MINIREVIEW

Selected Features of Nonendocrine Pancreatic Cancer

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We survey some interesting features of gene expression in nonendocrine pancreatic cancer, the response to some less widely known agents as they impact on pancreatic cell proliferation and programmed death, and several developing approaches to therapy. The proliferative and cellular suicide responses of Panc-1 cells to the free radical spin trap, NTBN, and to the 5-lipoxygenease inhibitor, MK 886, the latter assessed with CLONTECH Atlas Human cDNA Array 1, are reviewed. Difficulties in identifying those factors whose suppression or augmentation could result in inhibition of malignantly transformed cell properties are considered. [Exp Biol Med Vol. 226(6):521–537, 2001]

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Introduction

Compared with many hematopoietic cancers, achieving a response to therapy in solid cancers is much more intractable, and nonendocrine pancreatic cancers are among the least responsive. It is estimated that during this year in the United States, some 28,000 new patients will be diagnosed with pancreatic cancer, and a comparable number will die from this disease, which has a 5-year survival of 1% to 2% (1). An inability to induce programmed cell death (PCD) in a sufficient number of malignantly transformed cells is one current explanation for therapeutic failure (2). It can be suggested from evidence with cell lines that induction of a "classic" type 1 PCD in hematopoietic cells is more often achieved than in cells from solid cancers in which type 2 forms of PCD seem more common (3, 4). Therapy-induced inhibition of proliferation and induction of cellular suicide, as they are modulated by countervailing therapy-induced oxidative stress and DNA damage and repair responses, contributes to the selection of progressively more malignant cells. It is a reasonable supposition that augmenting PCD by inhibiting countervailing activity could reduce the residual cancer cell number, which might then be controlled by immune, anti-angiogenic, or other mechanisms.

A number of recent reviews emphasizing nonclinical aspects of pancreatic cancer have been published (5–11). The three main cell lineages include duct cell, and acinar cell and endocrine cell benign and malignant cancers. Malignant nonendocrine pancreatic ductal cancer, which includes about 85% to 90% of pancreatic cancers, is the focus of our consideration. These include ductal nonendocrine pancreatic cancer cell variants—mucinous, adenosquamous, and anaplastic carcinomas, among others (12). There is not as much information available about the less common nonductal serous and mucinous cystadenocarcinomas, anaplastic, and acinar cell carcinomas (12), and carcinomas believed to originate from pancreatic stem cells in the islets of Langerhans (13), and no attempt is made to consider them separately. The pathology of all these forms of pancreatic cancer are considered in detail in Reference 12 and in several of the cited monographs.

Karyotypic Changes in Ductal Pancreatic Cancer

Allelic chromosomal loss in ductal pancreatic carcinomas has been reported for 1p, 9p, 17p, and 18q (all > 60%) and for 3p, 6p, 8p, 10q, 12q, 13q, 18p, 21q, and 22q (40%–50%) (14). With comparative genomic hybridization of 27 pancreatic carcinomas, 23 showed abnormalities, including gains in 16p, 20q, 22q, and 17q, and reduction in 9p (15). Also amplified were regions 1p32, p34, 6q24, 7q22, 12p13, and 22q. The region 6q24 was associated with overexpression with amplification of c-myb, or with its deletion. In-

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cluded within the deleted 9p21 region is a cyclin-dependent kinase inhibitor 2 (CDKN2), a pancreatic tumor suppressor gene.

In a study of 62 primary pancreatic adenocarcinomas, 44 exhibited clonally abnormal karyotypes (16). Eight tumors gained chromosome 8, but loses were more common, including numbers 18, 13, 12, 17, and 6, and 209 breakpoints were identified. The chromosomal arms most frequently involved were 1p, 6q, 7q, 17p, 1q, 3p, 11p, and 19q. Overexpression of 12p coincides with an RAS protooncogene and a cyclin CND 2 gene. Double minute chromosomes were present in eight cases, and this observation is considered to be possibly the first report of gene amplification in primary pancreatic cancer. Chromosome 18 is believed to be modified early in pancreatic carcinogenesis (17).

Such deleted or amplified regions represent loci of candidate suppressor genes or oncogenes. While variations among these reports are present, the general theme is that characteristic patterns of chromosomal abnormalities in pancreatic cancers identifying pathogonomic genomic alterations as they are further classified in molecular terms will be discovered.

An Example of Homeotic Gene Expression in Pancreatic Cancer

The homeo-domain containing gene PDX1 (also designated as a glucose-sensitive factor) is a transcription factor involved in the embryogenesis of the pancreas, whose absence results in pancreatic agenesis (18). One early role may be in the recognition by the epithelium of signals from the mesenchyme. Acinar cells arise from ductal cells, both associated with PDX1 expression. A subsequent function is associated with elastase I promotor-activation in acinar cells, forming a trimeric complex with homeodomain proteins PBX1b and MRG1, members of five isoforms forming complexes with the Q50 class of HOX proteins. This shift in function emphasizes the several purposes that products of a gene may serve, depending upon pre- and postembryonic development, position in the cell cycle, state of differentiation, or mechanism of cell death. Whether selective inhibition of PDX1 might modify pancreatic cancer cell biology is not known.

Hereditary Forms of Pancreatic Cancer

Discussion of several forms of pancreatic cancer with a hereditary component is presented in References 8 and 10. These include diseases associated with BRCA-2 germ-line mutations occurring in Ashkanazi Jewish patients, altered p16 in the melanoma and pancreatic cancer FAMMM syndrome, and mutations identified as STK11/LKB1 in the Peutz Jeghers syndrome (19). In a study of white blood cells from 102 pancreatic cancer patients, the contribution from family history and germ-line mutations in p16, BRCA-1, BRCA-2, hMSH2, and hMLH1 genes were compared (20). Thirty-eight percent of the patients were considered "high risk," and among them, germ-line mutations were found in five (13%), including one p16 mutation, one BRCA1, and three BRCA2 mutations. All the BRCA mutations were present in Ashkanazi Jewish patients, a reported association (21). The lack of germ-line mutations in the majority of these patients with pancreatic cancer implies involvement of additional unidentified genes during the development of the disease. An association between pancreatic adenocarcinoma and melanoma that may harbor germ-line CDKN2A mutations has been reported (22).

Global Assessments of Pancreatic Cancer Gene Expression

It is now generally believed that of 30,000 potentially active mammalian genes, normal cells express 10% to 20% in specific patterns. In cancer, a relatively small number, amounting to some hundreds of these genes, are believed to be altered due to primary or secondary events.

The serial analysis of gene expression (SAGE) technique was applied to the analysis of pancreatic gene expression (23). Sequence tags were isolated from pancreas, concatenated, cloned, and 1000 expressed sequence tags (ESTs) were manually sequenced. A gene expression pattern that characterized pancreatic transcription and included new transcripts was identified. In a second report, ESTs corresponding to 45,000 genes in pancreatic cancer were examined, 183 of which were considered overexpressed (24). The metalloproteinase inhibitor TIMP-1 was overexpressed in pancreatic cancer cells, and combined with carcinoembryonic antigen and C19-9 in serum studies of expressed proteins, detected 65 (76%) of 85 patients with pancreatic cancer.

In a study employing grided cDNA library clones and differential hybridization of pancreatic cancer, 369 ESTs (25 and reviewed in Ref. 6) from one cDNA library probed with polyA mRNAs from 10 pancreatic cancers were classified into 11 functional categories (cytoskeleton, metabolism, etc.) and two additional categories of 240 ESTs with unknown function. A number of novel genes were identified, including an mRNA coding for four K-homologous (KH) domains, denoted as koc and found in a subset of RNA-binding proteins, a tumor-associated antigen L6 (TM4SF5, trans-membrane 4 superfamily 5), and a gene denoted as kop with two Kunitz serine protease inhibitor domains. Kunitz domains are found in amyloid B protein of Alzheimer's disease, inter-α-trypsin inhibitor, and tissue factor pathway inhibitor. At the time of this report, some 500 genes differentially expressed in pancreatic cancer had been identified.

