Interferon γ inhibits growth of human pancreatic carcinoma cells via caspase-1 dependent induction of apoptosis

K M Detjen, K Farwig, M Welzel, B Wiedenmann, S Rosewicz

Abstract

***Background and aims*—The poor prognosis of pancreatic cancer is partly due to resistance to a broad spectrum of apoptotic stimuli. To identify intact proapoptotic pathways of potential clinical relevance, we characterised the effects of interferon γ (IFN-γ) on growth and survival in human pancreatic cancer cells.**

***Methods*—IFN-γ receptor expression and signal transduction were examined by reverse transcriptase-polymerase chain reaction (RT-PCR), immunoprecipitation, western blot analysis, and transactivation assays. Effects on cell growth and survival were evaluated in terms of cell numbers, colony formation, cell cycle analysis, DNA fragmentation, and poly-(ADP ribose) polymerase (PARP) cleavage.**

***Results*—All four pancreatic cancer cell lines examined expressed functional IFN-γ receptors and downstream effectors, including the putative tumour suppressor interferon regulatory factor 1 (IRF-1). IFN-γ treatment profoundly inhibited anchorage dependent and independent growth of pancreatic cancer cells. Cell cycle analyses revealed subdiploid cells suggesting apoptosis, which was confirmed by demonstration of DNA fragmentation and PARP cleavage. Time and dose dependency of apoptosis induction and growth inhibition correlated closely, identifying apoptosis as the main, if not exclusive, mechanism responsible for growth inhibition. Apoptosis was preceded by upregulation of procaspase-1 and accompanied by proteolytic activation. Furthermore, the caspase inhibitor z-vad-fmk completely prevented IFN-γ mediated apoptosis.**

***Conclusions*—These results identify an intact proapoptotic pathway in pancreatic cancer cells and suggest that IRF-1 and/or procaspase-1 may represent potential therapeutic targets to be further explored.**

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Keywords: interferon γ; apoptosis; caspase-1;interferon regulatory factor; pancreatic cancer

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The type II cytokine interferon γ (IFN-γ) is capable of eliciting potent growth inhibitory effects in a number of tumour models in vitro and in vivo.[1–5] Direct actions of IFN-γ on tumour cells as well as indirect mechanisms such as immunomodulation[6] and antiangiogenesis[7] contribute to antiproliferative actions. However, direct effects appear to be highly tissue and cell type specific. As IFN-γ receptors are relatively ubiquitously expressed in different cell types,[8] specific loss of biological responsiveness in the context of malignant transformation has been suggested and has subsequently been demonstrated in lung tumour, melanoma, and endothelial cells.[9–11]

IFN-γ exerts its biological effects via cross linking of a heterodimeric receptor, thereby causing tyrosine phosphorylation of the receptor associated tyrosine kinases Jak-1 and Jak-2. These in turn phosphorylate the latent cytosolic transcription factor Stat-1 at tyrosine residues (reviewed by Bach and colleagues[8] and Platanias and Fish[12]). On phosphorylation, Stat-1 proteins convert from the latent to the DNA binding state, dimerise, and translocate to the nucleus where they activate transcription from IFN-γ response elements (GAS) and thus induce changes in gene expression that are thought to account for the pleiotropic cellular effects of IFN-γ.[13]

Key regulators of cellular growth control have been shown to be differentially regulated by IFN-γ in diverse cell models, leading to disparate biological responses such as G1 arrest,[14–17] S phase retardation,[14 18] or induction of apoptosis.[19] In some models, the antimitogenic actions of IFN-γ have been further dissected at the cell cycle level and cell cycle arrest in the early or late G1 phase was linked to accumulation of the hypophosphorylated growth restrictive form of the tumour suppressor protein retinoblastoma protein (Rb). This functional activation of Rb was attributed to downregulation of G1 cyclins[18] and/or induction of cyclin dependent kinase inhibitors (CKIs) such as $p21^{cip1}$[16 20 21] and/or $p27^{kip1}$.[22 23] In addition, induction of the double stranded RNA activated protein kinase (p68 PKR)[24] and downregulation of cellular c-myc[25] levels were reported, although these events were not associated with phase specific cell cycle changes.

Abbreviations used in this paper: RT-PCR, reverse transcriptase-polymerase chain reaction; PARP, poly(ADP ribose) polymerase; TNF-α, tumour necrosis factor α; DTT, dithiothreitol; IRF, interferon regulatory factor; Rb, retinoblastoma protein; CKI, cyclin dependent kinase inhibitor; PBS, phosphate buffered saline; PBST, phosphate buffered saline+0.1% Tween; FCS, fetal calf serum; PVDF, polyvinyl difluoride; SDS-PAGE, sodium dodecyl sulphate-polyacrylamide gel electrophoresis.

Recent interest has focused on IFN-γ mediated induction of apoptosis. Apoptosis or programmed cell death is an active tightly regulated process characterised by a set of morphological and biochemical changes, including chromatin condensation, internucleosomal DNA cleavage, membrane blebbing, and the formation of apoptotic bodies.[26 27] In spite of diverse stimuli, apoptotic pathways eventually converge in the activation of a cascade of well conserved cysteine proteases (caspases) which act as principle executioners of the apoptotic process.[28 29] Caspases in general are expressed as inactive proenzymes and become proteolytically activated in response to proapoptotic stimuli. Under physiological conditions, apoptotic cell death is induced predominantly by stimulation of death domain containing receptors such as Fas (CD 95) or tumour necrosis factor α (TNF-α) which have been demonstrated to be regulated by IFN-γ.[30 31] Also, modulation of HLA expression by IFN-γ indirectly regulates apoptosis by targeting immunosurveillance mechanisms.[32] In contrast, the relevance of direct proapototic actions of IFN-γ in tumour cells remains controversial, based on the striking diversity of biological effects in different tumour entities which range from potent apoptosis induction[33 34] to antiapototic protection[21 35] or IFN-γ resistance.[10 15] Moreover, the molecular determinants of either apoptosis or cell cycle arrest as alternative biological outcomes of IFN-γ treatment are poorly understood and likely intimately connected to the tumour specific set of oncogenic alterations. As defects in G1 cell cycle checkpoint control and/or apoptotic pathways contribute to a varying extent to the transformed phenotype of individual tumours, it has proved difficult to predict IFN-γ effects on a given tumour cell. Accordingly, the therapeutic potential of IFN-γ so far remains to be evaluated for each tumour entity individually.

Pancreatic cancer is characterised by defective G1 control as a result of loss of p16 and deficiency of apoptotic pathways due to loss of the tumour suppressor p53.[36] Additional defects of apoptotic pathways appear likely in view of the fact that pancreatic cancer cells are resistant to a broad variety of proapototic stimuli, such as most chemotherapeutic approaches and radiation therapy.[37] As a consequence, patients with advanced non-resectable pancreatic cancer face a dismal prognosis with median life expectancy measured in the range of 4–6 months, which ranks pancreatic cancer fifth as a cause of cancer related deaths in Western countries.[38]

Thus an improved understanding of the prevalent defects in cell cycle control and apoptosis signalling as well as detection of intact proapoptotic pathways may help to delineate new improved strategies in the treatment of pancreatic cancer. In view of the clinical availability and known antiproliferative and proapototic potential of IFN-γ, we tested the actions of this cytokine in a representative panel of human ductal pancreatic carcinoma cell lines and defined the growth regulatory mechanisms.

