation or primary sclerosing cholangitis; alternatively they may be the result of the different methodologies used in the studies. Importantly, there are regional differences in the incidence of K-ras mutations depending on the site of the tumour within the biliary tree. In one Japanese study [13] for example, K-ras mutations were found in only 33% of peripheral intrahepatic tumours, with the incidence steadily rising with tumours located closer to the hilum (56%) and further increasing to over 80% of distal extrahepatic cholangiocarcinomas. Mutations in the p53 tumour suppressor gene have also been found in cholangiocarcinomas, albeit at a lower frequency (35%) than in pancreatic cancers and resistance to apoptosis may be further exacerbated by overexpression of Bcl-2 [14].

Genetic intervention for pancreaticobiliary cancers

Utilising our existing knowledge of tumour biology has led to a number of potential therapies which aim to either correct the genetic alterations that occur in cancers or, more commonly, exploit them as therapeutic targets (Table 2). Some of these novel techniques, their advantages and disadvantages are discussed below.

Tumour suppressor gene replacement and pro-apoptotic therapies

The theory of tumour suppressor gene (TSG) replacement has so far been more appealing than its practical success in vivo. Pancreaticobiliary malignancies may demonstrate a number of different alterations in TSGs, expressed at disparate time points in tumourigenesis and so at varying locations within the same tumour mass. There may even be a resulting narrow 'therapeutic window' caused by the transient involvement of a defective tumour suppressor mechanism in carcinogenesis. Consequently, a single TSG replacement strategy is unlikely to be a wholly effective therapy for pancreaticobiliary cancer in vivo, notwithstanding any difficulties in efficient gene delivery to the target site. These problems, however may not be insurmountable, and there is demonstrable evidence of successful replacement of TSG function in the laboratory. Perhaps the most attention has been paid to correcting the effects of p53 mutations. Retroviruses [15] or replication-deficient adenoviruses [16, 17] have been used as vectors to carry wild-type p53, suppressing tumour growth in a variety of different cancers including pancreatic adenocarcinoma [18]. Furthermore, the restoration of p53 function can markedly increase the sensitivity of a tumour to 'conventional' chemotherapeutic drugs [19].

Whilst perhaps not 'gene therapy' in its strictest sense, selectively-replicating adenovirus strains such as the ONYX-015 virus have been used to target p53-deficient tumours [20], with cytolytic effects that can augment standard chemotherapy. Preliminary reports of clinical trials in head and neck cancer are said to be encouraging [21].

The functional replacement of other defective tumour suppressor genes is being explored. For example, transfection of pancreatic tumour cell lines with p21\textsuperscript{WAFI} using a recombinant adenoviral construct has been shown to cause cell cycle arrest at G\textsubscript{1}/S and significant growth inhibition of cultured cells [22]. A number of groups have also pursued the restoration of functional p16 in cancer cells in vitro. Increased apoptotic cell death was observed in non-small cell lung cancer, colonic and hepatic cell lines transfected with p16 adenoviral constructs, particularly if p53 was also overexpressed [23, 24]. Like p21\textsuperscript{WAFI}, p16 inhibits the effects of cyclin-dependent kinase (CDK) enzymes. Normally, the retinoblastoma gene product acts to discourage DNA synthesis and cell-division, but it can be inactivated by a CDK-mediated phosphorylation. As most pancreatic cancers express a functional retinoblastoma protein, a p16 replacement strategy could be a useful approach for this disease. Interestingly, in a rat model of proliferative cholangitis, direct adenovirus-mediated transfer and overexpression of the retinoblastoma gene to the biliary epithelium led to significant inhibition of epithelial proliferation [25]. Ultimately this technology might be developed into adjunctive therapy for malignant biliary strictures, but further studies are clearly required.

Other strategies to induce tumour cell apoptosis in pancreatic cancer have included transfection with a gene encoding the R55 tumour necrosis factor (TNF) receptor, followed by the administration of mfeutin TNF [26]. An alternative approach has been to transfect pancreatic tumour cell lines with a cDNA encoding a fusion protein between Fas and the ligand-binding region of the oestrogen receptor. Following transfection, cell death could be induced simply by the addition of oestrogens [27].

