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Inhibitor of Apoptosis Proteins and Associated Factors in Pancreatic Cancer

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INHIBITOR OF APOPTOSIS PROTEINS AND ASSOCIATED FACTORS IN PANCREATIC CANCER

Thesis Submitted to the
School of Graduate Studies,
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By

Jacynthe Bastien

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Abstract

Since the phenomenon was first identified, apoptosis has been proposed to serve as a barrier to the development of cancer in metazoans. Over the years, the inability to carry out apoptosis has been implicated in the initiation, formation and progression of tumors. Moreover, acquired resistance to apoptosis is now believed to represent a major obstacle to the successful application of oncotherapies. Caspases play a central role in the induction and execution of apoptosis. As such, their activity must be tightly regulated. To date, inhibitor of apoptosis proteins (IAPs) are the only known intrinsic regulators of caspase function. The present study focused on the identification of molecular targets differentially expressed in normal and cancer cells that could serve to facilitate the rational design of anti-cancer therapies. We hypothesized that variations in the levels of key apoptotic regulators such as caspases, IAPs and their antagonists could conceivably contribute to the acknowledged resistance of pancreatic cancer cells to cytotoxic therapies.

Our first specific aim was to derive an expression profile of apoptotic modulator/ effector genes in pancreatic cancer cell lines. Our analysis uncovered a tendency towards up-regulation of IAP expression (namely cIAP-2) and down-regulation of pro-apoptotic factors such as caspases and the Xiap antagonist Xaf-1 in these cell lines. In particular, Xaf-1 protein expression appeared to be completely repressed in neoplastic cell lines. Moreover, over-expression of one or more IAPs was observed in several solid malignancies. Lastly, while Xiap expression and subcellular localization were not altered in evolving intraductal lesions and pancreatic tumors, immunohistological surveys uncovered over-expression and nuclear redistribution of cIAP-1, cIAP-2 and survivin in pancreatic adenocarcinomas.

Our second objective was to determine if differential expression of IAPs influenced the sensitivity of three human pancreatic cancer cell lines to drug-induced apoptosis. *In vitro*

studies uncovered a good correlation between transcriptional up-regulation of IAPs, caspase-dependent cleavage of Xiap, activation of downstream effector caspase-3 and rapidity of onset of etoposide-induced apoptosis in these cell lines. In particular, endogenous levels of cIAP-2 mRNA appeared to be good predictors of etoposide-responsiveness. However, attempts at sensitizing pancreatic cancer cells to etoposide by down-modulating Xiap expression via over-expression of Xaf-1 or siRNA-mediated degradation of Xiap were unsuccessful.

Our last objective was to characterize the expression of a novel candidate IAP-interacting partner, FLN29/Xaf-2, mapping to 12q23-q24, a region prone to loss of heterozygosity in pancreatic cancer cell lines. The 2.8 Kbp FLN29/Xaf-2 mRNA transcript appears to contain 11 exons distributed over 28.8 Kbp of genomic DNA. The FLN29/Xaf-2 protein (582 aa) has a predicted molecular weight of 64.8 kDa and a pI of 5.01. Domains searches uncovered a Traf-like RING zing finger, a PEST sequence and two putative caspase cleavage sites within the FLN29/Xaf-2 sequence. Our studies revealed that the FLN29/Xaf-2 genomic locus does not appear to be prone to gross structural rearrangements in pancreatic cancer cell lines while FLN29/Xaf-2 transcript levels were found to be mildly over-expressed in pancreatic cancer cell lines and other solid malignancies.

Taken together, the findings presented in this thesis indicate that IAPs (particularly cIAP-2 and survivin) are differentially expressed in normal and cancer cells; the latter tending to over-express IAPs. Over-expression of IAPs may lead to an elevation in the apoptotic threshold and contribute to the intrinsic resistance of pancreatic cancer cells to oncotherapies. Combination strategies involving down-regulation of IAP expression and judiciously chosen oncotherapeutics warrant further investigation and should prove beneficial to pancreatic cancer patients.

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Dedication

À mon mari Daniel, pour ton support et ta confiance en moi

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List of Abbreviations

aa	amino acid
AIF	apoptosis-inducing factor
Ala	alanine
AML	acute myelogenous leukemia
Apaf-1	apoptosis activating factor-1
Asp	aspartic acid
ATP	adenosine triphosphate
BIR	baculoviral IAP repeat
BMP	bone morphogenetic protein
bp	base pairs
BSA	bovine serum albumin
CAD	caspase-activated DNase
CAM	cell-cell adhesion molecules
CARD	caspase recruitment domain
CDK	cyclin-dependent kinase
cDNA	complementary deoxyribonucleic acid
CDTA	trans-1,2-diaminocyclohexane-N,N,N',N',-tetraacetic acid monohydrate
cIAP-1	cellular inhibitor of apoptosis protein-1
cIAP-2	cellular inhibitor of apoptosis protein-2
CO_2	carbon dioxide
DAPI	4',6-diamidino-2-phenylindole dihydrochloride
dATP	deoxyadenosine triphosphate

DCC	deleted in colorectal cancer
dCK	deoxycytidine kinase
dCTP	deoxycytidine
DED	death effector domain
DEPC	diethyl pyrocarbonate
DFF45	DNA fragmentation factor-45
dGTP	deoxyguanidine
DIABLO	direct IAP binding protein with low pI
DIAP1	*Drosophila* inhibitor of apoptosis protein-1
DIAP2	*Drosophila* inhibitor of apoptosis protein-2
DISC	death inducing signaling complex
DMEM	Dulbecco's modified Eagle medium
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
DPC4	deleted in pancreatic cancer-4
dTTP	deoxythymidine
EBV	Epstein-Barr virus
ECL	enhanced chemiluminescence
EDTA	ethylenediaminetetra-acidic acid disodium salt
EGFR	epidermal growth factor receptor
ERK	extracellular signal-regulated kinase
FADD	Fas-associated death domain
Fam	carboxyfluorescein
5-FU	5-fluorouracil

GAPDH	glyceraldehyde-3-phosphate dehydrogenase
GST	gluthatione-S-transferase
GTP	guanine triphosphate
γ-irradiation	gamma irradiation
HA	hemaglutinin
HCl	hydrochloric acid
HEPES	N-2-hydroxethylpiperazine-N-2-ethane sulphonic acid
Het	heterozygosity
Hex	4,7,2',4',5',7',-hexachloro-6-carboxyfluorescein
Hiap1	human inhibitor of apoptosis protein-1
Hiap2	human inhibitor of apoptosis protein-2
hid	head involution defective
HNPCC	hereditary non-polyposis colorectal cancer
HPV	human papilloma virus
HRP	horseradish peroxidase
IAPs	inhibitor of apoptosis proteins
iCAD	inhibitor of caspase-activated DNase
ICE	interleukin-converting enzyme
IFN-β	interferon beta
IGF	insulin growth factor
I-κB	inhibitor of kappa B
IKK	inhibitor of kappa B kinase complex
IL	interleukin

Ile	isoleucin
IRES	internal ribosome entry site
JNK	jun kinase
Kbp	kilo basepairs
KCl	potassium chloride
kDa	kilo dalton
KH_2PO_4	potassium dihydrogen orthophosphate (monobasic)
K-SFM	keratinocyte serum-free medium
LOH	loss of heterozygosity
MALT	mucosa-associated lymphoid tissue
MAP	mitogen-activated protein
MAPK	mitogen-activated protein kinase
MDR	multidrug resistance
MEK	mitogen-activated extracellular kinase
$MgCl_2$	magnesium chloride
$MgSO_4$	magnesium sulfate
mL	milliliter
MOI	multiplicity of infection
mM	millimolar
mm	millimetre
MOPS	3-(N-morpholino)propanesulfonic acid
mRNA	messenger ribonucleic acid
MRP	multidrug resistance-associated protein
µg	microgram

μL	microliter
μM	micromolar
MW	molecular weight
Na_2HPO_4	disodium hydrogen orthophosphate anhydrous (dibasic)
NaH_2PO_4	sodium phosphate anhydrous (monobasic)
NAIP	neuronal apoptosis inhibitory protein
NaOH	sodium hydroxide
NCBI	National Center for Biotechnology Information
NCI	National Cancer Institute
NF-κB	nuclear factor kappa B
$(NH_4)_2SO_4$	ammonium sulfate
NIK	nuclear factor kappa B-inducing kinase
PAK2	p21-activated kinase-2
PanIN	pancreatic intraepithelial neoplasia
PARP	poly-ADP ribose polymerase
PCR	polymerase chain reaction
PEG	polyethylene glycol
pI	isoelectric point
PI3-kinase	phosphatidyl inositol-3 kinase
PBS	phosphate-buffered saline
PMSF	phenylmethylsulfonyl fluoride
Pro	proline
PVDF	polyvinylidene fluoride

RDA	representational difference analysis
REX	rapid extraction buffer
Riap	rat inhibitor of apoptosis protein
RIP	receptor-interacting protein
RNA	ribonucleic acid
RPA	RNase protection assay
RPMI-1640	Roswell Park Memorial Institute medium-1640
rpr	reaper
RT-PCR	reverse transcription polymerase chain reaction
RZF	ring zinc finger
SCID	severe combined immunodeficiency
SDS	sodium dodecyl sulfate
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
Ser	serine
siRNAs	short interfering ribonucleic acids
skl	sickle
SMA	spinal muscular atrophy
Smac	second mitochondria-derived activator of caspase
smn	survival motor neuron
SSC	saline sodium citrate
STR	short tandem repeat
TBE	tris borate-EDTA buffer
TBST	tris buffered saline and Tween 20
TCA	trichloroacetic acid

TGF-α	transforming growth factor alpha
TGF-β	transforming growth factor beta
TNF	tumor necrosis factor
TNF-α	tumor necrosis factor alpha
TNF-R	tumor necrosis factor receptor
TRADD	tumor necrosis factor receptor-associated death domain
Traf	tumor necrosis factor receptor associated factor
TRAIL	tumor necrosis factor-related apoptosis-inducing ligand
ts-IAP	testis-specific inhibitor of apoptosis protein
Tween 20	polyoxyethylene-sorbitan monolaurate
U	unit
UTR	untranslated region
UV	ultraviolet
Val	valine
VEGF	vascular endothelial growth factor
w/v	weight with respect to volume
Xaf-1	Xiap-associated factor-1
Xaf-2	Xiap-associated factor-2
Xiap	X-linked inhibitor of apoptosis protein
z-vad.fmk	carbobenzoxy-val-ala-asp-fluoromethyl ketone

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Chapter 1: General Introduction

Over the last decade, great strides in cancer research have identified better therapeutic targets thereby improving prognosis. Nonetheless, for the majority of solid tumors, cancer treatment remains problematic and unsuccessful at times thus warranting a clear understanding of the reasons for treatment failure. Effective therapeutic approaches are of particular importance in the treatment of pancreatic cancer since early detection of this malignancy is hampered by the absence of symptoms and the invasive nature of the detection methods. Chemoresistance represents a major hurdle to the successful outcome of cytotoxic therapies. Many anti-cancer therapies are designed to introduce DNA damage in rapidly dividing cells and trigger apoptosis. As such, evasion of apoptosis has become crucial for the survival of tumor cells. Caspases play a central role in the induction and execution of the apoptotic cascade. Inhibitor of apoptosis proteins (IAPs) are currently believed to be the most potent intrinsic regulators of caspases. In fact, it has been shown that over-expression of IAPs leads to an increase in the apoptotic threshold believed to contribute to the resistance of cancer cells to treatment. In the present study, we uncovered a moderate tendency towards over-expression of IAPs in pancreatic adenocarcinoma cell lines which we propose accounts for the reduced sensitivity of these cells to apoptotic triggers.

1.1. Carcinogenesis

It is currently accepted that cancer results from the accumulation of several mutations and usually develops over extended periods of time (Vogelstein and Kinzler, 1993). In other words, cancer arises from a progression of genetic alterations that modify the genetic makeup of the cell rendering it increasingly less responsive to the body's normal regulatory

machinery thereby enabling invasion and destruction of normal tissues (Weinberg, 1996). It has been proposed that malignant transformation results from a series of molecular alterations, each conferring a growth advantage to the affected cell (Foulds, 1954; Nowell, 1976; reviewed in Weinberg, 1996). Indeed, a single change or mutation can lead to genomic instability and confer a selective growth advantage to the growing tumor population resulting in neoplastic proliferation (Nowell, 1976). Initiated cells are able to escape normal growth control mechanisms resulting in hyperplasia or an abnormal increase in the number of cells of a tissue. Cells in the growing tumor population accumulate a series of molecular changes such as structural aberrations and loss of cellular differentiation ultimately resulting in the development of well-defined tumor masses or carcinoma *in situ.* Finally, cancer cells detach from the tumor and spread to surrounding, healthy tissues or organs through the lymphatic system or bloodstream resulting in invasive or metastatic cancer.

A recent review has proposed that six essential alterations in cell physiology are necessary for malignant growth (Hanahan and Weinberg, 2000). First, cancer cells possess self-sufficiency in growth signals, that is, they exhibit reduced dependence on exogenous growth stimuli. Indeed, many neoplastic cells are capable of synthesizing growth factors to which they are responsive, resulting in a positive autocrine feedback loop (Fedi et al., 1997). Alternatively, deregulations in the expression of receptors that transduce growth-stimulatory signals occur during tumorigenesis (Fedi et al., 1997). For instance, malignant cells favor the expression of integrin, a type of extracellular matrix receptor that transmits pro-growth signals (Lukashev and Werb, 1998; Giancotti and Ruoslahti, 1999). In addition, cancer cells may acquire growth autonomy via the constitutive activation of pathways responsible for the transduction of growth signals. For example, mammalian ras genes (H-ras, N-ras and Ki-ras) are frequently activated in human malignancies (Bos, 1989; Barbacid, 1987; Bishop, 1991).

Mutations in the ras oncogene occur predominantly in the active site of the protein resulting in a constitutively active GTP-bound ras that transduces cell proliferative signals (Bos, 1989). Signaling via the Raf/MEK/ERK cascade, that is, the sequential phosphorylation of MAP kinase kinase kinase (Raf), MAP kinase kinase (MEKs) and MAP kinase (ERKs) is important for ras transformation (Schaeffer and Weber, 1999; Bonner et al., 1985; Leevers et al., 1994; Stokoe et al., 1994; Sebolt-Leopold et al., 1999). Alternatively, ras signaling can be mediated by PI3-kinase (Rodriguez-Viciana et al., 1996a, 1996b, 1997) which regulates several downstream effectors such as the Akt/PKB kinase (Chan et al., 1999). In turn, Akt can decrease the expression of the pro-apoptotic proteins Bad and caspase-9 thus preventing cell death (Datta et al., 1997; Cardone et al., 1998).

A second alteration is that cancer cells become insensitive to anti-proliferative signals. The interpretation of such signals is largely mediated by cell cycle-regulatory proteins. For instance, pRb governs transit through the G1/S cell cycle checkpoint. In fact, the hyperphosphorylation of pRb by cyclin-dependent kinases (CDKs) relieves the inhibition of pRb on E2F transcription factors rendering cells insensitive to anti-growth signals (Weinberg, 1995). The signaling molecule TGF-β acts to prevent phosphorylation of pRb thereby blocking progression through the cell cycle; in particular, TGF-β stimulates the synthesis of $p15^{INK4B}$ and p21 proteins which block cyclin:CDK complexes (Hannon and Beach, 1994; Datto et al., 1997). It has been reported that cancer cells lose TGF-β responsiveness via down-regulation or mutations of TGF-β receptors (Fynan and Reiss, 1993; Markowitz et al., 1995) thereby forcing progression through the cell cycle and stimulating cell proliferation.

A third factor pertains to the mounting evidence of an acquired resistance to

apoptosis in cancer cells. In fact, apoptosis has long been proposed to serve as a barrier to cancer (Kerr et al., 1972). In addition, suppression of apoptosis was postulated to facilitate the accumulation of mutations necessary for malignant transformation (Green and Evan, 2002). Cancer cells have evolved several mechanisms conferring resistance to apoptotic triggers. In fact, the pro-apoptotic factor most commonly inactivated in human cancer is the p53 tumor suppressor gene; approximately 50% of tumors possess two inactivated copies of p53 (Nigro et al., 1989; Harris, 1996). In particular, germline mutations at the p53 locus are observed in Li-Fraumeni syndrome where patients are at very high risk for developing early onset cancer (Malkin et al., 1990; reviewed in Varley, 2003). As several apoptotic pathways are dependent on p53 function, its inactivation favors resistance to apoptosis (Levine, 1997). Alternatively, activation of the PI3 kinase-AKT/PKB pathway, responsible for the transmission of anti-apoptotic signals, has been observed in tumors; this pathway may be activated by extracellular factors such as IGF-1/2 or IL-3 (Evan and Littlewood, 1998), by ras-mediated intracellular signaling (Downward, 1998) or by loss of the pTEN tumor suppressor gene (Cantley and Neel, 1999). Interestingly, wild-type p53 was deemed to be necessary for the chemosensitizing effects of Akt down-regulation in cisplatin-induced apoptosis of ovarian cancer cells (Fraser et al., 2003).

A fourth element is that cancer cells display a limitless replicative potential. Indeed, *in vitro* propagation of tumor cells has uncovered their apparent immortality (Hayflick, 1997). It has recently been discovered that shortening of the telomeres (chromosome ends) occurs with each cell division as DNA polymerases are unable to completely replicate the 3' ends of chromosomal DNA during each S phase ultimately resulting in cell death (Counter et al., 1992). In contrast, telomere maintenance has been observed in most malignant cells (Shay and Bacchetti, 1997); an observation postulated to be mainly achieved by up-

regulating the expression of telomerase which confers unlimited replicative potential to the cells (Bryan and Cech, 1999).

A fifth characteristic is that tumors depend on an extensive network of capillaries and blood vessels thus favoring the activation of genes involved in angiogenesis (Diaz-Flores et al., 1994; Bouck et al., 1996; Hanahan and Folkman, 1996; Folkman, 1997; reviewed in Semenza, 2003). Angiogenic signals are mediated, in part, by vascular endothelial growth factors (VEGF) which bind to transmembrane receptors and induce neovascularization (Veikkola and Alitalo, 1999). Anti-angiogenic agents have been shown to inhibit the growth of tumor cells inoculated subcutaneously in mice (Folkman, 1997). In addition, tumors arising in cancer-prone transgenic mice are similarly susceptible to angiogenic inhibitors (Bergers et al., 1999). For instance, the inhibition of VEGF signaling using anti-VEGF antibodies or dominant-negative VEGF receptors led to impaired angiogenesis and reduced tumor growth in mice (Kim et al., 1993; Millauer et al., 1994).

Lastly, cancer cells evolve an ability to invade adjacent or distal tissues where they can form new tumor masses. These metastases account for 90% of human cancer deaths (Sporn, 1996). It has been proposed that cells metastasize to escape the constraints of the primary tumor mass where nutrients have become limited (Hanahan and Weinberg, 2000). Cells that possess the ability to invade and metastasize have altered expression of cell-cell adhesion molecules (CAMs) such as cadherins and integrins (Aplin et al., 1998). For instance, E-cadherin function is lost in a majority of epithelial cancers (Christofori and Semb, 1999). Normally, E-cadherin relays anti-growth signals via cytoplasmic contacts with β-catenin to intracellular signal transduction pathways involving the Lef/Tcf transcription factor (Christofori and Semb, 1999). In addition, invasive and metastatic capability has been

shown to involve extracellular proteases (Coussens and Werb, 1996; Chambers and Matrisian, 1997). In fact, up-regulation of matrix-degrading proteases, down-regulation of protease inhibitors and conversion of inactive protease zymogens has been reported in neoplastic cells (Werb, 1997; Stetler-Stevenson, 1999).

The six aforementioned capabilities of tumor cells are mainly acquired through changes in the nucleotide sequence of the nuclear genome of the cell. The identification of defective hMSH2 and hMLH1 repair genes in HNPCC suggested a link between such genes and widespread microsatellite DNA instability (Peltomaki et al., 1993; Ionov et al., 1993). In fact, inherited defects in DNA repair systems have been associated with several human cancer predisposition syndromes (reviewed in Duker, 2002; Boultwood, 2001; Oliver, 2000; Ford and Hanawalt, 1995). For example, the increased incidence of lymphomas, leukemia and breast cancer encountered in Ataxia Telangiectasia patients appears to be a consequence of defective double strand break repair linked to an inability to activate the p53 tumor suppressor protein via ATM/ATR kinase signaling (Canman and Lim, 1998). Unfortunate individuals who suffer from Xeroderma Pigmentosum are characterized by defective base excision repair of UV dimers and 6,4-photoproducts rendering them susceptible to the development of melanomas in sunlight-exposed areas of the skin (reviewed in Friedberg, 2004). In Li-Fraumeni syndrome, fibroblasts of subjects heterozygous for p53 mutations exhibit defective kinetics of inhibition of post-gamma irradiation *de novo* DNA synthesis and anomalous repair of UV base damage. This suggests that predisposition of Li-Fraumeni patients to early onset breast and soft tissue sarcomas is linked to inheritance of a mutant p53 allele which compromises genomic integrity (Ford and Hanawalt, 1995; Mirzayans et al., 1996). Furthermore, it has been proposed that, in addition to mediating double-strand break repair, the breast cancer susceptibility genes BRCA1 and BRCA2 function to facilitate

orderly homologous recombination thereby maintaining genomic integrity (Jasin, 2002; Tutt and Ashworth, 2002; Shamoo, 2003). Very recently, the crucial role of DNA repair systems in cancer protection has been demonstrated in animal models of cancer susceptibility syndromes including Li-Fraumeni and Xeroderma Pigmentosum (Ishikawa et al., 2004).

Recently, epigenetically-mediated alterations in gene expression have become an important feature of human cancer. Epigenetic events alter the heritable state of gene expression without modifying the DNA sequence (reviewed in Garinis et al., 2002). Hypermethylation of CpG islands in the promoter region of tumor suppressor genes are the most common epigenetic modifications of the human genome (reviewed in Worm and Guldberg, 2002; Garinis et al., 2002; Baylin, 2002; Nephew and Huang, 2003). Methylation of CpG islands in gene regulatory regions marks them for transcriptional silencing. Genes which confer a selective advantage to cancer cells such as tumor suppressor genes, inhibitors of angiogenesis and repair enzymes have been reported to be targeted for silencing (reviewed in Szyf, 2003). As aberrant *de novo* methylation of CpG islands has been observed in up to 10% of islands during tumor development (Costello et al., 2000), using inhibitors of methylation to reverse DNA methylation and re-express tumor suppressor genes has emerged as an attractive option for oncotherapy (Nephew and Huang, 2003).

1.2. Apoptosis or Programmed Cell Death

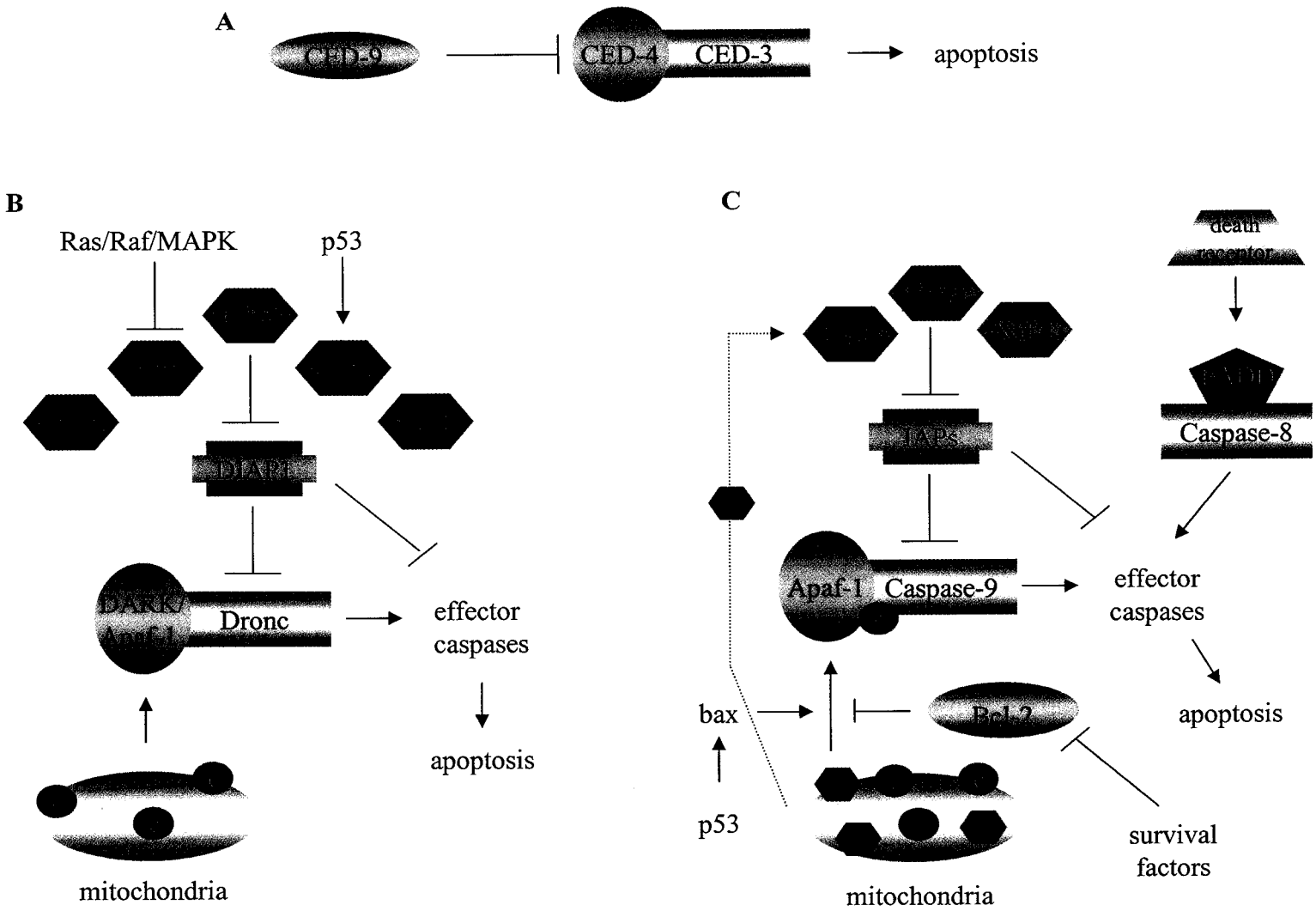
All cells have a limited lifespan. Cell death usually occurs by one of two general physiological mechanisms: necrosis and apoptosis. Necrosis has been defined as a rapid death characterized by cytoplasmic swelling, membrane rupturing and organelle dissolution without marked nuclear changes (Kerr et al., 1972). In contrast, cells dying by apoptosis

exhibit profound morphological changes leading to the disposal of unwanted cells in an orderly fashion (Kerr et al., 1972). Apoptotic cell death is characterized by cell shrinkage, membrane blebbing, chromatin condensation, dissociation from surrounding cells and prominent nuclear fragmentation resulting in the exposure of new surface molecules such as phosphatidylserine targeting apoptotic bodies for phagocytosis (Kerr et al., 1972; Wyllie et al., 1981; Martin et al., 1996; Savill, 1998). It is now widely accepted that apoptosis plays a crucial role in normal development and the maintenance of cellular homeostasis making it imperative for apoptosis to be tightly regulated (Kerr et al., 1972; Vaux et al., 1994; Steller, 1995). In fact, deregulation of apoptosis can lead to disorders such as auto-immune and neurodegenerative diseases (Huntington's, Alzheimer's Disease, spinal muscular atrophy and ischemia) characterized by an abundance of apoptotic death or in cancer where cells, in contrast, become increasingly unresponsive to apoptotic triggers (Thompson, 1995).

Apoptosis was originally identified in natural mutants of the nematode *Caenorhabditis elegans* that did not express the death genes CED-3 and CED-4 (Ellis and Horvitz, 1986; Shaham and Horvitz, 1996). In fact, at birth, these mutant worms contained 131 extra cells resulting from a lack of cell death. In contrast, the protective role of CED-9 against unwanted death was uncovered in CED-9 mutants (Ellis and Horvitz, 1986; Hengartner and Horvitz, 1994a, 1994b; Hengartner, 1996; Chinnaiyan et al., 1997; Wu et al., 1997); CED-9 serving to inhibit apoptosis induced by CED-3 and CED-4 (Hengartner et al., 1992). Over the last decade, homologues of the *C. elegans* death genes have been discovered in numerous vertebrate species including *Drosophila*, mouse and humans suggesting a strong conservation of the apoptotic process through evolution. Comparison of programmed cell death in *C. elegans*, *Drosophila melanogaster* and mammals (Fig.1.1) revealed that the apoptotic machinery is strikingly similar in both invertebrates and

Fig. 1.1. The apoptotic machinery is more complex in vertebrates than in invertebrates.

Although fundamental components of the apoptotic machinery are conserved from *C. elegans* (A) to *Drosophila melanogaster* (B) to mammals (C), it is clear from this diagram that apoptosis is more tightly regulated in mammals than in the other two systems. Proteins presumed to share function between the three systems are identically depicted. Adapted from Meier et al., 2000.


A
CED-9
CED-4
CED-3
apoptosis
B
Ras/Raf/MAPK
p53
DIAP1
DARK/
Apaf-1
Dronc
effector
caspases
apoptosis
mitochondria
C
death
receptor
IAPs
Caspase-8
Apaf-1
Caspase-9
effector
caspases
apoptosis
bax
Bcl-2
p53
survival
factors
mitochondria

vertebrates, although it is far more complex and variable in higher organisms (Steller, 1995).

1.3. Regulators of apoptosis

1.3.1. Caspases

The discovery that defective cell death in *C. elegans* resulted from mutations in the CED-3 gene sparked interest in the identification of mammalian counterparts of this gene. The first report of a mammalian caspase was the CED-3 homologue interleukin-1β converting enzyme (ICE; Cerretti et al., 1992, Thornberry et al., 1992; Yuan et al., 1993) which was subsequently renamed caspase-1 and shown to play a role in cell death and inflammation (Miura et al., 1993). The importance of caspases in apoptosis was further confirmed by the demonstration that caspase-3 is a critical mediator of apoptosis in mammalian cells (Nicholson et al., 1995).

Caspases are cysteine-dependent aspartate specific proteases which, as their name implies, cleave their substrates after aspartic acid residues (Alnemri et al., 1996). It has been determined that caspases function as heterotetramers resulting from the association of two heterodimers (small and large subunits) containing two active sites (Walker and Sikorska, 1994; Wilson et al., 1994; Rotonda et al., 1996; Earnshaw et al., 1999). Substrate specificity is conferred by the four N-terminal amino acid residues (Margolin et al., 1997). In fact, caspases can be divided into 2 subgroups based on their preferred cleavage sites. Group I caspases (1, 2, 4, 5, 6, 9) show preference for (W/L)EHD while group II caspases (3, 7) preferentially cleave the DEVD motif (reviewed in Earnshaw et al., 1999).

To date, more than 10 human caspases have been identified (Alnemri et al., 1996). Given the variability in caspase numbers among species, ranging from 4 in *C. elegans* to 7 in

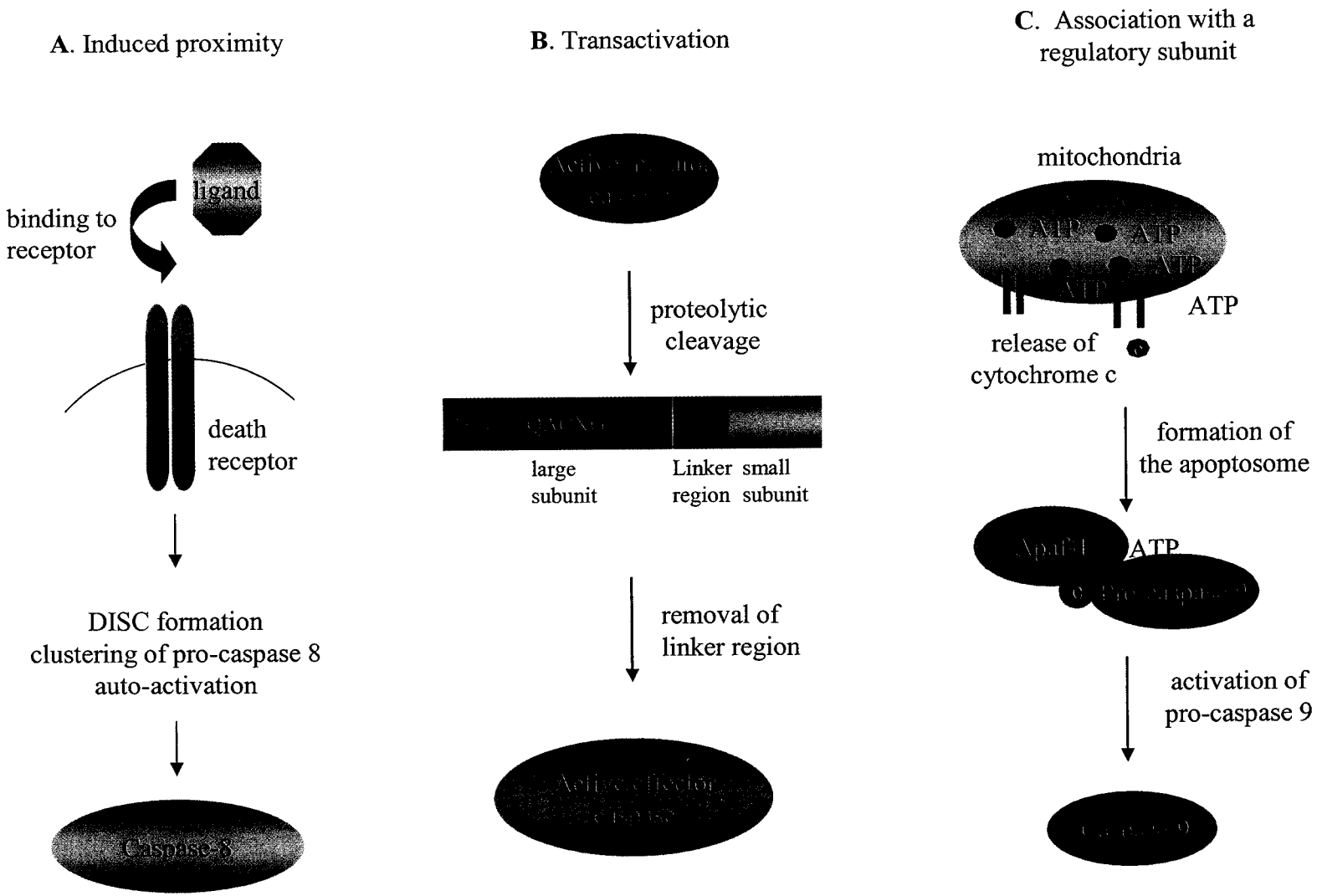
Drosophila and more than 10 in humans, it was suggested that, over phylogenetic time, there is a strong tendency to increase the number of caspases, probably to provide enhanced regulatory mechanisms in vertebrates (Lamkanfi et al., 2002).