Materials and methods

MATERIALS

Dulbecco's modified eagle medium, RPMI 1640 medium, phosphate buffered saline (PBS), and Lipofectamine transfection reagent were purchased from Gibco BRL (Berlin, Germany). Fetal calf serum (FCS), trypsin/EDTA, penicillin, and streptomycin were from Biochrom (Berlin, Germany). Recombinant human IFN-γ and the Cell Death Detection ELISA were obtained from Boehringer Mannheim (Mannheim, Germany). Z-vad-fmk and the anti-poly(ADP ribose) polymerase (PARP) antibody were purchased from Calbiochem/Oncogene Research Products (Bad Soden, Germany). Other primary antibodies were from Santa Cruz Corporation (Santa Cruz, California, USA) and secondary antibodies from Dianova (Hamburg, Germany). Reagents for western analysis were from BioRad Laboratories (Munich, Germany), except for protein A-sepharose beads which were from Sigma Chemical Co (Deisenhofen, Germany), polyvinyl difluoride (PVDF) membranes from NEN (Köln, Germany), and nitrocellulose membranes from Pharmacia Biotech (Freiburg, Germany). Reverse transcriptase-polymerase chain reaction (RT-PCR) reagents were from Promega (Heidelberg, Germany). All other reagents were obtained from Merck (Darmstadt, Germany).

CELL CULTURE

Human pancreatic carcinoma cell lines AsPc-1, Capan-1, and Capan-2 were obtained from the American Type Tissue Culture Collection. Dan-G cells were provided by the Deutsches Krebsforschungszentrum (Heidelberg, Germany). AsPc-1, Capan-1, and Dan-G cells were grown as subconfluent monolayer cultures in RPMI 1640 medium supplemented with FCS (AsPc-1, 20%; Capan-1, 15%; Dan-G, 10%), and Capan-2 cells in Dulbecco's modified eagle medium supplemented with 10% FCS. All media contained 100 U/ml penicillin/streptomycin and 2 mM L-glutamine. Cells were maintained in 95% air and 5% CO_2 at 37°C. Experiments were carried out in the log phase of growth after cells had been allowed to attach overnight.

RNA PREPARATION AND RT-PCR

Total RNA was prepared using RNAzol R reagent (WAK Chemie, Bad Soden, Germany) according to the manufacturer's instructions. RNA was submitted to DNAse digestion and aliquots of 1 μg were used for reverse transcription using Moloney murine leukaemia virus RT. The following 5'-primers and 3'-primers were designed complementary to the nucleotide sequence of the human IFN-γ-receptor α-chain: 5'-ACG-CAGAAGGAAGA TGATTGTGACG-3' and 5'-TCTATTGGA GTCAGATGGCTG-CCC-3'. The expected size of the PCR amplificate was 559 bp. The reaction was carried out in 10 mM Tris HCl buffer (pH 8.3) containing 50 mM KCl, 0.01% Triton X 100, 1 mM $MgCl_2$, 200 μM of each dNTP, 50 μM of each primer, and 2.5 U of Thermus aquaticus DNA polymerase in a final

volume of 50 µl. Amplification conditions for 35 cycles were as follows: denaturation for 30 seconds at 92°C, annealing at 60°C for 90 seconds, and extension for 90 seconds at 72°C. An additional 10 minute extension period was added to the final cycle.

IMMUNOPRECIPITATION AND IMMUNOBLOTTING

Approximately 5×10^6 cells were washed twice with PBS and lysed in 1 ml of immunoprecipitation buffer (20 mM Tris (pH 7.8), 150 mM NaCl, 2 mM EDTA, 50 mM β-glycerophosphate, 0.5% TNP 40, 1% glycerol, 1 mM Na_3Vo_4, 1 mM DTT, 10 IU/ml aprotinin, 10 mM NaF, 2 µM leupeptin, and 2 mM phenylmethylsulphonylfluoride). Lysates were sonicated and left at 4°C for 30 minutes followed by centrifugation at 15 000 *g* for 10 minutes. Aliquots of 1 mg of protein were then pre-cleared for one hour with protein A-Sepharose beads. Immunocomplexes were collected on protein A-Sepharose coated with saturating amounts of antibodies to Jak-1, Jak-2, and Stat-1, respectively. Beads were subsequently washed five times with ice cold immunoprecipitation buffer, boiled in Laemmli sample buffer, separated by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE), and transferred to nitrocellulose membranes. Non-specific binding was blocked with wash buffer (10 mM Tris HCl, pH 7.6, 150 mM NaCl, EDTA, 0.05% Tween 20) containing bovine serum albumin (1%) and ovalbumin (2%) for two hours at room temperature. Incubation with the antiphosphotyrosine antibody (1 µg/ml) was carried out overnight at 4°C and was followed by three washes in wash buffer at room temperature. After incubation with goat anti-mouse horseradish peroxidase conjugated secondary antibodies, membranes were washed 3×10 minutes in wash buffer and bands were visualised by enhanced chemiluminescence (ECL; Amersham). Membranes were stripped in a solution of 62.5 mM Tris HCl, pH 6.8, 2% SDS, and 100 mM β-mercaptoethanol for 30 minutes at 55°C and resubmitted to immunoblotting with the appropriate concentration of primary antibody.

For analysis of Rb, pro-Caspase-1, and interferon regulatory factor 1 (IRF-1), 20 µg aliquots of the above lysates were submitted to SDS-PAGE and blotted onto PVDF membranes. Immunodetection was carried out essentially as described, except that 5% non-fat dry milk in PBS supplemented with 0.1% Tween (PBST) was used for blocking and PBST for all washing procedures.

TRANSIENT TRANSACTIVATION ASSAYS

Cells were plated in 35 mm culture dishes at approximately 60% confluency. Transient transfections were then carried out for 18 hours in Ultraculture medium (Bio Whittaker, Walkersville, Maryland, USA) utilising 10 µl of lipofectamine and 1.5 µg of the GAS-luciferase construct per well. Cells were allowed to recover for six hours in FCS supplemented medium followed by stimulation with IFN-γ in Ultraculture medium. After cell lysis, luciferase activity was determined using luciferin, ATP, and coenzyme A (Promega Systems, Mannheim, Germany), as recommended by the manufacturer.

GROWTH ASSAYS

Anchorage dependent growth

Cells were plated in 24 well culture dishes at a density of 15 000 cells/well and vehicle or IFN-γ was added. At the indicated times, cells were washed with PBS and harvested by trypsinisation. Viable cells were identified by trypan blue exclusion and counted in a haemocytometer. Triplicate wells were analysed for each condition.

Anchorage independent growth

Clonal growth of pancreatic tumour cells was examined based on colony formation in agar suspension, exactly as previously described.[39] Briefly, 10^3 cells were plated as a single cell suspension in methylcellulose and incubated over 10 days. Colonies were scored microscopically with an arbitrary cut off set at 30 cells minimum.

FLOW CYTOMETRY

A total of 10^6 cells per condition were washed in PBS and fixed in 70% ethanol at 4°C for one hour followed by resuspension in 500 µl of PBS. After addition of 10 µl RNase (10 mg/ml), cells were left for 30 minutes at 37°C and stained with 10 µl propidium iodide (1 mg/ml). Cellular DNA content was determined for 10 000 cells on a FACScan utilising "Cellquest" software (Becton Dickensen, Heidelberg). Cells with subdiploid DNA content were quantitated.

DETERMINATION OF APOPTOSIS

Cells were plated in 15 cm dishes and treated as indicated. Floating and adherent cells were harvested, combined, and counted to be used in apoptosis assays as follows.