Blockade of dominant gene expression by antisense technology

This technique involves the use of nucleic acids that are complementary to a particular sequence of DNA or RNA. The hybridisation of these oligonucleotides to their target can effectively block the transcription or translation of the gene product. The most commonly-used constructs utilise DNA molecules of 15-20 nucleotides in length. The binding of the construct to its mRNA counterpart may cause steric hindrance of ribosomal 40S subunit binding, block the initiation codon, or cause mRNA cleavage by Rnase H depending on the chosen target site. Alternative approaches include manipulating oligonucleotides to irreversibly bind genes prior to transcription (forming a triple helix with loss of gene expression), targeting transcription factors or using ribozymes to cleave RNA sequences.

Antisense technology has been used to reduce K-ras expression and tumour growth in a lung cancer model [28]. Subsequently, the liposome-mediated gene transfer of an antisense K-ras construct has been used to treat murine pancreatic cancer in vivo [29] by intraperitoneal injection, resulting in a significant suppression of tumour growth without systemic toxicity. Mutant p53 has been targeted by a ribozyme-based antisense strategy in lung cancer cells, reducing the level of mutant p53 and leading to a slowing of proliferation [30]. Antisense oligonucleotides directed against ERBB2 have been shown to reduce the growth of ERBB2-positive breast cancer cell lines [31] and this may be applicable to pancreatic cancers where the mechanism of
ERBB2 transformation is similar. In cholangiocarcinoma cell lines, antisense oligonucleotides directed against the overexpressed Bcl-2 lead to both a reduction in Bcl-2 expression and a marked lowering of the threshold for apoptosis induction [32].

Antisense strategies are still developing and their continues to be some controversy over their specificity and precise mode of action. For example, oligonucleotides directed against p53 in human pancreatic cancer cell lines have antiproliferative effects that are not related to the expression of p53 [33]. Oligonucleotides that contain unmethylated cytosine-phosphate-guanine (CpG) motifs seen in some bacterial DNA sequences can act as immunomodulators and cause increased humoral and cellular immune activation [34].

Genetic prodrug activation therapy (GPAT)

GPAT employs the selective expression of so-called 'suicide' genes within tumour cells, their different metabolic profile now enabling pro-drugs to be delivered to the tumour without significant systemic side effects. The presence of these transferred enzymes can confer sensitivity to antiviral drugs or dramatically increase the therapeutic index of cytotoxic agents. Many studies have involved transferring the Herpes simplex (or varicella zoster) thymidine kinase gene to confer sensitivity to ganciclovir. Others have used E.coli cytosine deaminase, which converts non-toxic 5-fluorocytosine into the cytotoxic 5-fluorouracil (5-FU). The aim of most GPAT studies has been to achieve truly tumour-specific expression of the enzyme construct. This can be achieved by using transduction and/or transcription targeting of tumour cells.

Transduction targeting preferentially delivers genes by selecting a particular cellular phenotype. In the case of brain tumours, for example, this may be as simple as using a retrovirus to selectively infect dividing cells in the central nervous system. An alternative is to target gene delivery using constructed ligands that recognise tumour-specific cell surface markers [35,36]. Transcription targeting uses tumour-specific promoters to drive the expression of the therapeutic gene only in those cells which bear the transcription factors required to activate the promoter. The amylase promoter, for example, has been used to direct expression of an adenoviral construct in the pancreas [37]. Malignant cells can be targeted by utilising the promoter and enhancer elements of tumour-associated genes or oncogenes, such as carcinoembryonic antigen [38] or the MUC1 gene which encodes the polymorphic epithelial mucin [39] overexpressed in some breast cancers and cholangiocarcinomas [40]. As this latter mucin is a normal component of the exocrine pancreas, a more suitable pancreatic therapy might target the abnormal expressions of MUC2, MUC4 and MUC5 found in adenocarcinomas [41,42]. The ERBB2 promoter has similarly been exploited to deliver 'suicide genes' to cancer cells [43, 44] and this mechanism has recently been studied in a phase I clinical trial of 5-fluorocytosine treatment of skin metastases in patients with breast cancer [45]. This latter study demonstrated that tumour-specific expression of the cytosine deaminase gene without systemic side effects was a feasible result in vivo. Further trials are in progress.