Caspase precursors (also known as pro-caspases) are composed of three domains: the N-terminal pro-domain, a large subunit containing the conserved QACXG motif and a C-terminal small subunit (reviewed in Earnshaw et al., 1999). In addition, the pro-domain is separated from the large subunit by an aspartate cleavage site while the small and large subunits are linked by an interdomain linker containing, at most, two aspartate cleavage sites. Caspases containing large pro-domains typically function as initiator or upstream caspases as they are thought to be involved in the initiation of the apoptotic response. In contrast, effector caspases which are activated by initiator caspases contain short pro-domains. Although quite divergent, pro-domains contain two related motifs: the death effector domain (DED; Chinnaiyan et al., 1995) encountered in caspases-8 and -10 only and the caspase recruitment domain (CARD; Hofmann et al., 1997) found in caspases-1, -2, -4, -5, -9, -11 and –12. The DED motif appears to be involved in interactions with adapter molecules while the CARD motif is presumed to mediate protein-protein interactions.

Caspase precursors are synthesized as inactive zymogens. Currently, three mechanisms of caspase activation have been suggested: autoactivation through induced proximity, transactivation or association with a regulatory subunit (Fig.1.2). In the induced proximity model, best investigated for caspase-8, high enzyme concentration may lead to autoactivation since caspase zymogens have been shown to possess low proteolytic activity (Yamin et al., 1996; Muzio et al., 1998; Orth et al., 1996). The induced proximity of zymogen molecules with low intrinsic activity allows them to autoactivate (Salvesen and Dixit, 1999). Briefly, upon ligand binding, death receptors such as Fas/CD95 aggregate and

Fig. 1.2. Mechanisms of caspase activation.

A. In the induced proximity model, binding of an extracellular ligand to an intracellular death receptor triggers oligomerization of the receptor, formation of the DISC and recruitment of pro-caspase-8 molecules which can activate one another by virtue of their low intrinsic protease activity. **B.** Upstream initiator caspases usually activate downstream effector caspases by proteolytic cleavage of the interlinker domain, a process known as transactivation. In turn, effector caspases can cleave initiator caspases resulting in amplification of the caspase cascade. **C.** Activation of pro-caspase-9 is unique since it requires its association with an adaptor molecule, Apaf-1. The mitochondrial release of cytochrome c allows for the ATP-dependent oligomerization of the Apaf-1/pro-caspase-9 complex known as the apoptosome. The association of pro-caspase-9 with Apaf-1 increases its protease activity and leads to the cleavage and activation of pro-caspase-9 at Asp135.


A. Induced proximity
ligand
binding to receptor
death receptor
DISC formation
clustering of pro-caspase 8
auto-activation
Caspase-8
B. Transactivation
proteolytic cleavage
large subunit
Linker region
small subunit
removal of linker region
C. Association with a regulatory subunit
mitochondria
ATP
ATP
ATP
ATP
ATP
release of cytochrome c
formation of the apoptosome
ATP
activation of pro-caspase 9

form membrane-bound complexes which then recruit procaspase-8 molecules thereby restricting their mobility and increasing local enzyme concentration. Procaspase-8 molecules can then activate one another.

The transactivation model usually involves processing of caspase zymogens by other active caspases. In this case, caspases are activated by proteolytic cleavage of the interlinker domain between the large and small subunits followed by the removal of the pro-domain (Ramage et al., 1995; Yamin et al., 1996). What becomes very attractive in this model is that these cleavages all occur at candidate caspase cleavage sites (aspartate residues) reinforcing the notion that caspases are part of an amplification cascade. In support of this model, activated caspase-9 (initiator) has been shown to cleave caspase-3 (effector) whereas the reverse also holds true making for positive feedback loops leading to the amplification of the apoptotic signal (Srinivasula et al., 1998a; Fujita et al., 2001; Slee et al., 1999).

Lastly, the most complex mechanism of caspase activation involves association with a regulatory subunit as is the case for caspase-9. Indeed, the activation of procaspase-9 is quite unique in that proteolytic cleavage is neither necessary nor sufficient for its activation (Rodriguez and Lazebnik, 1999; Stennicke et al., 1999, Renatus et al., 2001). In short, the interaction between procaspase-9 and Apaf-1 via their respective CARD domains allows for autolytic activation of caspase-9 and the initiation of apoptotic cell death via the mitochondrial death pathway (see below).

Nicholson and Thornberry (1997) suggested that caspases participate in apoptosis by disabling major cellular processes and breaking down cellular structures. In fact, caspases, in particular effector caspases, are responsible for several of the morphological changes typical of apoptosis. For instance, it was recently demonstrated that the DNA ladder nuclease responsible for cutting genomic DNA between nucleosomes (aka CAD for caspase-activated

DNase) is complexed with an inhibitory subunit (iCAD/DFF45) which maintains it inactive. Caspase-3 mediates cleavage of the inhibitory subunit resulting in the release and activation of CAD and subsequent nucleosomal degradation (Nagata, 2000; Enari et al., 1998; Mitamura et al., 1998). Furthermore, the caspase-mediated cleavage of lamins results in nuclear shrinking and budding (Rao et al., 1996; Buendia et al., 1999). Lastly, the caspase-mediated cleavage of PAK2, a kinase involved in the regulation of the actin skeleton, helps in the formation of apoptotic bodies and results in the typical membrane blebbing observed during apoptotic cell death (Rudel and Bokoch, 1997).

Since their identification, the primordial role of caspases in apoptosis has clearly been established (reviewed by Degterev et al., 2003). Caspase knock-out mouse models have helped elucidate the essential role of individual caspases and their cofactors in programmed cell death. For example, mice deficient in caspase-9, caspase-3 and Apaf-1 exhibit profound defects in the developing brain (ectopic cell masses, defects in neural tube closure and protusion of cranial brain tissue) due to a lack of apoptosis (Kuida et al., 1996, 1998; Woo et al., 1998, Hakem et al., 1998; Yoshida et al., 1998). In addition, caspase-9 mutant mice revealed that caspase-9 is essential for the activation of caspase-3 since a lack of caspase-3 cleavage was observed in these animals. In contrast, reintroducing *in vitro*-translated caspase-9 into caspase-9-deficient lysates restored cleavage of caspase-3 (Kuida et al., 1998). Moreover, Apaf-1, caspase-9 and caspase-3 deficient mouse embryonic stem cells were shown to be resistant to drug-and radiation-induced apoptosis (Woo et al., 1998; Hakem et al., 1998; Yoshida et al., 1998) highlighting the role of these proteins in mitochondrial apoptosis. On the other hand, mice harbouring null mutations in caspase-8 exhibited abnormal development of cardiac tissues and died during embryogenesis while caspase-8 deficient cells were resistant to apoptosis induced by death receptors but sensitive to death

triggered by DNA damage (Varfolomeev et al., 1998; Yeh et al., 1998; Zhang et al., 1998) supporting a role for caspase-8 in death receptor-mediated apoptosis.

1.3.2. Inhibitor of Apoptosis Proteins

Inhibitor of apoptosis proteins (IAPs) were first discovered in baculoviruses based on their ability to suppress the host cell death response to infection (Crook et al., 1993; Birnbaum et al., 1994). The first mammalian IAP, NAIP (neuronal apoptosis inhibitory protein) was identified in a positional cloning effort to isolate the causative gene for spinal muscular atrophy (SMA), a neurodegenerative disorder leading to the progressive loss of motor neurons and wasting of the voluntary muscles (Roy et al., 1995). However, it was later demonstrated that the smn gene (survival motor neuron), also isolated in the SMA deletion region, was the causative gene for this disorder (Lefebvre et al., 1995). Nevertheless, although NAIP does not directly cause SMA, it has been proposed that its deletion may serve to modulate the severity of the disease (Gendron and Mackenzie, 1999). Given that over-expression of NAIP in mammalian cells has been shown to suppress apoptotic cell death, deletion of NAIP could enhance the loss of motor neurons characteristic of SMA (Liston et al.; 1996; Xu et al., 1997; Maier et al., 2002). Following isolation of NAIP, cIAP-1 (Hiap2), cIAP-2 (Hiap1) and Xiap (X-linked IAP) were identified as mammalian IAPs (Rothe et al., 1995; Duckett et al., 1996; Liston et al., 1996; Uren et al., 1996). Given that cIAP-1 and cIAP-2 lie in tandem within approximately 7 Kbp of each other on human chromosome 11q22-23, it has been suggested that they arose from a gene duplication event (Rajcan-Separovic, 1996; Young et al., 1999). The IAP family was further expanded with the identification of survivin (17q25; Ambrosini et al., 1997, 1998), livin (Lin

et al., 2000; Vucic et al., 2000; Kasof and Gomes, 2001) and testis-specific IAP (ts-IAP; Lagacé et al., 2001; Richter et al., 2001).

In addition to the human IAP gene family, homologs of the baculoviral IAPs (Cp-IAP and Op-IAP) were isolated in *Drosophila* (DIAP1 and DIAP2; Hay et al., 1995), mice (mNAIP1, mNAIP2, MIAP1, MIAP2, msurvivin, MIAP3; Yaraghi et al., 1998; Scharf et al., 1996; Liston et al., 1997; Farahani et al., 1997), rat (RIAP1, RIAP2, RIAP3; Holcik et al., 2002), pig (PIAP; Stehlik et al., 1998a) and chicken (ITA, ch-IAP; Digby et al., 1996; You et al., 1997).

Structurally, the IAPs are composed of one to three tandem copies of a 70 amino acid domain termed the baculoviral IAP repeat (BIR) domain that are found in the N-terminal region of all mammalian members (Crook et al., 1993; Birnbaum et al., 1994). Recently, the solution structure of the BIR domain has been solved (Hinds et al., 1999). The BIR domain forms a compact globular structure containing four to five alpha helices and anti-parallel beta sheets. In addition, the cysteine and histidine-rich protein folding BIR domain has been shown to coordinate a zinc ion. The presence of the BIR domain and the ability to suppress apoptosis confers membership to the IAP family (Miller, 1999; Hinds et al., 1999; C. Sun et al., 1999). Another defining motif frequently encountered in IAPs is a C-terminal RING zinc finger (RZF) domain (Freemont et al., 1991). RZFs are usually involved in DNA-protein interactions (Borden and Freemont, 1996). Additionally, RZFs can function as E3 ubiquitin ligases that can target proteins for proteosomal degradation by recruiting them to a multi-protein complex containing a ubiquitin-conjugating enzyme (reviewed in Pickart, 2001; see below for further details). Lastly, in addition to three N-terminal BIR domains and a C-terminal RZF domain, both cIAP-1 and cIAP-2 contain a CARD domain located between BIR3 and the RING zinc finger domain.

One way to regulate caspase activity is to prevent their activation. Nonetheless, once activated, both initiator and effector caspases can be inhibited by IAPs. In addition to their role as endogenous caspase inhibitors, IAPs are thought to participate in cell cycle regulation and in modulation of death receptor-mediated signaling. Over-expression of IAPs suppresses apoptosis triggered by a variety of death stimuli (Duckett et al., 1996; Liston et al., 1996; Ambrosini et al., 1997, J. Li et al., 1998). Indeed, IAPs, in particular Xiap, have been shown to directly inhibit the activity of recombinant caspases *in vitro* (Deveraux et al., 1997; Roy et al., 1997; Maier et al., 2002). Two different Xiap domains (BIR2 and BIR3) can suppress caspase activity (Deveraux et al., 1999). The Xiap BIR3 domain can bind directly to the small subunit of caspase-9 thereby inhibiting its activity (Sun et al., 2000; Srinivasula et al., 2001). This interaction is thought to prevent homodimerization of caspase-9 and maintain it in an inactive state (Shiozaki et al., 2003). Interestingly, the cleavage of caspase-9 at Asp330 by caspase-3 has been proposed to remove the Xiap binding site thereby facilitating caspase activation (Srinivasula et al., 2001). However, further studies have suggested that the BIR3 of Xiap can still interact with processed caspase-9 (Zou et al., 2003). Moreover, the BIR3 of cIAP-1 and cIAP-2 and the single BIR of livin and ts-IAP can also bind to and inhibit caspase-9 (Deveraux et al., 1998; Bratton et al., 2001; Vucic et al., 2000; Richter et al., 2001). In contrast, a small segment N-terminal to the BIR2 of Xiap is required for interaction with caspase-3 and -7 (Huang et al., 2001; Riedl et al., 2001; Chai et al., 2001). Indeed, high-affinity binding of Xiap BIR2 to caspases–3 and –7 results in steric occlusion of the active site of the caspases. In short, the BIR1-2 linker of Xiap is exclusively needed to achieve inhibition of caspase-3 while Xiap BIR2 is also needed for successful inhibition of caspase-7 (Chai et al., 2001; Huang et al., 2001; Riedl et al., 2001; C. Sun et al., 1999, 2000).

The RING zinc finger domain is thought to target IAPs and their binding partners for degradation. As such, targeting caspases to the proteosome for degradation may be another mechanism by which IAPs exert their anti-apoptotic effects. Proteosomes are multiprotein complexes which are responsible for the degradation of polyubiquitinated proteins (Weissman, 1997). Briefly, protein ubiquitination requires the sequential activation of three enzymes: ubiquitin-activating enzyme (E1), ubiquitin-conjugating enzyme (E2) and ubiquitin ligase (E3; Hershko and Ciechanover, 1998). It has previously been suggested that the E3 activity may be dependent on RZFs (Lorick et al., 1999; Iwai et al., 1999; Lisztwan et al., 1999, Waterman et al., 1999; Joazeiro et al., 1999; Fang et al., 2000). By incubating GST-fusion proteins (GST-Xiap and GST-cIAP-1) with recombinant E1, E2 and radio-labelled ubiquitin, Yang et al. (2000) demonstrated that both Xiap and cIAP-1 have intrinsic E3 ligase activity that is ablated by the removal of the RZF suggesting that proteosome-mediated degradation of IAPs may be necessary for cells to actually undergo apoptosis. In addition, cIAP-2 and Xiap have been shown to trigger ubiquitination of caspases-3 and –7 (Huang et al., 2000; Suzuki et al., 2001a). Interestingly, it was recently demonstrated that cIAP-1 and cIAP-2 function as ubiquitin ligases for the pro-apoptotic Smac/DIABLO protein thereby promoting the degradation of this strong Xiap antagonist (Hu and Yang, 2003).

The IAPs are also involved in signal transduction. Indeed, Xiap has been identified as an adaptor protein linking the BMP receptors and TAB1-TAK1 in the bone morphogenetic protein (BMP) signaling pathway involved in mesoderm induction and patterning in *Xenopus* embryos (Yamaguchi et al., 1999). This function of Xiap has been suggested to be mediated by the RING zinc finger domain. On the other hand, cIAP-1 and cIAP-2 were first identified by virtue of their association with the TNF-RII receptor complex although they do not directly contact the receptor (Rothe et al., 1995). In fact, cIAP-1 and –2

are presumed to be recruited to this complex via an association of their N-terminal BIR with Traf1/Traf2 heterocomplexes (Rothe et al., 1995). In addition, exposure to TNF-α leads to an up-regulation of cIAP-1, cIAP-2 and Xiap (Stehlik et al., 1998b). In turn, cIAP-1 mediates the ubiquitination and degradation of Traf2 resulting in sensitization to TNF-induced apoptosis (X. Li et al., 2002). It has further been suggested that cIAP-2 is required to suppress TNF-induced apoptosis via its involvement in TNF signaling events that induce NF-κB (Chu et al., 1997). Interestingly, stimulation of NF-κB by TNF-α results in the activation of cIAP-2 while cIAP-2 itself can reportedly activate NF-κB.

Last, but not least, a dual role was suggested for the 16 kDa cytoplasmic protein survivin: suppression of apoptosis (Ambrosini et al., 1997; F. Li et al., 1998; Altieri et al., 1999; Tamm et al., 1998) and regulation of cell division (Fraser et al., 1999; Li et al., 1999; Miller, 1999; Reed and Reed, 1999; Uren et al, 1999; Reed and Bischoff, 2000; Fortugno et al., 2002). Survivin has been found to co-immunoprecipitate with and suppress apoptosis induced by caspases-3, -7 and –9, albeit with less potency than Xiap (Tamm et al., 1998). Recently, it has been proposed that binding of the IAP antagonist Smac/DIABLO by survivin relieves Smac repression of Xiap thereby allowing Xiap to interact with caspases and effectively block taxol-induced cell death (Song et al., 2003). Furthermore, survivin is unique among IAPs in that it has been shown to be involved in cell cycle regulation (F. Li et al., 1998). Indeed, survivin is expressed at the G2/M checkpoint in a cell cycle-dependent manner and is associated with microtubule formation in the mitotic spindle via its C-terminal (Li et al., 1999). In addition, survivin promotes genetic stability and genome integrity during chromosomal segregation and cytokenesis (Uren et al., 2000). Recently, survivin has been shown to be up-regulated by oncogenic c-H-ras in ras-transformed cell lines; in fact, survivin

expression closely followed that of c-H-ras, with a 2h delay in a dose-dependent, cell cycle progression-independent manner while inhibition of the ras pathways successfully decreased survivin expression resulting in induction of apoptosis (Sommer et al., 2003).

1.3.2.1. Negative regulators of Inhibitor of Apoptosis Proteins in *Drosophila*

In *Drosophila*, embryos lacking the loci coding for the pro-apoptotic proteins head involution defective (hid), grim and reaper (rpr) die with an excess of cells in late embryogenesis (White et al., 1994). More recently, a fourth pro-apoptotic protein, sickle (skl), was identified as a *Drosophila* death gene (Srinivasula et al., 2002). The deletion of hid, grim, rpr or skl can suppress apoptosis in *Drosophila* embryos (White et al., 1994; Grether et al., 1995; Chen et al., 1996a, 1996b; Srinivasula et al., 2002) while over-expression of any one of these genes can induce apoptotic cell death (Grether et al., 1995; Chen et al., 1996b; White et al., 1996; Srinivasula et al., 2002). Recently, the thioredoxin peroxidase Jafrac2 was identified as a DIAP1-interacting protein (Tenev et al., 2002). Mature Jafrac2 can interact genetically and biochemically with DIAP1 and promote cell death, presumably by competing with the *Drosophila* caspase Dronc for binding to DIAP1 (Tenev et al., 2002). The *Drosophila* death genes bind on the surface of DIAP1 BIR2 thereby blocking caspase-binding sites and promoting caspase activation and cell death (Goyal, 2001; Song et al., 2000; Wu et al., 2001; Srinivasula et al., 2002). Interestingly, it was recently demonstrated that IAP antagonists in *Drosophila*, namely rpr, grim and Jafrac2 exhibit non-redundant modes of action as they display differential and selective binding to DIAP1 BIR domains (Zachariou et al., 2003).

While rpr and grim appear to be expressed solely in cells doomed to die (White et al., 1994; Chen et al., 1996a; Robinow et al., 1997), hid is expressed in both cells that survive and cells that undergo apoptosis (Grether et al., 1995) suggesting that hid may participate in

a cell survival pathway that is distinct from that of rpr and grim. Indeed, hid was reported to be a molecular target of ras-dependent cell signaling in that ras can down-regulate hid expression thus promoting cell survival (Bergmann et al., 1998; Kurada and White, 1998). In fact, mutations in genes that over-activate the ras/MAPK signaling pathway suppress hid-induced apoptosis (Bergmann et al., 1998). Consistent with these findings, increased ras activity decreased hid mRNA levels and/or resulted in the inactivation of the hid protein via phosphorylation (Kurada and White, 1998).

The fact that the *Drosophila* proteins hid, grim, rpr and skl can induce apoptosis in mammalian cells (McCarthy and Dixit, 1998; Haining et al., 1999; Srinivasula et al., 2002) once again supports the notion that apoptosis is a highly conserved mechanism.

1.3.2.2. Negative regulators of Inhibitor of Apoptosis Proteins in mammals

To this day, biochemical studies have uncovered three proteins capable of binding to and inhibiting the anti-apoptotic properties of mammalian IAPs: Smac/DIABLO, Omi/HtrA2 and Xaf-1.

The IAP-interacting protein Smac/DIABLO was identified simultaneously in humans and mice. The search for novel proteins capable of promoting cytochrome c/Apaf-1-dependent caspase activation led to the identification of the human mitochondrial protein Smac (Du et al., 2000) while the murine protein DIABLO was isolated based on its ability to associate with MIHA, the mouse homologue of Xiap (Verhagen et al., 2000). Database searches further revealed that the human Smac sequence is identical to that of mouse DIABLO. The authors of these back-to-back publications independently demonstrated that, in response to apoptotic stimuli, Smac/DIABLO is released from the mitochondria into the cytosol, interacts with IAPs and mediates reversal of the anti-apoptotic effects of Xiap. In fact, the N-terminal of Smac/DIABLO contains a 55 amino acid mitochondrial localization

sequence that is proteolytically removed for the translocation of the protein to the cytosol (Du et al., 2000; Verhagen et al., 2000). Removal of the mitochondrial targeting sequence exposes a new N-terminal sequence for Smac (Ala-Val-Pro-Ile) which fits a surface groove in the BIR3 of Xiap (Srinivasula et al., 2000). Consistent with these findings, the solution structure of Xiap BIR3 complexed with a functionally active 9 aa peptide derived from the N-terminal of Smac established that the four N-terminal residues of Smac mediate its interaction with the BIR3 of Xiap (Liu et al., 2000). Interestingly, these residues are homologous to the residues mediating the interaction between caspase-9 and Xiap BIR3 suggesting that Smac/DIABLO may displace Xiap from caspase-9 allowing for activation of caspase-9 and initiation of the apoptotic cascade (Sun et al., 2000). Indeed, Smac/DIABLO is thought to potentiate the activation of caspase-9 by disrupting the interaction between a linker peptide of caspase-9 and Xiap BIR3 since binding of the caspase-9 linker peptide and Smac to the BIR3 of Xiap are mutually exclusive (Srinivasula et al., 2001). In addition, recent data has suggested that the ability of Xiap to bind to caspase-3, caspase-9 and Smac/DIABLO is necessary to inhibit UV/etoposide-induced apoptosis (Silke et al., 2002). Given that the presence of Smac/DIABLO alone is sufficient to antagonize Xiap-mediated suppression of cell death, IAP antagonists may play a crucial role in relieving caspase inhibition (Silke et al., 2002).

Shortly after the identification of Smac/DIABLO, Omi/HtrA2 was identified as a second Xiap-binding protein (Suzuki et al., 2001b; Hedge et al., 2002; Martins, 2002; van Loo et al., 2002; Verhagen et al., 2002). In response to apoptotic stimuli, the serine protease Omi/HtrA2 is released from the mitochondria to the cytosol where it can interact with Xiap and effectively sensitize cells to apoptosis (Q. Yang et al., 2003). In fact, like Smac/ DIABLO, Omi/HtrA2 contains an IAP-binding motif consisting of a short stretch of

hydrophobic amino acids (Ala-Val-Pro-Ser) allowing it to bind competitively to the BIR3 of Xiap (Suzuki et al., 2001b; Hedge et al., 2002). Nevertheless, contrary to Smac/DIABLO, whose active entity is a homodimer, Omi/HtrA2 functions as a homotrimer (Chai et al., 2000; W. Li et al., 2002). Enzymatic substrates of Omi/HtrA2 were shown to include IAPs (Q. Yang et al., 2003). Interestingly, in etoposide-induced cell death, p53 can reportedly up-regulate Omi/HtrA2 resulting in the cleavage of cIAP-1 (Jin et al., 2003). As the cleavage of cIAP-1 can be inhibited by serine protease inhibitors, it has been suggested that Omi/HtrA2 is capable of cleaving cIAP-1. In addition, Omi/HtrA2-mediated cleavage of IAPs has been proposed to efficiently promote cell death by irreversibly reducing the inhibition of IAPs on caspase activity. On the other hand, Omi/HtrA2 can induce apoptosis in a caspase-independent manner, although this mechanism is not well understood. It appears that this pathway relies on the protease activity of Omi/HtrA2, potentially via the cleavage and inactivation of apoptotic inhibitors or via the activation of precursor proteins necessary for caspase-independent cell death (Cilenti et al., 2003).

Yeast two-hybrid screening identified another IAP binding partner and antagonist termed Xaf-1 (Xiap-associated factor 1; Fong et al, 2000). Xaf-1 is a 33 kDa, zinc-finger rich protein mapping to human chromosome 17p13.2 (Fong et al., 2000), a region prone to loss of heterozygosity in a variety of human cancers (Casey et al., 1993; Cornelis et al., 1994; Fujii et al., 1998; Phillips et al., 1993, 1996; Konishi et al., 1998). Moreover, Xaf-1 is ubiquitously expressed in normal human tissues (Liston et al., 2001) but low to non-existent in a majority of the NCI 60 cancer cell lines panel (Fong et al., 2000). This observation suggests that loss of Xaf-1 expression in cancer cell lines may allow for unrestricted Xiap activity and result in suppression of apoptosis (Fong et al., 2000). In addition, Xaf-1 has been shown to directly bind to Xiap and interfere with Xiap-mediated caspase-3 inhibition.

Indeed, co-infection of cells with adeno-Xiap and adeno-Xaf1 constructs resulted in the reversal of Xiap-mediated protection suggesting that Xaf-1 can sensitize cells to apoptotic stimuli, presumably through the sequestration of Xiap (Liston et al., 2001). Moreover, the nuclear Xaf-1 protein triggers the relocalization of cytoplasmic Xiap to the nucleus as a means of sequestering it away from the apoptosome. In contrast, depleting cells of Xaf-1 appears to increase their resistance to etoposide-induced cell death further suggesting that down-regulation of Xaf-1 expression may provide cancer cells with a survival advantage (Liston et al., 2001).

Xaf-1 was recently reported to be an interferon-inducible protein contributing to IFN-β-dependent sensitization of cells to TRAIL-induced apoptosis (Leaman et al., 2002). This observation is not surprising given previous reports that Xiap can confer resistance to TRAIL-induced apoptosis in melanoma cells (Zhang et al., 2001), that inhibiting the activity of Xiap appears to be necessary for TRAIL-induced apoptosis (Deng et al., 2002) and that interferon can sensitize cells to TRAIL-induced apoptosis (Kumar-Sinha et al., 2002; Chawla-Sarkar et al., 2002). Further studies in human gastric adenocarcinomas revealed that Xaf-1 mRNA expression was significantly down-regulated by aberrant hypermethylation of the Xaf-1 promoter (Byun et al., 2003). The fact that Xaf-1 expression is low or non-existent in cancer cell lines, potentially due to hypermethylation of the promoter region raises the possibility that Xaf-1 could be a tumor suppressor gene, although further studies are needed to substantiate this claim.

1.3.2.3. Translational regulation of Inhibitor of Apoptosis Proteins

In addition to its regulation by antagonists, the unusually long 5'UTR of Xiap suggested that it may be regulated at the translational level. Initiation is thought to be the

critical regulatory step in protein translation. In fact, initiation can either be cap-dependent (the more classical model) or cap-independent. In response to cellular stress, such as apoptotic triggers, cap-dependent protein synthesis usually shuts down. Internal ribosome entry site elements (IRES) were first discovered in picornaviruses as a means of initiating translation of uncapped viral mRNAs (Pelletier and Sonenberg, 1988; Jang et al., 1998). These IRES elements were proposed to directly bind to and recruit ribosomes for the initiation of cap-independent translation (reviewed in Reynolds et al., 1996). An IRES element located upstream of the initiation codon was identified in Xiap (Holcik et al., 1999). Further characterization of the Xiap IRES motif revealed that it is 162 nucleotides long and can bind the La autoantigen (Holcik and Korneluk, 2000). It has therefore been proposed that up-regulating Xiap protein via IRES-dependent translation is an elegant mechanism by which cells can protect themselves from stress-induced apoptosis (Holcik et al., 2000).

1.4. Apoptotic pathways: intrinsic vs. extrinsic

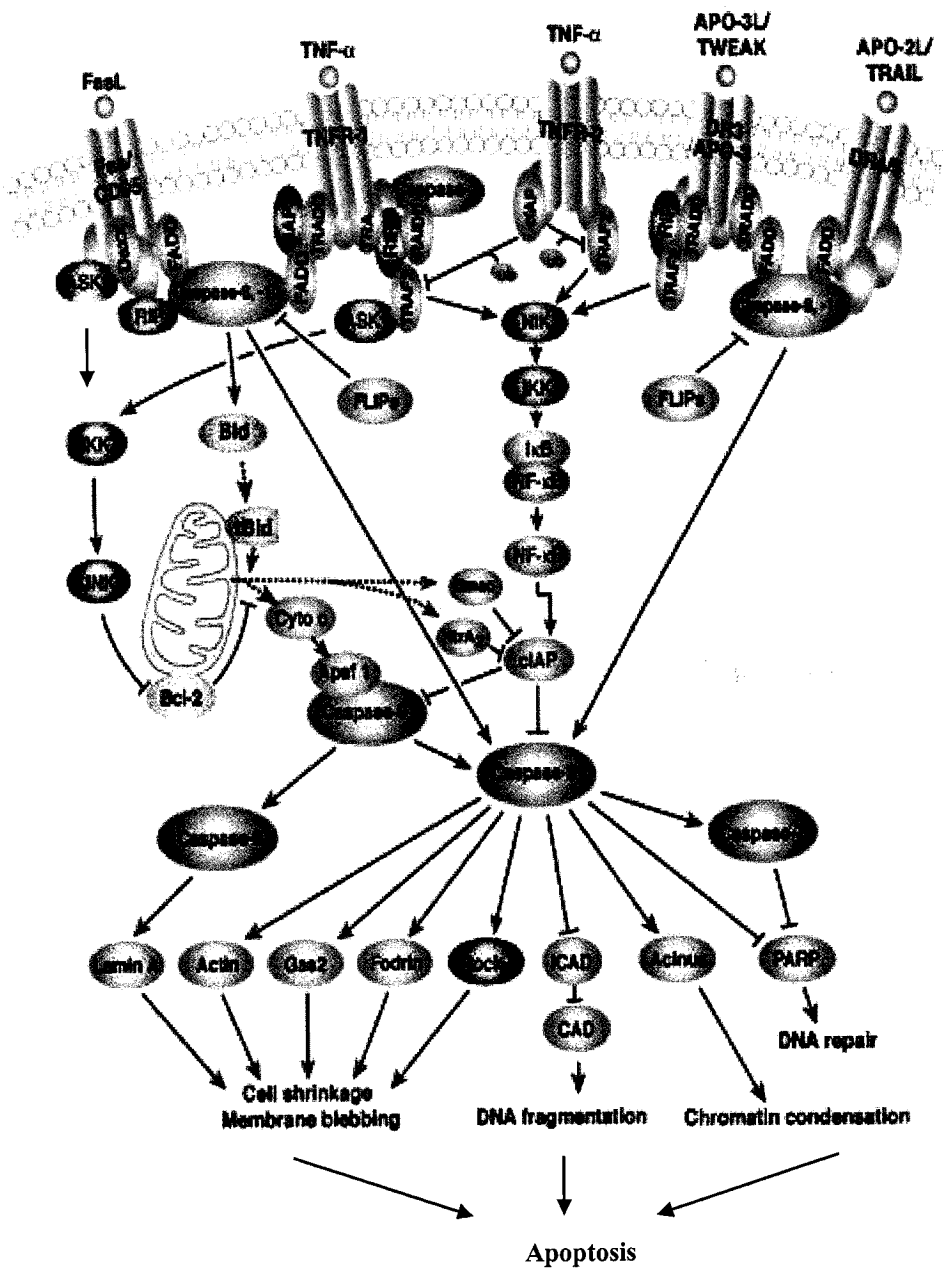
Apoptosis can be divided into two distinct but overlapping processes known as the extrinsic or intrinsic apoptotic pathways.

1.4.1. Death receptors and the extrinsic apoptotic pathway

The extrinsic apoptotic pathway relies on binding of an external ligand to a transmembrane receptor to trigger apoptosis (Fig.1.3). Death receptors involve a subset of tumor necrosis factor receptor (TNF-R) family members defined by cysteine-rich extracellular domains (Smith et al, 1994; Gruss and Dower, 1995). Typically, death

Fig.1.3. Death receptor-mediated or extrinsic apoptotic pathway.

In this system, binding of an extracellular ligand to a transmembrane receptor induces oligomerization of the receptor, formation of the death-inducing signaling complex (DISC) to which adaptor molecules are recruited to facilitate activation of caspase-8. In turn, activated caspase-8 can cleave and activate pro-caspase-3. Active caspase-3 is responsible for several hallmarks of programmed cell death such as cell shrinkage, membrane blebbing, DNA fragmentation and chromatin condensation. Modified from the website cellsignal.com


FasL
TNF-α
TNF-α
APO-3L/
TWEAK
APO-2L/
TRAIL
TNFR-1
TNFR-2
DR3/
APO-3
DR4/
APO-2
FADD
TRADD
RIP
Caspase-8
cFLIP
JNK
IκB
NF-κB
Bid
tBid
Cyto c
Apaf-1
Caspase-9
Bcl-2
cIAP
Caspase-3
Caspase-6
Caspase-7
Lamin A
Actin
Gas2
Fodrin
DFF45
CAD
ICAD
Actin
PARP
DNA repair
Cell shrinkage
Membrane blebbing
DNA fragmentation
Chromatin condensation
Apoptosis

receptors such as CD95/Fas/Apo1 transduce death signals. It appears that signaling by various members of the death receptor family follows the same sequence of events (reviewed in Krammer, 2000). First, ligand binding clusters the receptor and induces the formation of a death-inducing signaling complex (DISC). Adaptor molecules are then recruited to the DISC where they facilitate an association with procaspase-8. Cleavage and activation of the initiator caspase-8 ensues leading to the activation of effector caspase-3.

Signaling through the CD95/Fas receptor leads to apoptotic cell death (Krammer, 1999). In fact, binding of Fas ligand to Fas induces trimerization of the receptor (Huang et al., 1996) resulting in the rapid recruitment of cellular factors to an activated Fas receptor to form the basis of the DISC (Kischkel et al., 1995). The adaptor molecule FADD (Fas-associated death domain, aka Mort1) binds to the Fas receptor, an interaction mediated by their respective death domains (Chinnaiyan et al., 1995; Boldin et al., 1995). The DED domain of FADD can then recruit procaspase-8 to the DISC by means of an interaction with its own DED domain (Boldin et al., 1996; Muzio et al, 1996). This increases the local concentration of procaspase-8 and results in its activation, presumably via the induced proximity model described previously. Activated caspase-8 can then cleave and activate caspase-3 thereby ensuring completion of the apoptotic cascade.

Signaling via TNF-R1 activates the expression of the transcription factors NF-κB and AP-1 thereby inducing pro-inflammatory and immuno-modulatory genes (Tartaglia and Goeddel, 1992). Upon binding, TNF induces trimerization of TNF-R1 (Smith et al., 1994; Gruss and Dower, 1995). The adaptor molecule TRADD (TNF-R-associated death domain) subsequently binds TNF-R1 (Hsu et al., 1995) and recruits several cellular factors to the activated receptor. Among these, Traf2 (TNF-R-associated factor 2; Rothe et al., 1995; Hsu

et al, 1996a) and RIP (receptor-interacting protein; Hsu et al., 1996b; Ting et al., 1996) stimulate pathways leading to the activation of NF-κB and AP-1/JNK. Together, Traf2 and RIP activate NIK (NF-κB-inducing kinase) which itself activates the I-κB (inhibitor of κB) kinase complex IKK (Malinin et al., 1997; Regnier et al., 1997; DiDonato et al., 1997; Mercurio et al., 1997; Zandi et al., 1997). The phosphorylation of I-κB by IKK at serines 32 and 36 targets I-κB for polyubiquitination and degradation. NF-κB can then translocate to the nucleus and activate gene transcription. In fact, the activation of NF-κB can suppress cell death via the up-regulation of cIAP-1/-2, Xiap, Trafs and bcl-2 homologs (reviewed in Barkett and Gilmore, 1999).