Cleavage of poly (ADP-ribose) polymerase (PARP)

Aliquots of 10^6 cells were lysed in 250 µl of buffer containing 62.5 mM Tris/HCl (pH 6.8), 6 M urea, 10% glycerol, 2% SDS, 0.00125% bromophenol blue, and 5% β-mercaptoethanol. After sonication for 15 seconds and incubation at 65°C for 15 minutes, lysates were separated by SDS-PAGE. PARP immunodetection was carried out as described above using the anti-PARP antibody at a dilution of 1:1000.

DNA fragmentation

A total of 10^6 cells were digested at 37°C in 300 µl of lysis buffer (10 mM Tris HCl (pH 8.2), 400 mM NaCl, 2 mM EDTA, 1% SDS, and 300 µg/ml protease K). An equal volume of 6 M NaCl was added and samples were vigorously vortexed. After brief centrifugation at 2000 *g*, nucleic acids were precipitated from supernatants by addition of two volumes of ethanol. Pellets were solved in TE (10 mM Tris HCl (pH 7.5) and 0.2 mM EDTA) and incubated for 30 minutes at 37°C with 20 µg/ml RNase. DNA was separated on a 2% agarose gel.

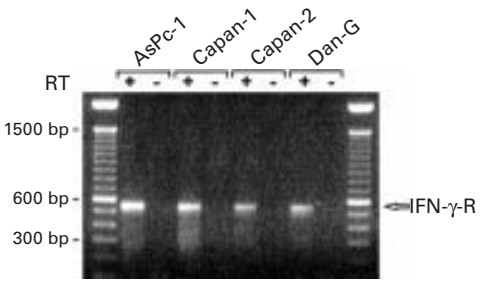

Figure 1 Pancreatic cancer cells express interferon γ receptor (IFN-γ-R) mRNA transcripts. Total mRNA was extracted, reverse transcribed into cDNA, and amplified using polymerase chain reaction with specific primers directed against the IFN-γ-R α chain. Alternating lanes represent reaction with (+) or without (−) addition of reverse transcriptase (RT). The expected size of the amplification product was 559 bp and a 100 bp DNA standard was included for size determination (lane 1).

Cell Death Detection ELISA

Cells were incubated in lysis buffer supplied with the Cell Death Detection ELISA plus (Boehringer Mannheim, Mannheim, Germany) and nuclei were separated from the cytosolic fraction by centrifugation (five minutes, 2000 *g*). Cytoplasmic histone associated DNA fragments were determined as described by the manufacturer.

STATISTICAL ANALYSIS

Statistical analysis was performed by one way ANOVA using the Newman-Keuls test (Prism, Graph pad software, San Diego, California, USA). Differences were considered significant at $p<0.01$.

Results

HUMAN PANCREATIC CANCER CELLS EXPRESS FUNCTIONAL IFN-γ RECEPTORS THAT ACTIVATE THE Jak–Stat SIGNALLING PATHWAY

The responsiveness of tumour cells to IFN-γ may vary due to loss or functional inactivation of components of the IFN-γ signal transduction pathway. Therefore, IFN-γ signalling was investigated in a panel of human pancreatic cancer cell lines: AsPc-1, Capan-1, Capan-2, and Dan-G.

Initially, we examined expression of IFN-γ receptors by RT-PCR. Using specific primers against the α-chain of the IFN-γ receptor, we demonstrated PCR amplificates of the expected size, confirming that IFN-γ receptor mRNA transcripts were present in all cell lines. Additional PCR without prior reverse transcription did not reveal amplificates, excluding genomic contamination (fig 1).

To confirm that expression of IFN-γ receptor mRNA translates into functionally competent surface receptors, ligand initiated signal transduction was analysed next. Activation of Jak kinases by IFN-γ was examined on immunoprecipitates of Jak-1 and Jak-2 using antiphosphotyrosine antibodies (fig 2A, B; top panel). Bands of 130 kDa, representing phosphorylated Jak-1 and Jak-2 proteins, were first apparent after one minute of IFN-γ stimulation and further increased at five minutes. Although phosphotyrosine bands in Capan-2 cells were weak compared with the other cell lines, a moderate increase in Jak1 and Jak2 phosphorylation was also observed in response to IFN-γ stimulation. Using an analogous immunoprecipitation approach, increased Stat-1 tyrosine phosphorylation was evident at one, five, and 15 minutes (fig 2C; top panel). Both the 91 kDa (Stat-1α) and the 84 kDa (Stat-1β) splice variants were phosphorylated, albeit the relative abundance of the 84 kDa form was considerably lower and barely detectable in Capan-2 cells. To ensure that equivalent amounts of protein had been analysed in the immunoprecipitations, immunoblots were reprobed with anti-Jak-1, anti-Jak-2, and anti-Stat-1 antibodies, respectively (fig 2A, B, C; bottom panels).

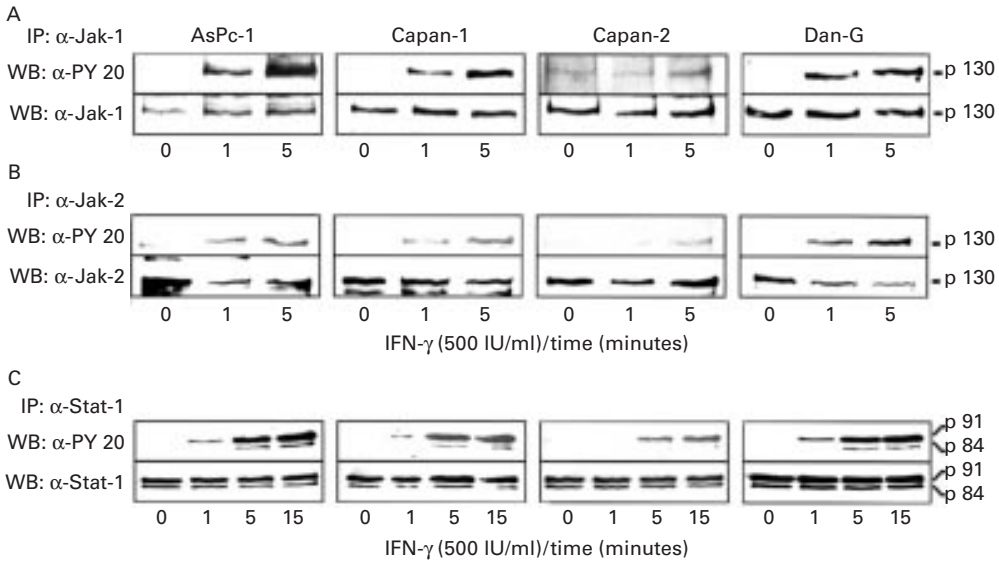

Figure 2 Interferon γ (IFN-γ) activates the Jak-Stat signal transduction pathway in human pancreatic cancer cells. Cells were stimulated with 500 IU/ml IFN-γ for the indicated time periods and Jak-1 (A), Jak-2 (B), and Stat-1 (C) were immunoprecipitated (IP) from cell lysates. Immunoblots were performed to determine IFN-γ induced changes in the phosphotyrosine content of Jak and Stat complexes (top panels). To ensure that equal amounts of protein had been examined, the western blots (WB) were subsequently stripped and reprobed with Jak-1, Jak-2, or Stat-1 antibody, respectively (bottom panels). Molecular masses were deduced from a molecular size marker electrophoresed in parallel.