Five-lipoxygenase inhibitors cultured with a variety of cells inhibit their proliferation and induce several variants of PCD (26–28). In a study employing a commercially available cDNA array, Panc-1 cells were cultured with the inhibitor of 5-lipoxygenase, MK 886, for 24 hr, total RNA was prepared and labeled with 32P-dATP by reverse tran-

scriptase, and the cells were stringently washed and hybridized against CLONTECH human cDNA arrays (Fig. 1 and Table I) (28, 29). After analysis with Image Quant and Excel 97, it was concluded that this inhibitor induced oxidative stress; that DNA damage and repair mRNAs were activated; that some mRNAs expected to promote proliferation were also activated; and that cellular suicide mRNAs were altered. The most interesting initial conclusion was that numerous countervailing mRNAs were inadvertently activated by treatment with this nonselective agent. This represents a qualitatively different form of unavoidable therapeutic resistance, when unselective agents including ionizing radiation are employed.

Specific Examples of Gene Expression

Oncogenes. The majority, perhaps 90%, of pancreatic cancers contain mutated k-ras genes, as can tissue from chronic pancreatitis (30). Although mutant K-ras can transform cultured cells, additional genomic events are probably required *in vivo*. Several downstream pathways include mitogen-activated protein (MAP) kinases, protein kinase C, COX-2 and TGF-β signaling events. Activation of the Raf-1 MAPKKK- (MAP kinase, kinase, kinase) MEK (MAPKK) ERK (extracellular-regulated protein kinase) (MAPK) signal transduction pathway can be present in both K-ras mutant and K-ras wild-type ductal pancreatic cancer (31). Point mutations in ras codon 12, which cause its GTP-binding site to be continuously active, occur in 75% to 90% of pancreatic cancers. Patients undergoing pancreatic resection (Whipple operation) and with a normal K-ras-2 genotype had significantly longer survival times than patients with mutated genes (32).

An RNA differential display method was used to examine the mRNA expression profile in the AsPC-1 pancreatic cancer cell line and in antisense K-ras-transduced AsPC-1 cells (33). Twenty cDNA fragments were compared. Mitochondrial genes were upregulated in 11 transduced clones, including mitochondrial 16s tRNA, mitochondrial cytochrome C subunit, NADH dehydrogenase, and ATP synthetase. Nine clones were downregulated and included PTH-1 (prostate antisense-transduced gene-1), MMP-7, B3 chain of laminin-5, lysosomal-associated membrane protein-2, the H chain of apoferritin, ribosomal protein S6, proteosome subunit XAPC7, and two fragments

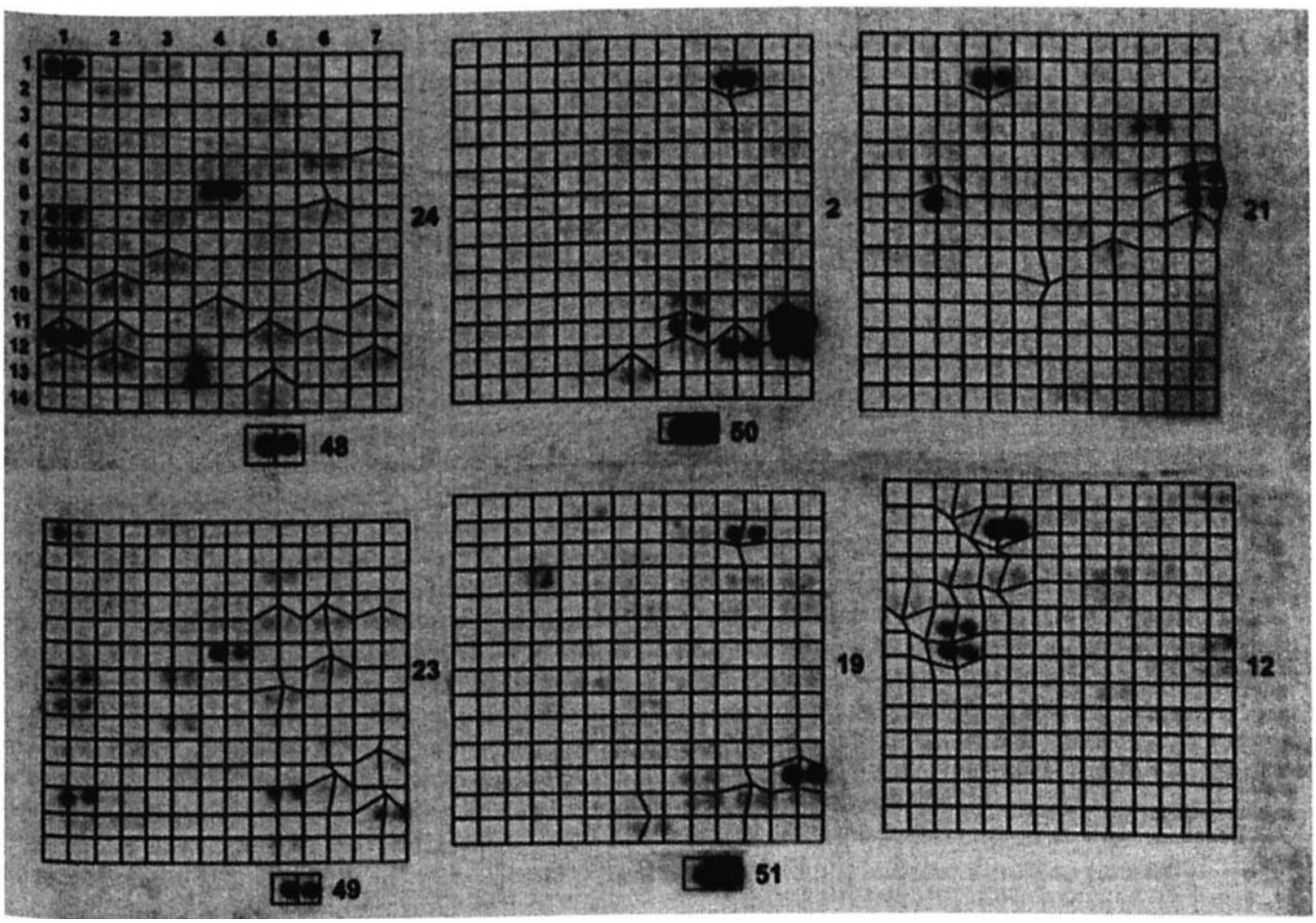

Figure 1. Control and experimental CLONTECH gene "chips" exposed to reverse transcriptase ^{32}P-transcribed RNA from Panc-1 cells incubated with 20 μM MK 886 (28). Panc-1 cells were cultured for 24 hr with MK 886, RNA prepared, incubated with reverse transcriptase and ^{32}P-dATP, hybridized against the "chips," stringently washed, and analyzed with Image-Quant and Excel 97 according to instructions included with the CLONTECH Human Atlas Array kit. Each cDNA in columns A through G is represented as a "doublet." Control chip at the top, MK-886 sample below (28, 29).

Table I. Augmented mRNA Syntheses in PANC-1 Cells Cultured for 48 hr with 40 μM MK 886

Cell cycle	Stress related	DNA syn/repair	Cell suicide
cyclin D2,G2	heat shock 27,40,60,70	replic fact 48,40 kD	Bcl-2
myb	prot kin JNK2,3	replic pro A1	Bcl-w
jun D	diox cyto P450	replic fact 37kD	Bcl-x
myeloblastin	glutathione reduc	excis/rep ERCC-5,6	FASL recep
p21	NK enhanc fact	topo II *a* iso-enz	TNF recep 1
MAP kin3,4,6		uv excis/rep RAD 23	TNF *B*
N-myc		DNA-dep protein kin	ICH-1,2

Note. Expression of some loci from subsections A, B, and C of CLONTECH human cDNA arrays that were upregulated in the experimental array compared with the control (modified after Table 1 of Ref. 29).

without homology with the GenBank data source. PTI-1 and MMP-7 genes were overexpressed in three and four (out of five) human pancreatic cancer samples, respectively. This study begins the identification of the subset of genes modulated in pancreatic cancer by overactive K-ras.