1.4.2. Mitochondria and the intrinsic apoptotic pathway

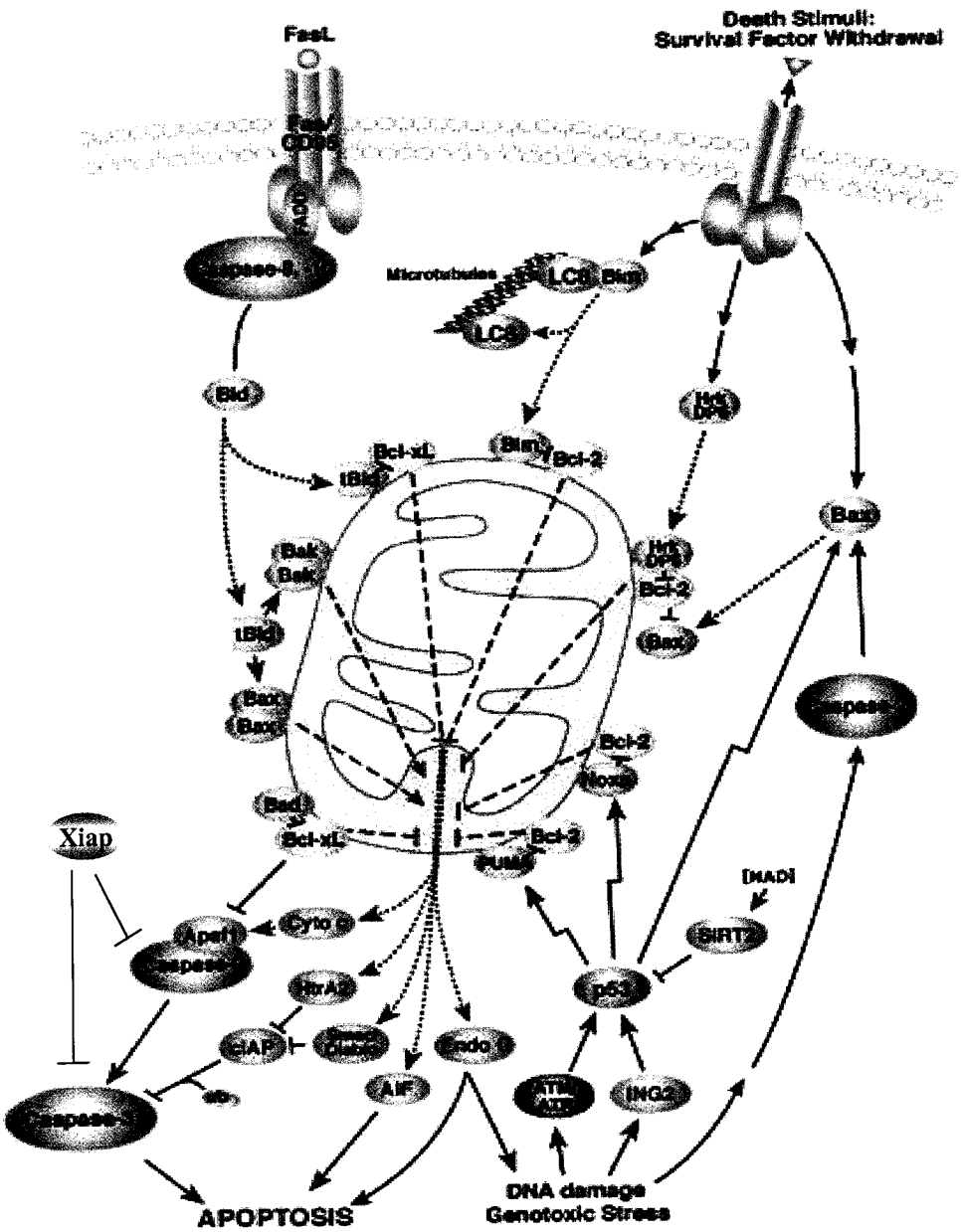
In addition to their role as energy suppliers, mitochondria are now believed to contain a potent arsenal of apoptogenic proteins (Hengartner, 2000). The anti-apoptotic members of the bcl-2 family are similar in sequence and function to the *C. elegans* anti-death gene CED-9 (Jacobson and Evan, 1994; Hengartner and Horvitz, 1994a, 1994b; Vaux et al., 1992). It has been proposed that members of the bcl-2 protein family regulate the release of mitochondrial proteins into the cytosol as a first step in the initiation of the intrinsic pathway of apoptosis (Green and Reed, 1998). The bcl-2 protein family contains both pro- and anti-apoptotic members of which anti-apoptotic members such as bcl-2 usually localize to the mitochondria (Hockenbery et al., 1990; Krajewski et al., 1993) whereas pro-apoptotic members including bax localize to the cytosol (Hsu et al., 1997a; Gross et al., 1998, Puthalakath et al., 1999). Following a death signal, a conformational change in pro-apoptotic

proteins allows them to integrate into the outer mitochondrial membrane thereby altering the exposure of their N-terminal domain (Wolter et al., 1997; Gross et al., 1998, Goping et al., 1998; Desagher et al., 1999).

The disruption of the outer mitochondrial membrane in response to death signals allows for the release of cytochrome c from the mitochondria (Fig.1.4; Goldstein et al., 2000). Cytosolic cytochrome c can then bind Apaf-1 thereby exposing its CARD domain to facilitate recruitment of procaspase-9 (Liu et al., 1996; Li et al., 1997; Green, 1998). In fact, in the presence of dATP, the Apaf-1/cytochrome c complex triggers its oligomerization and recruits procaspase-9 to form the apoptosome (Zou et al., 1999). It has now been demonstrated that the apoptosome is a multiprotein complex comprised of equal molar ratios of Apaf-1, caspase-9 and cytochrome c (Zou et al., 1999). The apoptosome consists of seven Apaf-1/cytochrome c heterodimers oligomerized to form a symmetrical "wheel" in which procaspase-9 can bind Apaf-1 via their respective CARD domains (Qin et al., 1999; Acehan et al, 2002). The binding of procaspase-9 to Apaf-1 leads to the cleavage of procaspase-9 at Asp315 to form a large and small subunit which will combine to form an active heterotetramer (Srinivasula et al., 1998b; Stennicke et al., 1999; Rodriguez and Lazebnik, 1999). In addition, the association of Apaf-1 and caspase-9 results in an increase in protease activity of the caspase (Rodriguez and Lazebnik, 1999; Renatus et al., 2001). Apoptosome-bound active caspase-9 can subsequently activate downstream effector procaspase-3 by proteolytic cleavage at Asp175 (Rodriguez and Lazebnik, 1999; Srinivasula et al., 2001). Interestingly enough, active caspase-3 can activate procaspase-9 by cleavage at Asp330 thereby increasing the activity of the apoptosome and resulting in a positive feedback loop between the two caspases and the amplification of the apoptotic signal (Srinivasula et al., 1998b; Fujita et al., 2001; Slee et al., 1999). In turn, active caspase-3 triggers the proteolysis

Fig.1.4. Mitochondrial or intrinsic apoptotic pathway.

Among other consequences, death stimuli and genotoxic stress such as DNA induce the expression of the pro-apoptotic protein bax which is thought to contribute to the disruption of the outer mitochondrial membrane. As a result, pro-apoptotic factors (cytochrome c, Smac/DIABLO, Omi/HtrA2, AIF) are released from the mitochondria to the cytosol where they facilitate activation of caspases, namely initiator caspase-9. Active caspase-9 is then able to cleave and activate caspase-3 resulting in apoptosis. Adapted from the website cellsignal.com.


FasL
Death Stimuli:
Survival Factor Withdrawal
Microtubules
LC8
Bid
Bcl-xL
Bcl-2
Bak
Bax
Bad
Xiap
Cyto c
Apaf1
SIRT2
p53
Endo G
AIF
ING2
[NAD]
DNA damage
Genotoxic Stress
APOPTOSIS

of several nuclear proteins including PARP and gelsolin resulting in nuclear degeneration and cell death (Lazebnik et al., 1994; Y. Geng et al., 1998).

1.5. Apoptosis and cancer

The development of long-term or even permanent therapeutic options for cancer has been hampered by several factors. Current cancer treatments impart a toxicity to normal cells while often failing to efficiently kill cancer cells. For this reason, therapies need to specifically target cancer cells (both the primary tumor and the metastases) without affecting survival of normal cells. Next, the presence of a molecular transporter capable of actively expelling chemotherapeutic drugs from cancer cells can also contribute to treatment failure (Persidis, 1999). For example, the MDR1 gene products, multidrug resistance-associated protein (MRP) and P-glycoprotein, can protect cells from chemotherapy and caspase-dependent death triggers (Cole et al., 1992; Johnstone et al., 1999; Smyth et al., 1998). Another consideration is that many anti-cancer drugs are currently designed to trigger an apoptotic response by introducing DNA damage (eg. double-strand breaks) into rapidly dividing cells (Kaufmann and Earnshaw, 2000; Dive, 1997; Makin and Hickman, 2000, Haq and Zanke, 1998). Furthermore, apoptosis limits the expansion of cell population early in the process of tumor growth, probably at the stage when angiogenesis occurs (Naik et al., 1996; O'Reilly et al., 1996). It has also been suggested that cancer arises from an accumulation of genetic events, namely the deregulation of cell proliferation and the suppression of apoptosis, resulting in uncontrolled cell growth (Green and Evan, 2002). Therefore, inhibition of apoptosis is crucial for a tumor's formation, progression and resistance to therapy (LaCasse et al., 1998; Hickman, 2002; Reed, 1999). An attractive way for tumors to evade apoptosis

is to increase the apoptotic threshold via the up-regulation of anti-apoptotic proteins or the repression of pro-apoptotic factors resulting in decreased sensitivity to apoptotic stimuli. Indeed, the elevated expression of anti-apoptotic proteins has been associated with the chemoresistance of tumors (Minn et al., 1995). For these reasons, anti-cancer strategies should focus on lowering the apoptotic threshold of cancer cells either through the enhanced expression of pro-apoptotic proteins or the repression of apoptosis inhibitors.

1.5.1. Expression of pro-apoptotic genes to induce apoptosis in human tumors

Gene therapy strategies aimed at lowering the apoptotic threshold via the delivery of apoptosis-inducer genes have been explored in mouse models and human cancer cell lines. Indeed, over-expression of the pro-apoptotic bax protein appeared to reduce tumor growth and restore sensitivity to a variety of apoptosis-inducing agents in severe combined immuno-deficient (SCID) mice (Bargou et al., 1996). Moreover, gene transfer of the Fas ligand has been shown to induce tumor regression *in vivo* (Arai et al., 1997). In addition, caspase-8 gene therapy has been tried in human glioma cells (Komata et al., 2002). Lastly, induction of apoptosis in A-172 cells that are resistant to p53-mediated apoptosis has been observed upon transduction with Apaf-1 or caspase-9 (Shinoura et al., 2000).

A recent study by L. Yang and colleagues (2003) suggested that the sole expression of apoptosis-inducer genes in a variety of cancer cell lines (including pancreas, colon and breast) is not restricted to cancer cells and is not sufficient to induce apoptosis. It was further proposed that tumor cell lines possess intact apoptotic machinery, as evidenced by the presence of caspase-3 activity in cancer cell lines; an activity possibly repressed by high levels of survivin and Xiap suggesting that down-regulation of IAPs may sensitize cells to

apoptotic triggers. Indeed, infection of cancer cells with survivin T34A (inactive) and Xaf-1 adenoconstructs to target the inhibition of survivin and Xiap, respectively, reduced the viability of cancer cells, probably via an increase in caspase-3 activity. Unfortunately, the data presented does not fully support this claim since the activity of both caspase-3 and caspase-9 is only minimally increased upon infection with AdsurvivinT34A and AdXaf-1.

1.5.2. Increasing the apoptotic threshold confers resistance to apoptosis-inducing agents

Evidence of an increase in the apoptotic threshold of cancer cells is mounting. Over the years, elevated levels of IAPs have been documented in a variety of human malignancies (Ambrosini et al., 1997; Satoh et al., 2001, Imoto et al., 2001, 2002; L. Yang et al., 2003). Indeed, the human survivin gene, which is not expressed in most normal differentiated tissues, is over-expressed in tumor samples and correlates with shorter disease survival (Ambrosini et al., 1997; Adida et al., 1998; Lu et al., 1998; Kawasaki et al., 1998). In some tumor cell lines, the use of antisense or mutant vectors to reduce the expression of survivin resulted in sensitization to cytotoxic agents (Ambrosini et al., 1998). Furthermore, over-expression of survivin in breast cancer tissues was associated with an increased apoptotic threshold and longer survival of these cells (Tanaka et al., 2000). Lastly, survivin over-expression in colorectal cancer negatively correlated with five-year survival rates for these patients (Sarela et al., 2001).

Although the expression of survivin in cancer is, by far, the best characterized, genetic studies have also focused on Xiap, cIAP-1 and cIAP-2 status in human malignancies. For instance, in approximately 50% of marginal cell lymphomas of the mucosa-associated lymphoid tissue (MALT), cIAP-2 is affected by the translocation t(11;18)(q21;q21) leading

to its over-expression (Dierlamm et al., 1999). In addition, over-expression of cIAP-2 in bladder cancer cells conferred resistance to TRAIL- and drug-induced apoptosis (Jonsson et al., 2003). Moreover, higher Xiap levels correlated with shorter remission times (52.5 vs 87 weeks) and overall survival (52.5 vs 133 weeks) in acute myelogenous leukemia patients treated with the nucleoside analog cytarabine (Tamm et al., 2000). Finally, endometrial cancer cells expressing active Akt were shown to over-express cIAP-1 but not cIAP-2 nor Xiap (Gagnon et al., 2003). The over-expression of Akt was paralleled by the up-regulation of cIAP-1 suggesting a direct link between the PI-3 kinase/Akt signaling pathway, cIAP-1 expression and resistance to apoptosis. Taken together, these findings suggest a role for IAPs in the resistance of tumor cells to oncotherapies.

1.6. Model system: pancreatic cancer

1.6.1. Pancreatic cancer statistics

Pancreatic cancer is the fourth leading cause of cancer-related deaths in North America; it is more prevalent in men than in women, as well as in African Americans versus Caucasians (Landis et al., 1998; Lowenfels and Maisonneuve, 2002; Coughlin et al., 2000). Estimates suggest an overall mortality rate for pancreatic cancer of 99%; median survival is approximately 6 months from the time of diagnosis with a one-year survival rate under 10% and minimal 5-year survival (2003 Canadian Cancer Statistics). This dismal prognosis is mainly due to the fact that, at the time of diagnosis, more than 85% of pancreatic tumours have metastasized, principally to the liver, spleen and lymph nodes (Eriksson et al., 1990; Gonzalez-Campora et al., 1995; Staley et al., 1996). As a result, less than 20% of pancreatic

tumours are resectable by surgery (Slavin et al., 1999). Finally, pancreatic tumours exhibit a notoriously poor response to therapeutic agents.

1.6.2. Risk factors and symptoms of pancreatic cancer

No definite cause of pancreatic cancer has yet been identified. Pancreatic cancer cases are mostly sporadic although some familial clustering has been observed. Smoking and coffee drinking have both been linked to this type of malignancy although no direct behavior-effect has been confirmed (Howe et al., 1991; Doll et al., 1994; Harnack et al., 1997; Zatonski et al., 1993). In addition, patients with a well-documented history of chronic pancreatitis have a 15-fold increased risk for pancreatic cancer (Lowenfels et al., 1993) while those suffering from hereditary pancreatitis show a 50-fold increase in their risk of developing the malignancy (Lowenfels et al., 2000). Other risk factors include obesity, diabetes, a familial history of the disease, occupational exposure to organics solvents and a diet low in fruits, vegetables and fibers and high in fat (Baghurst et al., 1991; Silverman et al., 1999; Wolk et al, 2001; Wideroff et al., 1997)

The absence and vagueness of symptoms associated with pancreatic cancer greatly hampers early diagnosis. The major symptoms of this disease include jaundice (caused by liver metastases), pain (particularly in the back and upper abdomen) and a sudden onset of diabetes. Indigestion, lack of appetite, nausea, diarrhea and weight loss are less frequently observed symptoms (Warshaw and Fernandez-Del Castillo, 1992; Sanfey et al., 1983).

1.6.3. Diagnosis and treatment of pancreatic cancer

In addition to the lack of symptoms, the inaccessibility of the pancreas in the abdomen contributes to diagnosis being made at a very late stage in the disease (Lowenfels and Maisonneuve, 2002). Routine diagnostic exams include biopsy, blood tests, ultrasound, CT scan, laparoscopy and endoscopic retrograde cholangiopancreatography. The tardy diagnosis of pancreatic cancer often limits its treatment to palliative care as the metastatic behavior of the disease at the time of diagnosis renders most tumors unresectable. Nevertheless, surgery is sometimes possible when the tumor is well defined and located in the head of the pancreas; the Whipple procedure (a pancreaticoduodenectomy) is then performed. Unfortunately, patients undergoing apparently successful resection procedures will often have a recurrence of the disease (Yeo et al., 1998; Allison et al., 1998; Cooperman et al., 2001).

Over the years, the response of pancreatic tumors to chemotherapy and radiation treatment has been minimal although recent advances have shown promise. Early on, the standard chemotherapy for pancreatic cancer was the pyrimidine analog 5-fluorouracil (5-FU) which inhibits *de novo* DNA synthesis. Unfortunately, disappointing median survivals of 5 months were achieved with 5-FU therapy (Maisey et al., 2002; Ducreux et al., 2002). Median survival of 4 months were observed with cisplatin-based therapies aimed at introducing double-stranded breaks in DNA in pancreatic cancer patients (Wils et al., 1993).

Recently, a nucleoside analog, gemcitabine (difluorodeoxycytidine) was developed and shown to have strong activity against solid tumors (Plunkett et al., 1996). Gemcitabine is a cell cycle-dependent anti-metabolite which must first be transported into the cell and be phosphorylated; incorporation of gemcitabine triphosphate into DNA blocks elongation of

the DNA strand and is required for the cytotoxic effect of the drug (Heinemann et al., 1995). The rate-limiting enzyme in this pathway is deoxycytidine kinase (dCK) which is responsible for the first phosphorylation step (Hapke et al., 1996). In fact, in murine tumors and human tumor xenografts, dCK levels were used as predictors of gemcitabine sensitivity (Kroep et al., 2002). Moreover, dCK activity is essential for the radiosensitizing effect of gemcitabine in mice and in human cancer cell lines (Grégoire et al., 2002). Alternatively, gemcitabine can induce apoptosis (Bouffard and Momparler, 1995; Gruber et al., 1996). The efficacy of gemcitabine therapy for the treatment of pancreatic cancer, assessed using "clinical benefit response" or an improvement in the quality of life, was shown to be significantly higher in gemcitabine-treated patients than in 5-FU-treated patients (23.8% vs 4.8%, respectively; Burris and Storniolo, 1997). Furthermore, gemcitabine treatment also favored longer median survival than 5-FU treatment (5.65 vs 4.41 months, respectively). The low toxicity of gemcitabine also makes it a good candidate for palliative therapy (Kozuch et al., 2002).

Multiple drug combination regimens have also been evaluated for the treatment of pancreatic cancer. For example, 5-FU-based combinations such as FAM (5-FU, doxorubicin, mitomycin C) and FAM-S (FAM + streptozocin) appear to offer a survival advantage compared to single agent therapies (Glimelius et al., 1996; Moertel et al., 1986). Since gemcitabine as a single-agent showed promising results, using this agent in combination therapy appears warranted. In fact, several 2, 3 and 4 agent combinations including gemcitabine are currently being tested. The first completed phase III trial involving gemcitabine +/- bolus 5-FU showed median survival of 5.4 and 6.7 months favoring the gemcitabine/5-FU arm (Berlin and Rothenberg, 2001). A synergestic effect was also observed when combining cisplatin and gemcitabine with a median survival of 8.3 months (Heinemann et al., 1999). A median survival of 9.5 months in 31 patients with

metastatic pancreatic cancer was obtained using a 4-drug combination therapy including 5-FU, cisplatin, epirubicin and gemcitabine (Reni et al., 2001). In addition, the radiosensitizing effect of gemcitabine has clearly been established in colon and pancreatic cancer cell lines (Lawrence et al., 1996; Shewach et al., 1994, Shewach and Lawrence, 1996). In spite of all these advances, the standard of care for metastatic pancreatic cancer remains single-agent gemcitabine.

1.6.4. The genetics of pancreatic cancer

1.6.4.1. Animal models

Animal models are commonly used in research to provide insight into the molecular biology of diseases and/or to test potential therapeutic agents. Animal models of pancreatic cancer have been described in several species. In hamsters, the nitrosamine BOP(N-nitriso-bis(2-oxo-proplyl)amine) is a very potent inducer of pancreatic tumors bearing striking similarities to the human pathology (Pour et al., 1980). Indeed, these tumors have a ductal phenotype and elicit a pronounced desmoplastic reaction. In addition, mutations in Ki-ras and p53, deletion of p16 and the Y chromosome and over-expression of EGF-R and TGF-α have been reported in the hamster model, making it remarkably similar to established human disease (Cerny et al., 1990; Fujii et al., 1990; Okita et al., 1995; Schmied et al., 1999).

Rat and mice models of pancreatic cancer have also been described (Longnecker, 1994; Hotz et al., 2000; Zimmerman et al., 1982; Rao et al., 1987, Rao, 1987; Cardiff et al., 1993; Glasner et al., 1992). However, these models differ both phenotypically and biologically from human disease. In fact, pancreatic tumors grown in rats and mice are usually of acinar origin, well differentiated, metastasize infrequently (Hall and Lemoine,

1993; Torrisani and Buscail, 2002; Watanapa and Williamson, 1993; Rao, 1987, Rao et al., 1987, Longnecker, 1983) and do not exhibit EGF-R and p53 mutations (Terhune et al., 1994, 1998). However, human xenografts of a pancreatic tumor can grow and metastasize in nude and SCID mice making them useful to study candidate therapeutic approaches (Giovanella et al., 1972; Shimosato et al., 1976; Sharkey and Fogh, 1984; Schmied et al., 2000).

1.6.4.2. Allelotype studies

Allelotype studies in pancreatic cancer samples are confounded by the presence of a strong desmoplastic reaction, that is, pancreatic tumors are surrounded by infiltrating stroma making it difficult to distinguish between normal and tumor pancreatic tissue. However, comparing expression profiles derived from pancreatic adenocarcinoma, normal pancreas and chronic pancreatitis can help evaluate the contribution of neoplastic cells (Gress et al., 1997; M. Geng et al., 1998; Logsdon et al., 2003). In spite of these drawbacks, several gene profiling approaches including representational difference analysis (RDA), serial analysis of gene expression and microarrays have been used to identify molecular markers of pancreatic cancer (Gress et al, 1997; Logsdon et al., 2003; Crnogorac-Jurcevic et al., 2002; Iacobuzio-Donahue et al., 2002; Han et al., 2002). These studies revealed multiple anomalies, for instance, whole chromosome gains on chromosomes 7 and 20, recurrent structural abnormalities involving chromosomes 1p, 1q, 3p, 6q, 11p, 17p and 19q and frequent allelic losses on chromosomes 6, 12, 13, 17 and 18 (Griffin et al., 1995). In addition, chromosomes 17p, 18q and 9p which are known to carry major tumor suppressor genes are affected by a high frequency (> 60%) of allelic loss while moderately frequent (40-60%) allelic losses were observed on chromosomes 1p, 3p, 6p, 6q, 8p, 10q, 12q, 13q, 18p, 21q and 22q (Hahn et al., 1995; Hilgers et al., 1999a, 1999b).

1.6.4.3. Molecular pathology

Over the last decade, the genetic nature of pancreatic cancer has been established by the presence of inherited and acquired mutations in cancer-related genes (Hahn and Schmiegel, 1998). Tumor suppressor genes, namely p16, p53 and DPC4 are frequently inactivated in pancreatic cancer. p16/MTS1/INK4A/CDKN2 on chromosome 9p is inactivated in approximately 95% of pancreatic tumors either due to homozygous deletion, LOH coupled to an inactivating mutation in the second allele or hypermethylation (Caldas et al., 1994; Rozenblum et al., 1997; Schutte et al., 1997; Bartsch et al., 1995; Hu et al., 1997; Naumann et al., 1996). Normally, p16 prevents the phosphorylation of pRB by interfering with the ATP-binding activity of CDK4 and CDK6 therefore its loss promotes G1/S transition by allowing phosphorylation of pRB (Russo et al., 1998). Transfecting pancreatic cancer cell lines with wild-type p16 resulted in decreased tumor cell proliferation both *in vivo* and *in vitro* (Kobayashi et al., 1999; Ghaneh et al., 2001). In addition, inactivation of p16 was found to be associated with shorter survival times (Gerdes et al., 2002). Next, inactivation of one p53 allele coupled with loss of the second allele occurs in 50-75% of pancreatic cancers (Redston et al., 1994; Rozenblum et al., 1997; Boschman et al., 1994; Coppola et al., 1998; Lundin et al., 1996; Ruggeri et al., 1992; Scarpa et al., 1993). p53 inactivation affects both cell cycle progression and apoptosis since p53 usually causes a G1/S arrest in the cell cycle in response to DNA damage, stabilizes the G2/M checkpoint and activates the transcription of apoptogenic genes (Kastan et al., 1991; Kern et al., 1991; Levine, 1997; Vogelstein and Kinzler, 1992; Bunz et al., 1998; Yonish-Rouach et al., 1991). Furthermore, inactivation of the DPC4/Smad4 gene at 18q21.1 via homozygous deletion or LOH coupled with an inactivating mutation of the second allele occurs in approximately 55% of pancreatic adenocarcinomas (Hahn et al., 1996).

Other tumor suppressor genes inactivated in a minority of pancreatic tumors include BRCA2 in 7% of cases (Goggins et al., 1996), MAP kinase kinase 4 on 17p (Su et al., 1998; Teng et al., 1997), STK11/LKB1 on 19p (a serine threonine kinase responsible for Peutz Jeghers syndrome; Su et al., 1999), ACVR1B/Alk4 on 12q (Su et al., 2001), DCC on 18q (deleted in colorectal cancer; Hilgers et al., 2000) and TGF-β receptors 1 and 2 (Goggins et al., 1998a). Lastly, inactivation of suppressor genes by hypermethylation of CpG islands has been reported in 60% of 45 pancreatic cancer samples (Ueki et al., 2001).

Amplification or constitutive activation of oncogenes is also encountered in pancreatic cancer. For example, activation of the Ki-ras oncogene in up to 95% of pancreatic tumors via point mutations in codon 12 and occasionally in codons 13 and 61 has been reported by many investigators (Hruban et al., 1993; Almoguera et al., 1988; Scarpa et al., 1994; Smit et al., 1988; Wilentz et al., 2000a). However, no correlation between Ki-ras status and survival times has been established (Hruban et al., 1993; Kawesha et al., 2000). Also, amplification of the AKT2 gene on 19q and the c-myc oncogene on 6q24 has been reported in approximately 10% of pancreatic tumors (Cheng et al., 1996; Ruggeri et al., 1998; Solinas-Toldo et al., 1996; Wallrapp et al., 1997). A role for the Akt/PI-3 kinase pathway in the intrinsic resistance of pancreatic cancer cells to chemotherapeutic agents was suggested by an increased apoptotic response of gemcitabine-resistant cell lines when treated with a combination of PI-3 kinase inhibitors and gemcitabine (Ng et al., 2000).

Inactivation of DNA mismatch repair genes by promoter hypermethylation was also observed in pancreatic carcinomas (Ueki et al., 2000). Mutations in DNA mismatch repair genes can lead to microsatellite instability which occurs in 3-13% of pancreatic tumors (Goggins et al., 1998b; Yamamoto et al., 2001). In addition, over-expression of HER-2/neu,

a member of the EGF-R family, is found in approximately 70% of pancreatic carcinomas (Day et al., 1996, Hall et al., 1990; Satoh et al., 1993).

To our knowledge, systematic profiling of apoptosis inducers and repressors in pancreatic cancer has not yet been performed. However, the analysis of their expression and contribution to poor prognosis are slowly emerging. For example, the levels of both bcl-2 (anti-apoptotic) and bax (pro-apoptotic) mRNAs were shown to be elevated (30% and 61%, respectively) in pancreatic tumors (Friess et al., 1998). In addition, prolonged survival was observed in patients with high levels of apoptosis promoting factors (bax) supporting the biological significance of apoptotic pathways in the treatment of pancreatic cancer. Next, caspase-1 over-expression correlated with the expression of cyclin D1, EGF and EGF-R, all of which contribute to a poor prognosis (Gansauge et al., 1998). Furthermore, caspase-3 mRNA was documented to be over-expressed in pancreatic cancer cells when compared to normal tissue and, when found in the cytoplasm, proposed to contribute to the invasiveness of the tumor (Satoh et al., 2000). It has also been suggested that survivin levels may be up-regulated early in the development of pancreatic cancer (Satoh et al., 2001). Lastly, recent data have suggested that mutations in p53 and NF-κB contribute to the chemoresistance of pancreatic tumors. For instance, mutations in the p53 tumor suppressor gene result in loss of normal growth control and inhibition of cell death (Lowe et al., 1994). Moreover, expression of NF-κB may also confer chemo- and radio-resistance to pancreatic cancer cells suggesting that inhibition of this pathway could sensitize cancer cells to cytotoxic treatment (W. Wang et al., 1999; C. Wang et al., 1999; Arlt et al., 2001). Given that all of these proteins play a crucial role in apoptotic cell death, it becomes clear that understanding apoptosis at the molecular level may offer new, attractive targets for pancreatic cancer therapy.

1.6.4.4. Progression model for pancreatic cancer

The early events leading to pancreatic carcinogenesis are not well defined. The identification of intraductal proliferative lesions adjacent to infiltrating pancreatic cancer supports a progression model from intraductal lesions to invasive cancer (Cubilla and Fitzgerald, 1976; Kozuka et al., 1979). A universal classification system was recently adopted which should prove useful in establishing the sequence of events involved in pancreatic carcinogenesis. Based on the degree of dysplasia observed within them, Pancreatic Intraepithelial Neoplasia (PanIN) lesions are classified as PanIN-1A, -1B, -2 and –3 (Hruban et al., 2001). In spite of being extremely common, less that one in 500 PanIN-1 lesions will become malignant while PanIN-2 lesions which occur in less that 10% of people very seldom become malignant. In contrast, PanIN-3 lesions are usually found in the vicinity of invasive cancers (Kern, 1998).

If PanIN lesions are indeed precursors to infiltrating cancer, they should harbor some of the mutations encountered in invasive cancer. Therefore, analysis of precursor lesions for genetic alterations should help establish the sequence of events leading to pancreatic carcinogenesis. Interestingly, PanIN-1 lesions are characterized by a shortening of the telomeres which is thought to contribute to genomic instability (van Heek et al., 2002). Next, analysis of precursor lesions for alterations in Ki-ras, HER-2/neu, p16, p53, DPC4 and BRCA2 revealed differences in genetic anomalies (DiGiuseppe et al., 1994; Yamano et al., 2000; Day et al., 1996; Wilentz et al., 1998, 2000a, 2000b; Moskaluk et al., 1997; Goggins et al., 2000). Indeed, 82% of flat ductal lesions (PanIN-1), 86% of papillary ductal lesions without atypia (PanIN-2) and 92% of atypical papillary duct lesions (PanIN-3) over-expressed HER-2/neu (Day et al., 1996). Moreover, Ki-ras mutations occured in all degrees of PanIN (Sugio et al., 1997; Tada et al., 1996; Luttges et al., 1999; Moskaluk et al., 1997)

while loss of p16 protein expression were found in PanIN-2 (Wilentz et al., 1998). In contrast, p16 mutations were exclusively observed in PanIN-3 (Moskaluk et al., 1997). Low to moderate allelic losses at 9p (p16), 17p (p53) and 18q (DPC4) are seldom encountered in PanIN-1; however, allelic loss of chromosome 9p was detected in PanIN-2 while LOH at all of these alleles is very frequent in PanIN-3 (Heinmoller et al., 2000; Luttges et al., 2001). Although no alterations in DPC4/Smad4 were detected in PanIN-1, they were encountered in approximately 50% of PanIN-3 lesions (Wilentz et al., 2000b). Lastly, a study by Goggins et al. (2000) revealed that BRCA2 mutations were more frequent in high grade ductal lesions. Based on these observations, a progression model for pancreatic cancer was proposed (Fig.1.5). In this model, HER-2 over-expression and Ki-ras mutations are early genetic events while p16 mutations occur at an intermediate stage during pancreatic carcinogenesis. Finally, p53, DPC4 and BRCA2 mutations occur late in the development of this malignancy.

1.7. Statement of hypothesis and objectives

In summary, pancreatic tumors are diagnosed at a very late stage and are extremely resistant to all forms of therapy. Unfortunately, in spite of the recently described progression model for pancreatic adenocarcinoma, no definite cause for this disease has been established making it quite difficult to monitor "at risk" patients for early detection. The observed benefit from the current standard of care, gemcitabine chemotherapy, is still relatively minimal. For these reasons, the identification of new therapeutic targets is warranted. Given the recent correlation between an increased apoptotic threshold and chemoresistance, we hypothesized that variations in the levels of key apoptotic players including caspases, inhibitor of apoptosis proteins and their regulators contribute to the intrinsic resistance of

Fig. 1.5. Progression model for pancreatic ductal cancer.

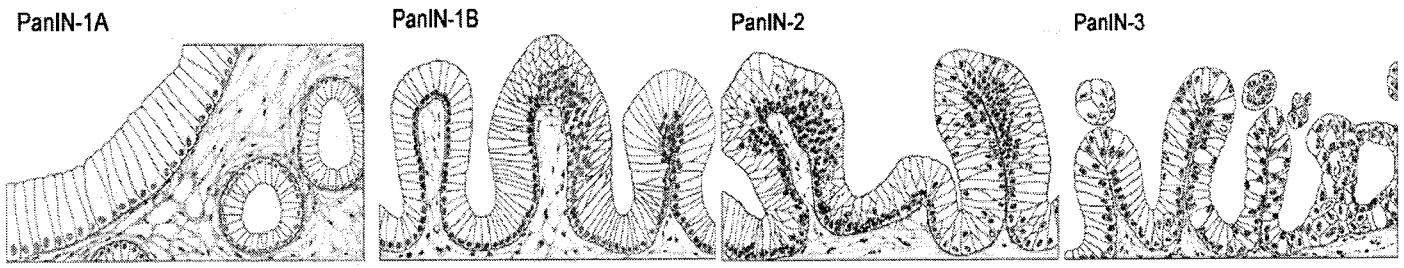
Pancreatic intraepithelial lesions are the precursors of invasive pancreatic adenocarcinoma and are characterized by specific genetic alterations (detailed in the lower part of the figure). In addition, PanIN lesions accumulate cellular abnormalities (summarized in the upper part of the diagram) during pancreatic tumorigenesis. Adapted from Hruban et al., 2000 and Real, 2003.