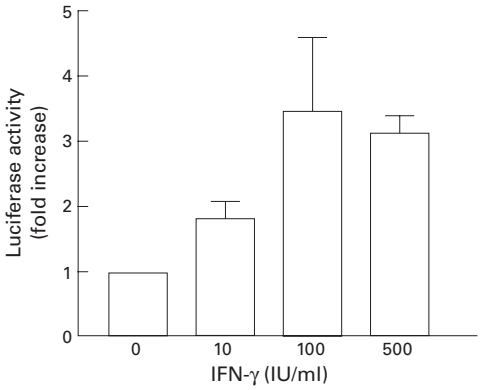

Figure 3 Interferon γ (IFN-γ) stimulation results in transactivation of a GAS driven reporter construct. AsPc-1 cells were transiently transfected with pGL2-GAS and relative luciferase activity was measured after a 24 hour period of IFN-γ stimulation with the indicated doses. Data represent mean (SEM) values from at least three separate experiments conducted in triplicate.

To verify that activation of the Jak-Stat pathway results in activation of IFN-γ regulated genes, transactivation of a GAS-luciferase reporter construct was evaluated in transient transfection assays. AsPc-1 cells were used as a representative cell line in these experiments as we repeatedly failed to achieve acceptable transient expression levels of the reporter construct in the other cell lines. IFN-γ treatment dose dependently induced luciferase activity (fig 3), indicating that IFN-γ was capable of stimulating GAS dependent gene expression in human pancreatic cancer cells.

IFN-γ INHIBITS ANCHORAGE DEPENDENT AND ANCHORAGE INDEPENDENT GROWTH OF HUMAN PANCREATIC CANCER CELLS

Having established functional IFN-γ signalling in the cell lines investigated, we evaluated the effects of IFN-γ on pancreatic cancer cell growth. To examine anchorage dependent growth, cell numbers were determined over a period of four days. During this time, control cells grew exponentially whereas proliferation of IFN-γ treated cultures (500 IU/ml) was profoundly reduced in all four cell lines (fig 4A). Growth inhibition was notably absent during the first two days of IFN-γ treatment but became apparent on day three in all cell lines. However, once growth inhibition was detectable, two cell lines (Capan-1 and Capan-2) presented a complete block in proliferation.

To determine if growth inhibition could be achieved with therapeutically relevant concentrations of IFN-γ, a dose-response relation was established after four days of IFN-γ treatment. The antiproliferative action of IFN-γ was dose dependent in all four cell lines although sensitivity to IFN-γ varied with EC_{50} values below 10 IU/ml in AsPc-1 and approximately 50 IU/ml in Dan-G cells (fig 4B). Despite modest Jak/Stat1 activation, proliferation of Capan 2 cells was strongly inhibited (15.7 (2.3)% of control; EC_{50} 32.6 (1.1) IU/ml).

Because growth of malignant tumours in vivo is influenced by unique properties of transformed cells more accurately examined in anchorage independent growth assays, colony

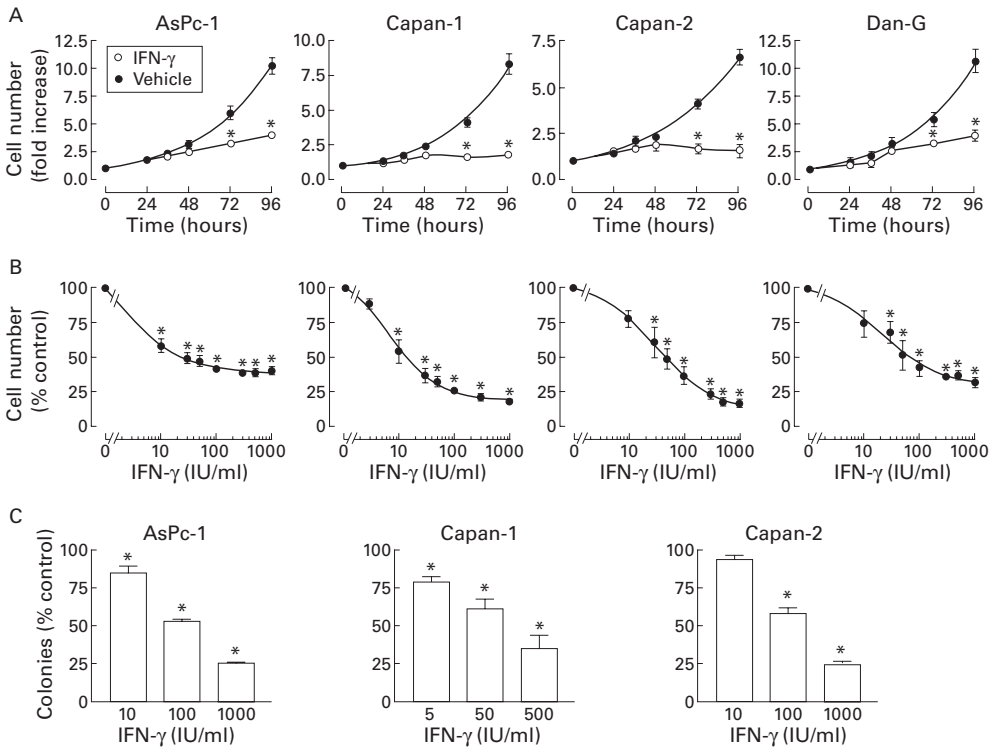

*Figure 4 Interferon γ (IFN-γ) inhibits anchorage dependent and independent growth of human pancreatic cancer cells. Subconfluent cells were treated with 500 IU/ml IFN-γ or vehicle for the indicated time periods (A), or with the indicated final concentrations of IFN-γ for 96 hours (B) and cell numbers were determined. (C) A single cell agar suspension containing 1×10^3 cells was incubated with the indicated doses of IFN-γ for 10 days and colony formation was evaluated. Data represent mean (SEM) values from at least three separate experiments, each conducted in triplicate (*p<0.01).*

formation in agar suspension was also evaluated. IFN-γ treatment profoundly reduced the number of colonies in the pancreatic cancer cell lines studied (fig 4C), except for Dan-G cells which do not form colonies. Again, growth inhibition was dose dependent with maximal decreases in colony number to 25.3 (0.6)% of control in AsPc-1 and 24.7 (3.2)% in Capan-2 achieved with 1000 IU/ml and to 35.2 (8.7) in Capan-1 cells with 500 IU/ml IFN-γ.

THE ANTIPROLIFERATIVE ACTION OF IFN-γ IN PANCREATIC CANCER CELLS IS DUE TO INDUCTION OF APOPTOSIS

To identify potential mechanisms of IFN-γ mediated growth inhibition, cell cycle distribution was monitored by flow cytometry (fig 5). During a four day period the fraction of cells in the individual cell cycle phases did not change in untreated controls. In contrast, IFN-γ treated cultures revealed an increased percentage of cells with subdiploid DNA content over time, suggesting induction of apoptosis (fig 5A). A quantitative analysis of the DNA histograms confirmed a substantial amount of cells with subdiploid DNA content in IFN-γ treated cultures with percentages ranging from 19.7 (3.1)% in AsPc-1 to 47.3 (5.8)% in Capan-1 cells after 96 hours of IFN-γ stimulation (fig 5B). In good agreement with the kinetics of growth inhibition observed in substrate dependent proliferation assays (correlation coefficient r=0.9892 for Capan-1) this putative population of apoptotic cells was first observed after two days and then dramatically increased with prolonged treatment (fig 5A, B). Also, the extent and dose dependence of substrate dependent growth inhibition and the appearance of the pre-G1 population were almost identical (r=0.9563 for Capan-1), suggesting that apoptosis might fully account for the reduction in cell numbers (fig 5C).