Erb-B (Her-2-neu) overexpression has been detected by immunohistochemistry in 50% to, more recently, 17% of the pancreatic cancers examined (34, 35). Overexpression of this oncogene was associated with reduced survival from eight to five months. EGF-2 encompasses the HER-2 or c-erb-B2, HER-3 (c-erb-B3), and HER-4 (c-erb-B4) receptors, with intracellular components exhibiting tyrosine kinase activity against various substrates, including phospholipase C. *In vitro* overexpression of this oncogene can malignantly transform cultured cells.

The AKT2 gene (protein kinase B), amplified in 10% of pancreatic cancers, can malignantly transform cultured cells (36). The Raf kinase-MEK-ERK pathway "cross-talks" with the phosphoinositol-3-kinase- (PI-3-Kinase) Akt kinase pathway (37), while Akt inhibits Raf by phosphorylating a regulatory site on that protein, as reported for breast cancer cells (38). It was speculated that this interaction regulates the response to insulin-like growth factor. C-Myb at 6q24 is amplified in a small number of pancreatic cancers (39).

Suppressor Genes. Four major suppressor genes have been implicated in pancreatic cancers: p16 (MTS1, INK4A, CDKN2, DPC30), P53, DPC4 (SMAD4), and BRCA2 (DPC1/2); others undoubtedly exist.

The p16 gene, located at 9p21 as part of the RB (retinoblastoma)/p16 pathway, was inactivated in 82% of samples studied, due to homozygous deletion or loss of heterozygosity with a mutation in the second allele (40). The p16 gene was also inactivated by hypermethylation of a 5′-CpG "island" in the promotor region (41). In 49 of 50 pancreatic cancers examined, the Rb/p16 pathway was inactivated. The gene product serves as a cyclin-dependent kinase (cdk) inhibitor, binding to cdk4 and cdk6 and negatively regulating cyclin D-dependent phosphorylation of the Rb protein, which in its less phosphorylated form normally inhibits the G1 to S transition. In another study, the Rb/p16 pathway was abrogated in more than 90% of pancreatic cancer patients, due to inactivation of the p16 (CDKN2) gene (42). More than 40% of pancreatic adenocarcinomas studied showed a loss of p16 expression associated with a worse histologic grade, increased metastases, and a shortened survival of patients lacking this expression. Disruption of the RB/E2F phosphorylation/dephosphorylation cycle due to loss of inhibition of cyclin-dependent kinase activity allows expression of E2F and DP-1 transcription factors, promoting proliferation.

In response to DNA damage, the wild-type P-53 gene limits passage through the cell cycle and can initiate PCD. P53 gene mutations at 17p13, generally point mutations in exons 5 to 8, rather than deletions, are perhaps the most widespread of all human cancer mutations (43). Serum concentrations of P53 may be a useful marker for measuring progression of pancreatic adenocarcinoma, and increases correlate with the accumulation of mutant P53 protein (44).

The products of DPC4 (deleted pancreatic cancer, also SMAD4) mutations at 18q21.1, related to the Drosophila MAD genes and deleted in one-half the pancreatic samples examined, function in TGF-β signal transduction (45). Inactivation is due to homozygous deletion or loss of heterozygosity, coupled with intragenic mutation of the other allele. P21(waf1), which normally limits cell cycle progression, is one of the downstream genes under positive control by DPC4 (46). Loss of DPC4 expression occurs late in the progression of intraepithelial pancreatic neoplasia (47). This loss may provide a useful marker for risk of subsequent overt invasive development. A high concordance of DPC4 and P16 inactivation has been noted in pancreatic cancer, suggesting that genetic inactivation of the latter increases the selective survival advantage of subsequent mutation in DPC4 (48). However, no significant clinical or physiologic associations were evident.

BRCA2 on 13q12.3 is inherited as a germ-line mutation in about 7% of sporadic pancreatic cancers (49). This compares with less than 4% in sporadic breast or other cancers.

In one study, infrequent mutations of RB1 and DCC tumor suppressor genes were detected (50). The genes DCC (deleted in colon cancer), SMAD2, B-catenin, and the APC (polyosis coli) genes are rarely affected in pancreatic cancers. The met oncogene (a hepatocyte growth factor) is expressed in a small number of pancreatic cancers, but the significance of this is not evident (51). FHIT (fragile histidine triad) gene expression in the regions of 3p14.2, suggested as a candidate suppressor gene, was examined in two

normal, 21 primary pancreatic ductal carcinomas, and 19 pancreatic cancer cell lines (52). A complex pattern of its presence, abnormal expression, or absence occurred, leading to the conclusion that FHIT protein expression in pancreatic cancer correlated with absence or alteration of its mRNA, often with FHIT gene anomalies.

MAPK Kinase 4 (MKK4) may serve as a tumor suppressor, and this is based on evidence from a variety of cancers, including those of the pancreas (53). It phosphorylates JNK1 and p38, but not ERK1. Homozygous deletions were present in 2% of 92 pancreatic adenocarcinomas, and one somatic missense mutation was present in 45 screened pancreatic carcinomas. MKK4 can be activated by MEKK, a component of a Ras-dependent cytokine/stress-induced signaling cascade involving JNK1 and p38. The MKK4 pathway is believed to be distinct from the P53, p16, DPC4, and BRCA2 signaling pathways.

Other Cell Cycle Modulators. Altered p16 (CDKN2, MTS1) gene expression affecting a subset of pancreatic intraductal lesions that contain K-ras gene mutations may identify high-risk precursors of invasive malignancy (54). The loss of $p27^{Kip1}$, a cdk inhibitor that normally regulates cell cycle progression from G1 to the S phase by binding to the cyclin E/cdK2 complex, independently predicts poor prognosis in patients with resectable adenocarcinoma (55). In immunostained pancreatic cancer samples, the presence of p21 was associated with earlier clinical stage and strong p53 staining was associated with an advanced clinical stage (56). Adjuvant chemotherapy or radiation improved survival if tumors expressed $p21^{WAF/1CIP}$ without P53. The conclusion was that following DNA damage, wild-type P53 protein activates the WAF/CIP-1(p21) gene, leading to G1 arrest.

Overexpression of cyclin D1 in human pancreatic adenocarcinoma is associated with poor prognosis (57), while its inhibition leads to reduced proliferation, increased chemosensitivity, and decreased expression of multiple chemoresistance genes (58). Stable transfection of a cyclin D1 antisense construct in Panc-1 and COLO-357 human pancreatic cancer cells was used to reach this conclusion. In MiaPaCa-2 and Panc-1 cells, a constitutively active FRAP-$p70^{s6K}$ signal transduction pathway regulated cell growth and cyclin D1 expression (59). Rapamycin, a selective FRAP inhibitor, reduced p70 kinase activity, p33 CDK2, and p34 CDC2, and led to dephosphorylation of p70 kinase and 4E-BP1. Proliferation, whether anchorage-dependent or -independent and cyclin D1 expression were strongly inhibited, suggesting that disruption of this pathway could provide a new approach to therapy of this disease.

The transforming, membrane-associated Src gene was overexpressed in 33 (70%) of 47 patients with pancreatic ductal adenocarcinoma, in conjunction with IGF-1R-dependent increase in cell proliferation in 30 patients (64%) (60). It was suggested that Src- and IGF-1R- related phosphorylation of the transcription factor STAT resulted in increased antiapoptotic protein Bcl xL, inhibiting the pro-apoptotic factor, APAF-1, thereby reducing activation of pro-caspase 9 to the active pro-apoptotic protease, caspase 9 (11).

DNA Methylation: DNA Repair. Methylation of CpG islands of DNA bases at selected sites represents a common mechanism of gene silencing. Ukei *et al.* (61) report the hypermethylation of at least one of the genes assessed in 60% of 45 pancreatic cancer samples studied by PCR. Genes studied included RABB (20% of those studied methylated), p16 (18%), CACNAIG (16%), TIMP-3 (11%), E-cad (7%), THBS1 (7%), hMLH1 (4%), DAP kinase (2%), and MGMT (0%). Hypermethylation was chiefly present in pancreatic cancers and only three loci, E-cad, DAP kinase, and MINT2, were methylated in some normal pancreata (36%, 21%, and 14%, respectively). The simultaneous expression of four loci was observed in five of 36 (14%) samples, defining a subgroup as "CpG island-methylator-phenotype positive," or the CIMP + phenotype. Two of four samples with microsatellite instability exhibited hypermethylated hMLH1 and were CIMP positive.