The effects of cytotoxic GPAT are not absolutely specific, however. Many investigators have reported the so-called 'bystander effect'. Administering the prodrug to a mixed population of transfected and untransfected cells may lead to an unexpectedly high rate of cell death. In one example, transfection of just 10% of a cell population led to more than 50% of cells being killed by the addition of the prodrug [46]. Different mechanisms have been suggested to explain this phenomenon; in the case of 5-FU generation, the cytotoxic agent itself is able to diffuse into adjacent cells from those killed by activation of the prodrug. With thymidine kinase/ganciclovir therapy, cell to cell contact is required for the bystander effect to occur and it now seems likely that this phenomenon is mediated via intercellular gap junction communications [47].

Immunotherapeutic approaches

The field of immunotherapy has received new impetus recently, driven by advances in our understanding of basic immunology and the molecular biology of cancer. For many years, scientists have recognised that an appropriately activated immune response can be a powerful weapon against cancer cells. In many ways, immunological therapies could be considered as theoretically 'ideal' cancer therapies - searching out and destroying malignant cells throughout the body with the specificity to leave normal tissues unharmed. This clinical potential has yet to be fulfilled, but important recent advances have once again given cause for optimism.

The traditional concept of tumour immunology has centred on the immune surveillance hypothesis [48] with the belief that a self/non-self discrimination would allow for the recognition and elimination of tumour cells that expressed 'foreign' antigens. However, it is clear that antigenic tumours do evade the immune system, despite being recognised by T lymphocytes and the current concept held by many immunologists is that an effective immunological response against a presented antigen requires the appropriate costimulatory signals in order to trigger activation as opposed to tolerance [49]. According to this 'danger' model of immunity, tumour cells that multiply and grow in an apparently 'healthy' way without necrosis or tissue damage are unlikely to be rejected and instead induce tolerance by presenting their tumour 'antigens' to T cells without the costimulatory signals that occur with an inflammatory response. It is hypothesised that even large tumours which develop ischaemic necrosis at their centres may still evade the immune system as any tumour specific T cells will presumably have already induced tolerance to the large mass of tissue [50]. Many of our current attempts to break this tolerance and tip the balance of immunity against tumour cells will still involve combinations of conventional debulking treatments (surgery, radiotherapy or chemotherapy for example) to reduce the tumour burden. Immunotherapies that involve the induction of costimulatory 'danger' signals are then more likely to succeed. Recent work has suggested that the mechanism of cancer cell death, whether apoptotic or non-apoptotic, can determine whether an inflammatory signal is generated. A possible mechanism for this is that non-apoptotic cell death can induce the expression of heat shock proteins thereby increasing the immunogenicity of the tumour [51]. The mycobacterial vaccine BCG has been
successfully used as a topical treatment for superficial bladder cancer, presumably reflecting the non-specific induction of inflammation and development of costimulation. In a recent modification of this strategy, patients with stage II/III colon cancer were given intradermal vaccinations of irradiated autologous tumour cells mixed with BCG in addition to surgical resection of the tumour. A five year follow-up has recently reported a 44% reduction in cancer recurrence [52]. More attention however has been focused on determining specific tumour 'antigens' and in particular defining epitopes which may be presented to (and recognised by) T cells via the MHC class I route. Melanoma antigens such as gp100 or tyrosinase have provided the greatest number of HLA-A2 presented epitopes for study so far [reviewed in reference 53] but there are also a number of possible tumour antigens that associate with pancreatic cancer and cholangio-carcinoma. Theoretically, this could encompass the protein products of any of the mutant genes described earlier. Examples that have been identified as possible targets for cytotoxic T cells to date include the HLA-A2 restricted epitope of MUC2 [54], mutated p53 [55] and codon 12 mutants of K-ras [56, 57]. Early clinical studies have shown that cytotoxic T cells can be generated against cells bearing ras mutations after peptide vaccination [58].

Clinical trials of cytokine therapy in patients with melanoma have shown encouraging results in increasing tumour immunogenicity, but problems with systemic toxicity prompted the development of gene transfer strategies to express and deliver cytokines at tumour sites. One approach has been the adoptive transfer of cytokine genes (such interleukin-2) into ex-vivo cultured tumour-infiltrating lymphocytes which are then infused into the patient [59]. Alternatively, tumour cells can be transduced ex-vivo with immunostimulatory cytokines such as GM-CSF, irradiated and returned to the host [60]. A clinical trial using this latter method in patients with pancreatic cancer is now under way [61].