As the pre-G1 peak described above does not provide conclusive evidence of apoptosis, we performed experiments to corroborate IFN-γ mediated apoptosis. Firstly, DNA integrity was evaluated after four days. Whereas IFN-γ treated cultures of all four cell lines presented the typical DNA laddering, essentially no DNA

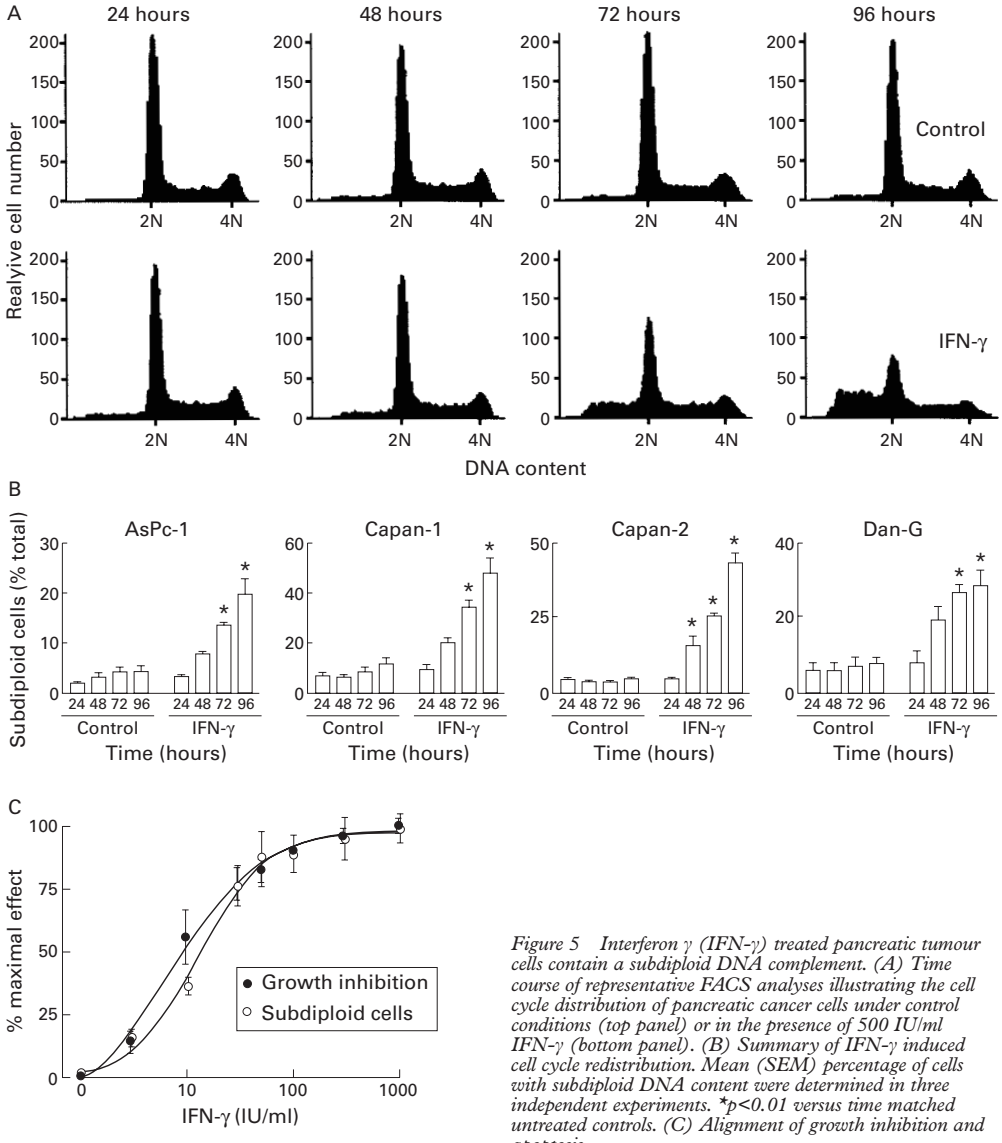

*Figure 5 Interferon γ (IFN-γ) treated pancreatic tumour cells contain a subdiploid DNA complement. (A) Time course of representative FACS analyses illustrating the cell cycle distribution of pancreatic cancer cells under control conditions (top panel) or in the presence of 500 IU/ml IFN-γ (bottom panel). (B) Summary of IFN-γ induced cell cycle redistribution. Mean (SEM) percentage of cells with subdiploid DNA content were determined in three independent experiments. *p<0.01 versus time matched untreated controls. (C) Alignment of growth inhibition and apoptosis.*

degradation was observed in untreated AsPc-1 and Capan-2 cells (fig 6A). Corresponding to their relatively high rates of spontaneous apoptosis (see also fig 5), Capan-1 and Dan-G cells revealed faint DNA laddering under control conditions, which was however greatly enhanced by IFN-γ treatment.

DNA fragmentation was further confirmed and quantitated in a complementary approach using a histone ELISA with antibodies directed against the DNA or the histone component of the nucleosomes, respectively. Starting at 24 hours, cytoplasmic extracts of IFN-γ treated cells revealed a distinct increase in DNA-histone complexes compared with control cultures (fig 6B). Thus the onset of DNA fragmentation preceded the appearance of the pre-G1 population observed in the FACS analyses, most likely reflecting more sensitive detection of cells that initiate DNA fragmentation in the histone ELISA.

PARP is an early target of active caspases in apoptosis and the resulting 85 kDa cleavage product may be used as a separate monitor of apoptosis. In immunoblot analyses of control cultures, PARP was detected nearly exclusively as a 116 kDa band, representing the intact PARP protein. In contrast, a prominent 85 kDa band was time dependently induced in IFN-γ treated cells (fig 6C). Again, the first effects were noted after 24 hours of IFN-γ treatment. Similar to the DNA fragmentation assay, few individual control cultures also revealed a weak but distinct 85 kDa fragment indicative of spontaneous apoptosis under tissue culture conditions.

IFN-γ-MEDIATED APOPTOSIS DOES NOT REQUIRE pRb REGULATION

In response to IFN-γ, pRb appears to function as a determinant of G1 cell cycle arrest and as a caspase substrate. Therefore, IFN-γ effects on cellular content and phosphorylation status of pRb were examined (fig 7). Under control conditions, pRb was detected primarily in its hyperphosphorylated growth permissive form. In none of the four cell lines did IFN-γ affect either expression or phosphorylation of pRb within the first 12 hours. At later time points, AsPc-1 and Capan-1 displayed marked hypophosphorylation and a distinct reduction of overall protein content consistent with apoptotic degradation. In contrast, no alteration in pRb was observed in Capan-2 or Dan-G cells. Thus IFN-γ mediated apoptosis of pancreatic cancer cells appeared to be independent of