Univariate analysis has shown that histologic grade, nuclear grade, ploidy (diploid versus aneuploid), and DNA index are negatively correlated with prognosis (62). An incompletely characterized group of enzymes, including hMSH2, hMLH1, PMS1, and PMS2, which some classify as tumor suppressors, are responsible for DNA mismatch repair. Their clinical importance was first noted in hereditary nonpolyposis colon cancer and in some instances of pancreatic carcinoma. Following loss of function, mutations occur in repeated sequences (RER, replication errors), leading to "microsatellite instability" (63). These cancers express poor differentiation, a syncytial growth pattern with expansive borders, are frequently diploid, and express wild-type K-ras genes. This may represent a pathway distinct from most ductal carcinomas. DNA replication errors are thought to occur in about 4% of pancreatic carcinomas. However, in one study three out of nine pancreatic cancers examined exhibited microsatellite instability for two or more dinucleotide repeat markers, suggesting that such instability may contribute to some pancreatic cancers (64). It is of interest that BRCA1 protein can function as a mismatch repair protein with hRad50 in a complex with hMer 11-p95/nibrin complex (65).

In a study of 42 pancreatic cancers compared with 24 control samples from organ donors, DNA adducts, oxidative damage, and genetic polymorphisms of genes associated with response to these activities, including CYP1A1, CYP2E1, NAT1, NAT2, GSTM1, MnSOD, and hOGG1 genes, were assessed by PCR (66). The number of adducts/10^8 nucleotides and 8-OH-deoxyguanine content were increased in the cancer samples. DNA adducts and polymorphism of CYP1A1 was correlated; the level of 8-OH-dG did not correlate with any genetic polymorphism. These results are consistent with the hypothesis that DNA damage and oxidative stress are involved in the genesis of human pancreatic cancer and that polymorphisms of CYP1A1 affect

the extent of adduct formation. GADD-45, the growth-arrest and DNA-inducible protein that functions downstream from p53, was studied in 63 cases of intraductal pancreatic carcinoma with paraffin-embedded immunostained samples (67). Fifty-two percent were positive for the protein, which was not correlated with age, gender, clinical stage, or histologic grade. Negative expression did correlate with slightly longer survival and better response to chemotherapy.

Contemporaneous Expression of These Genomic Alterations. Of interest is the extent to which different subsets of these genes are expressed in pancreatic cancer, but few studies are available. In an interesting study, the incidence of mutations affecting p16, p53, DPC4, and k-ras in 42 pancreatic adenocarcinomas was scored (47). Seventy-six percent of the cancers contemporaneously expressed either three or four mutations, including an abnormal K-ras in all of them.

Growth Factors, Cytokines, Their Ligands, and the Interplay with Other Genomic Elements

Epidermal Growth Factor Receptors and Ligands. Pancreatic cancers are reported to express increased concentrations of EGF receptor, and EGF and TGF-α ligands. These ligands, and others including amphiregulin, B-cellulin, and heparin-binding EGF-like growth factors, activate the receptors and increase proliferation of pancreatic cancer cells (68, 69). They can also increase expression of the EGF receptor. This and other evidence (HER-2 neu expression, presence of increased cognate mRNAs, etc.) support the belief that pancreatic cancer cells utilize these mechanisms to provide autocrine and paracrine stimulation. Signal transduction mediated by the receptor-associated intracellular tyrosine kinases results in phosphorylation of intermediary molecules, leading to augmented enzyme activities including phospholipase C. In an interesting combination of chemotherapy and biological therapy, human pancreatic carcinoma L3.6p1 cells in nude mice treated with gemcitabine and an anti-EGF receptor antibody exhibited reduced proliferation, increased apoptosis, and fewer metastases (70).

Tumor Growth Factor Receptors and Ligands. Tumor growth factor receptors-β types 1, 2, and 3 and their cognate growth factors are present in most mammalian epithelial cells. Generally, they reduce cell growth rate and can alter differentiation, angiogenesis, intercellular matrix components, and immune function (68, 69, 71). Messenger RNAs for several of these components are increased in pancreatic cancer cells, which, however, do not reduce their growth rate when challenged with TGF-β. Normally, the ligand increases cyclin-dependent kinase inhibitors p15 (Ink4B), p21(Cip1), p27(Kip1), and TGF-β 1, mRNA and protein levels. Type 1 TGF receptor subclass ALK3 is the major receptor component involved in signal transduction in pancreatic cancer cells. The tumor suppressor gene DPC4 (Smad4) functions as a transcription factor in the TGF pathway. Deletion of DPC4 in about one-half of the pancreatic cancer samples examined suggests that lack of the negative feedback promotes overexpression of dysregulated downstream functions (39, 40). The TGF-β-inducible gene (TIEG) is expressed in pancreatic ductal epithelial cells as an early response gene, which when overexpressed in the TGF-β sensitive Panc-1 cell line, induces apoptosis (72).

Cytokines and Chemokines. Tumor necrosis factor α upregulates platelet-derived growth factor (PDGF) in pancreatic cancer cells, which is suggested as one of the reasons for the extensive connective tissue response to that cancer (69, 73–75). TNF receptors type 1 and 2 are present, and activation of at least the former results in upregulation of protein kinase C, phospholipase A2, and lipoxygenases—all components of TNF signaling pathways. Additional factors reported to be synthesized by pancreatic cancer cells include G-CSF, GM-CSF, IL-1β, interferon 1γ, and IL-6, with complex relationships among them. For example, TNF-α can upregulate EGF receptor expression, with a positive effect on proliferation. Although the expression of IL-4 and IL-13 receptor complexes in pancreatic cancer cell lines is not consistent, those expressing IL-4 receptor may provide a target for therapy with an IL-4 toxin (76). Inhibition of EGF-induced interleukin-1-β-converting enzyme (or "ICE") with several inhibitors reduced proliferation of the pancreatic carcinoma cell line AsPC-1, but without apoptosis (77).

Other Growth Factors and Modulators of Pancreatic Cancer. The concentrations of insulin-like growth factor (78, 79) and acidic and basic fibroblast proteins (80) are increased in a significant number of pancreatic cancers, as is the expression of cholecystokinin-A and -B receptors in the rat AR4-2 pancreatic cancer cell line, compared with A-type receptors in normal rat pancreas (74). In a study of human tissues, type B receptor mRNA was present in all pancreatic and most extrapancreatic tissues, while type A receptor mRNA was present in a more restricted set, including pancreatic adenocarcinomas, but not in normal pancreatic cells (81). There is a positive correlation between the presence of either FGF or *b*FGF in cancer cells and tumor stage and the presence of *b*FGF with reduced survival of patients with pancreatic cancer. Insulin receptor substrate-2 (IRS-2) protein is a multi-site docking protein implicated in mitogenic signaling following activation by insulin of insulin-like growth factor (IGF-1) receptors. IRS-2 is overexpressed in human pancreatic cancer, which may contribute to increased mitogenic signaling via the phosphatidyl inositol-3-kinase pathway and excessive growth stimulation (69). Nitric oxide induced apoptosis in four human pancreatic cell lines and is associated with a G1-arrest and increased $p21^{WAF1/CIP1}$ cyclin-dependent kinase inhibitor (82). Arachidonic acid and its metabolites exert a positive effect on the growth of various types of cultured cells (83). The enzyme 12-lipoxygenase and its products including 12-HETE increase pancreatic cell proliferation via activation of the ERK-MAP kinase pathway, while its inhibition leads to

apoptosis (84). Tyrosine kinase, ERK, and p38 MAP kinase were activated by 12-HETE, while the JNK/SAPK pathway was not. Whether lipoxygenase inhibitors will have any role in clinical pancreatic cancer has not been established, but is being investigated.