Flat epithelial esions
Tall, columnar cells
Small nuclei

Papillary/micro-
papillary lesions
Pseudostratified structure

Flat/papillary lesions
Enlarged nuclei
Hyperchromatism
Nuclear crowding

Papillary/micropapillary lesions
Cells bud off into lumen
Loss of nuclear polarity
Abnormal mitoses
Nuclear irregularities
Macronucleoli



K-ras
HER-2/neu
Telomere shortening
Genomic instability

p16
LOH on 9p

p53
DPC4
BRCA2
LOH on 17p, 18q

pancreatic cancer cells towards therapeutic agents.

To verify this hypothesis, our first objective was to derive a systematic expression profile of apoptosis regulator genes in the normal pancreas, immortalized ductal cell lines and pancreatic adenocarcinoma cell lines. We next attempted to elucidate the molecular events involved in drug-induced apoptosis. Indeed, our second objective was to determine if differential expression of IAPs could influence the response to DNA damage caused by the chemotherapeutic agent etoposide. Finally, our last objective was to characterize the expression of a candidate IAP-interacting protein, FLN29/Xaf-2, in pancreatic cancer cell lines and begin investigating its potential involvement in mediating cell survival.

Chapter 2: Materials and Methods

2.1. Description of materials

2.1.1. Cell lines

Human pancreatic adenocarcinoma cell lines CRL1420, CRL1469, CRL1682, CRL1687, CRL1837, HTB79, HTB80, HTB134 and HTB147 were purchased from the American Type Culture Collection (ATCC, Manassas, VA). GM9948 (EBV-immortalized lymphoblast cell line), GM38 and GM43 (human primary fibroblast strains) were purchased from the NIGMS Human Genetic Mutant Cell Repository (Camden, NJ). HPDE4, HPDE6 early, HPDE6 late and HPDE6 C7FR5 are pancreatic ductal cell lines immortalized by transfecting the E6E7 gene of human papilloma virus 16 resulting in the proteosomal degradation of p53 (Furukawa et al., 1996). PK1, PK8 and PK9 are primary ductal cancer cell lines; PK1 was isolated from a liver metastasis of the PK9 primary pancreatic tumor. HPDE and PK cell lines were generously provided by Dr. M.S. Tsao (Dept. of Pathology, University of Toronto, Toronto, ON).

Cells were grown as subconfluent monolayers (except for GM9948 which grows in suspension) at 37°C according to the media formulations specified by the suppliers in a humidified (80% R.H.) atmosphere containing 5% CO_2 in air. Medium (DMEM, RPMI 1640, K-SFM), antibiotics (10 000 U/mL penicillin/10 000 µg/mL streptomycin, 200 mM L-glutamine), 0.5% trypsin-5.3 mM EDTA and fetal bovine serum were purchased from Invitrogen (Burlington, ON).

2.1.2. Isolation of high molecular weight genomic DNA

High molecular weight genomic DNA was isolated free of contaminating RNA and

protein using an adapted version of a method previously described by Fourney et al. (1989). Briefly, adherent cells were washed 3X with cold PBS (Appendix I) to remove excess serum and lysed in rapid extraction (REX) buffer (Appendix I) prior to digestion with RNAse A and proteinase K at 56°C for 1.5 and 2h, respectively. Following a series of phenol: chloroform extractions and ethanol precipitations, DNA pellets were resuspended in 10 mM Tris-HCl pH 8.0, 1 mM EDTA buffer and stored at 4°C until used. Genomic DNA from normal pancreatic tissue was isolated from a cryopreserved sample of pancreas resected from an organ donor (according to ethical standards and procedures for procurement) as described above except that the tissue was pulverized in liquid nitrogen prior to lysis in REX buffer. DNA concentration and purity were determined spectrophotometrically and verified on 1% (w/v) agarose gels.

2.1.3. Isolation of total RNA

Subconfluent monolayer cultures were washed 3X in cold PBS prior to RNA extraction using the acid guanidinium thiocyanate phenol chloroform method (Chomczynski and Sacchi, 1987). Alternatively, cells were lysed in Trizol (Invitrogen) for RNA extraction as instructed by the supplier. RNA pellets were dissolved in 0.5% SDS/1 mM Tris-CDTA by heating for 10 min at 65°C and stored at -80°C until used. Normal pancreatic tissue was crushed in liquid nitrogen prior to RNA extraction. Concentration and purity were determined spectrophometrically and verified on 1% (w/v) denaturing agarose gels.

2.1.4. Isolation of protein extracts

Protein extracts were obtained from fresh cell cultures lysed in protein extraction buffer containing protease inhibitors (Appendix I) following a series of washes in cold PBS.

Protein lysates were boiled for 5 min prior to storage at -20°C until further use. Protein lysates from normal pancreatic tissue were extracted from pulverized donor pancreas. To determine protein concentration, an aliquot of the protein lysates was TCA-precipitated to remove traces of reducing agents which interfere with the assay reagents. Briefly, aliquots of protein lysates were transferred to a 1.5 mL microcentrifuge tube containing 1 mL chilled 15% trichloroacetic acid (TCA, BDH, Montreal, Qc), incubated on ice for 15 min and centrifuged for 1 min at 13 000 x g. Pellets were further washed twice with 1 mL of chilled 15% TCA. TCA precipitates were then dissolved in 0.1N NaOH (BDH) by vortexing. TCA-precipitated protein extracts were quantified against a BSA standard using the Micro BCA Protein Assay Reagent kit (Pierce, Rockford, Il) as instructed by the supplier.

2.2. Nucleic acid hybridizations

2.2.1. Digestion of genomic DNA and Southern transfer

Three to five μg of genomic DNA were digested to completion with 10 units of restriction endonuclease (New England Biolabs (NEB), Mississauga, ON) per μg of DNA in the buffers provided by the supplier. Digestions were allowed to proceed for 4-6h at 37°C, at which point half the initial amount of restriction enzyme used was added to the reaction mix. To ensure complete digestion of genomic DNA, reactions then proceeded overnight at 37°C in the presence of 4 mM spermidine-trihydrochloride (Sigma, Oakville, ON). Digests were resolved on 1% (w/v) agarose gels in 1X TBE buffer (Appendix I) and alkali-blotted to Pall Biodyne B nylon membranes (Invitrogen). Forensic DNA analysis marker (FDAM, Invitrogen) was included on the gel for sizing. Following transfer, membranes were

neutralized in 0.2 M Tris-HCl pH 7.5/2X SSC, baked for 30 min at 80°C and processed immediately or stored sealed at 4°C.

2.2.2. Isolation of mRNA from total RNA and Northern transfer

Messenger RNAs were extracted from 100 μg of total RNA using a paramagnetic mRNA purification kit (Dynal, Delaware, NY) as per the manufacturer's instructions except that mRNAs were eluted in 1X RNA loading buffer (Appendix I). Isolated mRNAs or 7-10 µg of total RNAs were electrophoresed on a 1% (w/v) denaturing agarose gel in 1X MOPS buffer (Appendix I) and blotted to Pall Biodyne A nylon membranes (Invitrogen) in 15X SSC O/N. RNAs were UV-crosslinked to the membrane (Stratalinker™, Stratagene, La Jolla, CA). Membranes were processed immediately or stored frozen at -80°C.

2.2.3. Preparation and radiolabelling of probes from constructs and PCR amplicons

A recombinant construct containing the coding region of Xiap cloned into the pcDNA3.6 myc vector was obtained from Drs. LaCasse, Liston and Korneluk (University of Ottawa and Aegera Technologies, Ottawa, ON). To liberate the Xiap insert, 2.5 µg of the construct was digested with 25U each of *Bam* HI and *Xho* I (NEB) under standard conditions. Digests were resolved by electrophoresis on 1% (w/v) agarose gels in 1X TBE buffer. A band of ≈ 1.5 kb corresponding to the Xiap insert was extracted from the gel using the QIAquick gel extraction kit (Qiagen, Mississauga, On) as instructed by the supplier and stored at -20°C until used.

Xaf-1, Xaf-2, caspase-3, caspase-9, cIAP-1, cIAP-2, Smac/DIABLO and survivin probes were generated by PCR amplification. Primers were synthesized (Invitrogen) based on sequences (Table 2.1) designed with Primer Designer 4 v4.20 (Scientific and Educational

Table 2.1. Primers used to generate cDNA probes for nucleic acid hybridization

Probe (accession #)	Primer pair	Position	Product size
Xiap-1 (X74806)	F: 5'-cttactgcctgcggttcctg-3' R: 5'-aagaccaccacagcaagtag-3'	Nt 74-93 Nt 989-970	915 bp
Naip-2 (NM_006709)	F: 5'-gactcacatggctgcagaac-3' R: 5'-cagttctgctgcggtagttg-3'	Nt 210-229 Nt 1719-1700	1490 bp
caspase-8 (NM_033340)	F: 5'-ctcggtctggtacagatgtc-3' R: 5'-ctgcagcatgagagtaggtc-3'	Nt 423-442 Nt 1192-1173	751 bp
caspase-9 (NM_001229)	F: 5'-gccactgcctcattatcaac-3' R: 5'-gactgcaggtcttcagagtg-3'	Nt 577-596 Nt 1259-1240	664 bp
cIAP-1 (NM_001166)	F: 5'-gttgaacacttgaagccatc-3' R: 5'-atgtttctcacaaactaggg-3'	Nt 3066-3085 Nt 3480-3461	415 bp
cIAP-2 (NM_001165)	F: 5'-aaccaaaacatcgtctaaac-3' R: 5'-caccatgtccctaaaatgtc-3'	Nt 2545-2564 Nt 3115-3096	570 bp
Smac/DIABLO (NM_019887)	F: 5'-cgcgcagcgtaacttcattc-3' R: 5'-tcttctcggtgcacagacag-3'	Nt 45-64 Nt 838-819	794 bp
Survivin (NM_001168)	F: 5'-catgggtgccccgacgttgc-3' R: 5'-tggaagtggtgcagccactc-3'	Nt 49-68 Nt 526-507	478 bp

Software, Durham, NC) to ensure specific amplification of the target sequences. In particular, cIAP-1 and cIAP-2 probes were designed in the 3'UTR of the gene to distinguish between the two closely related sequences. The DNA template used for PCR amplification differed among probes. Twenty ng of Xaf-1 pCDNA3.1 HA or Xaf-2 pBluescript SK$^-$ constructs were used as template to generate Xaf-1 and Xaf-2 probes, respectively. In contrast, 0.5 ng of Marathon Ready cDNA™ (Clontech, Palo Alto, CA) from human liver, pancreas or foetal pancreas were used to amplify cIAP-1/2, caspases-3/9, survivin and Smac/DIABLO sequences. For Xaf-1/2, PCR reactions were performed in 20 μL volumes containing DNA template, 160 μM each dATP, dCTP, dGTP and dTTP, 500 μM each forward and reverse primers, 2.5U AmpliTaq DNA polymerase (Applied Biosystems (AB), Mississauga, ON) in 1X Gene Amp PCR buffer (10 mM Tris-HCl pH 8.3, 50 mM KCl, 1.5 mM $MgCl_2$; AB). Amplifications were carried out in a PTC 200 thermocycler (MJ Research, Watertown, MA) under the following conditions: 5 min at 94°C followed by 35 cycles of 30 sec at 94°C, 60 sec at 60°C and 45 sec at 72°C and a final extension of 5 min at 72°C. For cIAP-1/2, caspases-3/9, Smac/DIABLO and survivin, 5U of AmpliTaq Gold DNA polymerase (AB) were used to amplify the PCR products while all other components of the reaction remained constant. However, AmpliTaq Gold DNA polymerase requires a hot start for its activation, therefore the PCR conditions were modified as follows: 15 min at 95°C followed by 33 cycles of 45 sec at 95°C, 45 sec at 58-64°C and 45 sec at 72°C with a final extension of 10 min at 72°C.

Purified probe fragments (25-50 ng) were labelled to high specific activity using a commercially available random priming kit (Prime-a-Gene labelling kit, Promega, Madison, WI) and α-^{32}P-dCTP according to manufacturer's instructions. FDAM probe was prepared

in a similar fashion in agreement with the protocol suggested by the supplier. Non-incorporated nucleotides were removed by exclusion chromatography through Sephadex G-50 columns (Probe Quant, Amersham, Baie D'Urfé, Qc). Prior to hybridization, probes were boiled for 5 min to allow for denaturation and immediately cooled on ice.

2.2.4. Hybridization protocol and probe removal

Membranes were pre-hybridized in Hybsol (Appendix I) supplemented with 200 μg/mL heat denatured sonicated Herring sperm DNA (Boehringer Mannheim Canada, Laval, Qc) for 2-4h at 65°C. Hybridizations were carried out overnight at 65°C; blots were washed to a final stringency of 0.1X SSC/0.1% SDS then exposed to X-ray film (Biomax MS, Kodak, New Haven, CT) between two Dupont Cronex Lightning Plus Intensifying screens at -76°C. Length of exposure depended on signal intensity, typically 1-5 days.

For stripping, DNA membranes were incubated in 0.4N NaOH for 30 min at 45°C followed by 15 min washes in 0.1X SSC/0.1% SDS and 0.2M Tris-HCl pH 7.4 at room temperature. In contrast, RNA membranes were boiled in DEPC-treated H_2O for 5 min. Membranes were exposed to X-ray film overnight to confirm adequate removal of probe.

2.2.5. Specialized blots

To generate zooblots, genomic DNAs from human, mouse, dog, rhesus monkey, rat, pig, chicken, rabbit and fly were purchased from Clontech. Three μg of genomic DNA were digested with *Hind* III or *Eco* RI and processed as detailed for the Southern blot except that washes (final wash 0.1X SSC/0.1% SDS) were performed at three different temperatures (55°C, 60°C and 65°C) in succession. Membranes were exposed to X-ray film overnight following hybridization at each temperature.

Membranes (Human Multi Tissue Northern Blot I, III and IV) containing 2 μg of mRNA from several human tissues (Clontech) were processed for hybridization in ExpressHyb™ solution provided by the supplier. Membranes were probed with radiolabelled Xaf-1, Xiap, Xaf-2 and survivin cDNAs successively. Membranes were washed to a final stringency of 0.1X SSC/0.1% SDS and exposed to X-ray film. Probes were stripped from the blot by incubating the membrane in sterile H_20 containing 0.5% SDS at 90-100°C for 10 min. Human β-actin cDNA probe provided by the supplier was used as a loading control.

Cancer Profiling Arrays (CPA) containing normalized SMART™-amplified cDNA from tumors and corresponding normal tissues from individual patients were purchased from Clontech. One array (CPAIa) was probed, in succession, with Xaf-1, Xiap, cIAP-1 and cIAP-2 while a duplicate array (CPAIb) was hybridized with Xaf-2. Finally, a similar array (CPAII) was probed with survivin, caspase-3, caspase-9 and Smac/DIABLO. Hybridizations were carried out in ExpressHyb™ solution as recommended by the supplier. Blots were washed to a final stringency of 0.2X SSC/0.5% SDS. For stripping, membranes were boiled for 10 min in DEPC-treated H_2O containing 0.5% SDS. Human ubiquitin cDNA, included with the array, served as a loading control.

2.3. Microsatellite allelotyping

Short tandem repeat (STR) markers D17S796 (5'-Hex tagged; het.index 0.82; 144-174 bp), D17S1881 (5'-Fam tagged; het.index 0.78; 216-230), D12S105 (5'-Fam tagged; het.index 0.72; 137-155 bp) and D12S369 (5'-Hex tagged; het.index 0.80; 201-225 bp) were purchased from Research Genetics (Huntsville, AL (now Invitrogen)). STR markers were

amplified from 40 ng of genomic DNA in a 20 μL reaction volume containing 1.5 mM $MgCl_2$, 160 μM each dATP, dCTP, dGTP and dTTP, 500 μM each of forward and reverse primers and 2.5U of AmpliTaq DNA polymerase in 1X Gene Amp PCR buffer. DNA was amplified under the following conditions: 2 min at 94°C followed by 27 cycles of 10 sec at 94°C, 30 sec at 55°C and 45 sec at 72°C and a final extension of 5 min at 72°C in a thermocycler (PTC200, MJ Research, Watertown, MA). PCR amplicons were first diluted 20-fold to reduce signal intensity and then diluted 1:8 in 12 μL of deionized formamide containing a DNA size standard (GS350, AB) labelled with the fluorescent dye N,N,N',N',-tetramehyl-6-carboxyrhodamine. PCR products were resolved and detected using a semi-automated capillary electrophoresis genetic analysis system (Applied Biosystems Model 310 Genetic Analyser). Data was exported to an analysis software (GeneScan™ v2.1, AB); data files were filtered to remove extraneous peaks and allele sizes were assigned using Genotyper™ v2.0 software (AB). Based on the presence of one or two peaks, samples were classified as homozygous or heterozygous, respectively.

2.4. RNAse Protection Assay

hAPO1C and hAPO5 human apoptotic modulator/effector gene templates were purchased from BD Biosciences (Mississauga, ON). Multi-probe template sets can be used for the T7 polymerase-directed synthesis of radio-labelled anti-sense RNA probes capable of hybridizing with target human RNAs. RPAs were essentially carried out as recommended by the manufacturer. Typically, 10 μg of total RNA from the cell lines of interest were used for hybridizations and probe dilutions were done as suggested for each template set. RNase-

protected fragments were resolved on 5% acrylamide gels in 1X TBE buffer which were then dried prior to exposure to X-ray film. Short exposure times (typically 6-24 hours) were satisfactory to obtain bands of adequate intensity for analysis. Given that the RNA derived from normal pancreatic tissue was of poor quality, we used HPDE6 late as our baseline level. In order to calculate the fold increase relative to the expression in HPDE6 late, RPA results were analyzed by densitometry to determine the relative intensity of each band and normalized to an internal standard (the housekeeping gene L32) to control for loading.

2.5. RT-PCR of Xaf-2

First-strand cDNAs were synthesized from 5 μg of total RNA (SDS-free) using the Superscript™ preamplification system for first-strand cDNA synthesis (Invitrogen) as per the manufacturer's instructions. No reverse transcriptase (no RT) controls were included to control for genomic DNA contamination. PCR reactions were set up in 20 μL volumes to include 2 μL of first-strand cDNA or an equivalent quantity of template total RNA, 160 μM each dATP, dCTP, dGTP and dTTP, 500 μM each forward and reverse primers, 2.5U AmpliTaq DNA polymerase in 1X Gene Amp PCR buffer. Primers sequences were as follows: 5'-tccatgagatccactgtcaa-3' and 5'-ccacaaacttcagggtgagt-3' for forward and reverse primers, respectively. PCR amplifications were performed on a PTC100 thermocycler (MJ Research) under the following cycling conditions: 5 min at 94°C followed by 35 cycles of 1 min at 94°C, 1 min at 60°C and 1 min at 72°C and a final extension of 5 min at 72°C. PCR amplicons (expected size = 350 bp) were sized against a 1 kb DNA ladder (Invitrogen) on a 2% (w/v) agarose gel in 1X TBE buffer.

2.6. Sequencing of the cIAP-2, Xaf-2 and Xiap coding regions in pancreatic adenocarcinoma cell lines

First-strand cDNAs from human adult and foetal pancreatic tissue, HPDE4, HPDE6, CRL1420, CRL1469, CRL1682, CRL1687, CRL1837, HTB79, HTB80, HTB134 and HTB147 were synthesized from 3 μg of total RNA as described previously. Two μL aliquots of cDNAs were then PCR-amplified using primers (Table 2.2) designed to span the entire coding region of the gene of interest. Full-length Xaf-2 mRNA was obtained via the amplification of two overlapping PCR products, referred to as the ATG and the STOP fragments. PCR amplifications were carried out in either of two conditions. First, the majority of fragments were obtained by amplifying the DNA template in a 20 μL reaction mixture containing 300 μM each dATP, dCTP, dGTP and dTTP, 1 μM each forward and reverse primers, 1.25U Proof Start DNA polymerase™ (Qiagen) in 1X Proof Start PCR buffer™ (Tris-HCl pH 8.7, KCl, $(NH_4)_2SO_4$, 1.5 mM $MgSO_4$, Triton X-100). Cycling conditions were: 5 min at 95°C followed by 32 cycles of 1 min at 95°C, 1 min at 56°C (ATG) or 58°C (STOP) and 1 min (ATG) or 1 min 30 sec (STOP) at 72°C with a final extension of 5 min at 72°C. Alternatively, PCR reactions were done in a final volume of 20 μL containing 1.5 mM $MgCl_2$, 200 μM each dATP, dCTP, dGTP and dTTP, 250 μM each forward and reverse primers, 2.5U of AmpliTaq Gold DNA polymerase in 1X Gene Amp PCR buffer. In this case, cycling conditions were: 15 min at 95°C followed by 33 cycles of 1 min at 95°C, 1 min at 56°C and 1 min at 72°C with a final polishing of 90 min at 72°C to ensure A-tailing of product ends.

To generate full length coding sequences corresponding to cIAP-2 and Xiap, three overlapping PCR fragments (arbitrarily termed a, b and c) were amplified. PCR reactions

Table 2.2. Primers used to sequence the cIAP-2, Xaf-2 and Xiap cDNAs

Gene fragment	Primer pair	Position	Product size
[illegible]	F: 5'-gtccctttcttccccattc-3' R: 5'-taggctgagaggtagcttcc-3'	Nt 698-716 Nt 1058-1039	362 bp
[illegible]	F: 5'-agctgcagattcgttcagag-3' R: 5'-ggcagcattaatcacaggag-3'	Nt 998-1017 Nt 1879-1860	882 bp
[illegible]	F: 5'-agctgctatccacatcagac-3' R: 5'-cacacctggcttcatgttcc-3'	Nt 1755-1774 Nt 2905-2886	1151 bp
[illegible]	F: 5'-ggcgcgaaaaggtggacaag-3' R: 5'-tctcagatggcctgtctaag-3'	Nt 91-110 Nt 531-512	441 bp
[illegible]	F: 5'-aatagtgccacgcagtctac-3' R: 5'-tctgcaaccagaacctcaag-3'	Nt 426-445 Nt 1366-1347	941 bp
[illegible]	F: 5'-cttgaggagtgtctggtaag-3' R: 5'-tctttccaccagcatggaac-3'	Nt 1170-1189 Nt 2081-2062	912 bp
[illegible]	F: 5'-atggctgaatttctagatgac-3' R: 5'-agactggtcggcctcacata-3'	Nt 33-52 Nt 848-829	815 bp
[illegible]	F: 5'-aatgaaggccaagcctccag-3' R: 5'-gtctctggagacaccattac-3'	Nt 780-799 Nt 17971778	1017 bp
[illegible]	F: 5'-cgttgtaaaacaacggccag-3' R: 5'-agcggataacaatttcacacagg-3'	N/A	N/A
[illegible]	F: 5'-caggaaacagctatgac-3' R: 5'-gtaaaacgacggccagt-3'	N/A	N/A

contained 5 μL of cDNA template, 300 μM each dATP, dCTP, dGTP and dTTP, 1 μM each forward and reverse primers, 2.5U Proof Start DNA polymerase™ in 1X Proof Start PCR buffer™ in a final volume of 50 μL. Cycling conditions were: 5 min at 95°C followed by 32 cycles of 1 min at 95°C, 1 min at 58°C and 1 min at 72°C with a final extension of 5 min at 72°C. Following amplification, PCR products were ethanol precipitated, resuspended in 15.5 μL of DEPC-treated H_2O and single A-tailed in the presence of 200 μM dATP, 2.5U AmpliTaq Gold DNA polymerase in 1X Gene Amp PCR buffer for 90 min at 72°C. All PCR reactions were carried on a PTC200 thermocycler.

PCR amplicons were resolved against a 1 kb DNA ladder (Invitrogen) on a 1% (w/v) agarose gel in 1X TBE buffer to confirm amplification of the expected products. PCR products were then excised from the gel using the QIAquick gel extraction kit (Qiagen) as per the manufacturer's recommendations. Purified PCR products were cloned into pPCR-Script (Stratagene) or pPCR2.1 (Invitrogen) and transformed into XL-2 Blue or XL-10 Gold ultracompetent bacterial cells (Stratagene) under standard conditions. Colonies were PCR-screened to identify colonies bearing an insert. Briefly, a small fraction of the colony was frozen in 200 μL of DEPC-treated H_2O at -76°C for 20-30 min, thawed, vortexed and PCR amplified using one of two primer pairs: lacZ or M13 (Table 2.2). PCR reactions were set up in 20 μl volumes containing 5 μL of template, 200 μM each dATP, dCTP, dGTP and dTTP, 1 μM each forward and reverse primers, 2.5 U AmpliTaq Gold DNA polymerase in 1X Gene Amp PCR buffer. Cycling conditions were: 15 min at 95°C followed by 32 cycles of 1 min at 95°C, 1 min at 55°C and 1 min at 72°C with a final extension of 5 min at 72°C in a PTC200 thermocycler. M13 and lacZ sequences are contained within the cloning vectors therefore amplification of insert-containing colonies should generate larger amplicons than

vector alone. Positive colonies were then grown overnight in 3 mL of SOB medium (Appendix I) supplemented with ampicillin and magnesium at 37°C with agitation. Plasmids were extracted with the QIAprep plasmid extraction kit (Qiagen) as instructed by the supplier.

Three to five clones were independently sequenced for each locus using the Big Dye Sequencing reaction kit v3.1 (AB). Typically, 20 μL sequencing reactions contained 500 ng of plasmid, 3.6 pmol each forward and reverse primers (M13), 2 μL of Terminator Ready™ reaction mix in 1X sequencing solution™ (AB). Sequencing reactions were run in a PTC200 thermocycler under the following conditions: 27 cycles of 1.0°C/sec to 96°C, 10 sec at 96°C, 1.0°C/sec to 50°C, 5 sec at 50°C and 1.0°C/sec to 60°C, 4 min at 60°C followed by an indefinite hold at 4°C. Samples were then cleaned using the Dye-Ex spin kit (Qiagen) according to manufacturer's instructions, dried and resuspended in 20 μL of terminator suppressant reagent (TSR™; AB) or HiDi formamide (AB). Sequences were resolved on an ABI Prism Model 3100 (samples resuspended in HiDi formamide) or Model 310 (samples resuspended in TSR) genetic analyser. Sequencing data was exported to the Sequencher™ v4.0.5 analysis software (Gene Codes Corporation, Ann Arbor, MI) for trimming of ambiguities and vector ends from the sequence. Sequences were then aligned against the reference sequence to check for mutations using Align Plus 4 v4.10 (SECentral). Reference sequences were downloaded from the NCBI website and their accession numbers are: NM_006700 for Xaf-2 (FLN29), NM_001165 for cIAP-2 (BIRC3) and NM_001167 for Xiap (BIRC4).

2.7. Western immunoblotting

Ten to thirty μg of protein were diluted 1:3 in 3X reducing buffer (NEB) before resolution on 8, 12 or 15% SDS polyacrylamide gel electrophoresis according to Laemmli (1970). Polyacrylamide concentrations were chosen based on the size of the protein to be detected. Gels were transfered to a PVDF membrane (Immobilon-P, Millipore Canada, Nepean, On) and blocked in 1% blocking reagent (Roche Applied Science, Laval, Qc) in 1X TBST (Appendix I) for a minimum of 1h at room temperature. Membranes were then incubated with primary antibodies diluted in 0.5% blocking reagent as recommended by the suppliers. Suppliers, incubation conditions and expected sizes of all antibodies used are summarized in Table 2.3. Antigen-primary antibody complexes were detected using a 1:2000 dilution of horseradish peroxidase-conjugated secondary antibody (stock 100 μg/mL; Amersham Biosciences). For bcl-2, caspase-7 and PARP only, sheep-anti-mouse IgG was used as secondary antibody while all other antibodies were raised in rabbit and therefore required donkey-anti-rabbit IgG as a secondary antibody. Complexes were revealed in ECL solution (Amersham) and exposed to autoradiography film. Membranes were stripped by incubating in stripping solution (Appendix I) in a 50°C H_20 bath for 30 min and washing extensively in 1X TBST. Lane-to lane loads were corrected by re-hybridizing membranes with an anti-α-actin rabbit polyclonal antibody.

Band intensities were analysed using a personal densitometer (Molecular Dynamics™, Amersham Biosciences). To determine fold induction of protein expression, the intensity of the target protein band was normalized to that of the actin control. Protein expressions were then graphed as fold increases or decreases relative to the appropriate control (HPDE6 late or normal pancreas).

Table 2.3. Description of the antibodies used for Western immunoblotting

Antibody	Incubation conditions	Expected band size
α-actin[a]	1h RT; 0.35 μg/mL	42 kDa
Apaf-1[b]	1h RT; 1 μg/mL	130 kDa
bax[c]	1h RT; 0.5 μg/mL	19 kDa
bcl-2[d]	1h RT; 1 μg/mL	25 kDa
caspase-3[e]	O/N 4°C; 1 μg/mL	32 kDa (inactive) 17 kDa (active)
caspase-7[f]	1h RT; 1 μg/mL	35 kDa (inactive) 17 kDa (active)
caspase-9[g]	O/N 4°C; 1 μg/mL	46-48 kDa (inactive) 37 kDa (active)
cIAP-1[h]	O/N 4°C; 0.5 μg/mL	70 kDa
cIAP-1[i]	1h RT; 0.5 μg/mL	70 kDa
cIAP-2[h]	O/N 4°C; 1.5 μg/mL	66 kDa
PARP C-2[d]	1h RT; 1:1000	116 kDa (uncleaved) 85 kDa (cleaved)
Rixp[h]	1h RT; 0.5 μg/mL	70 kDa (cIAP-1) 66 kDa (cIAP-2)
Smac/DIABLO[h]	O/N 4°C; 1 μg/mL	25 kDa
Survivin[d]	O/N 4°C; 1 μg/mL	16 kDa
Xaf-1[h]	O/N 4°C; 1 μg/mL	33 kDa
Xiap[h]	1h RT; 0.5 μg/mL	55 kDa
Xiap[i]	O/N 4°C; 0.5 μg/mL	55 kDa

[a]Sigma (Oakville, ON)
[b]Chemicon Int. (Temecula, CA)
[c]Santa Cruz Biotech Inc. (Santa Cruz, CA)
[d]Oncogene Research Products (Hornby, ON)
[e]BD Biosciences/Pharmingen (Mississauga, ON)
[f]R&D Systems (Minneapolis, MN)
[g]Biomol (Hornby, ON)
[h]Generously provided by Drs. Korneluk and LaCasse (University of Ottawa and Aegera Technologies, Ottawa, ON)
[i]Imgenex (San Diego, CA)

2.8. Tissue micro-array construction and immunohistochemistry

Pancreatic tissue originated from a bank of snap-frozen and paraffin-embedded pathology specimens established at the Princess Margaret Hospital and Toronto General Hospital after approval by the Research Ethics Board of the University Health Network. Tumor tissues were obtained from patients who had undergone primary pancreatic cancer resection without pre-surgical radiation or chemotherapy. Corresponding non-neoplastic pancreatic parenchyma was obtained from a subset of these patients. H&E stained slides were reviewed by two pathologists to establish stage and grade. The best representative slides were selected and the respective paraffin blocks were retrieved.

Tissue micro-arrays were constructed using a manual tissue arrayer (Beecher Instruments, Silver Spring, MD). On average, three to four cores, obtained from each tumor, were arrayed into four recipient tissue micro-array blocks. Each block also included non-pancreatic tissue cores to serve as controls and for orientation purposes.

Serial 4-μm-thick sections from the tissue micro-array blocks were cut and dried in a 60°C oven overnight. Sections were de-waxed in xylene and rehydrated through graded alcohol to water changes. Endogenous peroxidase activity was suppressed by incubation with 3% hydrogen peroxide. After performing microwave antigen retrieval in 10 mM citrate buffer (pH 6.0) in a pressure cooker, slides were blocked for endogenous biotin (Vector Labs, Burlington ON). After blocking with pre-immune serum, sections were covered in primary antibody for 16h at room temperature in a humidified chamber. Antibodies against human cIAP-1, cIAP-2, Xiap and survivin, purchased from R&D Systems were used at 1:200 dilutions. After washing in PBS, secondary antibody incubation was carried out using the multi-species link reagent (Ultra-Streptavidin detection system; Signet Pathology

System, Dedham, MA), which was followed by incubation with streptavidin-horseradish peroxidase. Immuno-reactivities were revealed by incubation in Nova Red substrate (Vector Labs) for 5 min. Slides were counterstained in Mayer's hematoxylin and mounted in Permount. Staining intensities were evaluated independently by two pathologists and scored in three grades: 0 for complete absence of staining, 1 for weak staining, and 2 for strong staining. The extent of positively stained nuclei was scored into four grades: 0 for <10% nuclei staining, 1 for 10% to <25%, 2 for 25% to <50%, and 3 for tumors with 50% or greater tumor cell nuclei staining positive. The final score for each tumor sample represents the sum of staining intensity and extent. Colon cancer samples served as internal positive controls.

2.9. *In vitro* exposure of pancreatic cancer cell lines to 5'-aza-2-deoxycytidine

CRL1420, CRL1469 and CRL1682 human pancreatic adenocarcinoma cells were seeded in T75 culture flasks (Nunc) in complete medium and allowed to attach overnight. The next day, the medium was replaced with fresh medium supplemented with 1-10 μM of the DNA methyl transferase inhibitor 5'-aza-2'-deoxycytidine (5azadC; Sigma). Cells were passaged regularly and maintained in 5azadC-containing medium for 11 days. Cells were then allowed to recover from the treatment in fresh culture medium (without 5azadC) for 2-3 days and subsequently seeded in 100 mm dishes (Nunc) and grown to confluence for DNA or RNA extraction in 3 mL of REX buffer or 1 mL of solution D (Appendix I), respectively. Following nucleic acid hybridizations, band intensities were determined as described in section 2.7 except that the expression was normalized to that of the GAPDH control.

2.10. Etoposide challenge of pancreatic cancer cell lines

CRL1420, CRL1469 and CRL1682 pancreatic cancer cells were seeded at a density of 50 x 10^3 cells/well in 24-well plates (Corning24, Fisher Scientific, Ottawa, ON) and allowed to attach at 37°C/5% CO_2 overnight. Etoposide stock solutions (100 mM) were prepared by solubilizing etoposide in DMSO (Fisher Scientific). Cells were then challenged or not with 2, 5, 10, 15 and 20 μM of etoposide (Sigma) for 6h; cells were also treated with a volume of DMSO equivalent to the highest volume of etoposide used to control for the effect of the vehicle. At the end of 6h, cells were washed with 1X PBS, placed in fresh, etoposide-free culture medium and allowed to recover at 37°C/5% CO_2 for 48h. At this time, 10 μL/well of a 1:1 mixture of serum free-medium and 1X alamarBlue™ (Trek Diagnostic Systems Inc., Cleveland, OH) were added and incubated at 37°C/5% CO_2 for 1h. Readings were performed on a Cytofluor 2300 automated fluorescence detection system (Millipore) and exported to the SoftMax analysis software (Molecular Devices Corporation, Sunnyvale, CA). Filters were set as follows: EX filter C530/25 and EM filter C590/25. Survival curves were then graphed from the absorbance readings. Triplicates from a single experiment were used to determine the standard error of the mean. Statistical analyses (ANOVA; one factor analysis) were performed using Microsoft™ Excel Software. Results were deemed to be statistically significant when the student t test value was superior to the critical t (2.77 when n=3) and the probability was at least 95% ($p \leq 0.05$).