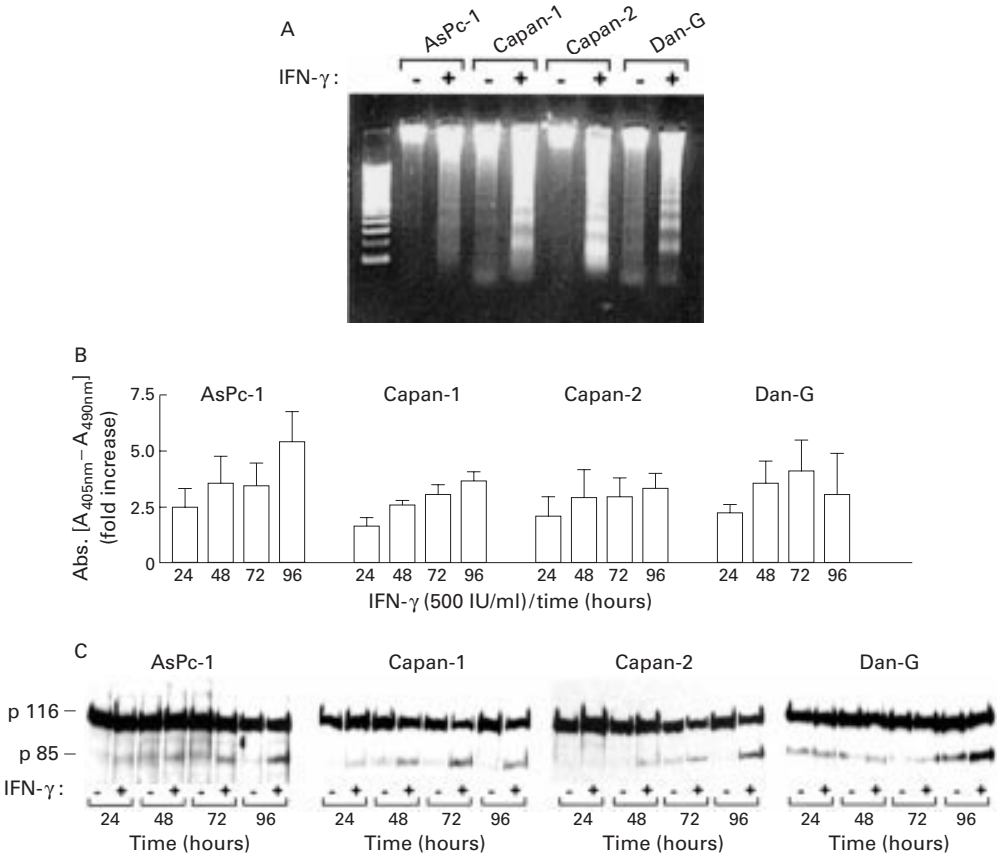

Figure 6 Interferon γ (IFN-γ) induced DNA fragmentation and poly(ADP ribose) polymerase (PARP) cleavage in human pancreatic cancer cell lines. (A) Cells were incubated for four days with 500 IU/ml IFN-γ (lanes 3, 5, 7, and 9) or left untreated (lanes 2, 4, 6, and 8) and genomic DNA was subsequently analysed for oligonucleosomal fragmentation. For size determination a 100 bp DNA standard was used (lane 1). (B) Cells were treated with vehicle or IFN-γ (500 IU/ml) for the indicated time periods and examined using a specific histone DNA ELISA to quantitate DNA fragmentation. Mean (SEM) absorbance at 405 nm relative to untreated controls was determined in three independent experiments, each performed in triplicate. (C) Immunoblot demonstrating expression of PARP (p 116) and its apoptosis related cleavage product p 85 in untreated controls and cells that had been treated with 500 IU/ml IFN-γ for the time periods indicated. Alternating lanes represent IFN-γ treated (+) and untreated (−) cells. Whole cell lysates were separated by 7.5% sodium dodecyl sulphate-polyacrylamide gel electrophoresis.

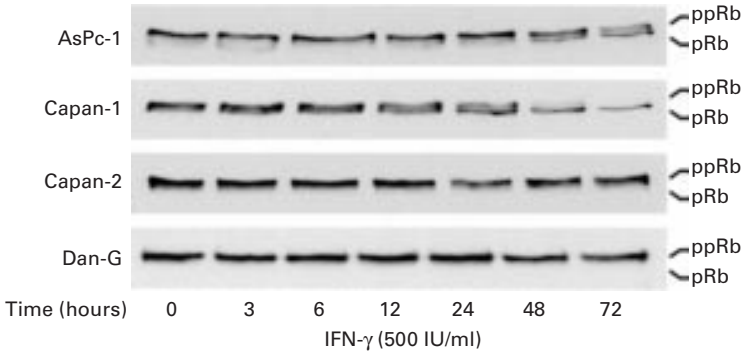

Figure 7 Interferon γ (IFN-γ) treatment regulates retinoblastoma protein (pRb) phosphorylation and abundance in human pancreatic cancer cells. Immunoblot analysis demonstrating the time course of changes in pRb expression and phosphorylation status in control and IFN-γ stimulated human pancreatic cancer cells. In each lane, 20 μg of whole cell lysates were separated by 7.5% sodium dodecyl sulphate-polyacrylamide gel electrophoresis. Shown is a representative of two experiments yielding similar results. pRb, ppRb, hypo- and hyperphosphorylated forms of Retinoblastoma protein, respectively.

pRb. In addition, these data argue against a pRb mediated G1 specific cell cycle inhibition prior to the onset of apoptosis.

IFN-γ MEDIATED INDUCTION OF APOPTOSIS INVOLVES UPREGULATION AND ACTIVATION OF THE AMPLIFIER PROTEASE CASPASE-1

To evaluate the role of caspases in IFN-γ induced apoptosis, we next tested the ability of the caspase inhibitor z-vad-fmk to prevent apoptosis. Cells were treated with a combination of IFN-γ and the caspase inhibitor, and apoptosis was quantitated by flow cytometry after three days. Addition of z-vad-fmk alone did not change cell cycle distribution (data not shown). However, the IFN-γ induced pre-G1 peak was completely abrogated by the caspase inhibitor in all four cell lines (fig 8A, B). Furthermore, prevention of apoptosis by z-vad-fmk was dose dependent, as demonstrated in experiments on Capan-1 cells (fig 8B).

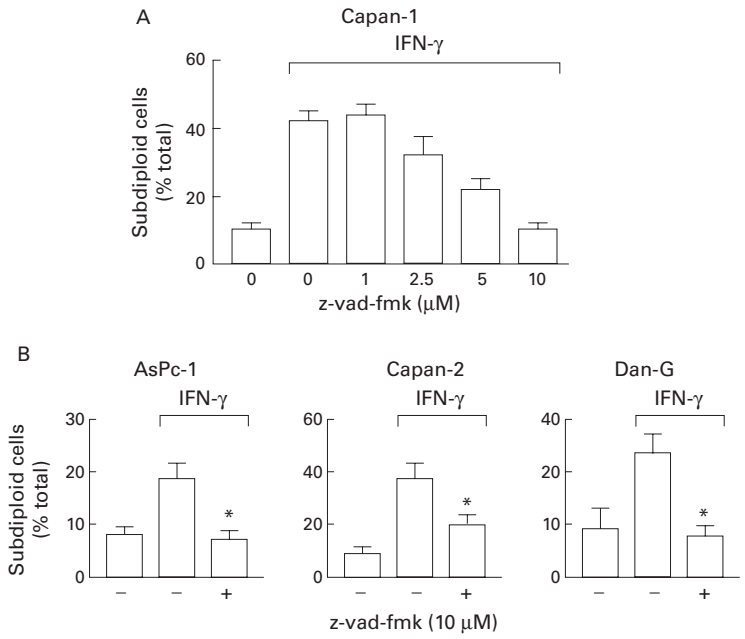

Figure 8 Interferon γ (IFN-γ) mediated apoptosis is prevented by the caspase inhibitor z-vad-fmk. Cells were stimulated for three days with either IFN-γ (500 IU/ml), a combination of IFN-γ and z-vad-fmk at various doses, or were left untreated. Subsequently, cells were analysed by flow cytometry and the fraction of cells with subdiploid DNA content was quantitated. Mean (SEM) percentage of cells with subdiploid DNA content was determined in three independent experiments.