Abnormalities in Pancreatic Cancer Cell Suicide

Therapeutic interventions can lead to several forms of non-necrotic PCD (85). The type 1 "apoptotic" (extrinsic, death domain) form is characterized by nuclear chromatin margination, disruption of DNA, engulfment in cytoplasmic vesicles, and their uptake by macrophages and adjacent cells, without eliciting an immune reaction. Intranucleosomal disruption of DNA by DNAses yields a characteristic DNA "ladder" upon agarose gel electrophoresis. Procaspases, pro-proteolytic enzymes whose activation as caspases yields many of these changes, are fundamental to these events. A variety of pro- and anti-PCD proteins, which modulate these processes, included in the BCL-2 family have been described. The type 2 (intrinsic, autophagic, mitochondria-dependent) form of PCD generally lacks several of the type 1 characteristics, instead exhibiting features of autophagy and the formation of multiple cytoplasmic vesicles without evident DNA laddering. Participation of mitochondria in this form, which is believed to be the major pathway activated by chemotherapy or ionizing radiation, is central to its expression. Recently, a third (lysosomal) type of PCD arising from activated endoplasmic reticulum and depending upon activated caspase 12 has been described.

Bcl-2, Bcl-X_L, and MCL-1 (anti-apoptotic proteins) and BAX and Bcl-X_S (pro-apoptotic proteins) are expressed in pancreatic cancer cells (86). As reviewed in Reference 11, Bcl-X_L expression reduces and co-expression of P53 and paradoxically of Bcl-2 protein is associated with increased survival of pancreatic cancer patients.

In a recent study, six pancreatic cancer cell lines were examined for their expression of Fas (CD95, apo 1)/FasL (Fas ligand), DR4 (death receptor)/Trail (tumor necrosis factor-related apoptosis-inducing ligand), and DR3/Trail receptor/ligand related "apoptotic" molecules (87). FasL and TRAIL are members of the TNF family. All cell lines expressed Fas, DR4, and DR3 receptors (assessed by RT-PCR, flow cytometry, and western blots), but none elaborated soluble FasL. PCD was not induced by activation of the Fas system and the "counter-attack model" of cancer cell-induced destruction of anticancer immune cells is excluded in these cells, due to their lack of FasL synthesis. Panc-1 cells, which overexpress the TGF-β-inducible TIEF gene, undergo apoptosis (72). The responses of four pancreatic cancer cell lines to gemcitabine correlated inversely with their Bcl-2 content (88). Bcl-xL protected Colo 357 pancreatic cells against TRAIL-induced apoptosis (89). Increased phosphorylation of the inhibitor releases NF-κB for translocation to cell nuclei. Actinomycin D can increase pancreatic cancer cell apoptosis by activating the JNK/SAPK pathway and by increasing expression of BAX (90).

We examined the response of Panc-1 cells to the 5-lipoxygenase inhibitors, MK 886 (Merck) and SC41661A (Searle) and to the free radical spin trap, NTBN (26, 91). The 5-LPOx inhibitors reduced cell proliferation and induced an atypical form of PCD characterized by a number of small "dark" cells, lack of DNA laddering, and an atypical nuclear structure with partially marginated chromatin (Fig. 2, SC) or absent characteristic nuclear changes and a

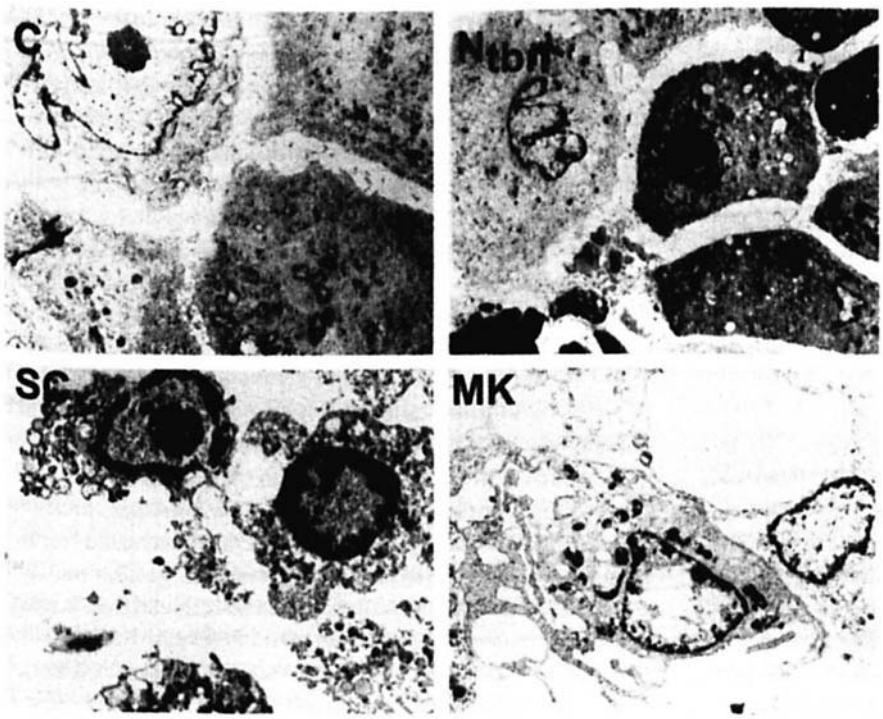

Figure 2. Electron photomicrographs of Panc-1 cells cultured with the free radical spin trap NTBN or the 5-lipoxygenase inhibitors, SC41661A or MK 886. Panc-1 cells were cultured with 20 mM NTBN or 40 μM of the lipoxygenase inhibitors MK886 or SC41661A for 24 hr and then cells were washed and prepared for ultrastructural studies according to standard methods (26, 91). (C) Control cells. (SC) SC 41661A-treated cells (Ntbn) Spin trap-treated cells. (MK) MK 886-treated cells. SC-treated cells exhibit nuclear chromatin margination, Ntbn-treated cells are characterized by the compact "dark" cells, and MK-treated cells are characterized by extensive vesicle formation in the cytoplasm and "washed out" nuclei containing residual clumps of chromatin.

widespread "cytoplasmic" form of death with vacuolization and widely separated smooth internal membranes (Fig. 2, MK). NTBN reduced proliferation and evoked small, "dark" cells without DNA laddering and ultrastructural changes more consistent with a non-type 1, probably type 2, "cytoplasmic" variant of PCD (Fig. 2, NTBN). Pinocytosis in a number of cells was increased. Panc-1 cells do not express Bcl-2 (92). MGI 114, a sesquiterpene analogue, increased PCD in six human pancreatic cell lines, with activation of caspases 3, 7, 8, and 9; the effect was inhibited by Z-VAD-fmk (93).

The NF-κB complex is constitutively activated in pancreatic cancer and can inhibit cell death (94). NK-κB can activate protective antiapoptotic proteins in at least some types of cells (95) or can promote the process in others (96) such as hepatic and lymphocyte B cells. The proteosome inhibitor PS-341 inhibited the growth of pancreatic cancer cells orthotopically implanted in nude mice, and reduced NF-κB, VEGF (vascular endothelial growth factor), and microvascular density (97). Cancers relying on NF-κB for antiapoptosis survival pathways were considered candidates for such therapy. Overexpression of FAP-1 (fas-associated phosphatase-1) and underexpression of Fas may be one mechanism for escape of pancreatic cells from FAS-mediated apoptosis (98). Bcl-xL was shown to protect human pancreatic carcinoma cells from TRAIL-induced apoptosis (89). Overexpression of autocrine IGF-1R and EGF-R ligand protected MIAPaCa-2 cells from apoptosis, while their suppression enhanced it (99). Inhibition of the phosphatidal inosital-3 kinase (PI-3-K) pathway increased the response of cultured pancreatic cancer cells to gemcitabine (100). Ras-related resistance to apoptosis in intestinal epithelial cells has been associated with constitutive phosphatidylinositol-3-kinase- (PI-3K)-mediated downregulation of Bak, a pro-apoptotic member of the Bcl-2 family (101). Detachment of nonmalignant human and rat intestinal epithelial cells resulted in reduced antiapoptotic Bcl-xL and promoted apoptosis; activated H or K-ras oncogenes abrogated this decline. Inhibition of Bcl-xL expression in ras-transformed cells promoted their apoptosis and a mechanism involving Ras-related formation of TGF-α was proposed—thus two distinct mechanisms were identified. Salicylates increased TNF-α-induced apoptosis of pancreatic cancer cells by inhibiting NF-κB due to reduced phosphorylation of its inhibitor, IκB-α (102).