CRL1420 cells were treated with 20 μM of etoposide for 6h as described above. Twenty-four, 48, 72 and 96 hours post-etoposide challenge, cells were fixed in a 3:1 methanol:acetic acid solution and deposited onto sterile glass microscope slides. Cells were stained with 10 μL of DAPI (0.24 μg/mL) in Antifade™ (Sigma) and visualized by

fluorescence microscopy with a Zeiss Axioscope fluorescence microscope for digital capture of images. In addition, cells exposed to etoposide were viewed by phase contrast microscopy with a Zeiss Axiovert 10 microscope. Images were captured on TMAX 400 ASA black and white professional film (Kodak). Alternatively, etoposide-treated cells were processed for immunoblotting with an anti-PARP antibody. Protein lysates were extracted in lysis buffer containing 6M urea (Invitrogen; Appendix I) and sonicated 2 x 20 sec on ice to break DNA and liberate PARP bound to single and double strand DNA breaks. Protein lysates were then processed as usual for western blotting on 8% SDS- PAGE gels.

To evaluate the effect of etoposide on Xiap, cIAP-1, cIAP-2 and survivin mRNA levels, pancreatic cancer cells were seeded at a density of 1 x 10^6 cells in 100 mm dishes and treated or not with 20 μM of etoposide as described above. Twenty-four, 48, 72 and 96h post-etoposide treatment, cells were lysed in 3 mL of Trizol for RNA extractions and processed for Northern hybridizations with Xiap, cIAP-1, cIAP-2 and survivin cDNA probes. Re-hybridizing the membranes with an actin probe served to assess loading. As detailed in section 2.7, band intensities were determined using a personal densitometer. To control for loading, the intensity of the target RNA band was normalized to that of the corresponding actin band. To evaluate the effect of etoposide on target RNA expression, normalized intensities (for vehicle control and 20 μM etoposide) were then expressed as relative fold inductions over that of the untreated control for each timepoint. In other words, 24h etoposide treatment is expressed as fold induction over 24h untreated control whereas 48h etoposide treatment is expressed as fold induction over 48h untreated control and so on.

To determine the effect of etoposide challenge on the expression of pro- and anti-apoptotic proteins, CRL1420, CRL1469 and CRL1682 cells were seeded at a density of 1 x

10^6 cells in 100 mm dishes and treated with 2 or 20 μM of etoposide. Cells were lysed in 1 mL of protein extraction buffer and processed for immunoblotting.

Finally, CRL1420 cells were seeded in 60 mm dishes (Nunc) and allowed to grow to a confluency of ≈ 60% before being treated or not with 50 μM of a pan-caspase inhibitor z-vad.fmk (Biomol, Hornby, ON) one hour prior to being challenged with 20 μM of etoposide for 6h. Cells were then washed with 1X PBS and placed in fresh culture medium ± 50 μM z-vad.fmk at 37°C/5% CO_2. Cells were lysed for protein extraction 24, 48, 72 and 96h post-etoposide challenge as per standard procedure and processed for immunoblotting with an anti-Xiap antibody.

2.11. Infection of CRL1420 and CRL1469 cells with adenoviral constructs

To determine the optimal multiplicity of infection (# of viral particle per cell, MOI) for Xaf-1 protein expression, CRL1420 and CRL1469 cells were seeded at a density of 1 x 10^5 cells/dish in 60 mm dishes and incubated overnight at 37°C/5% CO_2 to allow cells to attach and resume growth. The culture medium was then replaced with 3 mL of fresh culture medium containing a range of MOI (0, 5, 10, 25, 50 and 100) for adenoXaf-1. Adenoviral constructs were generously provided by Dr. P. Liston (University of Ottawa, Ottawa, ON). Cells were incubated in adenovirus-containing medium for 6h at 37°C/5% CO_2 then grown in fresh culture medium and lysed in protein extraction buffer 24h post-adenoviral infection. Equivalent amounts of protein lysates were resolved by 12% SDS-PAGE and immuno-detected with an anti-Xaf-1 polyclonal antibody.

To evaluate if cells expressing the Xaf-1 protein differed in their sensitivity to etoposide, 2 x 10^3 cells/well of CRL1420 and CRL1469 cells were seeded in 24-well plates

and incubated at 37°C/5% CO_2 overnight to allow attachment. Cells were then infected with adenoXaf-1 or adenolacZ constructs (MOI = 25 (CRL1420) or 50 (CRL1469)) as detailed above. Twenty-four hours post-adenoviral infection, cells were challenged or not with 20 μM of etoposide for 6h as described previously. Cell survival was determined using alamarBlue™ 48h post-etoposide challenge.

2.12. Down-regulation of IAPs using short interfering RNAs

CRL1420 and CRL1469 pancreatic cancer cells were seeded at a density of 2 x 10^5 cells/well in complete culture medium in 24-well plates (Costar, Fisher Scientific) and allowed to attach at 37°C/5% CO_2 overnight. Cells were then transfected or not with 50, 100, 250 or 500 nM of target-specific siRNAs obtained from Dharmacon Inc. (Lafayette, CO). In addition, cells were mock-transfected or transfected with 100 nM of a non-specific siRNA pool to control for the effect of the transfection reagent or of siRNA transfection, respectively. Briefly, 2 μL of *Trans*IT-TKO® transfection reagent (Dharmacon Inc.) were combined with 50 μL of Opti-MEM I® (Invitrogen) for each well receiving siRNA and incubated for 20 min at room temperature under sterile conditions. SMARTpool™ siRNA duplexes were then added to this mixture and allowed to complex with *Trans*IT-TKO® transfection reagent for 20 min at room temperature. Volumes were determined based on 10 μM stock concentrations of siRNAs dissolved in 1X universal buffer (20 mM KCl, 6 mM HEPES pH7.5, 0.2 mM $MgCl_2$; supplied with the siRNA). *Trans*IT-TKO® reagent/siRNA complex mixtures were then added to the cells (4 wells per siRNA concentration) in 500 μL of complete culture medium, mixed thoroughly and incubated at 37°C/5% CO_2 for 48h. At

this point, cells were either lysed in 750 μL of Trizol or 250 μL of protein extraction buffer for RNA and protein isolation, respectively. Identical samples (4 per siRNA concentration) were pooled from this point forward.

To determine the optimal siRNA concentration necessary to obtain at least 50% reduction in RNA expression, 10 μg of siRNA-treated RNAs were resolved on 1% (w/v) denaturing agarose gels, blotted to nylon membranes and hybridized with cIAP-2, survivin and Xiap cDNAs probes as described previously. Membranes were stripped and re-hybridized with β-actin to control for loading. On the other hand, the effect of RNA-mediated interference on target protein expression was assessed by resolving 10 μg of protein lysates on 12% SDS-PAGE and probing the membranes with cIAP-2, survivin and Xiap antibodies. Blots were stripped in 1X Western Re-Probe Solution (Oncogene Research Products, Hornby, On) and hybridized with a polyclonal anti-α-actin antibody to control for lane-to-lane loading.

To determine if down-regulating IAP expression influenced the sensitivity to etoposide, CRL1420 and CRL1469 cells were treated with siRNA as described above. For CRL 1420, the siRNA concentration used was 50 nM for both Xiap and survivin while for CRL1469, the siRNA concentrations used were 500 nM (cIAP-2) and 100 nM (survivin). Transfection with a non-specific siRNA pool (50 nM and 100 nM for CRL1420 and CRL1469, respectively) was performed as a control. At the end of the 48h siRNA transfection, cells were treated with a dose range (0-50 μM) of etoposide. Cell survival was assessed 48h post-etoposide challenge using Cell Proliferation Reagent WST-1 (Roche Diagnostics) as recommended by the supplier. O.D. readings (450 and 650 nm) were performed on a μQuant plate reader (Fisher). Readings were corrected for background by

substracting O.D. $_{650\ nm}$ and no cell readings from the O.D. $_{450\ nm}$ readings. Cell survival was then expressed relative to the "mock transfected, no etoposide" sample. Due to time constraints, triplicates of 2 independent experiments were considered as 6 independent samples to perform statistical analyses as detailed in section 2.9. Results were deemed to be statistically significant when the student t test value was superior to the critical t (4.96 when n=6) and the probability was at least 95% ($p \leq 0.05$).

Chapter 3: Systematic profiling of apoptosis regulators in pancreatic cancer

3.1. Introduction

Defective apoptotic pathways have been implicated in the initiation and progression of human malignancies (Hickman, 2002; Reed, 1999; LaCasse et al., 1998; Satoh et al., 2001). Furthermore, as many cancer therapies are currently designed to introduce DNA damage in rapidly proliferating cells to induce programmed cell death, decreasing the sensitivity to apoptotic signals may contribute to chemoresistance (Kaufmann and Earnshaw, 2000; Dive, 1997; Makin and Hickman, 2000; Haq and Zanke, 1998). The identification of apoptotic regulators that are differentially expressed in normal and cancer cells is therefore warranted to develop effective cancer therapies (L. Yang et al., 2003).

Currently, two major apoptotic pathways, known as intrinsic and extrinsic, are thought to govern programmed cell death. In the intrinsic, or mitochondrial, apoptotic pathway, the cytosolic release of mitochondrial proteins is thought to be mediated by pro-apoptotic members of the bcl-2 family (Green and Reed, 1998). Following death triggers, a conformational change in pro-apoptotic proteins such as bax allows them to integrate into the outer mitochondrial membrane (Gross et al., 1998; Goping et al., 1998; Deshager et al., 1999). In the presence of ATP, the ensuing mitochondrial leakage of cytochrome c leads to the formation of the apoptosome, a multimeric complex comprised of seven Apaf-1/cytochrome c heterodimers in which Apaf-1 recruits pro-caspase-9 via binding of their respective CARD domains (Rodriguez and Lazebnik, 1999; Qin et al., 1999; Acehan et al., 2002). This binding of pro-caspase-9 to Apaf-1 results in the cleavage of pro-caspase-9 at Asp135 and the formation of an active caspase-9 heterotetramer (Srinivasula et al., 1998b; Stennicke et al., 1999; Rodriguez and Lazebnik, 1999). Active initiator caspase-9 can

subsequently cleave downstream effector pro-caspase-3 at Asp175 (Rodriguez and Lazebnik, 1999; Srinivasula et al., 2001). The apoptotic signal is amplified via a positive feedback loop in which caspase-3 activates pro-caspase-9 by cleavage at Asp330 (Srinivasula et al., 1998b; Fujita et al., 2001; Slee et al., 1999). On the other hand, the death-receptor mediated, or extrinsic, apoptotic pathway relies on binding of an external ligand to a transmembrane death receptor to trigger apoptosis. This binding induces clustering of death receptors, formation of the DISC and recruitment of adaptor molecules like TRADD and Trafs which facilitate an association with procaspase-8 (reviewed in Krammer, 2000). This assembly allows for the autoactivation of procaspase-8 molecules via the induced proximity model due to the high concentration of caspase zymogens which have been shown to possess low proteolytic activity (Yamin et al., 1996; Muzio et al., 1998; Orth et al., 1996). In turn, activated initiator caspase-8 cleaves and activates effector caspase-3. Lastly, caspase-3 triggers the proteolysis of several targets substrates ultimately resulting in nuclear degeneration and cell death.

In both pathways, caspases play a central role in the induction of apoptosis (Alnemri et al, 1996; Hengartner, 2000). As such, inhibiting the activation and/or function of caspases should confer a survival advantage to the growing tumor population. According to the current evidence, IAPs are the main inhibitors of caspase activation and activity. Indeed, Xiap can directly bind to and inhibit caspases-3, -7 and –9 both *in vivo* and *in vitro* (Deveraux et al., 1997, 1998; Roy et al., 1997; Takahashi et al., 1998). In humans, three Xiap-interacting proteins, Smac/DIABLO, Omi/HtrA2 and Xaf-1 have been shown to relieve the caspase-inhibiting activity of Xiap providing a model for intricate control of apoptosis (Du et al., 2000; Verhagen et al., 2000, 2002; Martins et al., 2002; Hedge et al., 2002; Liston et al., 2001). Removal of the mitochondrial targeting sequence of Smac/DIABLO and

Omi/HtrA2 reveals a N-terminal sequence (AVPI/S) capable of binding to the BIR3 domain of Xiap (Srinivasula et al., 2000; Liu et al., 2000; Suzuki et al., 2001b; Hedge et al., 2002). As the residues mediating this interaction are homologous to those mediating binding of Xiap to caspase-9, it has been suggested that Smac/DIABLO and Omi/HtrA2 may displace Xiap from caspase-9 to allow initiation of the apoptotic cascade (Sun et al., 2000). In contrast, Xaf-1 is proposed to trigger relocalization of Xiap to the nucleus thereby effectively sequestering Xiap away from the apoptosome and relieving its inhibition of caspase activity (Liston et al., 2001). On the other hand, cIAP-1 and cIAP-2 are recruited to the TNF-RII receptor complex via an association of their N-terminal BIR domains with Traf1/Traf2 heterocomplexes (Rothe et al., 1995). Consequently, it is currently believed that cIAPs might play a role in preventing death receptor-mediated apoptosis. In fact, exposure to TNF-α up-regulates cIAP-1, cIAP-2 and Xiap (Stehlik et al., 1998b). Moreover, cIAP-2 is required to suppress TNF-induced apoptosis via its involvement in TNF signaling events that induce NF-κB (Chu et al., 1997). In turn, active NF-κB can prevent cell death by up-regulating IAPs, Trafs and bcl-2 homologs (reviewed in Barkett and Gilmore, 1999).

High levels of IAPs have been reported in a number of human cancers (Satoh et al., 2001; Ambrosini et al., 1997; Sarela et al., 2001, Tanaka et al., 2000). Of particular relevance to this thesis, survivin was shown to be up-regulated in 77% of pancreatic ductal adenocarcinomas while being undetectable in normal pancreatic tissue (Satoh et al., 2001). In addition, lower survival rates were observed in stage II colorectal cancer patients whose tumors were survivin-positive compared to survivin-negative patients (Sarela et al., 2001). Moreover, in human tumors, high Xiap levels have been shown to confer resistance to drug-induced apoptosis (Ferreira et al., 2001; Li et al., 2001; Sasaki et al., 2000). In light of this,

directly targeting IAPs as a means of overcoming resistance to apoptosis constitutes a promising therapeutic strategy.

The grim prognosis associated with pancreatic cancer (overall mortality rate estimated to be 99%) along with the acknowledged clinical resistance of these tumors to radiation and chemotherapy in general prompted us to investigate the underlying molecular cause of this resistance. As it was recently shown that, although to varying extents, apoptosis can be induced in pancreatic cancer cells (Ding and Adrian, 2002), we hypothesized that a deregulation of apoptotic modulator/effector genes may contribute to the intrinsic resistance of pancreatic adenocarcinomas to cytotoxic therapies. To identify key apoptotic regulators differentially expressed in normal and cancer cells, systematic profiling of caspases, IAPs and their regulators was extensively performed in normal pancreatic tissue, immortalized ductal cell lines and pancreatic adenocarcinoma cell lines.

In short, our study revealed that pancreatic cancer cell lines generally tend to over-express IAPs (cIAP-2 in particular) and frequently under-express Xaf-1, caspases-9 and -3 supporting the notion that such changes might lead to an elevation in the apoptotic threshold which, in turn, favors resistance to anti-cancer therapies. Immunohistological surveys of normal, intraductal lesions and adenocarcinomas of the pancreas also revealed over-expression of cIAP-1, cIAP-2 and survivin with redistribution of these IAPs to the nuclei of cancerous cells. By contrast, Xiap expression and subcellular localization appeared unaffected in adenocarcinomas. Furthermore, all pancreatic cancer cell lines tested appeared to repress Xaf-1 protein expression despite the presence of variable mRNA levels. Down-regulation of Smac/DIABLO mRNA and protein was also observed in pancreatic cancer cell lines. Lastly, analysis of matched normal/tumor RNA sets from different solid tumor types uncovered the same tumor-specific tendencies towards over-expression of one or more IAP

and down-regulation of Xaf-1 as well as caspases-3 and -9. Taken together, these findings suggest that successful treatment of malignant solid tumors might be achievable through selective targeting of affected components of the IAP/caspase cascade in combination with judiciously chosen apoptotic therapeutics.

3.2. Results

3.2.1. The loci encoding Xaf-1, Xiap, cIAP-1 and cIAP-2 are structurally intact in pancreatic adenocarcinoma cell lines

Structural rearrangements such as recombinations and deletions which can lead to aberrant mRNA and/or protein expression are often encountered in tumors. In light of this, we began by assessing the structural integrity of the Xaf-1, Xiap, cIAP-1 and cIAP-2 loci. To this end, *Hind* III-digested genomic DNAs from normal pancreatic tissue and pancreatic cancer cell lines were resolved by agarose gel electrophoresis and alkali-blotted to a nylon membrane for Southern bybridization. One membrane was probed with a partial cDNA encoding Xaf-1 (designed in the central portion of the mRNA) while a second membrane was probed with full-length Xiap cDNA. A third membrane was probed with partial cIAP-1 and cIAP-2 cDNAs biased to the 3' end in succession. To predict restriction fragment sizes, target genomic sequences were obtained using the MapView feature of the Entrez website (www.ncbi.nlm.nih.gov/mapview), imported into Clone Manager v6.0 and aligned against the corresponding mRNA sequences to ascertain gene boundaries using Align Plus4 v4.10. Approximately 3 Kbp of 5' and 3' untranslated sequences were included for mapping of *Hind* III restriction sites. Following alignment of the probe sequence against this map, the

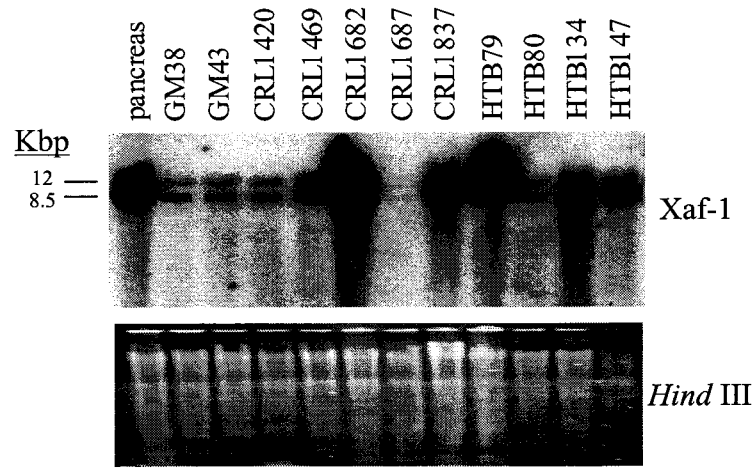
number and size of restriction fragments likely to be detected by the individual probes was determined.

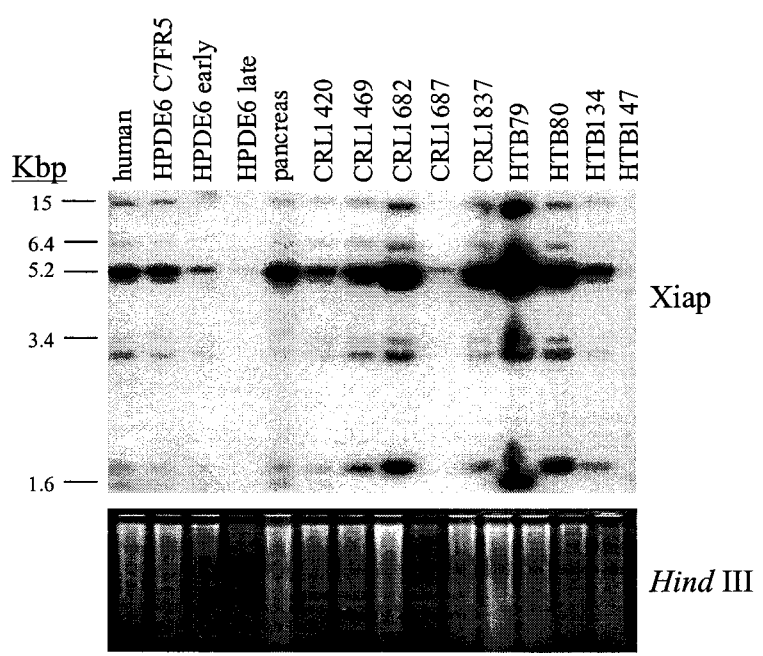
Two bands of approximately 8.5 and 12 Kbp (corresponding to the sizes predicted from the restriction map) were observed for Xaf-1 in all cell lines studied although the intensity of the signal was very weak in CRL1687 in spite of a good intensity on the agarose gel (Fig.3.1). According to our map predictions, the Xiap probe should detect 7 bands ranging in size from 1.7 to 16 Kbp. Indeed, 7 bands (ranging between approximately 1.6 and 15 Kbp) were detected in most cancer cell lines tested although the lower molecular weight band varied in size. In the control cell lines (human, HPDE6 and pancreas), we observed a doublet (1.6 and 1.7 Kbp) for the lower molecular weight band possibly due to length polymorphisms. Interestingly, all cancer cell lines tested had only one of the two lower molecular weight band; in most cases, it was the 1.7 Kbp band except for HTB79 which had the 1.6 Kbp band. Finally, we did not obtain a Xiap signal in HTB147 in spite of a good intensity on the agarose gel possibly due to DNA degradation.

To successfully detect the loci encoding cIAP-1 and cIAP-2, cDNAs probes were generated in the 3'UTR in order to distinguish between the two closely related genes. Two cIAP-1 bands (3.1 and 6 Kbp) could clearly be detected in all cell lines tested with the exception of HPDE6 late; however, these fragments did not agree with the predicted sizes of 2 and 3.1 Kbp. Based on map predictions, we expected to detect a single cIAP-2 band at 17.5 Kbp. However, in addition to the 17.5 Kbp cIAP-2 band, at least two additional bands of smaller molecular weight were uncovered in all cell lines tested except for HPDE6 late. Lack of signal for HPDE6 late is probably due to the relatively low amount of this DNA sample on the membrane as verified by agarose gel electrophoresis. Assuming roughly

Fig.3.1. The genes coding for Xaf-1, Xiap, cIAP-1 and cIAP-2 are structurally intact in pancreatic adenocarcinoma cell lines.

Three μg of *Hind* III-digested genomic DNAs were resolved on a 1% (w/v) agarose gel in 1X TBE buffer, alkali-blotted to a nylon membrane and hybridized with radiolabelled Xaf-1, Xiap, cIAP-1 and cIAP-2 cDNA probes. Blots were washed to high stringency and exposed to X-ray film. Fragments were sized against the BRL Forensic DNA Analysis Marker (corresponding reference size fragments are indicated beside each autoradiogram). Corresponding agarose gels are included below each autoradiogram to control for lane-to-lane loading and confirm complete digestion of genomic DNA samples.
* Unassignable bands (one for cIAP-1 and two for cIAP-2) possibly corresponding to non-specific hybridization were also revealed in our analysis.


pancreas
GM38
GM43
CRL1420
CRL1469
CRL1682
CRL1687
CRL1837
HTB79
HTB80
HTB134
HTB147
Kbp
12
8.5
Xaf-1
Hind III


human
HPDE6 C7FR5
HPDE6 early
HPDE6 late
pancreas
CRL1420
CRL1469
CRL1682
CRL1687
CRL1837
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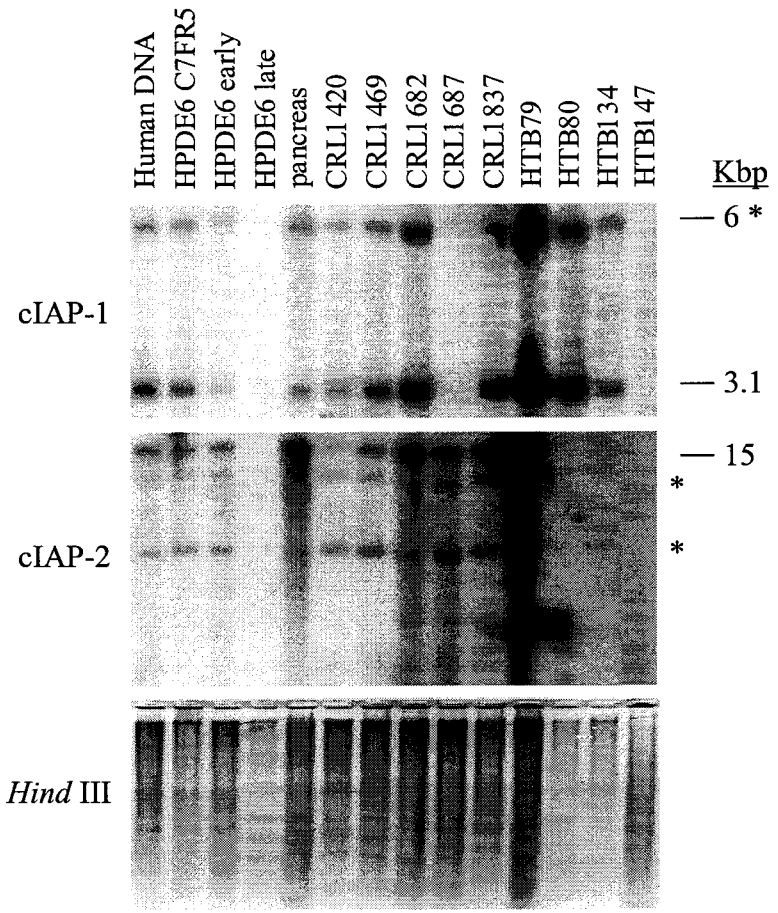
6.4
5.2
3.4
1.6
Xiap
Hind III


Human DNA
HPDE6 C7FR5
HPDE6 early
HPDE6 late
pancreas
CRL1420
CRL1469
CRL1682
CRL1687
CRL1837
HTB79
HTB80
HTB134
HTB147
Kbp
cIAP-1
— 6 *
— 3.1
cIAP-2
— 15
*
*
Hind III

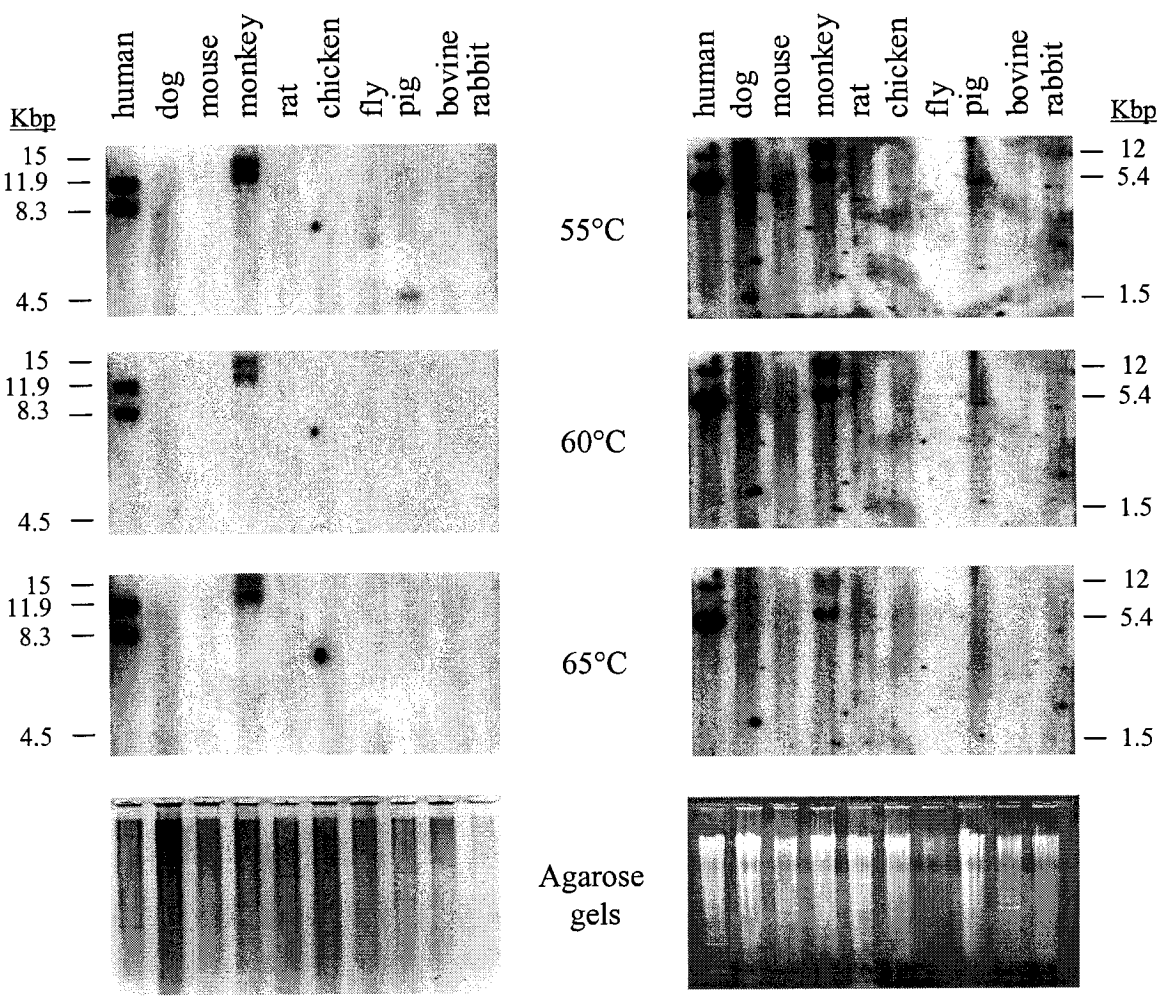
equivalent loading of digested DNA samples as visualized by agarose gel electrophoresis, some cancer cell lines (CRL1682, CRL1837 and HTB80) appear to have a higher copy number of cIAP-1. Copy numbers for Xaf-1, Xiap and cIAP-2 appear to be relatively equal.

3.2.2. The Xaf-1 coding region is conserved among animal species

At this stage of our study, we were interested in evaluating conservation of the Xaf-1 sequence among animal species. As a parallel experiment, *Hind* III- or *Eco* RI-digested genomic DNAs from ten different animal species were used to generate zooblots. In particular, we wished to verify if human Xaf-1 shared sequences with *Drosophila* as Xaf-1 was identified as a Xiap-interacting partner proposed to restrict IAP activity in a manner similar to that of *Drosophila* hid, grim and rpr (Fong et al., 2000, Liston et al., 2001). Fig.3.2 shows that, under conditions of low stringency (55°C), human Xaf-1 can hybridize strongly with rhesus monkey and weakly with pig. In addition, while two *Hind* III-digested Xaf-1 bands of 12 and 8.5 Kbp were detected in human (as previously observed), the size of the *Hind* III-digested Xaf-1 bands in rhesus monkey (11.9 and 15 Kbp) and pig (4.5 Kbp) differed from the human ones suggesting that, although the coding sequence of the human Xaf-1 gene may be conserved in other species, non-coding sequences (eg. introns) may differ. This observation is further supported in the *Eco* RI-digested samples where the two Xaf-1 bands (12 and 5.4 Kbp) observed in human differed from those observed in rhesus monkey (15 and 7.5 Kbp). Increasing the hybridization stringency resulted in the loss of signal for pig without affecting the signal in human and rhesus monkey implying that Xaf-1-like gene sequences appear to be most highly related between human and rhesus monkey.

Fig.3.2. The human Xaf-1 gene shares related sequences with other species.

Three μg of *Hind* III- or *Eco* RI-digested genomic DNAs from several animal species were resolved on a 1% (w/v) agarose gel in 1X TBE buffer, alkali-blotted to a nylon membrane and probed with radiolabelled Xaf-1 cDNA. Blots were hybridized under low (55°C), intermediate (60°C) and high (65°C) stringency conditions in succession. Fragments were sized against the BRL Forensic DNA Analysis Marker (corresponding reference size fragments are indicated beside each autoradiogram). Agarose gel electrophoresis confirmed complete digestion of genomic DNA samples and served as control for lane-to-lane loading. Strongest sequence similarity for human Xaf-1 was observed with the rhesus monkey although some hybridization occurred with pig at lower stringency.


human
dog
mouse
monkey
rat
chicken
fly
pig
bovine
rabbit
Kbp
15
11.9
8.3
4.5
55°C
60°C
65°C
Agarose gels
12
5.4
1.5
Hind III
Eco RI

3.2.3. Loss of heterozygosity and hypermethylation of CpG islands at the Xaf-1 locus in pancreatic adenocarcinoma cell lines

Even though no apparent rearrangement or deletion of the Xaf-1 gene could be detected in pancreatic ductal and adenocarcinoma cell lines by Southern blot, the possible loss of Xaf-1 alleles could not be excluded. The genomic region encoding the Xaf-1 locus (17p13.2) is frequently deleted in human cancer cell lines (Fong et al., 2000). In addition, the Xaf-1 gene lies distal to p53 (17p13.1), a tumor suppressor gene frequently inactivated in pancreatic adenocarcinomas (Redston et al., 1994; Johansson et al., 1992). In light of this, microsatellite marker typing using fluorescently-labelled STR markers (D17S796 and D17S1881) flanking the Xaf-1 locus was performed. DNAs from various sources were included as controls: GM38, GM9948, human pancreas (Hpan), total human DNA and the immortalized pancreatic ductal cell lines HPDE4 and HPDE6 while pancreatic cancer cell lines served as test DNA samples. Both markers used were informative, consistent with their reported heterozygosity indices in the normal population of 82% and 78% for D17S796 and D17S1881, respectively (www.resgen.com). A strong reduction to homozygosity was observed in pancreatic adenocarcinoma cell lines with only one of the cancer cell lines analysed being heterozygous at both markers tested (Table 3.1). In contrast, we observed a heterozygosity index of 66% in the control cell lines studied for each microsatellite marker used. The discrepancy with the reported heterozygosity indices (obtained from >100 alleles) can be explained by the small number of control samples tested in our study.

The absence of Xaf-1 mRNA expression observed by Fong et al. (2000) in a broad panel of cancer cell lines suggested that down-regulation of Xaf-1 may be an important event in the development of human malignancies. This, along with the reported Xiap-

Table 3.1. General trend towards loss of heterozygosity at the Xaf-1 locus in pancreatic adenocarcinoma cell lines.

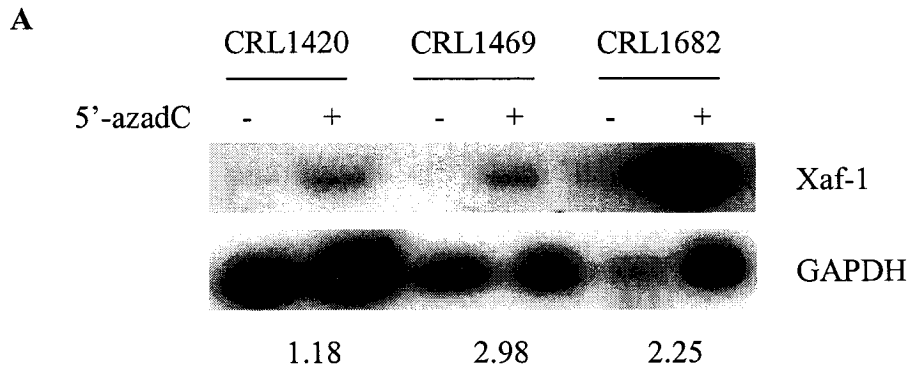
Forty ng of genomic DNA were amplified using fluorescently-labelled D17S796 and D17S1881 microsatellite markers. Amplicons were resolved by gel electrophoresis through a polymer-filled capillary on an ABI 310 gene sequencer and detected by charge-coupled device capture of laser-induced fluorescence. Data was analysed using GeneTyper v3.7. Peaks whose height was less than 32% of the highest peak were considered background and disregarded. Y indicates heterozygous cell lines while N applies to homozygous cell lines.