Z-vad-fmk potentially inhibits caspases 1, 3, 4, and 7. Of these, caspase-1 is unique in that it qualifies as an amplifier rather than an executioner caspase and is located most proximal within the caspase cascade. Therefore, changes in caspase-1 were investigated in AsPc-1 and Dan-G cells as representative cell lines. In view of the delayed onset of apoptosis, changes in caspase expression rather than activity were examined (fig 9A). In untreated controls, weak bands of 45 kDa were detected, corresponding to procaspase-1. Following IFN-γ treatment, a time dependent distinct induction of the 45 kDa band was observed starting at six hours in AsPc1 and at 12 hours in Dan-G cells, which then persisted throughout the observation period of 72 hours. Compared with their time matched controls, IFN-γ treated cells displayed not only increased abundance of the proform but also bands of approximately 35 kDa (fig 9B), indicative of enzyme activation.[40] As the caspase-1 antibody used does not recognise the 20 kDa mature processing product and the 10 kDa subunit of the mature active complex has previously been shown to be highly instable,[40] these smaller subunits were not detected.

Studies on the regulation of procaspase-1 expression have identified various regulatory sites in the procaspase-1 promoter, including an IRF-1 binding site. Therefore, effects of IFN-γ on IRF-1 expression were investigated by immunoblotting (fig 9B). Bands of the appropriate size of 48 kDa were barely detectable in either AsPc-1 or Dan-G control cultures. Stimulation with IFN-γ resulted in a profound increase in IRF-1 protein starting as early as three hours. Subsequently, IRF-1 levels remained elevated until the end of the observation period at three days. Thus induction of IRF-1 preceded the manifestation of apoptosis. Similar IRF-1 upregulation was observed in Capan-1 and Capan-2 cells (data not shown).

Discussion

Induction of apoptosis represents a paramount goal in cancer therapy. In contrast with other common malignancies, pancreatic cancer is characterised by resistance to a broad spectrum of proapoptotic stimuli, such as chemotherapy and radiation.[37] Although prevalent genetic alterations in pancreatic cancer have been specified, the basis for the striking deficiency in apoptosis susceptibility remains to be elucidated.[36 41] Altered expression of Bcl 2 family members is variably observed in histochemical analysis of pancreatic tumour specimens and studies utilising permanent human pancreatic cancer cell lines, but the biological relevance of these observations has remained controversial.[42–45] In addition, pancreatic cancer was suggested to circumvent apoptosis mediated by immunsurveillance mechanisms by means of Fas ligand mediated counter attack.[46]

Using a representative panel of human pancreatic cancer cell lines, the current study investigated the antiproliferative actions of

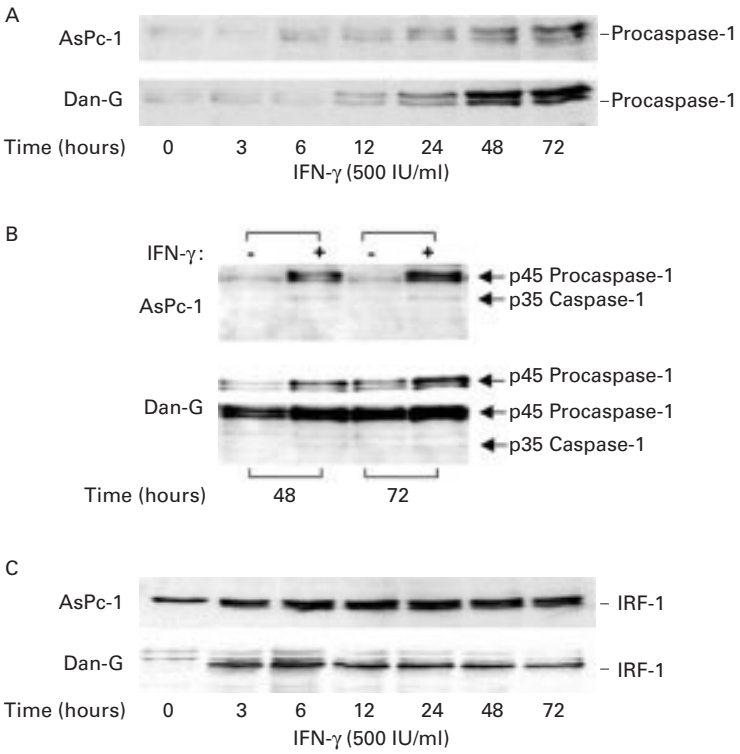

Figure 9 Interferon γ (IFN-γ) causes upregulation of procaspase-1 and interferon regulatory factor 1 (IRF-1) in pancreatic cancer cell lines. (A) Cells were incubated with vehicle or IFN-γ (500 IU/ml) for the indicated time periods. Aliquots of 20 μg of whole cell lysate were separated by 12% sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS PAGE) and subsequently incubated with an anticaspase antibody that recognises a double band of the procaspase precursor form at approximately 45 kDa. (B) Immunoblot revealing caspase-1 processing intermediates with the 35 kDa form indicative of activation. Lysates were from AsPc-1 and Dan-G cells treated for 48 and 72 hours with 500 IU/ml IFN-γ or time matched control cells. In Dan-G cells, top and bottom panels represent light and darker exposures of the same blot. (C) Immunoblot analysis for IRF-1 expression. Cells were incubated with vehicle or IFN-γ (500 IU/ml) for the indicated time periods. In each lane, 20 μg of whole cell lysates were separated by 12% SDS-PAGE and IRF-1 expression was determined using a monospecific antibody. Shown are representative blots of at least two experiments.

IFN-γ. Responsiveness to IFN-γ varies considerably between different tumours and cell types and IFN-γ resistance is common in some tumour entities—for example, in mesothelioma,[47] ovarian,[48] mammary,[15] lung, and melanoma cancer[10] cells. This may be due to reduced IFN-γ receptor expression during malignant transformation, as has been suggested in endothelial cells,[11] or to deficient downstream components in the IFN-γ signalling pathway. A careful analysis of IFN-γ resistant lung tumour cell lines revealed functional inactivation of Jak2, loss of Jak-1, and loss of the IFN-γ receptor α chain as underlying molecular defects.[10] Similarly, a comparison of IFN-γ responsive or resistant human mesothelioma cell lines connected the failure of IFN-γ to inhibit DNA synthesis with altered expression or activation of Jak-2 or Stat-1 and low induction of IRF-1, respectively.[49] As tumour specific defects occur at all steps of the IFN-γ signalling pathway, the individual signalling components were carefully analysed in the current study. Firstly, we demonstrated the presence of IFN-γ receptor mRNA transcripts. Secondly, we documented expression of key effectors of IFN-γ—that is, Jak-1, Jak-2, and Stat-1—in pancreatic cancer cells and their activation in response to ligand stimulation. Thirdly, transactivation of a GAS driven reporter construct and induction of an endogenous IFN-γ inducible gene product (IRF-1) were observed, establishing a functionally intact IFN-γ signalling pathway in all pancreatic cancer cell lines examined. Specifically, Stat-1 and IRF-1 downstream effectors are well established critical mediators of IFN-γ dependent growth inhibition[50 51]

When subsequently evaluated in anchorage dependent and independent growth assays, IFN-γ was indeed capable of profoundly inhibiting proliferation of pancreatic cancer cells. Remarkably, all four pancreatic cancer cell lines were highly sensitive towards IFN-γ and a significant antiproliferative effect was obtained in the range 8–30 IU/ml IFN-γ— that is, within the range of therapeutically achievable concentrations.[52] The reasons for minor differences between individual cell lines regarding sensitivity to and efficacy of IFN-γ action remain to be determined. Previous reports suggested that the extent or kinetics of activation of Jak-2 and Stat-1, respectively, accounted for differential responsiveness of mesothelioma cell lines towards IFN-γ.[49] In our study, we noted a comparably low efficacy of IFN-γ to induce activation of these effectors in Capan-2 cells. However, the strong induction of IRF-1 that was consistently observed in all four pancreatic cell lines contradicts a linear relation between Jak-Stat signalling and activation of growth relevant effectors. Furthermore, the growth inhibitory potential of IFN-γ in Capan-2 cells was well within the range observed in the other cell lines and specifically exceeded the antiproliferative action in AsPc-1 cells.