Potential Therapeutic Agents and Their Combinations in a Search for Synergism

Less Common Agents Alone or in Various Combinations. In addition to inhibiting cell proliferation, the inability of therapeutic agents to readily induce sufficient PCD in solid cancer cells is considered one of the important forms of "drug resistance," leading to evolution of drug-resistant cells (2, 103, 104; but see 105 and 106 for contrary suggestions). A number of agents modulate pancreatic cell growth and death by mechanisms that generally have not been identified. Most of these potential adjuncts to therapy have not been studied in combination with current standard chemotherapy of cis-platinum or gemcitabine, with or without radiation enhancement.

Polyunsaturated fatty acids and their congeners. Gamma linolenic acid (GLA) and dihydro-gamma-linolenic acid *in vitro* kill about 40 different types of human cancer cell lines (107). GLA in conjunction with 5-fluorouracil or with vinca alkaloids augmented the cell kill. Reduced motility and invasiveness have been related to an induced re-expression of E-cadhedrins on cancer cell surfaces. Lithium salts (to increase solubility) of GLA were administered to 250 patients with cancer, 150 of which affected the pancreas. With expected survival of the patients with inoperable pancreatic cancer of 2 to 6 months, patients receiving the highest amounts of lithium GLA, some 80 g over 8 to 15 days, were reported to survive about four times longer than those receiving the lowest concentrations (108).

Eicosapentaenoic acid induces cell cycle arrest and apoptosis in Panc-1 and other pancreatic cancer cell lines (109). Another potentially interesting agent, eicosatetraynoic acid (ETYA), an arachidonic acid analogue in which four triple carbon-carbon bonds replace the C to C double bonds of AA, exerts a variety of effects on cultured mammalian cells, including inhibition of proliferation, reduction in cholesterol synthesis, the possible inhibition of farnesylation, and the induction of PCD (110). At one time it was studied as a cholesterol inhibitor, but it exhibited significant hepato-toxicity.

The cholesterol synthesis inhibitor, Lovastatin, which impairs the activity of the enzyme 3-hydroxy-3-methylglutaryl coenzyme A (HMG CoA), reduces cell proliferation and induces apoptosis in various cell lines (111, 112). At least one component of its action is the inhibition of farnesylation of ras proteins.

In animal models, perillyl alcohol, a monoterpene, inhibits carcinogenesis and cell proliferation and increases differentiation (113). The agent is believed to inhibit cholesterol synthesis, Ras farnesylation, ubiquinone and DNA synthesis, and induces growth factor expression and apoptosis. It may affect isoprenylation of other proteins besides K-ras, including Rho B and has been studied with pancreatic cancer cells (114). Several clinical studies employing this agent are in progress (115). Synergism *in vitro* between gamma linolenic acid and the 5-lipoxygenase inhibitor MK 886 was observed (26).

Phenylacetate and phenylbutyrate. Phenylbutyrate and the less active phenylacetate cause cytostasis and limited differentiation of various human cancer cell lines (116). Mechanisms implicated include reduced protein prenylation, activation of proxisome proliferator-activated receptors, DNA hypo-methylation, depletion of glutamine, and increased sensitivity to radiation due to reduced antioxidant defenses. In combination with gemcitabine, MiaPaCa human pancreatic xenografts were reduced in size, although the actual extent did not seem striking (117). In a phase 1

study of 18 patients with various cancers who received 125 mg of phenylacetate twice daily for 2 weeks, limited dose-dependent CNS toxicity of depression occurred (118). One patient with carcinoma of the prostate experienced a reduction of prostate-specific antigen lasting 1 month, and a second patient with a refractory glioma exhibited a brief response.

Farnesyl and geranyltransferase inhibitors. GTP-binding proteins associated with the Ras protooncogene superfamily include rho, rap, ral, rho, rac, and rab (119). While the final determinants may be more complex, at first approximation farnesyl transferases recognize methionine or serine at the C-terminal of ras proteins and geranylgeranyl transferases recognize leucine (excepting possibly Rab). Ras proteins are farnesylated, but other family members are geranylgeranylated. These prenylated proteins cycle between a GTP-bound active and GDP inactive states. Prenylation allows binding to a membrane of Ras with activation by guanine nucleotide exchange proteins of its GTPase, followed by dissociation from the membrane. Inhibitors of HMG-CoA prevent the formation of mevalonate, subsequently converted to five carbon chain isopentenyl pyrophosphates and finally to farnesyl and geranyl-pyrophosphates.

The farnesyl transferase inhibitor, R115777 (Janssen Pharmaceuticals) is in a phase 2 trial, with anecdotal reports of several instances of colon, pancreatic, and non-small-cell lung cancer disease stabilization (120). Granulo and thrombocytopenias, rash, nausea, and vomiting have been associated with its use. Other inhibitors under study include SCH 66336 (Schering-Plough), L-778,123 (Merck), and BMS 2146621 (Bristol Myers Squibb) (121). There is some concern that in pancreatic cancer, because K-ras exhibits a lesser requirement for farnesylation and greater affinity for geranylgeranylation, farnesyl transferase inhibitors may not be successful (122). In addition, possible alternate sites of protein farnesylation such as Rho B, a protein modulating actin, cell adhesion, and proliferation may be implicated.

Cox 2 inhibitors. Cyclooxygenase 2 (Cox 2) inhibitors, a logical extension of employing nonsteroidal anti-inflammatory inhibitors in cancer prevention, have exhibited a remarkable ability to inhibit several forms of hereditary colon polyps, although they do not appear to affect established nonhereditary colon lesions.

In about 90% of pancreatic cancers, Cox 2 may be strongly or moderately overexpressed (123). In a recent study of pancreatic cancers and cell lines, increased Cox 2 protein was found in 67% (14 of 21) pancreatic adenocarcinomas, while its mRNA level exceeded that in a sample from normal pancreas (124). Several pancreatic cell lines exhibited increased concentrations of Cox 2 protein and mRNA. Both selective (NS398) and nonselective (sulindac sulfide) Cox 2 inhibitors reduced proliferation, arresting cells in G1 (125). Cox-2 mRNA concentrations correlated positively with differentiation of the cell lines and could be upregulated by EGF when grown in serum-free conditions. While sulindac sulfide and NS398 inhibited proliferation of the cell lines examined, this was unrelated to the extent of Cox 1 and 2 expression. Both drugs together induced Cox 2 expression. Sulindac sulfone, a metabolite of sulindac, lacks anti-cyclooxygenase activity, yet inhibits cell proliferation and, in animal models, exhibits antiproliferative, pro-apoptotic activities, and its chemopreventive activity raises additional questions concerning the mechanism of action of NSAI agents (126). The role of such agents in the prevention and even treatment of pancreatic cancer is yet to be established.

Metalloproteinases. Matrix metalloproteinases (MMP) include a number of zinc-containing enzymes that selectively degrade extracellular matrix proteins. These enzymes, which are directed against collagens, stromylsins, gelatins, and other less well-characterized membrane-bound proteins, participate in tissue remodeling, neovascularization, and local or distant cellular invasion (127–129). As is generally the case with biological control circuitry, another group of proteins, the tissue inhibitors of metalloproteinases (TIMPS) modulate their function. At least 16 are known, exhibiting specificity against collagenases and other extracellular matrix proteases. As an example, collagen IV, which predominates in the extracellular matrix (ECM), is degraded by gelatinase A (MMP2) and gelatinase B (MMP9). Both cancer cells and fibroblasts release such proteases. Loss of collagen and proteoglycans in the ECM surrounding pancreatic cancers commonly occurs, with overexpression of a number of MMTs and TIMPs. Numerous growth factors facultatively activate MMP mRNA synthesis, leading to c-fos and c-myc heterodimer formation, binding at the AP-1 site, selective activation of the genome, and subsequent formation of MMP mRNAs and proteins. These proteins are activated *in vivo* by serine proteases, plasmin, cathepsins, and other active MMPs, while TIMPS bind to MMPs to inactivate them.