Inducing tumour cells to express co-stimulatory molecules on their surface could enable them to activate T-cells directly, bypassing the requirement for antigen-presenting cells. Transfection of human tumour cell lines with the B7.1 molecule can invoke cellular immunity [62].

In addition to engineered tumour cell-based vaccines, attempts are being made to stimulate anti-tumour immunity with vaccines based on viral or bacterial vectors [63], DNA vaccines [64] or by the use of antigen-presenting dendritic cell populations [65]. These latter cells have a pivotal role in capturing and presenting antigens to T cells via Class I and II MHC molecules, as well as possessing a host of costimulatory and cell-adhesion molecules [reviewed in reference 65]. Dendritic cell-derived vaccines can be developed ex-vivo by pulsing the cells with tumour-derived peptides or transfecting them with the genes for tumour-associated antigens before transfecting them back into the patient. Clinical trials of dendritic cell vaccination are currently in progress for B-cell lymphoma, melanoma, prostate cancer and breast cancer.

Recent work has suggested that the antigen-presenting cytoplasmic vesicles ('exosomes') present within dendritic cells may themselves have the ability to activate specific CTL's and eradicate established tumours [66].

Possible future developments: combining treatments and solving problems

As with much of 'conventional' cancer therapy, many of the novel gene therapy-based treatments may prove more effective in combination, both with each other and with existing modalities. Examples include transfecting cells with two different cytokine genes, two different prodrug activating enzymes or combining cytokine gene transfection with GPAT. Combining different therapies may dramatically alter the responsiveness of cancer cells to existing treatments. Pederson et al recently demonstrated that cholangiocarcinoma cells could be made sensitive to radiotherapy, both in vitro and in vivo, by prior transfection with the cytosine deaminase GPAT gene [67]. In a further development, Freytag et al reported the use of three combined therapies - a selectively cytotoxic adenovirus FGR (similar to ONYX-015), double suicide genes (cytosine deaminase and HSV-1 TK in the same fusion gene construct) and tumour irradiation. Specificity was maintained, in that neither the FGR virus itself nor the suicide genes caused significant toxicity to normal cells. The addition of the double GPAT system not only enhanced the cytotoxic effects of the FGR virus, but also increased the tissue sensitivity to radiotherapy [68]. As mentioned earlier, immunotherapy is more likely to succeed if the tumour burden is relatively low. In addition to the currently available debulking treatments, combining immunotherapy with anti-angiogenic agents such as angiostatin or endostatin, which diminish tumour blood supply, may be effective [69]. Gene therapists continue to explore novel methods of gene delivery, as well as refining existing vectors. Some of the problems associated with adenoviral gene delivery to the pancreas in vivo may be overcome in the near future. For instance, the adenovirus itself can generate an immune response to itself that prevents further treatment courses [70], but recent work suggests that it may be possible to induce tolerance to adenoviral proteins in rats with pre-existing immunity [71]. Pancreatic adenoviral infection may also cause significant pancreatitis [72] and whilst different viral modifications are studied it may be possible to avoid this complication by using an alternative vector. Cationic liposomal gene transfer has recently been shown to be an efficient vector for delivering intraductal gene therapy to the pancreas in vivo, without causing pancreatic inflammation [73]. Ironically, a modification of this technique has been used to administer intraperitoneal IL-10 gene therapy as a treatment for acute pancreatitis [74]. Elsewhere, pharmacological developments should lead to a wider choice of 'suicide' pro-drugs. The E. coli uracil phosphoribosyl-transferase enzyme has been adenovirally-transfected into pancreatic, colonic, gastric and hepatocellular cancer cell lines and shown to overcome resistance to 5-FU therapy [75]. New adjuncts to these genetic therapies are also under development. Specific inhibitors of the C-terminal endopeptidase are being studied and have recently been shown to block the growth of ras-transformed cancer cells [76].

There is still considerable work to be performed before many of the techniques we have discussed bring benefits to patients with pancreatic and biliary cancer. However, the last few years have seen several of these treatments begin to
move from the laboratory into the clinical arena. The beginning of the 21st century will surely see further exciting developments in this intriguing field.

Acknowledgements

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References