IFN-γ treated pancreatic cancer cell cultures typically completed one round of replication without perceivable changes in cell cycle distribution prior to the subsequent almost complete block of proliferation. Thus their response differed from other cell models[22 53 54] where a CKI mediated inhibition of cyclin dependent kinase activity rapidly resulted in G1 arrest on the basis of pRb hypophosphorylation[15 22] or pRb independent mechanisms.[53] Of note, the lack of cell cycle redistribution did not result from loss of pRb, as we documented continued pRb expression in all cell lines. Instead of cell cycle arrest, the appearance of subdiploid cells in cell cycle analyses suggested apoptosis induction, which was confirmed by (i) demonstration of DNA fragmentation, (ii) detection of the apoptotic 85 kDa PARP cleavage product, and (iii) functional requirement for caspase activity. As the dose-response relationship of growth inhibition and induction of apoptosis closely correlated, apoptosis probably constituted the main if not the exclusive mechanism underlying IFN-γ mediated growth inhibition.

The proapoptotic actions of IFN-γ were late in onset, suggesting that delayed signalling events such as synthesis of intermediary products were involved. Consistent with this hypothesis we found IFN-γ mediated induction of caspase-1 prior to the onset of apoptosis. Within the caspase cascade, caspase-1

assumes an intermediary function between initiator and executioner caspases and has been suggested as an amplifier of proapoptotic signalling.[28] Proteolytic cleavage of the inactive procaspase-1 by upstream initiator caspases, such as caspase-8 or caspase-9, represents the prevalent mode of caspase-1 activation.[28 29]. However, autocatalytical cleavage following substantial endogenous or exogenous upregulation of proenzyme expression may bypass the requirement for upstream activators.[55 56] Thus IFN-γ mediated induction of procaspase-1 in pancreatic cancer cells may trigger its activation and thereby initiate activation of downstream executioner caspases such as caspase-3. An analogous IFN-γ mediated induction of caspase-1 has previously been reported in HeLa and A431 cells,[55] vascular smooth muscle cells,[57] leukaemia cells,[19] and Sertoli cells.[58] The functional relevance of caspase-1 activation for apoptosis initiation in pancreatic cancer cells was underlined by the ability of the caspase inhibitor z-vad-fmk to completely prevent IFN-γ effects. This is in good agreement with results from studies on primary splenocytes from caspase-1 (−/−) mice,[55] providing conclusive evidence for the requirement of caspase-1 in IFN-γ dependent apoptosis in these cells.

Analyses of the caspase-1 promoter have revealed binding sites for IRF-1. IRF-1 itself represents an immediate IFN-γ response product[59 60] critically involved in IFN-γ mediated apoptosis as IFN-γ induced caspase-1 activation was completely abrogated in IRF-1 deficient mice.[59] Furthermore, the growth inhibitory action of IFN-γ in KG1 human myelocytic leukaemia cells could be converted into a proliferative stimulus by retroviral expression of IRF-1 antisense.[61 62] Also, resistance to interferon induced apoptosis was associated with IRF-1 gene rearrangements[63] or defective IRF-1 binding to the caspase-1 promotor[64] in HCC cell lines or Daudi cells. In pancreatic cancer cells, we demonstrated sustained induction of IRF-1 protein content, consistent with IRF-1 constituting the functional link between IFN-γ initiated signalling events and upregulation of procaspase-1. In particular, the time course of IRF-1 induction placed this event between early activation of Stat-1 and delayed induction of procaspase-1.

Once procaspase-1 has been induced and converted into the active caspase, proteolytic activation of downstream effector caspases results. Caspase-3 represents one of the biologically most relevant downstream targets of caspase-1 as it functions to incapacitate or destroy essential homeostatic pathways during the effector phase of apoptosis. Substrates include endonuclease inhibitors,[65] PARP,[66 67] and most likely pRb.[68 69 70] Consistent with the caspase-1 dependent activation of caspase-3 described in the literature,[28] we were able to detect oligonucleosomal DNA fragmentation, PARP cleavage, and pRb downregulation in IFN-γ-treated pancreatic cancer cells. The reduction in pRb protein levels was preceded by a shift from the hyper to its hypophosphorylated form. Similar regulation of pRb during apoptosis has been extensively characterised in human leukaemia cell lines,[71] where it resulted from dephosphorylation and subsequent degradation triggered by an ICE-like protease. Given the established antiapoptotic function of pRb, this caspase mediated degradation was proposed as an important intermediate step in the execution of apoptosis. A role for pRb degradation in the context of IFN-γ induced apoptosis is supported by the observation that reconstitution of pRb prevented apoptosis in IFN-γ treated pRb deficient bladder carcinoma cells.[72] In the current study however only two of four cell lines investigated presented appreciable pRb reduction although they uniformly underwent apoptotic cells death, indicating that IFN-γ mediated apoptosis of pancreatic cancer cells can occur independent of pRb degradation.

Other investigators have identified the double stranded RNA dependent protein kinase PKR as a critical parameter of IFN mediated apoptosis induction.[24 73 74] However, PKR induction occurs in response to both type I and type II interferons. As IFN-α treatment was ineffective in inducing apoptosis in pancreatic cancer cell lines[75] but resulted in S phase cell cycle delay (unpublished observation), a decisive role of PKR induction for IFN-γ mediated apoptosis versus cell cycle effects appears unlikely. None the less, PKR induction may be a necessary intermediate step for either cell fate, as was demonstrated for IFN-α dependent G1 arrest in myeloid leukaemia cells,[76] as well as for IFN-γ dependent IRF-1 induction and stress induced apoptosis in mouse embryo fibroblasts of $PKR^{(-/-)}$ mice.[24]

Based on both clinical experience and in vitro data, the relative resistance of pancreatic cancer cells to conventional chemotherapy and/or radiation as well as to many biotherapeutic modalities is a disappointing although well known fact. The tremendous increase in our understanding of the regulation and execution of apoptosis has produced a considerable and successful effort in revealing and elucidating molecular changes that are responsible for this "apoptosis resistance". In accordance with this, the current study delineated an intact proapoptotic pathway in pancreatic cancer cells which was found to be activated in response to the clinically available cytokine IFN-γ. Specifically, IRF-1 and/or procaspase-1 may represent potential therapeutic targets to be further explored in the treatment of pancreatic cancer. Furthermore, IFN-γ should be evaluated in in vivo studies in animal models of pancreatic cancer such that a valid basis for considering its clinical use is obtained.

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