Of the 10 or so MMP inhibitors under development, Marimastat is probably the clinically most widely studied agent. In one study of 100 patients with inoperable pancreatic cancer receiving 25 mg of Marimastat for 28 days, as assessed by CT scan and 19.9 antibody serological level, stage II and II disease patients survived an average of 7 months compared with 3 months for controls (130). In a combination study, patients with nonresectable pancreatic cancer received Marimastat and Gemcitabine (Gemzar), achieving a median survival of 9.7 months compared with a 5.7 month median survival with Gemcitabine alone (three objective responses, 10 with stable disease, 14 with declining 19.1 antibody) (131). Marimastat was combined with 5-fluorouracil in a phase 1 dose-scheduling study (132). In a prospective study of 64 patients with advanced pancreatic cancer, Marimistat as a single agent in a phase I dose study achieved a median survival of 160 days and a 1-year survival of 21% (133). Doses of 5, 10, or 25 mg twice daily were well tolerated. However, a current view is that this agent, alone or in combination, has exhibited no advantage in the treatment of advanced pancreatic cancer. Bay 12-

9566 (Bayer) has a broader specificity against a range of MMPs, but to date, no therapeutic advantage seems to have been reported.

Retinoids and pancreatic cancer. Retinoids inhibit human pancreatic cancer cell lines, reduce xenotransplanted tumor proliferation, and increase the extent of differentiation and reduce adhesion to ECM components (134). Two retinoid receptor subtypes, RAR, which binds all-trans retinoic acids, and RXR, whose natural ligand is 9-cis retinoic acid, were detected in pancreatic cells and the expression of a protein kinase C isoenzyme was altered by retinoids. In a study of 24 pancreatic patients, no differences in RAR*a* was present. One-third of samples lacked RXR*b* and expressed less RAR*b* mRNA, as determined by *in situ* hybridization. Retinoid-sensitive lines expressed RAR*g*, and insensitive lines did not. In view of the antiproliferative *in vitro* synergism between retinoids and α-interferon, 22 pancreatic cancer patients were enrolled in a phase 2 study of cis-retinoic acid and that agent. One partial remission occurred and disease was stable (5 months) in 14 patients. The median survival of stage III and stage IV patients was 8.7 and 7 months, respectively (135).

Angiogenesis, anti-angiogenesis, and cell matrix factors. Pancreatic cancers express a number of angiogenic factors, including vascular endothelial growth factor-A (VEGF-A) and its receptors, VEGF-R1 (flt-1) and VEGF-R2 (kdr), FGF-2 (bFGF), and FGR-1, thymidine phosphorylase (PDECGF), angiogenin, and avB2 integrin (136–138). Several agents increase angiogenesis in various experimental settings, including IL-1 and IL-2, interferons, platelet factor 4, thalidomide, angiostatin, and endostatin. A number of angiogenesis inhibitors are in clinical investigation, including TNP-470, CM-101, recombinant human antivascular endothelial growth factor monoclonal antibody, SU 5416, and SU 6618. Other protease inhibitors, direct inhibitors of cell proliferation, angiogenesis growth factor inhibitors, integrin survival inhibitors, and chelators of copper, with or without penicillamine, are being evaluated in gastrointestinal and other malignancies (128, 129). PS-341, a dipeptide boronate, activated proteosome activity of pancreas (PaCa-2) or PC-3 prostate cells orthotopically implanted in nude mice, inhibited their proliferation, increased apoptosis, and reduced the ingress of new blood vessels associated with reduced VEGF and inhibition of NF-κB (94, 139). A phase I trial of this agent is reportedly underway. In a recent study, 10 of 15 pancreatic cells lines secreted high concentrations of VEGF (140). When transplanted into nude mice, those with high levels of secretion produced larger primary tumors and more numerous metastases, a site for potential interdiction. Tissue factor is overexpressed in advanced pancreatic cancer (141). It can upregulate the expression of plasminogen activator receptor and can facilitating tumor invasion and metastases.

CD44 includes a heterogeneous family of proteins with protective functions in cell-cell and cell-matrix adhesions. Splice variants containing exon v6 appear to be involved in tumor metastases. As CD44v6 is significantly reduced in pancreatic carcinoma, it may provide a prognostic marker for the disease, since patients with low serum concentrations had reduced median survivals (142).

Free radical spin traps. N-tertiarybutyl-phenylnitrone (NTBN), a free radical spin trap, at low concentration inhibited PCD in those cell lines in which it was studied (143). However, higher concentrations (10 mM) inhibit proliferation and induce an atypical form of PCD, primarily cytoplasmic in location (Panc-1 cells) (91), in contrast with U937 cells in which a "classic" form of type 1 PCD occurs (144). Free radical spin traps at low concentrations, which do not induce PCD, protect against ionizing radiation or therapy-induced oxidation events (145, 146). A large number of signal transduction and other biochemical events require free radicals at some point during their implementation (147).

Thalidomide. Thalidomide improves the clinical course of patients with multiple myeloma, and this occurs for a variety of potential reasons (148, 149). The agent inhibits ICAM, downregulates IL-6, a growth factor for myeloma, and downregulates TNF-α and VEGF, among many activities (150). To date, we know of no results from clinical trials of the agent in pancreatic cancer.

Tyrosine kinase inhibitors. Inhibitors of tyrosine kinases show promise for a highly selective reduction in malignant cell growth (151). The use of STI 571 in chronic myelogenous leukemia expressing the BCR- ABL hybrid gene coding for a fusion protein regularized the peripheral blood counts of 31 out of 31 patients for periods up to 8 months (152). The tyrosine kinase inhibitor CEP-701 (KT-5555) has antitumor activity against Panc 1 cell line xenografts and *in vivo* invasiveness in athymic nude mice and rat trachea. Ten milligrams subcutaneously twice daily for 21 days reduced tumor growth without morbidity or mortality (153). The possibility of selective inhibitors against specific receptors and their associated kinases, including EGF, FGF, PDGF, Src, Flk-1, and VEGF-R1 (Flt-1), the latter directed against neovascularization, represent potentially powerful agents against a variety of malignant and benign diseases.

Telomerase activity. The RNA-dependent DNA polymerase that maintains and extends teleomere length in normal immortalized stem cells and in malignantly transformed cells is considered normally to "record" the remaining number of potential cell divisions (154). With continued shortening of telomeres, cells enter senescence while continued activity with maintenance or extension confers a degree of immortality. Factors influencing expression of telomerase include c-myc, Bcl-2, p21(waf1), Rb, p53, PKC, Akt/PKB, and protein phosphatase 2A at various levels of gene expression and post-translational function. Transfection of telomerase genes extends the lifespan of otherwise senescing cells (155); inhibition of the enzyme with an antisense compound shortens cell survival (156). Telomerase overactivity was present in the majority of pancreatic cancers studied (157). Detection of telomerase activity in cells obtained by endoscopic retrograde pancreatic duct brushing

may aid in diagnosing the disease (158). We found no reports of clinical studies with such agents directed against pancreatic cancer telomerase. However, gemcitabine was found to inhibit telomerase in MIA-PaCa-2 cells, which inhibition correlated with reduced proliferation (159).

Other agents of note. Other potential therapeutic agents of potential interest include topoisomerase inhibitors such as 9-nitro-camptothecin (160), arsenic trioxide, originally employed in the therapy of leukemias, but more recently under study in renal, prostate, and cervical cancers (161), and inhibitors of specific phosphatases and protein kinases (37, 38, 100, 162, 163). At present, there is little preclinical information regarding such agents in pancreatic cancer.

Modulation of Pancreatic Cell Growth with Antisense Compounds. Examples *in vitro* in which antisense compounds have been employed to alter pancreatic cell growth with "therapeutic" intent have been reported. Gastrin and cholecystokinin are autocrine growth factors for human pancreatic cancer (74). *In vitro* and *in vivo* studies in which cells cultured for 48 hr with 0.5 to 10 μM concentrations of a 20-mer antisense phosphorothioate to gastrin exhibited reduced growth (88% *in vitro*) with reduction in size (about 50%) and secretion of the cells *in vivo* (164).

An antisense molecule to amphiregulin, a ligand for the EGF receptor, inhibited the growth of pancreatic cancer cells and reduced its rate of synthesis and amount detected by immunoassay (165). Inhibition of the EGF receptor/ligand and associated signal transduction pathway has been a popular site for interdicting cancer cell growth.