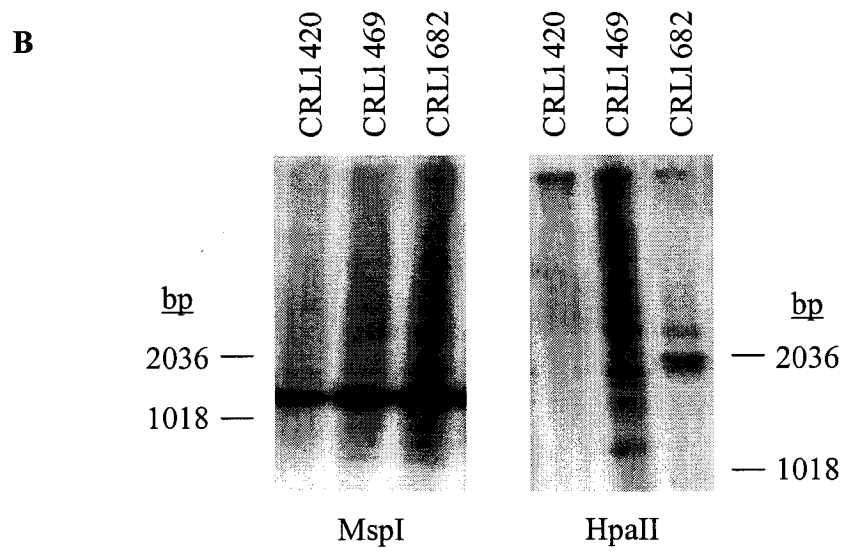
	Cell line	D17S796 (0.82)	D17S1881 (0.78)
Normal cell lines	Human	Y	Y
	GM38	Y	N
	GM9948	N	Y
	Hpan	Y	N
	HPDE4	Y	Y
	HPDE6	N	Y
Tumor cell lines	CRL1420	N	N
	CRL1469	N	N
	CRL1682	N	N
	CRL1687	N	N
	CRL1837	Y	Y
	HTB79	N	N
	HTB80	N	N
	HTB134	N	N
	HTB147	N	N
	PK1	N	N
	PK8	N	N
	% Het	0,09	0,09

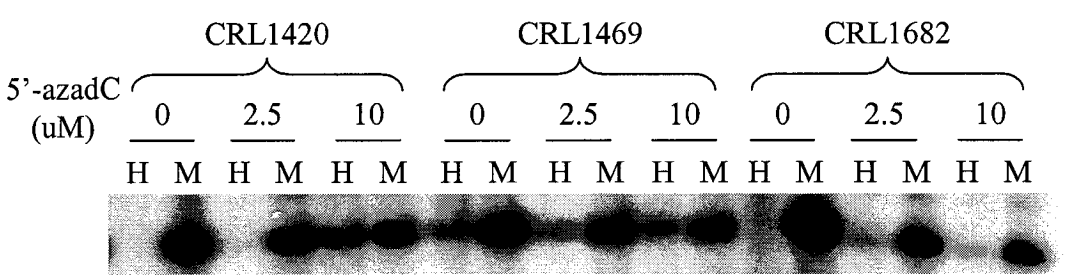
binding and sequestering properties of Xaf-1 suggest that Xaf-1 may function as a tumor suppressor gene. Inactivation of both alleles of a tumor suppressor gene is required to effectively suppress its expression and function (Knudson, 1971). The LOH observed in the Xaf-1 region can only explain the inactivation of one Xaf-1 allele; the remaining allele must be inactivated by a different mechanism. Epigenetic events such as methylation of cytosine residues within the context of CpG islands have been suggested to contribute to the silencing of tumor suppressor genes (reviewed in Garinis et al., 2002; Worm and Guldberg, 2002). To assess the methylation status of Xaf-1, the human pancreatic adenocarcinoma cell lines CRL1420, CRL1469 and CRL1682 (chosen because they are weak expressors of Xaf-1) were treated with the DNA methyl transferase inhibitor 5'-aza-2-deoxycytidine and processed for Northern analysis with a partial Xaf-1 cDNA probe followed by hybridization with GAPDH to control for lane-to-lane loading. Normalizing Xaf-1 mRNA expression to that of GAPDH revealed induction of Xaf-1 mRNA expression upon treatment with the demethylating agent (Fig.3.3A). Indeed, 1.18, 2.98 and 2.25 fold increases in Xaf-1 mRNA expression were observed for CRL1420, CRL1469 and CRL1682, respectively. To confirm this observation, genomic DNAs from the aforementioned cancer cell lines were digested with *Hpa* II or *Msp* I and processed for Southern blotting using a Xaf-1-specific cDNA probe. These restriction enzymes are isoschizomers, that is, they recognize the same consensus sequence (5' CC↓GG 3') except that *Msp* I is insensitive to CpG methylation while *Hpa* II cleavage of mammalian genomic DNA is blocked by CpG methylation. Interestingly, while a strong *Msp* I band was detected at approximately 1.2 Kbp in all cell lines tested, this band was undetectable in *Hpa* II-digested samples implying the presence of intragenic methylation of the Xaf-1 locus (Fig.3.3B). In addition, the appearance of a 1.2

Fig.3.3. Silencing of the Xaf-1 gene by CpG methylation in human pancreatic adenocarcinoma cell lines.

A. Pancreatic cancer cell lines were treated (+) or not (-) with 2.5 μM (CRL1420) or 1 μM (CRL1469, CRL1682) of the methylation inhibitor 5'-aza-2'-deoxyxcytidine (5'-azadC) for 11 days. Cells were then maintained in complete culture medium and grown to confluence for RNA isolation. Ten μg of total RNA were resolved on a 1% (w/v) denaturing agarose gel and transferred to a nylon membrane in 15X SSC. Blots were hybridized with a Xaf-1-specific cDNA probe, stripped and re-hybridized with a GAPDH-specific probe to control for lane-to-lane loading. Autoradiographic signal intensities for Xaf-1 were quantified by densitometry, normalized to GAPDH and are expressed as fold increases (treated/non-treated) below the corresponding signals. **B.** Three μg of genomic DNAs were digested with *Hpa* II or *Msp* I and processed for Southern blotting with a Xaf-1-specific cDNA probe. **C.** Genomic DNAs isolated from 5'-aza-2'-deoxycytidine-treated pancreatic cancer cells were digested with *Hpa* II (H) or *Msp* I (M) and processed for hybridization with radiolabelled Xaf-1 cDNA.


A
CRL1420
CRL1469
CRL1682
5'-azadC
-
+
-
+
-
+
Xaf-1
GAPDH
1.18
2.98
2.25


B
CRL1420
CRL1469
CRL1682
CRL1420
CRL1469
CRL1682
bp
2036
1018
bp
2036
1018
MspI
HpaII


C
CRL1420
CRL1469
CRL1682
5'-azadC
(uM)
0
2.5
10
0
2.5
10
0
2.5
10
H M H M H M H M H M H M H M H M H M

Kbp band in 5-aza-2'-deoxycytidine-treated/*Hpa* II-digested genomic DNA samples from CRL1420 and, to a lesser extent, from CRL1469 and CRL1682 is evident in Fig.3.3C further suggesting reversible intragenic methylation of Xaf-1.

Overall, although structurally intact, the Xaf-1 locus appears to be affected by loss of heterozygosity and hypermethylation in pancreatic adenocarcinoma cell lines.

3.2.4. Inhibitor of apoptosis protein mRNAs are over-expressed while caspase mRNAs are under-expressed in pancreatic cancer cell lines

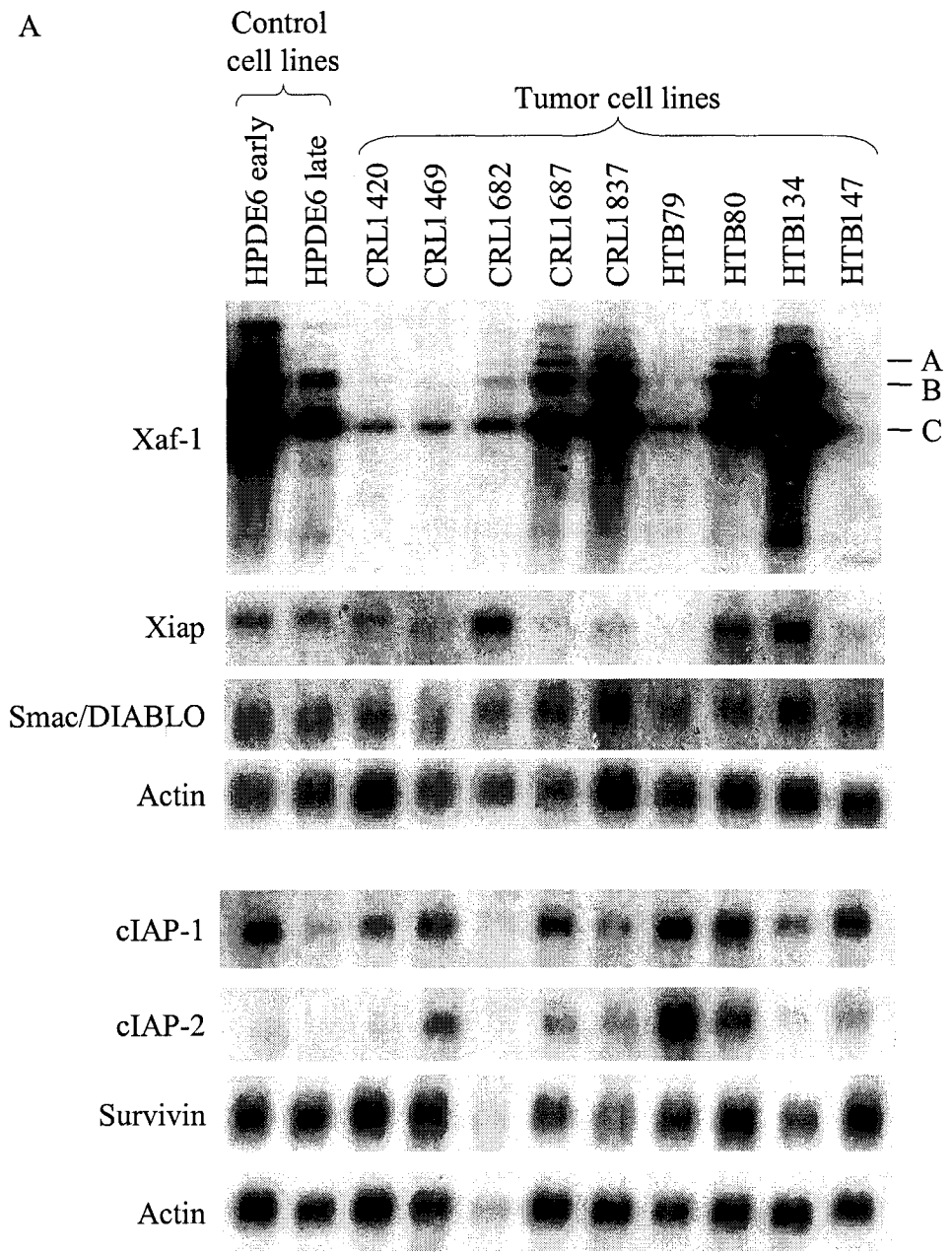
To determine the relative steady-state levels of IAPs and their antagonists in pancreatic cancer cell lines, mRNAs isolated from immortalized and neoplastic pancreatic cell lines were processed for Northern blot analysis. As it is extremely difficult to isolate, easily degraded and often of poor quality, we were unable to include mRNA from normal pancreatic tissue. Two membranes were generated simultaneously and probed with ^{32}P-labelled cDNAs; one membrane was probed with Xaf-1, Xiap and Smac/DIABLO in succession while a parallel membrane was sequentially probed with cIAP-1, cIAP-2 and survivin. Re-hybridizing the membranes with β-actin controlled for lane-to-lane loading.

Since Xaf-1 and Xiap can be viewed as positive and negative regulators of apoptosis, respectively, we were expecting to observe decreased expression of Xaf-1 mRNA and increased expression of Xiap mRNA in pancreatic cancer cell lines. The presence of three Xaf-1 transcripts (arbitrarily labelled A, B and C) can clearly be detected in ductal (HPDE) as well as pancreatic adenocarcinoma (CRL and HTB series) cell lines (Fig.3.4A). As revealed by densitometry (Fig.3.4B; refer to figure legend for details on calculations), two pancreatic cancer cell lines (CRL1687 and HTB134) had elevated levels of Xaf-1 mRNA

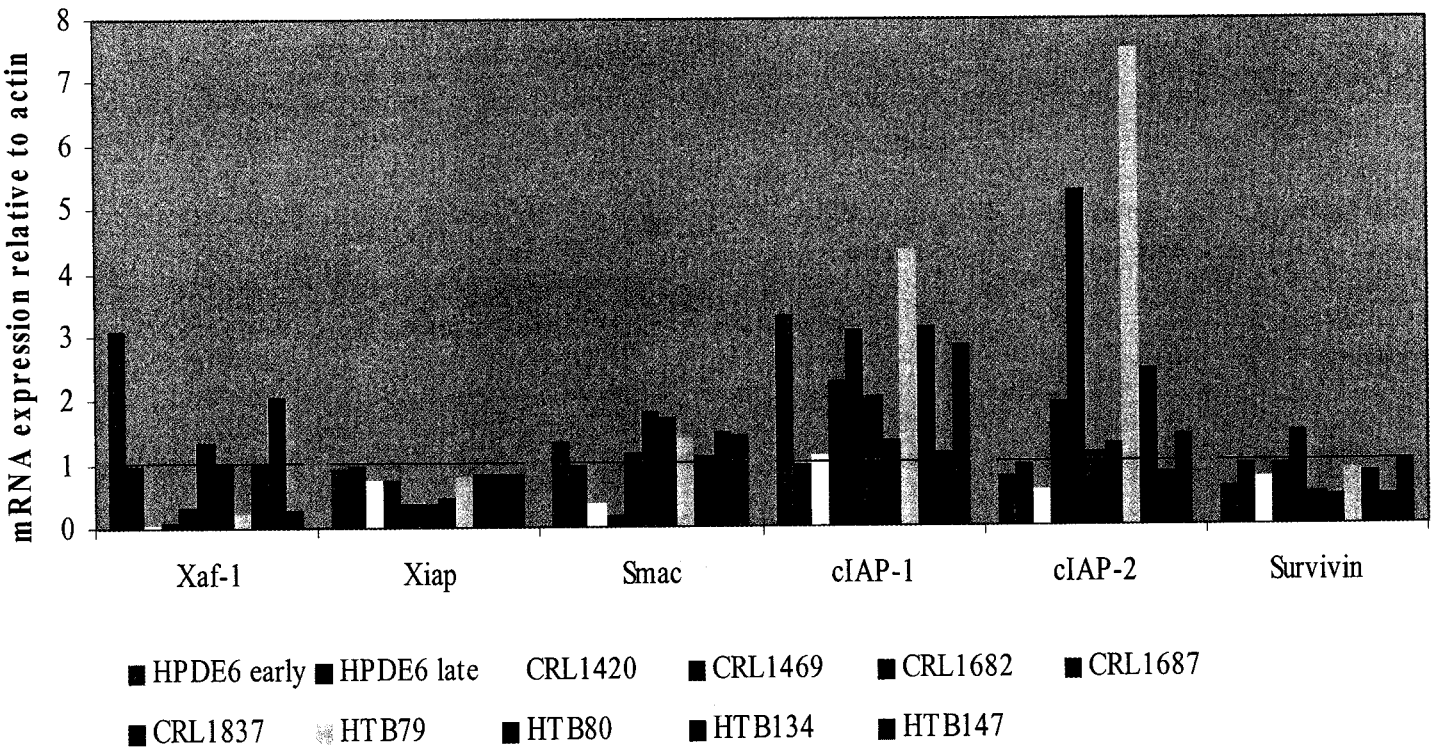
Fig.3.4. Variable expression of mRNAs encoding IAPs and their antagonists in pancreatic adenocarcinoma cell lines.

mRNAs from immortalized ductal and pancreatic cancer cell lines were isolated from 100 μg of total RNA, separated by 1% (w/v) denaturing agarose gel electrophoresis and blotted to a nylon membrane. One membrane was probed with radiolabelled Xaf-1 cDNA, stripped, probed with Xiap cDNA, stripped and re-probed with Smac/DIABLO cDNA. A duplicate membrane was generated and probed with radiolabelled cIAP-1, cIAP-2 and survivin cDNAs in sequence. Blots were washed to high stringency and exposed to X-ray film. β-actin was used as an internal loading control. All mRNAs tested were expressed, albeit at varying levels, in the majority of cancer cell lines. mRNAs coding for Smac/DIABLO, cIAP-1 and cIAP-2 appear to be over-expressed in pancreatic cancer cell lines while mRNAs coding for Xaf-1, Xiap and survivin appear to be down-regulated in most pancreatic cancer cell lines. **A.** Autoradiograms. **B.** Relative intensities of target RNA expression normalized to that of actin and expressed as fold inductions compared to HPDE6 late (control cell line).

A


Control cell lines
Tumor cell lines
HPDE6 early
HPDE6 late
CRL1420
CRL1469
CRL1682
CRL1687
CRL1837
HTB79
HTB80
HTB134
HTB147
Xaf-1
A
B
C
Xiap
Smac/DIABLO
Actin
cIAP-1
cIAP-2
Survivin
Actin

B


mRNA expression relative to actin
8
7
6
5
4
3
2
1
0
Xaf-1
Xiap
Smac
cIAP-1
cIAP-2
Survivin
HPDE6 early
HPDE6 late
CRL1420
CRL1469
CRL1682
CRL1687
CRL1837
HTB79
HTB80
HTB134
HTB147

when compared to the non-tumorigenic immortalized pancreatic ductal cell lines (HPDE6 late) while the other 7 had lower levels of Xaf-1 mRNA. Probing the membrane with Xiap revealed a single transcript (Fig.3.4A). Contrary to our predictions, none of the 9 cancer cell lines tested over-expressed Xiap mRNA relative to the HPDE ductal cell controls. In addition, Xaf-1 and Xiap mRNAs did not appear to be co-regulated as there was no correlation between the relative amounts of both mRNAs in individual cell lines although probe specific activities differed in each experiment. Next, we hypothesized that cancer cells might under-express Smac/DIABLO mRNA as this gene product is a strong Xiap antagonist. However, our densitometric analysis showed that the mRNA encoding Smac/DIABLO was slightly (1.2-2 fold) over-expressed in 7 of the 9 pancreatic cancer cell lines studied (Fig.3.4B). As cIAP-1, cIAP-2 and survivin are anti-apoptotic, we expected the mRNAs encoding these genes to be over-expressed in pancreatic cancer cell lines. Indeed, CRL1469, CRL1682, HTB79 and HTB80 strongly (2-7.5 fold) over-expressed cIAP-2 while a weak (1.2-1.5 fold) cIAP-2 over-expression was observed in CRL1687, CRL1837 and HTB147. Only CRL1420 appeared to down-regulate cIAP-2 mRNA expression. It is noteworthy that weak but quantifiable amounts of cIAP-2 mRNA were observed in our control cell lines (HPDE6) in spite of a strong actin signal (Fig.3.4A). On the other hand, cIAP-1 mRNA was over-expressed in all pancreatic cancer cell lines tested albeit at varying levels. Strong (2-5 fold) cIAP-1 over-expressors included CRL1469, CRL1682, CRL1687, HTB79, HTB80 and HTB147. Finally, contrary to our predictions, survivin mRNA was down-regulated in all but one of the pancreatic cancer cell lines studied. Nonetheless, we must be careful during this analysis as we are comparing IAP mRNA expressions in pancreatic cancer cell lines to an immortalized (i.e. initialized) cell line; as a result, we may be underestimating the real change in mRNA level.

Interesting observations can also be derived when comparing the expression of the mRNAs tested in HPDE6 early - an immortalized ductal cell line which is still in crisis - to the expression observed in this cell line post-crisis (HPDE6 late). Indeed, Xaf-1 and cIAP-1 mRNA expression is much stronger in HPDE6 early than in HPDE6 late suggesting that Xaf-1 and cIAP-1 mRNAs may be stress-inducible.

To expand and confirm this Northern analysis, we performed RNase protection assays on two human template sets (hAPO1C and hAPO5) using our panel of ductal and tumor pancreatic cell lines as targets. In addition, this analysis allowed us to survey for variations in components of parallel apoptotic pathways. The assignment of bands corresponding to the RNase-protected fragments tested is depicted in Fig.3.5. The results presented here are representative of two independent experiments; however, only one of the data sets was quantifiable by densitometry. As a result, error bars could not be included on this figure. Autoradiograms and analysis of the data set that was quantified can be found in Appendix II.

RPA profiles did not entirely agree with Northern profiles (Fig.3.6). In fact, while Northern hybridizations revealed a down-regulation of Xiap mRNA, RPAs uncovered a moderate (2.5-3 fold) over-expression of Xiap mRNA in CRL1420 and CRL1682 as well as a weak (≈1.5 fold) over-expression of Xiap RNA in CRL1687, HTB80, HTB134 and HTB147 while the other pancreatic cancer cell lines tested showed a down-regulation of Xiap (upper left panel of Fig.3.6). Furthermore, while Northern profiles established a tendency towards up-regulation of cIAP-1 and cIAP-2 mRNAs, RPA profiles did not confirm this. Indeed, of all the pancreatic cancer cell lines studied by RPA, only CRL1837,

Fig.3.5. Schematic representation of typical RPA results.

Two human apoptotic templates (hAPO1C and hAPO5) were probed with ductal and tumor RNAs for RNase protection assay. Depicted in this figure are typical results obtained showing appropriate resolution of bands for the gene expression tested.

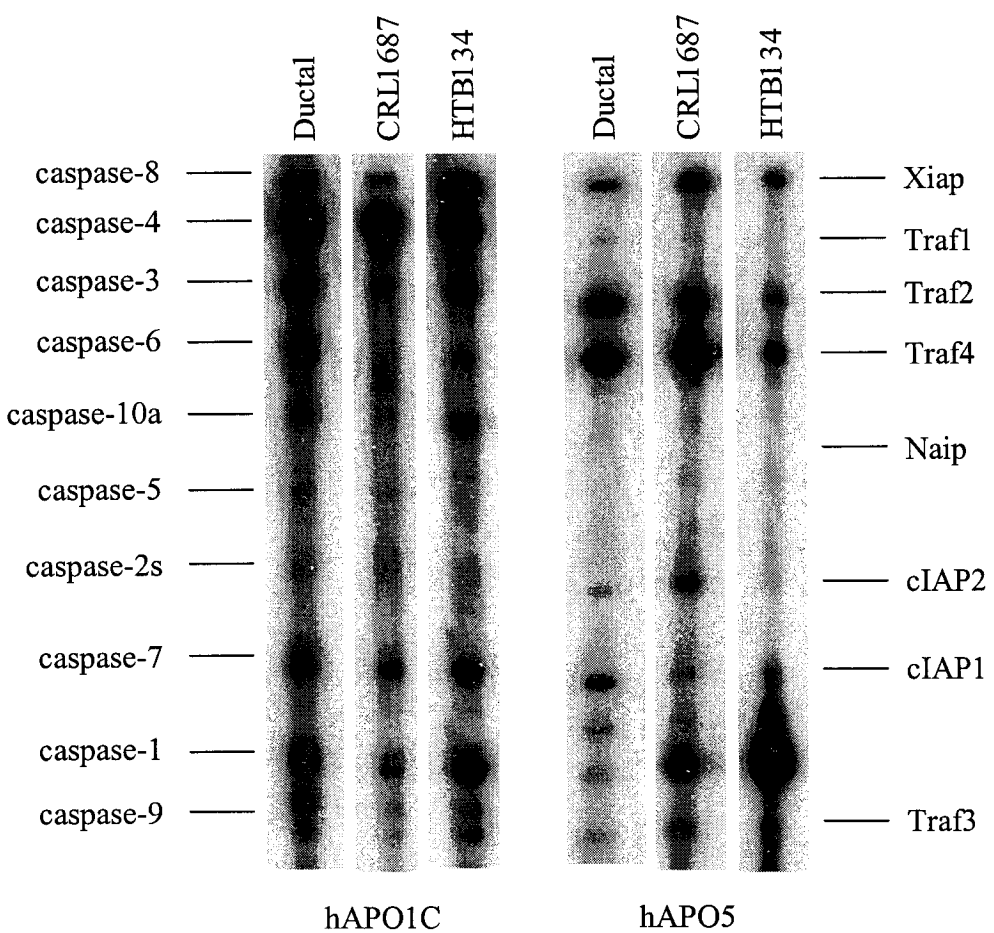
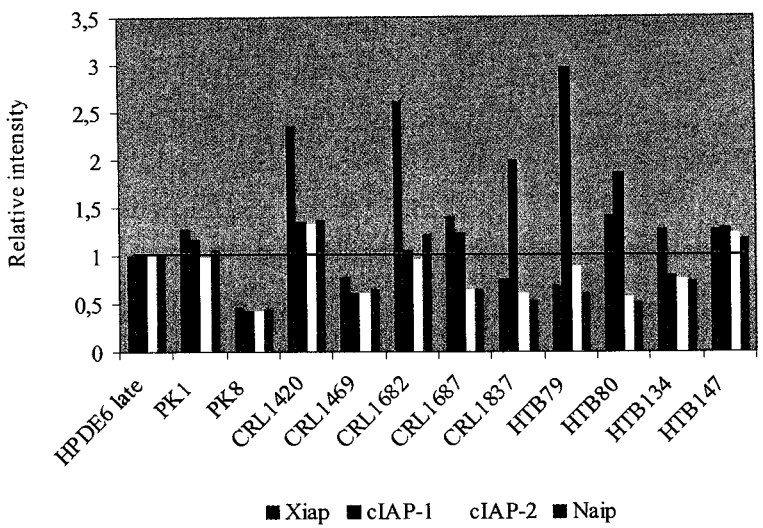
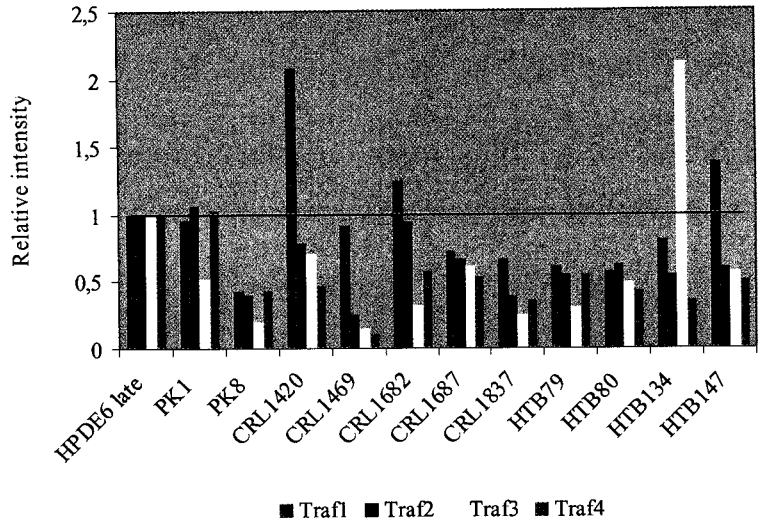
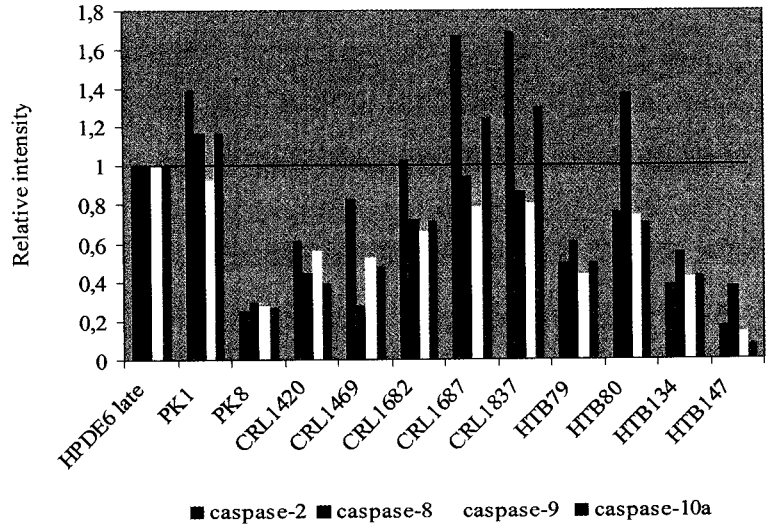

Ductal
CRL1687
HTB134
Ductal
CRL1687
HTB134
caspase-8
caspase-4
caspase-3
caspase-6
caspase-10a
caspase-5
caspase-2s
caspase-7
caspase-1
caspase-9
Xiap
Traf1
Traf2
Traf4
Naip
cIAP2
cIAP1
Traf3
hAPO1C
hAPO5

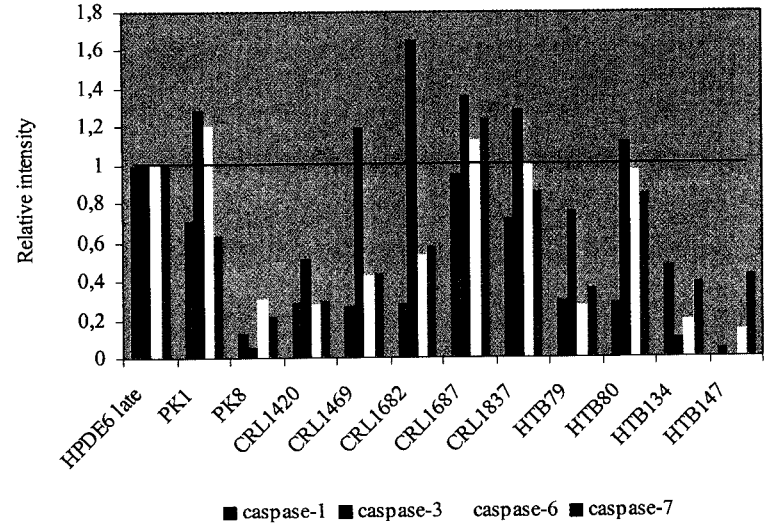
Fig.3.6. Steady-state levels of IAP and caspase mRNAs in pancreatic cancer cell lines.

RNase protection assays were performed on two human apoptotic templates (hAPO1C and hAPO5) using pancreatic ductal and tumor RNAs as targets. RNase protected fragments were resolved on 5% polyacrylamide gels and exposed to X-ray films. Results are representative of two independent experiments. Band intensities were determined by densitometry, normalized to the internal standard L32 and are expressed relative to the expression of HPDE6 late.


Relative intensity
3,5
3
2,5
2
1,5
1
0,5
0
HPDE6 late
PK1
PK8
CRL1420
CRL1469
CRL1682
CRL1687
CRL1837
HTB79
HTB80
HTB134
HTB147
Xiap
cIAP-1
cIAP-2
Naip


Relative intensity
2,5
2
1,5
1
0,5
0
HPDE6 late
PK1
PK8
CRL1420
CRL1469
CRL1682
CRL1687
CRL1837
HTB79
HTB80
HTB134
HTB147
Traf1
Traf2
Traf3
Traf4


Relative intensity
1,8
1,6
1,4
1,2
1
0,8
0,6
0,4
0,2
0
HPDE6 late
PK1
PK8
CRL1420
CRL1469
CRL1682
CRL1687
CRL1837
HTB79
HTB80
HTB134
HTB147
caspase-2
caspase-8
caspase-9
caspase-10a


Relative intensity
1,8
1,6
1,4
1,2
1
0,8
0,6
0,4
0,2
0
HPDE6 late
PK1
PK8
CRL1420
CRL1469
CRL1682
CRL1687
CRL1837
HTB79
HTB80
HTB134
HTB147
caspase-1
caspase-3
caspase-6
caspase-7

HTB79 and HTB80 moderately (2-3 fold) over-expressed cIAP-1 while only 2 cancer cell lines (CRL1420 and HTB147) very weakly (1.2-1.5 fold) over-expressed cIAP-2 mRNA. Finally, NAIP was shown to be down-regulated in most cancer cell lines tested. Unfortunately, Xaf-1 was not available on these templates therefore we cannot confirm Northern observations for this mRNA.

We also surveyed the expression of RNAs encoding caspases by RPA. As can be observed in Fig.3.6, there is a strong tendency towards down-regulation of both initiator (bottom left panel) and effector (bottom right panel) caspases at the mRNA level. Indeed, initiator caspases were found to be down-regulated in virtually all of the pancreatic cancer cell lines tested. The few cases of over-expression are very weak as they do not exceed a 1.7 fold induction. Moreover, there is a strong tendency towards down-regulation of effector caspases except for a few cases involving caspase-3 which is weakly (1.2-1.7 fold) over-expressed in 5 pancreatic cancer cell lines (PK1, CRL1469, CRL1682, CRL1687 and CRL1837).

Given that cIAP-1 and cIAP-2 are thought to associate with Traf1/Traf2 heterocomplexes thereby facilitating their recruitment to the TNF receptor complex and inhibition of apoptosis, it is reasonable to predict that Traf1/2 expression levels should be increased in pancreatic cancer cell lines to enhance the anti-apoptotic effects of cIAP-1 and cIAP-2. Unexpectedly, only 3 cancer cell lines (CRL1420, CRL1682 and HTB147) weakly (1.2-2 fold) over-expressed Traf1 while a cancer cell line (HTB134) weakly (2 fold) over-expressed Traf3 (upper right panel of Fig.3.6). All other cell lines studied showed a down-regulation of Trafs.

In summary, at the mRNA level, there appears to be a mild tendency towards an up-regulation in the transcription of mRNAs encoding IAPs and a down-regulation of caspase

mRNAs supporting a deregulation of apoptotic regulators in pancreatic cancer cell lines. However, as discrepancies exist between Northern and RPA profiles (possibly due to the increased sensitivity of RPA), further repeats and/or experiments are needed to allow for statistical analyses and confirm these observations.

3.2.5. IAPs appear to be important anti-apoptotic proteins in pancreatic cancer cell lines

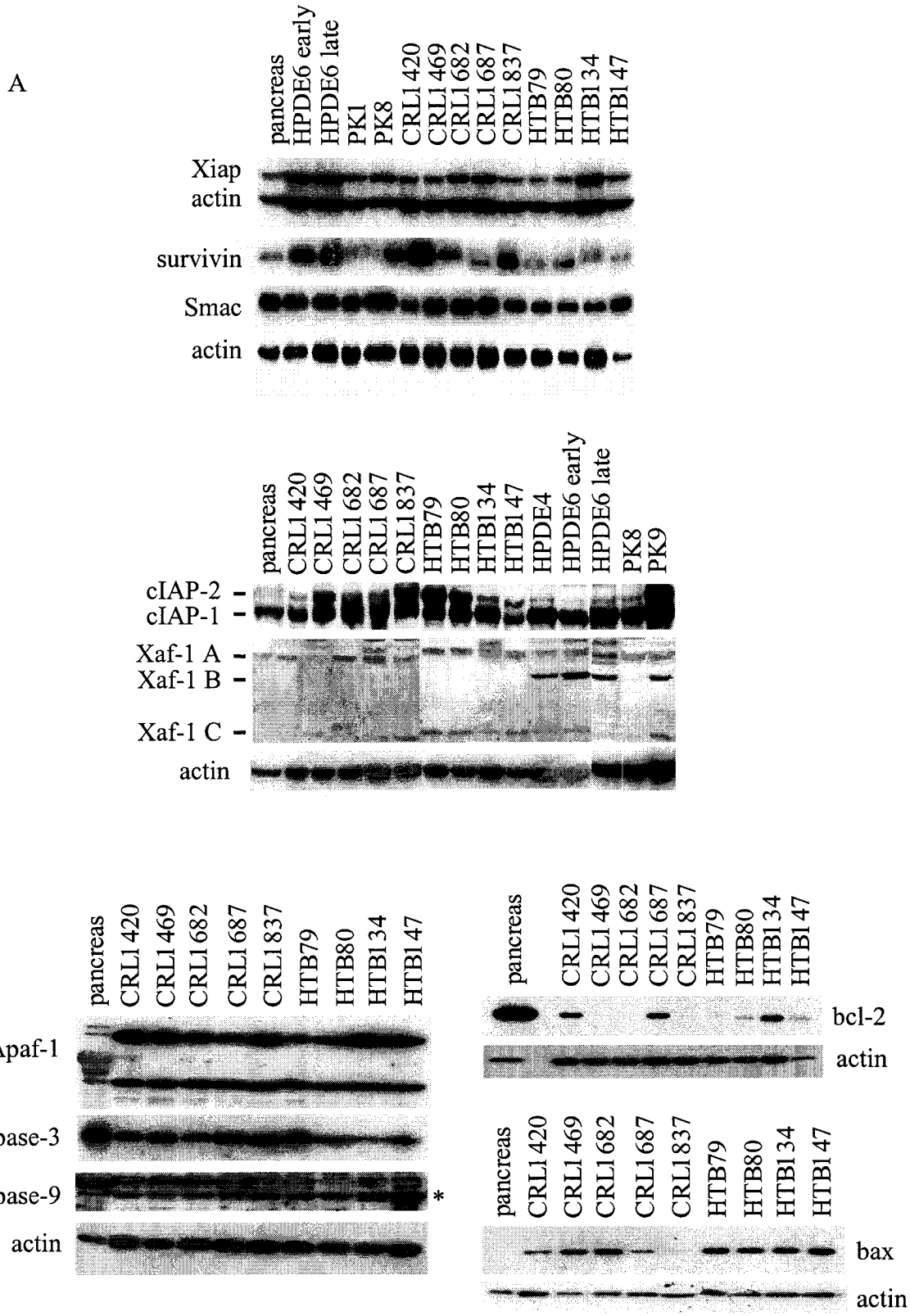
Having surveyed steady-state mRNA levels of IAPs and their antagonists in pancreatic adenocarcinoma and immortalized ductal cell lines, we sought to correlate them with protein levels. To this end, protein lysates from normal pancreatic tissue, immortalized ductal and pancreatic cancer cell lines were processed for immunoblotting with various antibodies. All proteins were detected at the expected size except for Smac/DIABLO which was detected at ≅ 20 kDa instead of the expected 25 kDa.

Based on mRNA levels, we expected the Xaf-1 protein to be expressed in all cell lines tested, albeit at varying levels. In HPDE cell extracts, three isoforms (arbitrarily termed A, B and C) of the Xaf-1 protein were detected (Fig.3.7A). Although unconfirmed, these isoforms are proposed to be derived from alternative splicing of the Xaf-1 mRNA (C. Lefebvre, personal communication); the full-length version of the Xaf-1 protein being Xaf-1B. In contrast, no Xaf-1B protein was detected in normal pancreatic tissue (pancreas) while both the C isoform (presumably a truncated version) and the A isoform of the Xaf-1 protein were detected in pancreatic tumor cell lines. If Xaf-1B truly corresponds to full-length Xaf-1 protein, it is quite interesting to speculate that translational regulation of Xaf-1 may be crucial to its function as Xaf-1 mRNA expression was detected in all cell lines tested. Next,

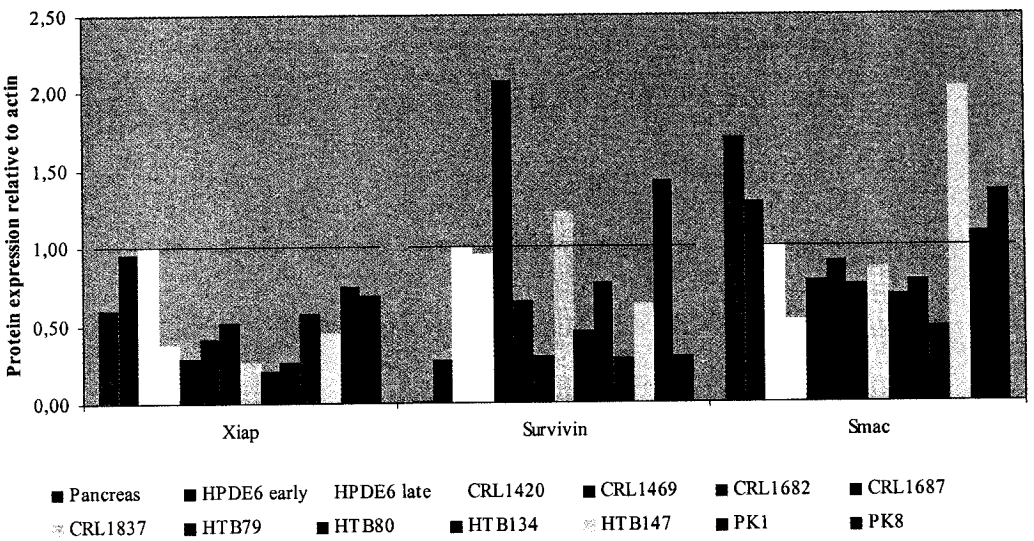
Fig.3.7. IAPs appear to be important anti-apoptotic proteins in pancreatic adenocarcinoma cell lines.

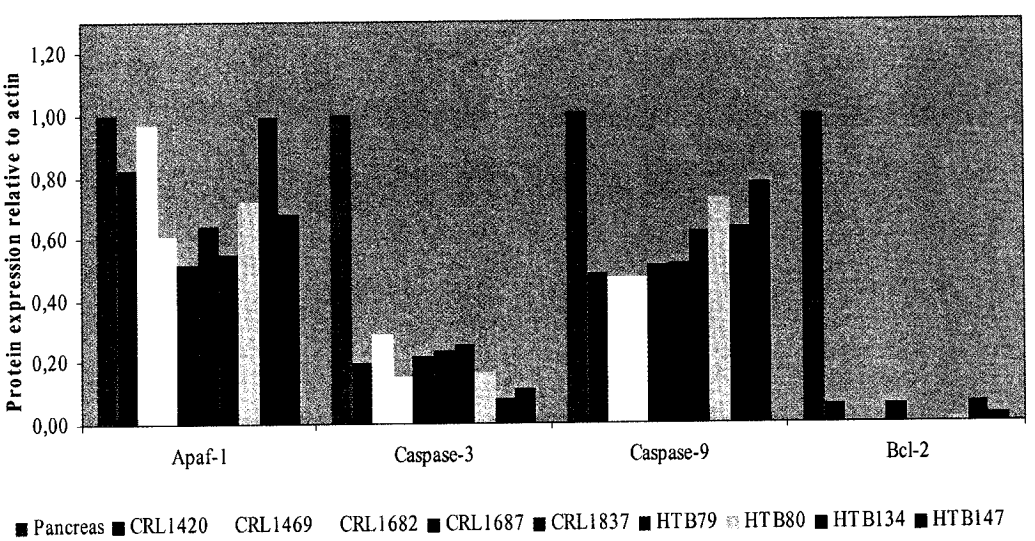
To determine steady-state levels of apoptotic proteins in pancreatic cancer cell lines, 10-30 μg of total protein were run on 8% (cIAP-1/2), 12% (Xaf-1, Xiap, bax, bcl-2, Apaf-1, caspases-3 and 9) and 15% (survivin, Smac/DIABLO and Omi/HtrA2) SDS-PAGE and transfered onto PVDF membranes. Blots were probed with the following antibodies: Xiap(a) (55 kDa), survivin (16 kDa), Smac/DIABLO (25 kDa), Riap1 (70 kDa for cIAP-2; 66 kDa for cIAP-1), Xaf-1 (A:36 kDa; B:33 kDa; C:16 kDa), Apaf-1 (130 kDa), caspase-3 (32 kDa), caspase-9 (46-48 kDa; band depicted by *), bcl-2 (25 kDa) and bax (19 kDa). For clarity reasons, molecular weights were not included on this figure. Nonetheless, all proteins were detected at the expected weight except for Smac/DIABLO and Apaf-1 (refer to results section for further details). Antigen-primary antibody complexes were detected with the appropriate HRP-conjugated secondary antibody and visualized by chemiluminescence. α-actin (42 kDa) was used to control for lane-to-lane loading. **A.** Autoradiograms. **B.** Where data permitted, band intensities were determined by densitometry (refer to text and discussion for further details). Relative intensities of target protein expression normalized to β-actin are expressed as fold induction over that of HPDE6 late (upper panel) or normal pancreas (bottom panel).

A


pancreas
HPDE6 early
HPDE6 late
PK1
PK8
CRL1420
CRL1469
CRL1682
CRL1687
CRL1837
HTB79
HTB80
HTB134
HTB147
Xiap
actin
survivin
Smac
actin
pancreas
CRL1420
CRL1469
CRL1682
CRL1687
CRL1837
HTB79
HTB80
HTB134
HTB147
HPDE4
HPDE6 early
HPDE6 late
PK8
PK9
cIAP-2
cIAP-1
Xaf-1 A
Xaf-1 B
Xaf-1 C
actin
pancreas
CRL1420
CRL1469
CRL1682
CRL1687
CRL1837
HTB79
HTB80
HTB134
HTB147
Apaf-1
caspase-3
caspase-9
*
actin
pancreas
CRL1420
CRL1469
CRL1682
CRL1687
CRL1837
HTB79
HTB80
HTB134
HTB147
bcl-2
actin
pancreas
CRL1420
CRL1469
CRL1682
CRL1687
CRL1837
HTB79
HTB80
HTB134
HTB147
bax
actin

B


2,50
2,00
1,50
1,00
0,50
0,00
Protein expression relative to actin
Xiap
Survivin
Smac
Pancreas
HPDE6 early
HPDE6 late
CRL1420
CRL1469
CRL1682
CRL1687
CRL1837
HTB79
HTB80
HTB134
HTB147
PK1
PK8


1,20
1,00
0,80
0,60
0,40
0,20
0,00
Protein expression relative to actin
Apaf-1
Caspase-3
Caspase-9
Bcl-2
Pancreas
CRL1420
CRL1469
CRL1682
CRL1687
CRL1837
HTB79
HTB80
HTB134
HTB147

in agreement with the Northern analysis, cIAP-1 protein was detected in all cell lines analyzed (Fig.3.7A). In contrast, very little cIAP-2 protein was detected in HPDE protein extracts while its expression varied in pancreatic cancer cell lines, in general concordance with the trend observed for mRNA expression (Fig.3.7A). Unfortunately, the immunoblot probed with Riap1 (antibody which recognizes both cIAP-1 and cIAP-2) was not subsequently re-probed with actin. The actin control depicted on the figure is that of a parallel membrane probed with Xaf-1. As a result, densitometric analysis of cIAP-1 and cIAP-2 protein expression could not accurately be performed. Likewise, the intensity of the Xaf-1 bands in contrast to the background precluded any reliable densitometric data for this blot.

In concordance with Northern profiles (Fig.3.4B) but in contradiction with RPA profiles (Fig.3.6), the expression of Xiap protein was down-regulated in all cancer cell lines tested. In contrast, expression of survivin protein followed a trend similar to the one observed by Northern hybridization, CRL1469 and PK1 being the only two pancreatic cancer cell lines to moderately (1.5-2.2 fold) over-express survivin protein. Lastly, in disagreement with mRNA profiles, protein expression of the Xiap antagonist Smac/DIABLO was down-regulated in 8 of the 11 pancreatic adenocarcinoma cell lines tested; CRL1420 and HTB134 showing the strongest repression. RNA profiles had suggested that CRL1420 and CRL1469 were the only two cancer cell lines to down-regulate survivin while there was a very modest over-expression of Smac/DIABLO in the other cell lines.

We also surveyed the expression of proteins involved in the mitochondrial apoptotic pathway, namely Apaf-1 and caspase-9 which constitute the apoptosome and caspase-3, a downstream effector of this pathway. Even though Apaf-1 is a 130 kDa polypeptide, the Apaf-1 antibody used revealed two bands (85 and 45 kDa) in all cell lines tested. Compared

to the normal pancreas, Apaf-1, caspase-3 and caspase-9 appeared to be under-expressed in pancreatic cancer cell lines. The protein profiles obtained for caspase-9 are mostly in agreement with RPA profiles. However, where RPAs had uncovered a very weak over-expression of caspase-3 in CRL1469, CRL1682, CRL1687 and CRL1837, protein profiles disagreed with these observations and revealed a down-regulation of caspase-3.

Lastly, to determine if IAPs were key apoptotic regulators in pancreatic cancer cell lines, we assessed the expression of the pro-apoptotic bax and anti-apoptotic bcl-2 proteins in these cell lines. While it was not expressed in normal pancreatic tissue, bax protein was strongly expressed in most cancer cell lines except for CRL1837 (Fig.3.7A). In contrast, we uncovered a significant repression of bcl-2 in pancreatic adenocarcinoma cell lines (Fig.3.7B). Taken together, the observed expression of bax and repression of bcl-2 suggest that the balance between these two apoptotic regulators cannot account for the intrinsic resistance of pancreatic tumors to apoptosis. However, these observations do support a deregulation of apoptotic proteins in pancreatic neoplastic cell lines. Indeed, the tendency of IAPs (eg. cIAP-1 and cIAP-2) to be over-expressed in pancreatic cancer cell lines suggests that they may compensate for varying levels of bcl-2 and that they are important anti-apoptotic regulators contributing to an increased apoptotic threshold.

3.2.6. The IAP/caspase cascade is frequently altered in human solid tumors

Thus far, we have observed a tendency to up-regulate some IAPs (namely cIAP-1 and cIAP-2) and down-regulate pro-apoptotic factors (Xaf-1, Smac/DIABLO and caspases) in pancreatic cancer cell lines. In an effort to investigate if these observations could be extended to other tumor types, we evaluated the expression levels of several apoptogenic

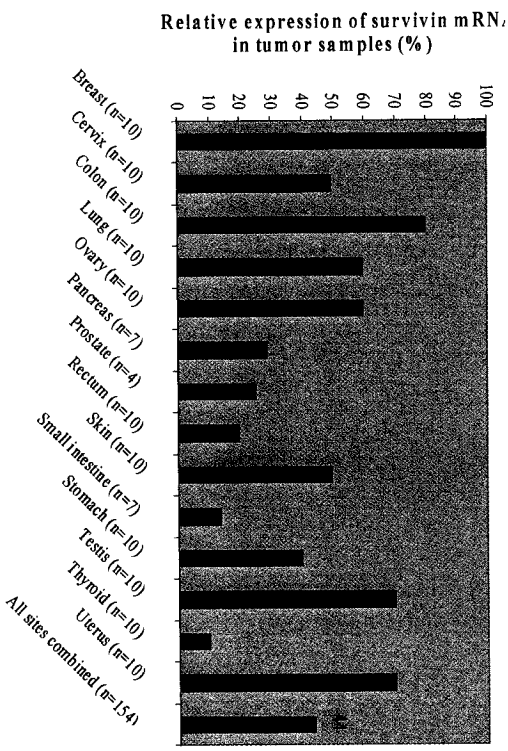
factors in matched normal/tumor RNA sets from a variety of human malignancies spotted on commercial "Cancer Profiling Arrays" (CPA, Clontech). Indeed, a first CPA containing 241 matched samples was hybridized with radiolabelled Xaf-1, Xiap, cIAP-1 and cIAP-2 cDNAs. Unfortunately, the presence of a low number of samples for the pancreas, cervix and small intestine (n=1,1 and 2, respectively) on the CPAI membrane prevented their inclusion in our analysis. In addition, a second tumor array (CPAII) containing 154 matched samples was probed with radiolabelled survivin, caspase-3, caspase-9 and Smac/DIABLO cDNAs. Raw data (autoradiograms and densitometric analyses) for both Cancer Profiling Arrays can be found in Appendix II.

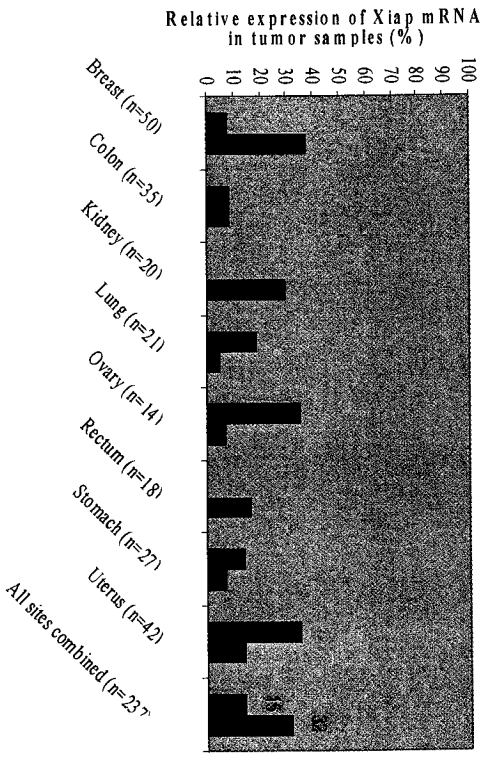
We expected mRNA levels of IAPs to be increased in tumor versus non-tumorigenic controls as high levels of IAPs have been reported in a number of human cancers (Satoh et al., 2001; Ambrosini et al., 1997; Sarela et al., 2001; Tanaka et al., 2000). In general agreement with our findings in pancreatic tumor cell lines, Xiap mRNA was down-regulated in 32% while it was up-regulated in only 15% of all tumors analysed (Fig.3.8A). Next, cIAP-1 mRNA was found to be up-regulated in only 5% of tumors but was down-regulated in 24% of tumors while cIAP-2 mRNA was up-regulated in a higher proportion (24%) of human solid tumors than it was down-regulated (18%). In contrast to our previous observations in pancreatic cancer cell lines, none of the tumors present on CPAII down-regulated survivin mRNA. In fact, survivin mRNA was up-regulated in 44% of the human solid tumors surveyed. Interestingly, we observed a tendency for IAP over-expression in a significant proportion of tumors originating in female reproductive organs such as ovary and uterus. In particular, survivin mRNA was up-regulated in 50-100% of breast, cervical, ovarian and uterine tumors although the number of samples tested was quite limited (n=10).

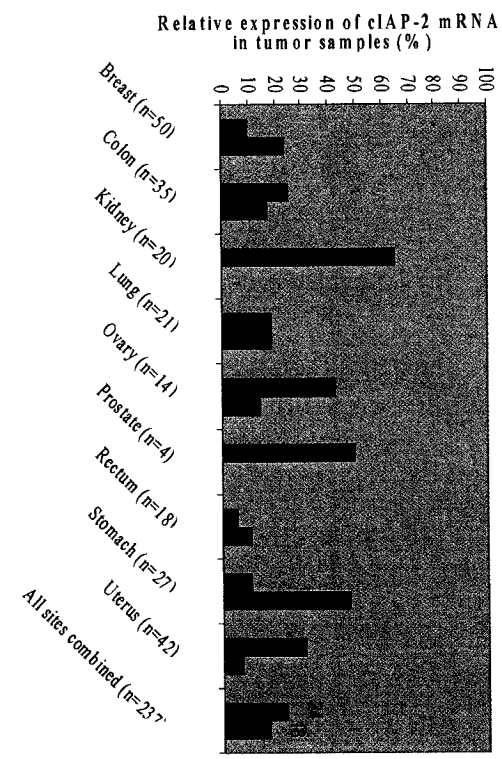
Fig.3.8. The IAP/caspase cascade is frequently altered in human solid tumors.

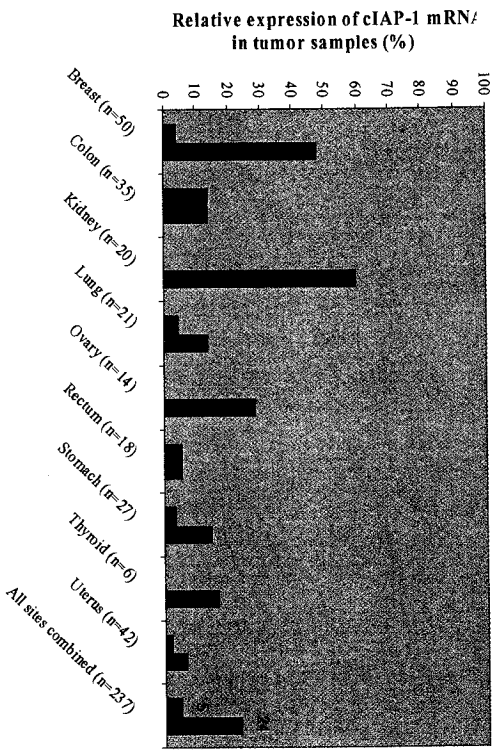
Cancer Profiling Arrays (Clontech) containing SMART™-amplified cDNA from tumors and corresponding normal tissues were hybridized with a series of probes. A first array (CPAI) containing 241 matched samples was probed with cDNA specific to Xaf-1 and Xiap and partial 3' cIAP-1 and cIAP-2 sequences in succession while a second array (CPAII) containing 154 matched samples was probed with survivin, caspase-3, caspase-9 and Smac/DIABLO. Ubiquitin served as an internal loading control. Hybridization intensities were determined by densitometry. Tumor samples were deemed to over-express target RNA when the intensity of the signal was twice that of the normal control (red bars). In contrast, tumors were deemed to down-regulate target RNA expression when the intensity of the signal was half that of the normal counterpart (blue bars). Relative expression of Xiap, cIAP-1, cIAP-2, Xaf-1, Smac/DIABLO, survivin, caspase-3 and caspase-9 mRNAs are expressed as percentages. When none of the tumors analysed up- or down-regulated target mRNA expression, this tissue was omitted from graph. Refer to text for further details. **A.** Anti-apoptotic factors. **B.** Pro-apoptotic factors.
Note: all sites combined refers to the total number of matched normal/non-tumorigenic RNA sets analysed on each CPA.
(Figure continues on next page)

A


Relative expression of survivin mRN
in tumor samples (%)
0
10
20
30
40
50
60
70
80
90
100
Breast (n=10)
Cervix (n=10)
Colon (n=10)
Lung (n=10)
Ovary (n=10)
Pancreas (n=7)
Prostate (n=4)
Rectum (n=10)
Skin (n=10)
Small intestine (n=7)
Stomach (n=10)
Testis (n=10)
Thyroid (n=10)
Uterus (n=10)
All sites combined (n=154)


Relative expression of Xiap mRNA
in tumor samples (%)
0
10
20
30
40
50
60
70
80
90
100
Breast (n=50)
Colon (n=35)
Kidney (n=20)
Lung (n=21)
Ovary (n=14)
Rectum (n=18)
Stomach (n=27)
Uterus (n=42)
All sites combined (n=237)


Relative expression of cIAP-2 mRNA
in tumor samples (%)
0
10
20
30
40
50
60
70
80
90
100
Breast (n=50)
Colon (n=35)
Kidney (n=20)
Lung (n=21)
Ovary (n=14)
Prostate (n=4)
Rectum (n=18)
Stomach (n=27)
Uterus (n=42)
All sites combined (n=237)


Relative expression of cIAP-1 mRN
in tumor samples (%)
0
10
20
30
40
50
60
70
80
90
100
Breast (n=50)
Colon (n=35)
Kidney (n=20)
Lung (n=21)
Ovary (n=14)
Rectum (n=18)
Stomach (n=27)
Thyroid (n=6)
Uterus (n=42)
All sites combined (n=237)

B

Relative expression of Smac/DIABLO mRNA
in tumor samples (%)
Breast (n=10)
Cervix (n=10)
Colon (n=10)
Kidney (n=10)
Lung (n=10)
Ovary (n=10)
Pancreas (n=7)
Rectum (n=10)
Skin (n=10)
Stomach (n=10)
Testis (n=10)
Thyroid (n=10)
Uterus (n=10)
All sites combined (n=152)
Relative expression of caspase-9 mRNA
in tumor samples (%)
Bladder (n=5)
Cervix (n=10)
Colon (n=10)
Kidney (n=10)
Lung (n=10)
Ovary (n=10)
Pancreas (n=7)
Skin (n=10)
Stomach (n=10)
Testis (n=10)
Uterus (n=10)
Vulva (n=5)
All sites combined (n=152)
Relative expression of Xaf-1 mRNA
in tumor samples (%)
Breast (n=50)
Colon (n=35)
Kidney (n=20)
Lung (n=21)
Ovary (n=14)
Prostate (n=4)
Rectum (n=18)
Stomach (n=27)
Thyroid (n=6)
Uterus (n=42)
All sites combined (n=237)
Relative expression of caspase-3 mRNA
in tumor samples (%)
Bladder (n=5)
Breast (n=10)
Cervix (n=10)
Colon (n=10)
Kidney (n=10)
Lung (n=10)
Ovary (n=10)
Pancreas (n=7)
Prostate (n=4)
Rectum (n=10)
Skin (n=10)
Small intestine (n=7)
Stomach (n=10)
Testis (n=10)
Thyroid (n=10)
Uterus (n=10)
Vulva (n=5)
All sites combined (n=152)

As Xaf-1 mRNA was previously reported to be down-regulated in a significant proportion of cancer cell lines (Fong et al., 2000), we expected its expression to be significantly repressed in human solid tumors compared to matched non-tumorigenic counterparts. However, our analysis revealed that, even though 22% of tumors under-expressed Xaf-1 mRNA, a similar proportion of tumors (17%) over-expressed this mRNA (Fig.3.8B). These observations make it quite difficult to conclude anything regarding the relative expression of Xaf-1 mRNA in human solid tumors. As Smac/DIABLO is another Xiap antagonist (Du et al., 2000; Verhagen et al., 2000), we hypothesized that its under-expression would confer a survival advantage to tumors. Contrary to our expectations, 29% of tumors analyzed over-expressed Smac/DIABLO mRNA while only 4% of tumor samples under-expressed it in agreement with our observations in pancreatic cancer cell lines.

Recent reports have suggested that tumors possess intact apoptotic machinery as evidenced by the presence of caspase-3 activity (L. Yang et al., 2003). Consistent with these findings, we found that caspase-3 mRNA was expressed in all tumors surveyed with 16% of tumors surveyed under-expressing and 21% of tumors over-expressing caspase-3 mRNA (Fig.3.8B). Surveying the same tumor samples for caspase-9 expression uncovered up-regulation of caspase-9 mRNA in 14% of tumor samples while 8% of tumors down-regulated caspase-9 mRNA, again leaving a significant proportion of tumor samples with unaltered caspase-9 expression. Taken together, these results suggest that variations in caspase-3 and caspase-9 expression are infrequent in human malignancies.

In summary, this analysis revealed that mRNAs encoding survivin and cIAP-2 are more frequently up-regulated than Xiap and cIAP-1 mRNAs in human solid tumors. However, we must stress that some tumor types tend to up-regulate select IAPs. In addition, as the mRNA expression of pro-apoptotic factors such as Xaf-1, caspase-3, caspase-9 and

Smac/DIABLO did not appear to be decreased in a significant proportion of tumors, we propose that tumor cells are capable of interpreting apoptotic signals but exhibit a reduced propensity to apoptosis due to an up-regulation in the expression of IAPs.

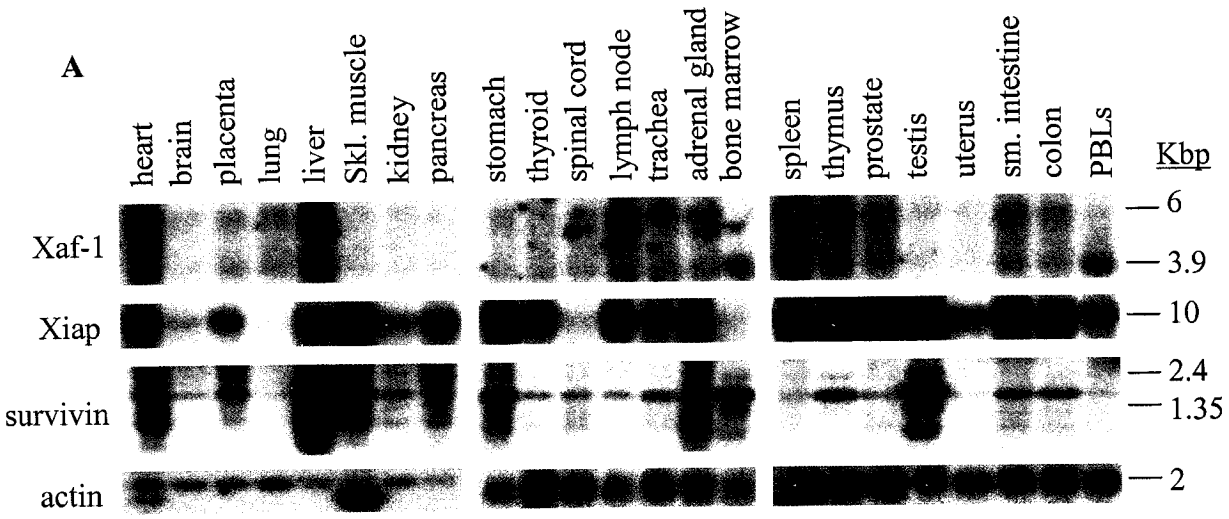
3.2.7. Steady-state levels of apoptotic proteins in non-diseased human tissues

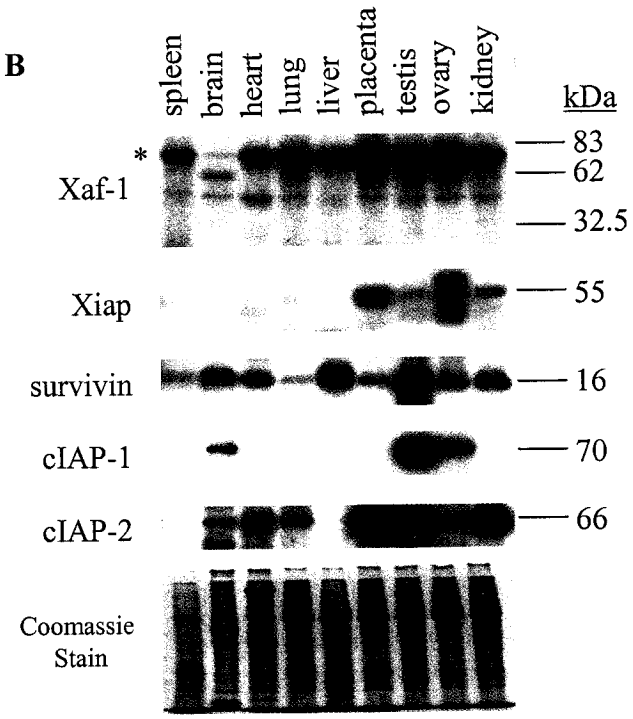
Rational therapeutic designs need to target cancer cells directly without affecting normal or non-neoplastic cells. To determine if targeting the apoptotic pathway by modulating IAP expression levels was feasible for the treatment of pancreatic tumors, we examined the tissue distribution of mRNAs and proteins encoding key IAPs in non-diseased human tissues. To this end, Multi Tissue Northern Blots were probed with target-specific cDNAs while protein medleys from non-diseased human tissues were processed for immunoblotting with antibodies directed against the protein of interest.

Previous reports suggested that Xaf-1 mRNA was ubiquitously expressed in normal adult tissues (Liston et al., 2001). Confirming these studies, we observed ubiquitous expression of two Xaf-1 transcripts (3.9 and 6 Kbp) in the majority of tissues tested (Fig3.9A). However, Xaf-1 mRNA levels did not correlate with Xaf-1 protein levels as we were unable to detect a protein of the appropriate size (33 kDa) in all tissues tested (Fig.3.9B). This discrepancy may be attributable to the poor quality of the Xaf-1 antibody or to translational regulation. We did observe a strong non-specific band at $\approx$ 66 kDa which we are unable to explain. Next, consistent with previous published reports (Liston et al., 1997; Lagacé et al., 2001), ubiquitous expression of a single Xiap transcript (10 Kbp) was observed in all tissues tested. However, the expression of Xiap protein was more restricted. In fact, we observed a lack of concordance between mRNA and protein profiles for Xiap as strong

Fig.3.9. IAPs are ubiquitously expressed in the majority of non-diseased human tissues.

A. Membranes containing 2 μg of polyA+ RNAs from normal human tissues were obtained from Clontech and hybridized with radiolabelled Xaf-1 or Xiap cDNA probes. Duplicate membranes were probed with survivin, cIAP-1 and cIAP-2. Blots were washed to high stringency and exposed to X-ray film. Transcript sizes (indicated at the right of the figure) were determined based on the migration of RNA size markers included on the membranes by the supplier. To control for lane-to-lane loading, blots were hybridized with β-actin cDNA. For actin, a single transcript (2 Kbp) is expected in all tissues tested except in heart and skeletal muscle where a second actin isoform (1.6-1.8 Kbp) should be detected. **B.** Thirty μg of total protein lysates (protein medleys obtained from Clontech) were resolved on 12% or 15% (survivin only) SDS-PAGE and electro-blotted onto PVDF membranes under standard conditions. Blots were probed with Xaf-1 (33 kDa), cIAP-1 (70 kDa), cIAP-2 (66 kDa), survivin (16 kDa) and Xiap (55 kDa; R&D Systems) antibodies. Antigen-primary antibody complexes were detected with an HRP-conjugated donkey-anti-rabbit secondary antibody and visualized by chemiluminescence. Coomassie stained gel is included to control for loading as actin levels were found to be variable among tissues. Tissues known to be prime metastatic sites for pancreatic cancer are highlighted in red.
* Unassignable Xaf-1 band (wrong molecular weight)


A
heart
brain
placenta
lung
liver
Skl. muscle
kidney
pancreas
stomach
thyroid
spinal cord
lymph node
trachea
adrenal gland
bone marrow
spleen
thymus
prostate
testis
uterus
sm. intestine
colon
PBLs
Kbp
Xaf-1
Xiap
survivin
actin
6
3.9
10
2.4
1.35
2


B
spleen
brain
heart
lung
liver
placenta
testis
ovary
kidney
kDa
*
Xaf-1
Xiap
survivin
cIAP-1
cIAP-2
Coomassie Stain
83
62
32.5
55
16
70
66

mRNA expressors (eg. spleen, heart, liver) did not express the protein. Whether this might possibly be due to IRES-mediated translational regulation of Xiap remains to be determined. The lack of expression of the Xiap protein is perplexing as it has been well documented that Xiap is ubiquitously expressed in non-diseased adult tissues (Liston et al., 1997; Lagacé et al., 2001). Our attempts at detecting cIAP-1 and cIAP-2 mRNA expression in non-diseased human tissues were unsuccessful to date but are currently being re-tested. However, immunoblotting experiments uncovered cIAP-1 expression in brain, ovary and testis while expression of cIAP-2 was also detected in heart, lung, placenta and kidney in addition to the aforementioned tissues. Lastly, contrary to previous reports by Ambrosini et al. (1997), we observed expression of survivin mRNA (major transcript of 1.9 Kbp) in all adult tissues tested which generally correlated with the expression of the 16 kDa survivin protein.