A transfected PKCα antisense in AsPc1 pancreatic cells partially inhibited retinoic acid-stimulated proliferation (166). Antisense K-ras-transduced AS-PC-1 cells inhibited proliferation of peritoneally disseminated pancreatic cancer (167). More recently, an anti-k-RAS-expressing adenovirus vector suppressed the hepatic growth of AcPC-1 pancreatic cancer cells in nude mice, as described previously (33). Particular genes that were up- or downregulated may represent potential targets for therapy, either from their inhibition or augmentation, respectively. ISIS 5132 is a phosphorothioate antisense compound directed against c-raf mRNA, the product of which is a downstream component of the k-RAS signaling system; an ECOG phase II trial of advanced pancreatic cancer is in progress (121).

Genomic Control as Massive Parallel Processing

Ideas of parallel distributive processing and complex adaptive systems have been invoked to describe the information-containing, -expressing, and perhaps even -generating aspects of cells (168). An analogy between the cellular response to metabolic "stress" and parallel nonlinear processing algorithms employed in neural networks with their connections via "nodes" and an ability to alter their internal structure through weighting procedures has been suggested (169, 170). The concept of "redundant complexity" is a further extension of this concept (171). These ideas apply to cells and their collectives over evolutionary time. Additional complexity is introduced by multiple uses of a particular protein, e.g., Bcl 2 can serve at least six different functions depending upon the developmental stage and type of the target cell. Utilization of molecular "cassettes" for different purposes has been proposed (172). It seems that as cancer cell karyotypes become increasingly disorganized, their ability to respond to metabolic stress should decline; why this does not happen is obscure. Redundant metabolic "circuitry" has evolved that allows mammalian cells to adapt within a comparatively narrow environmental "window"; chemotherapy of solid cancers is unable to exceed these limits for the N^{th} cell destined to survive therapy. The problem seems to be how to shift the balance toward cell death of these evolving variants or otherwise prevent their evolution.

Genetic Modifications of Pancreatic Cancers with Therapeutic Intent

This topic has been recently reviewed (173). We paraphrase the logic of this approach and cite some examples. In any cancer, replacement of defective suppressor genes or their function should represent one therapeutic avenue. In pancreatic cancer this might include provision of wild-type suppressor P53, DPC4, or p16 genes in the event they are defective, or augment expression of some normal gene that might compensate for their defective expression. Inhibition of oncogene expression in pancreatic cancer could encompass suppressing mutant K-ras or ERB2 gene-function. While replacement of defective suppressor gene function or inhibition of dominant, promalignant gene expression so far has not proven clinically useful, efforts to implement these approaches represent a major therapeutic effort.

Retroviral transfer of P53 in a replication-defective adenovirus reduced the growth of pancreatic cancer cells and increased their extent of apoptosis (174). The EB1 attenuated adenovirus, ONYX-015 caused tumor-specific cytolysis of pancreatic cells and augmented traditional chemotherapy (175). This indicates that one way to increase drug response is to combine it with an additional distinct, metabolically unrelated therapy and to look for synergism rather than focusing on modulating MDR protein function *per se* (176, 177). Adenovirus transduction of p21(waf1) to pancreatic cancer cells limited their passage past GoG1 and reduced their rate of proliferation (178); similar results were obtained in lung and other gastrointestinal cancer cells with overexpression of p16 (179). Both gene products inhibit the function of cyclin-dependent kinases required for passage into and through the cell cycle. In studies with lung and other cell lines, overexpression of TNF receptor or a receptor for FAS-estrogen receptor fusion protein reduced cell proliferation.

A cationic, liposome-encased β-galactosidase gene construct delivered intra-arterially or intra-ductally was expressed in lining ductal cells for up to 28 days (180). Studies with ribozyme-related anti-p53 RNA and ERB-2 antisense

compounds in lung and breast cancer do not yet seem to have been extended to studies of pancreatic cancer. Antisense compounds directed against Bcl-2 increased the extent of PCD, augmented by additional therapy (181, 182). Several anti-K-ras antisense compounds are active against Panc-1, MiaPaCaII, and other human cancer cells, in part through inhibition of MAP kinase activity and induction of apoptosis (183). A limited number of patients with different cancers, including those of the pancreas, have been treated with ras antisense compounds, but objective tumor responses seem to have been lacking. Other examples of antisense compounds inhibiting pancreatic cancer cell growth were cited earlier.

A further approach is represented by genetic pro-drug activation in which "suicide" genes are introduced into cancer cells, which can act selectively within the target cells on administered pro-drugs (184). Vectors have included the combination of herpes simplex thymidine kinase/ganciclovar or *Escherichia coli* cytosine deaminase/5-fluorocytosine. Targeting is attempted either by selection of a specific cell type, as in the case of dividing cancer versus nondividing CNS cells, or by development of ligands against tumor-specific cell surface markers. An adenovirus/amylase construct against pancreatic cancer cells has been developed (185), while a phase I clinical trial of lethally irradiated allogeneic pancreatic tumor cells transfected with GM-CSF in pancreatic cancer is in progress (186). Immunotherapy, including cytokine and costimulatory gene therapy, tumor antigen vaccine, and dendritic cell studies are outside the purview of these comments.

Overview

Absent radically innovative ideas and discoveries, the best hope for the near future appears to be to continue the present strategy of combining available agents in configurations that ideally limit toxicity while synergistically interdicting fundamental cancer cell properties.

Why do solid cancers usually fail chemotherapy (176)? At least one major impediment to a more successful response is believed to be the unintentional induction of multiple countervailing responses, e.g., by ionizing radiation, MK 886, or other unselective agents (28, 29), tending to reverse the desired therapeutic effect; an inadvertent sowing of "dragons teeth" (187). Furthermore, to the extent that chemotherapy or radiation-induced type 2 PCD is essential for a successful therapeutic response and participation of mitochondria is central to that outcome (188, 189), initial or subsequent reduction in mitochondrial mass, as cells become less differentiated, may strongly bias against successful therapy and may promote the emergence of resistant cells. With more focused therapies, blockade of specific protein kinases, and use of receptor antibodies, antiangiogenesis agents, etc., this should become less of a presence in therapy (152).

It may be true that specifically targeted therapy such as interferon or retinoic acid against acute promyelocytic leukemia or tyrosine kinase inhibitors against BCR-ABL in myelogenous leukemias will limit malignant hematopoietic cell proliferation for an extended period of time and may even induce a degree of differentiation. It is an open question as to whether a single highly selective "magic bullet," e.g., an anti-angiogenesis agent, a reagent directed against an important fusion protein, etc., can sufficiently confine solid cancer cell growth and spread or whether several agents, each addressing a different deleterious property of the malignant cell, are necessary. It would not be surprising if destruction of evolving solid cancer stem cells requires additional selectively lethal events, but this supposition certainly depends upon presently unknown details of cellular metabolic "wiring" and their context.

As a first approximation, employing a combination of agents directed against targets expressed by an individual patients' cancer, based upon data from cDNA (Fig. 1) or proteonomic arrays, and intending simultaneously and selectively to interdict biochemical pathways required for the expression of malignant properties, may synergistically impair their aggregate expression and limit the emergence of resistant clones. These agents could include antisense compounds, antibodies to receptors, vectors containing replacement genomic information, other agents as they become available, and even current chemotherapeutic drugs (190) capable of affecting dysfunctional targets expressed by cancer cells.

As a hypothetical example, if Her-2-neu were overexpressed, consider Rautuxamab; if a ras oncogene, employ farnesyl transferase inhibitors or possibly a semi-selective tyrosine kinase inhibitor; were both genes dysfunctional, employ both inhibitors simultaneously, etc. Therapeutic decisions would be based on the dysfunctional genomic expression of an individual patients' cancer. The combination of an agent that activates type I PCD caspases in pancreatic cancer cells, such as the sesquiterpene analogue MGI 114 (93) with a chemotherapeutic agent that instead activates the type 2 pathway (187, 188), might exhibit synergistic effects on cell proliferation and death.

The transition to identification and therapeutic modification of discrete pathways responsible for the malignant properties of cancer cells by more precisely targeted and therefore generally less randomly cytotoxic agents seems inevitable, and presently represents the major hope for future treatment of pancreatic and other cancers.

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