In conclusion, a significant proportion of non-tumorigenic adult tissues expressed Xaf-1, Xiap, cIAP-1/-2 and survivin mRNAs and proteins. Endogenous expression levels of IAPs need to be considered for the rational design of cancer therapies as altering them will undoubtedly affect the delicate IAP/caspase intracellular balance. For instance, if we modulate (whether it be up- or down-regulation) the expression of IAPs in order to increase the efficacy of current cytotoxic therapies, it is not unreasonable to suggest that normal cellular activities in other vital organs, in particular the liver, will be perturbed by these treatments.

3.2.8. The genomic regions encoding cIAP-2 and Xiap are not mutated in pancreatic adenocarcinoma cell lines

Having observed strong expression of Xiap and cIAP-2 in pancreatic cancer cell lines (by RPA), we wished to verify whether or not the genomic regions encoding these loci were mutated. In particular, we wanted to ascertain that the amino acid residues mediating IAP-caspase interactions or other structural/functional properties of IAPs were not subject to mutations which would hinder said interaction. To this end, sequencing of cIAP-2 and Xiap cDNAs using primers spanning the entire coding region did not uncover any mutations in the pancreatic cancer cell lines CRL1420 and CRL1469.

3.2.9. Expression and nuclear redistribution of Inhibitor of Apoptosis Proteins in developing pancreatic adenocarcinomas

To expand our analysis to *in vivo* samples, tissue arrays of pancreatic cancer and evolving intraductal neoplastic lesions (PanIN) were processed for immunohistochemistry using Xiap, cIAP-1, cIAP-2 and survivin antibodies. Staining intensities were evaluated as detailed in section 2.8 (Materials and Methods). Briefly, the higher the positive score, the greater the proportion of positive staining. Interpretable results are summarized in Table 3.2 while typical results obtained for each antibody tested are included in Appendix II.

This experiment uncovered a general tendency towards redistribution of cIAP-1, cIAP-2, Xiap and survivin from cytoplasm to nucleus in high grade pancreatic tumors. Briefly, weak cIAP-1 staining was observed in normal pancreatic tissue although nuclear staining was occasionally present in ductal cells. The number of nuclei showing distinct, positive cIAP-1 staining augmented in PanIN lesions, a tendency which was maintained in

Table 3.2. Expression and nuclear redistribution of IAPs in developing pancreatic adenocarcinomas.

Immunohistochemistry was carried out on tissue micro-arrays using 1:200 dilutions of cIAP-1, cIAP-2, Xiap and survivin antibodies. After blocking endogenous peroxidase activity and washing in PBS, secondary antibody incubation (using the multi-species link reagent) was followed by incubation with streptavidin-horseradish peroxidase. Immuno-reactivities were revealed by incubation in Nova Red substrate for 5 min. Slides were counterstained in Mayer's hematoxylin and mounted in Permount. Staining intensities were evaluated independently by two pathologists and scored in three grades: 0 for complete absence of staining, 1 for weak staining, and 2 for strong staining. The extent of positively stained nuclei was scored into four grades: 0 for <10% nuclei staining, 1 for 10% to <25%, 2 for 25% to <50%, and 3 for tumors with 50% or greater tumor cell nuclei staining positive. The final score for each tumor sample represents the sum of staining intensity and extent. Background staining was evaluated with pre-immune serum. Colon cancer samples served as internal positive controls.

Legend: PanIN – pancreatic intraductal neoplasia; ADC – adenocarcinoma of the pancreas; cyto – cytoplasmic; nuc – nuclear; SN - supranuclear

	cIAP-1 (cyto)	cIAP-1 (nuc)	cIAP-2 (cyto)	cIAP-2 (nuc)	Xiap (cyto)	Xiap (SN)	Xiap (nuc)	Survivin (cyto)	Survivin (nuc)
+ve scores	**0**	**1,2**	**2,3**	**1,2**	**2,3**	**+**	**1,2**	**1,2**	**1,2**
Normal	2/28	1/28	23/29	1/29	14/28	12/28	15/28	29/29	0/29
PanIN 1A	10/21	11/21	0/22	0/22	1/22	9/22	12/22	19/21	2/21
PanIN-1B	21/28	7/28	0/30	0/30	4/33	22/33	17/33	29/29	0/29
PanIN-2	5/15	10/15	9/19	0/19	7/22	14/22	11/22	14/20	6/20
PanIN-3	4/13	9/13	7/16	2/16	2/18	9/18	6/18	6/16	10/16
AD*	21/38	11/38	16/38	16/38	7/38	7/38	7/38	9/38	29/38

adenocarcinomas. Likewise, in normal pancreatic sections, cIAP-2 staining was predominantly cytoplasmic. However, staining intensity was stronger in apical membranes indicative of an association with cellular membranes. Variable cIAP-2 staining was observed in PanIN lesions, possibly due to interference with mucin or other components. In contrast, nuclear redistribution of cIAP-2 occurred in ≈50% of adenocarcinomas. Similarly, in normal pancreatic tissue, Xiap staining was revealed to be cytoplasmic, albeit with a frequent supranuclear distribution while nuclear staining was observed in a few ductal and centro-acinar cells and more frequently in islets of Langerhans. No notable changes in the subcellular distribution of Xiap were observed in PanIN lesions. However, the proportion of positively stained nuclei as well as in the intensity of cytoplasmic staining increased in adenocarcinomas. Finally, cytoplasmic staining, particularly intense in ductal cells, was observed for survivin in normal pancreatic sections. By contrast, the number of positive nuclei increased in PanIN-2/-3 lesions while strong nuclear staining was observed in adenocarcinomas.

3.3. Summary of results

The findings described in this chapter suggest that differential expression of apoptotic modulator/effector genes between normal and cancer cells may very well contribute to the intrinsic resistance to cytotoxic therapies. Indeed, we have observed a tendency towards up-regulation of IAP expression (namely survivin and cIAP-2) indicative of an increased apoptotic threshold in pancreatic cancer cell lines and several human malignancies. Nonetheless, the observation that both Xiap mRNA and protein were down-regulated in pancreatic cancer cell lines remains perplexing. On the other hand, the results presented here

suggest that the expression of pro-apoptotic factors (Xaf-1 and caspases) tends to be biased towards down-regulation. However, expression of the IAP antagonist Smac/DIABLO was biased towards up-regulation in pancreatic and other solid tumors. In addition, we show that the Xaf-1 locus lies within a region of loss of heterozygosity and appears to be affected by intragenic methylation in pancreatic adenocarcinoma cell lines, both observations being suggestive of gene silencing. Interestingly, while the mRNA encoding Xaf-1 was expressed at varying levels, protein expression appeared to be completely repressed in all pancreatic adenocarcinoma cell lines tested. Lastly, *in vivo* studies uncovered a tendency towards nuclear redistribution of IAPs, namely survivin, in high grade pancreatic adenocarcinomas. Taken together, the above results warrant investigating modulation of IAP expression as a means of targeting the apoptotic pathway and enhancing the efficacy of oncotherapies, with particular emphasis into the effect of Xaf-1 expression on Xiap levels.

Chapter 4: Modulation of IAP expression in drug-induced apoptosis

4.1. Introduction

The findings compiled in the previous chapter revealed a deregulation of apoptotic effector genes in pancreatic cancer cell lines. Indeed, we observed a tendency towards up-regulation of IAPs (namely survivin and cIAP-2) and down-regulation of pro-apoptotic proteins such as caspase-3, caspase-9 and Xaf-1. These observations lend credence to the hypothesis that an increase in the apoptotic threshold contributes to the intrinsic resistance of pancreatic tumors to cytotoxic therapies.

Since it was first identified, apoptosis has been proposed to serve as a barrier to cancer (Kerr et al., 1972). Moreover, evidence of an acquired resistance to apoptosis as a hallmark of cancer cells has accumulated over the years (reviewed in Hanahan and Weinberg, 2000). This resistance to apoptosis is thought to play an important role during tumorigenesis. Indeed, apoptosis has been reported to limit the expansion of the growing tumor population, most likely at an early stage in the progression of malignant transformation (Naik et al., 1996; O'Reilly et al., 1996). In addition, the accumulation of genetic lesions necessary for the development of malignant tumors supports a need for suppression of apoptosis (Green and Evan, 2002). Lastly, evidence implicating resistance to apoptosis as a cause of cancer therapy failure is mounting (Minn et al., 1995; X. Sun et al., 1999; Haq and Zanke, 1998; Kaufmann and Earnshaw, 2000; Dive, 1997; Makin and Hickman, 2000). As a result, selective pressures favor the survival of cancer cells which have evolved mechanisms to bypass apoptogenic signals.

Cytotoxic drugs such as etoposide have been shown to elicit both p53-dependent and independent apoptotic responses (Lowe et al., 1993a, 1993b; Perego et al., 1996; Bertrand et

al., 1993). Etoposide is a phyllotoxin analog capable of introducing single-strand DNA breaks in cells by interfering with the action of topoisomerase II-α (Kingma and Osheroff, 1998). The extent of the DNA damage forces cells exposed to etoposide to undergo apoptosis. Interestingly, over-expression of IAPs was shown to suppress etoposide-induced cell death (Uren et al., 1996, Deveraux et al., 1997, 1998). Moreover, several reports suggest that down-regulating the expression of IAPs sensitizes cancer cells to apoptotic triggers (Chow et al., 2003; Notarbartolo et al., 2002, Ng et al., 2002, Ng and Bonavida, 2002; Li et al., 2001).

In light of this, we wished to investigate the response of pancreatic cancer cells to etoposide, namely with regards to IAP expression. We hypothesized that differential IAP expression would influence etoposide sensitivity and that down-modulating IAP expression and/or compromising IAP function via the re-introduction of Xaf-1 could sensitize pancreatic cancer cells to etoposide. Our experiments uncovered a good correlation between the *in vitro* sensitivities of three pancreatic cancer cell lines to etoposide, the transcriptional up-regulation of IAPs, the caspase-dependent cleavage of Xiap protein, the activation of caspase-3 and the rapidity of onset of apoptosis. However, attempts at sensitizing pancreatic cancer cells to etoposide have been unsuccessful to date.

4.2. Results

4.2.1. Human pancreatic cancer cell lines differ in their sensitivity to etoposide

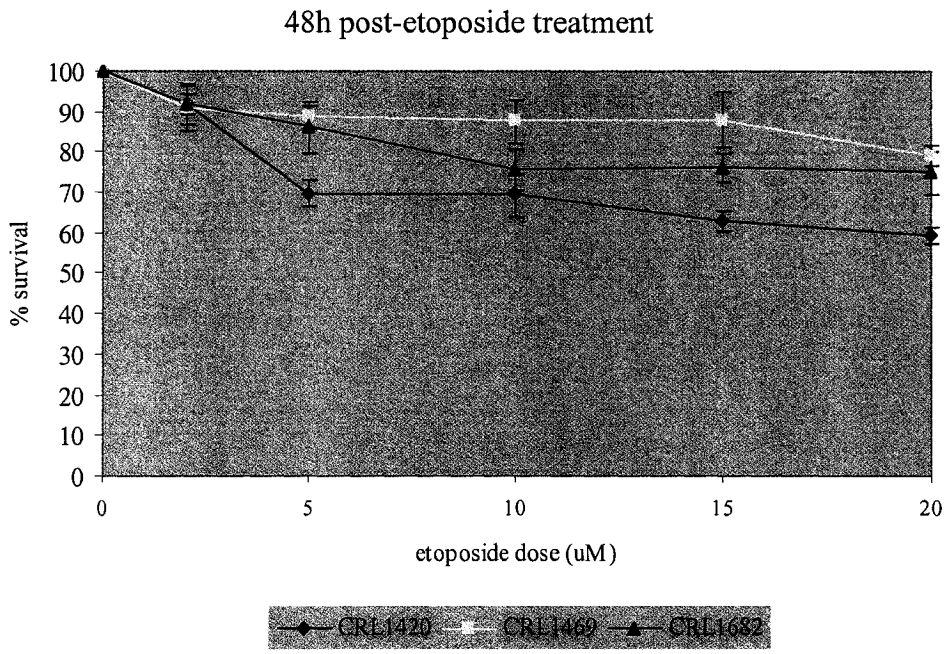
To determine the sensitivity of human pancreatic cancer cell lines to etoposide, log phase cultures of CRL1420, CRL1469 and CRL1682 cells were treated with doses of

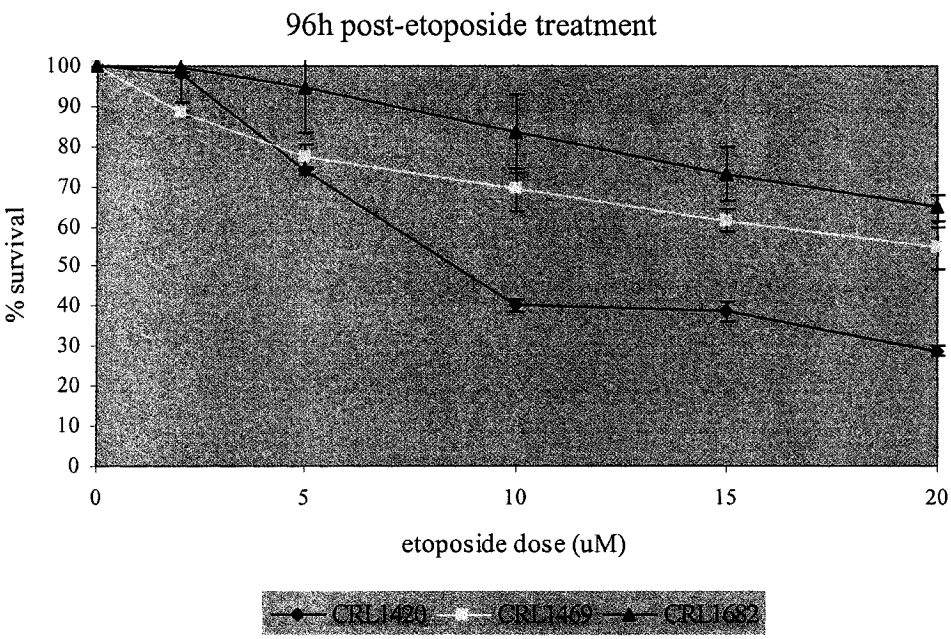
etoposide ranging from 0 (control) to 20 μM for a short period of time (6h). Following incubation in etoposide-containing medium, cells were allowed to recover in fresh, etoposide-free growth medium overnight. In addition, cells were exposed to DMSO, the vehicle used for solubilization of etoposide, to control for its effect on cell survival. Cell survival in all cell lines tested was assessed 48h and 96h post-etoposide treatment; the survival of individual cell lines in the absence of etoposide (untreated control) being set as baseline. The effect of DMSO on cell survival was minimal (< 10% cell death).

As can be observed in Fig.4.1, CRL1420, CRL1469 and CRL1682 cells differ in their sensitivity to etoposide (Fig.4.1). Even though all three cell lines exhibited decreased survival in response to increasing doses of etoposide, the extent of the response differed among cell lines. In fact, exposure of CRL1420 cells to etoposide revealed that the latter were more sensitive to this agent as evidenced by a steeper slope in the survival curve than that observed for CRL1469 and CRL1682. In addition, survival of CRL1420 cells was affected by lower concentrations of etoposide; for instance, exposure to a low dose of etoposide (5 μM) resulted in 69.7, 88.6 and 86.2% survival for CRL1420, CRL1469 and CRL1682 cells, respectively. This observation was also true at higher etoposide doses as the survival of CRL1420, CRL1469 and CRL1682 cells following challenge with 20 μM of etoposide was 59.4, 79.2 and 75%, respectively. The effect of etoposide on cell survival was even more pronounced 96h post-etoposide challenge. In fact, at this timepoint, cell survival upon challenge with 20 μM of etoposide was 28.8, 54.6 and 64.7% for CRL1420, CRL1469 and CRL1682, respectively. Comparing the extent of the response to etoposide challenge clearly established CRL1420 as the most sensitive cell line (as assessed by student t tests),

Fig.4.1. Human pancreatic cancer cell line CRL1420 displays sensitivity to etoposide.

Log phase adherent cultures of human pancreatic cancer cells were treated with a dose range (0-20 μM) of the topoisomerase II-α inhibitor, etoposide, for 6h. Cell viability was assessed 48h and 96h post-etoposide challenge by incubating the cells in 1X alamarBlue™ for 1h at 37°C/5% CO_2 and establishing absorbance readings on a Cytofluor 2300 automated fluorescence system. Data files were subsequently exported to the SoftMax software for analysis. The effect of the vehicle (DMSO) on cell death was minimal (<10%). Cell survival is expressed relative to that of the non-treated control. Standard errors of the mean (represented by error bars) were calculated from triplicates of one experiment.


48h post-etoposide treatment
% survival
100
90
80
70
60
50
40
30
20
10
0
0
5
10
15
20
etoposide dose (uM)
CRL1420
CRL1469
CRL1682


96h post-etoposide treatment
% survival
100
90
80
70
60
50
40
30
20
10
0
0
5
10
15
20
etoposide dose (uM)
CRL1420
CRL1469
CRL1682

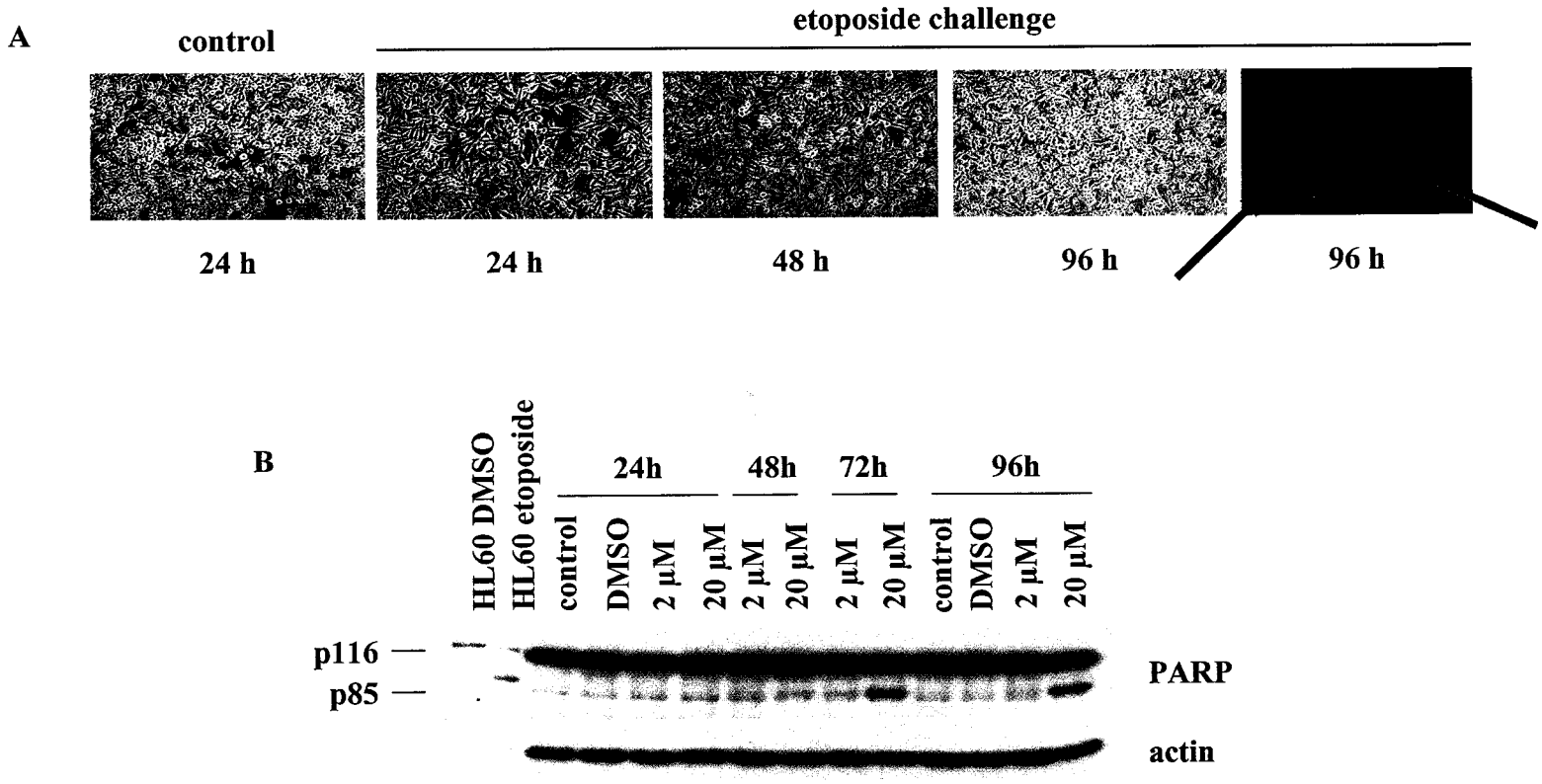
followed by CRL1682 and CRL1469. The difference between CRL1469 and CRL1682 was not statistically significant as t values comparing these two sets of data were below the critical t value (2.77; refer to section 2.10 of Materials and Methods for further details).

The shape of the survival curves further suggested that cancer cell populations are heterogeneous in their response to etoposide. This statement is particularly evident for CRL1420. Indeed, while there was a rapid induction of cell death in response to lower doses of etoposide, this death appeared to plateau after 5 or 10 μM of etoposide at 48h and 96h, respectively. In fact, exposing CRL1420 cells to 20 μM versus 5 μM of etoposide only enhanced cell death by 10-12% at both timepoints. These observations suggest the presence of at least two different etoposide-responsive cell populations; one that is highly responsive to etoposide and the second whose resistance to etoposide is greater than the former.

We assessed nuclear morphology and PARP cleavage (hallmarks of programmed cell death) in etoposide-treated CRL1420 cells to verify if the demise of this cell line was indeed apoptotic in nature. Under phase contrast microscopy, we observed that, over time, challenging CRL1420 cells with etoposide resulted in an increased proportion of dead cells coupled with a decreased proportion of round, detached cells (Fig.4.2A). In addition, staining of etoposide-treated CRL1420 cells with DAPI clearly established the presence of apoptotic bodies post-etoposide challenge (Fig.4.2A; indicated by arrows). Finally, the appearance of a p85 PARP cleavage product 72h post-treatment with 20 μM of etoposide was observed in CRL1420 cells (Fig.4.2B). As PARP cleavage is a caspase-3-dependent event, this observation further supports apoptosis as a cause of etoposide-induced cell death.

Fig.4.2. Etoposide induces apoptosis in CRL1420 cells.

Treatment with the topoisomerase II-α inhibitor, etoposide leads to a massive accumulation of single-strand DNA breaks resulting in the gradual demise of cancer cells. Log phase cultures of cells were treated with 2 or 20 μM of etoposide for 6h and allowed to recover in fresh etoposide-free culture medium. **A.** Etoposide-challenged cells were visualized under phase contrast microscopy; images were captured on Kodak TMAX 400 ASA black and white professional film. Etoposide-treated cells were also stained with DAPI and visualized by fluorescence microscopy (extreme right). Arrows point to apoptotic bodies. **B.** CRL1420 cells treated with etoposide were lysed in protein extraction buffer containing 6M urea and processed for immunoblotting with a polyclonal anti-PARP antibody capable of recognizing the p85 cleavage product of PARP. HL60 cell extracts treated or not with etoposide (lanes 1 and 2, respectively) were supplied with the anti-PARP antibody and are included as controls.


A
control
etoposide challenge
24 h
24 h
48 h
96 h
96 h
B
HL60 DMSO
HL60 etoposide
24h
48h
72h
96h
control
DMSO
2 µM
20 µM
2 µM
20 µM
2 µM
20 µM
control
DMSO
2 µM
20 µM
p116
p85
PARP
actin

4.2.2. Xiap, cIAP-1, cIAP-2 and survivin mRNA levels increase in response to etoposide

Having observed that pancreatic cancer cell lines responded distinctly to etoposide, we hypothesized that this effect may be due, in part, to a drug-induced transcriptional up-regulation of mRNAs encoding IAPs in the more resistant cell lines (CRL1469 and CRL1682). To verify this hypothesis, all three cell lines were challenged with 20 μM of etoposide for 6h and then allowed to recover in fresh complete medium. RNA lysates were extracted from cells 24, 48, 72 and 96h post-challenge and processed for Northern hybridization with Xiap, cIAP-1, cIAP-2 and survivin-specific cDNA probes. Fold inductions in IAP mRNA expression were then calculated as detailed in section 2.10 (Materials and Methods).

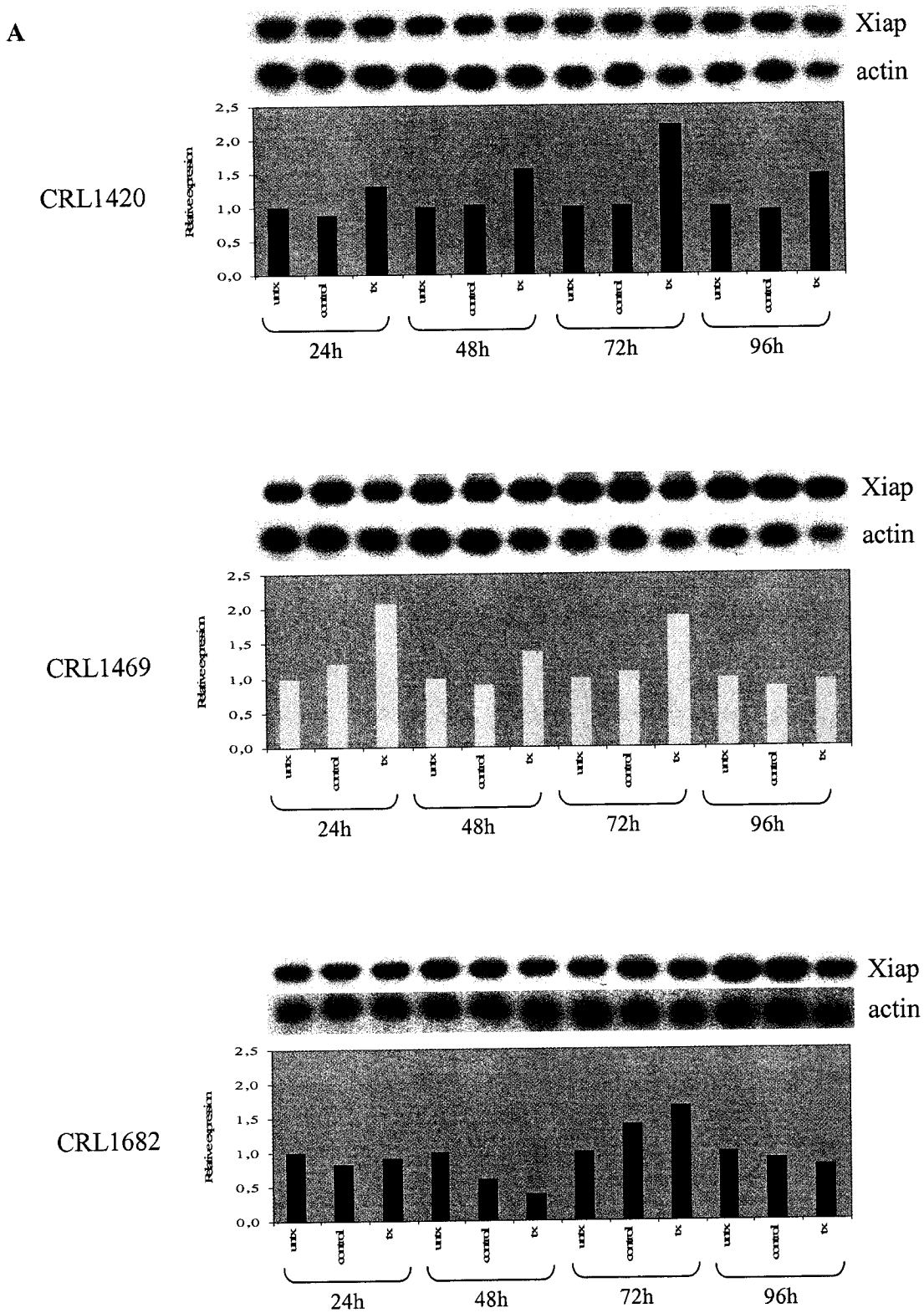
Contrary to our predictions, we observed a transcriptional induction of all IAPs tested in all three cell lines (Fig.4.3). However, the extent of the induction differed depending on the mRNA as well as among cell lines. Strongest induction of Xiap mRNA was observed 72h post-etoposide challenge in nearly all cell lines tested (Fig.4.3A). Nonetheless, the expression of Xiap mRNA was induced to a greater extent (2.2 fold) in the more etoposide-sensitive CRL1420 cells compared to the more etoposide-resistant CRL1469 (1.9 fold) and CRL1682 (1.7 fold) cells although Xiap induction occurred earlier in CRL1469. Nevertheless, the difference among cell lines is minimal suggesting that the transcriptional up-regulation of Xiap may not contribute significantly to the difference in etoposide sensitivity.

As was the case for Xiap mRNA, strongest induction of cIAP-1 and cIAP-2 mRNAs occurred 72h post-etoposide challenge (Figs.4.3B and 4.3C). Interestingly, we again observed the strongest mRNA induction in CRL1420 cells (6.7 and 23 fold for cIAP-1 and

Fig.4.3. Etoposide challenge induces the expression of IAP mRNAs in human pancreatic cancer cell lines, albeit at varying levels.

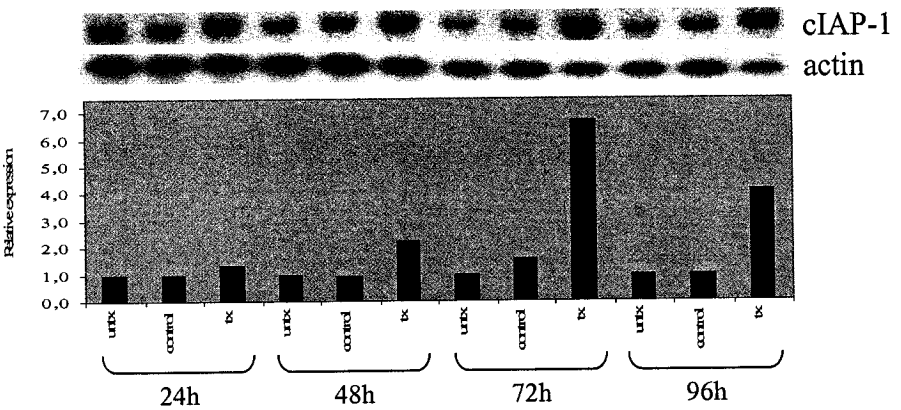
Log phase cultures of CRL1420, CRL1469 and CRL1682 cells were treated with 20 μM of etoposide for 6h and allowed to recover in fresh complete medium up to 96h. Every 24h post-etoposide treatment, cells were lysed in Trizol for RNA isolation. Seven μg of RNA were run on 1% (w/v) denaturing agarose gels and transferred to nylon membranes in 15X SSC overnight. Parallel membranes were hybridized with cIAP-1, cIAP-2, survivin and Xiap-specific cDNA probes. Lane-to-lane loading was assessed by stripping the membranes and re-hybridizing with actin. Band intensities were analyzed by densitometry and normalized to those of the internal loading control (actin). Expression of Xiap (**A**), cIAP-1 (**B**), cIAP-2 (**C**) and survivin (**D**) mRNAs in the presence of etoposide or vehicle control was normalized to that of untreated cells at the corresponding timepoints. Challenging pancreatic cancer cells with etoposide clearly induces the expression of IAP mRNAs albeit to different extents dependent on the mRNA and the cell line in question. Results presented here are representative of two independent experiments showing the same extent of fold inductions.
Note: in the graph legends, untx refers to untreated cells, control refers to cells mock-treated with DMSO (solubilization vehicle for etoposide) and tx refers to cells treated with 20 μM of etoposide.
(Figure continues on next 3 pages)

A

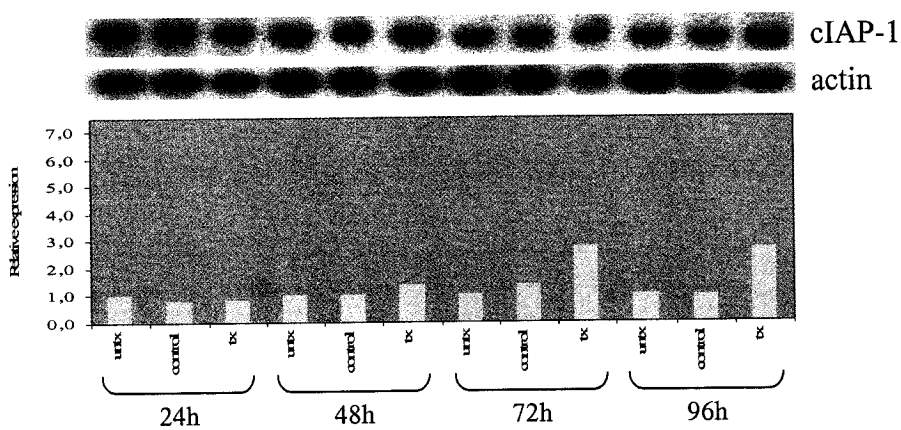

Xiap
actin
CRL1420
CRL1469
CRL1682
Relativeexpresin
2,5
2,0
1,5
1,0
0,5
0,0
untx
control
tx
24h
48h
72h
96h

B

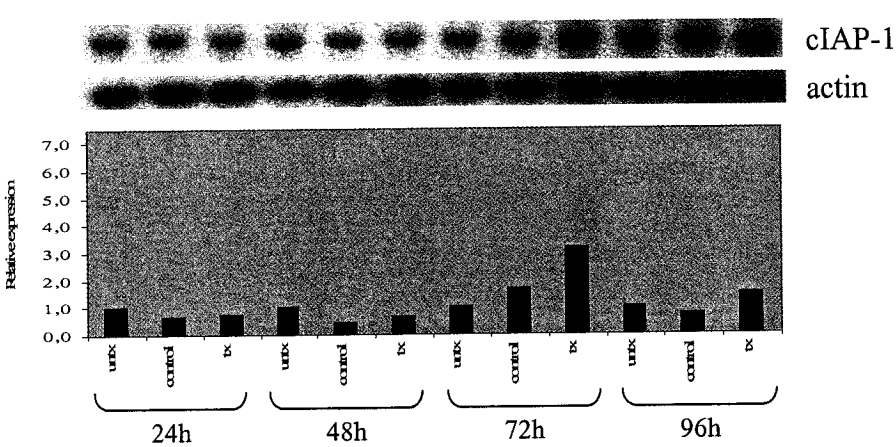
CRL1420



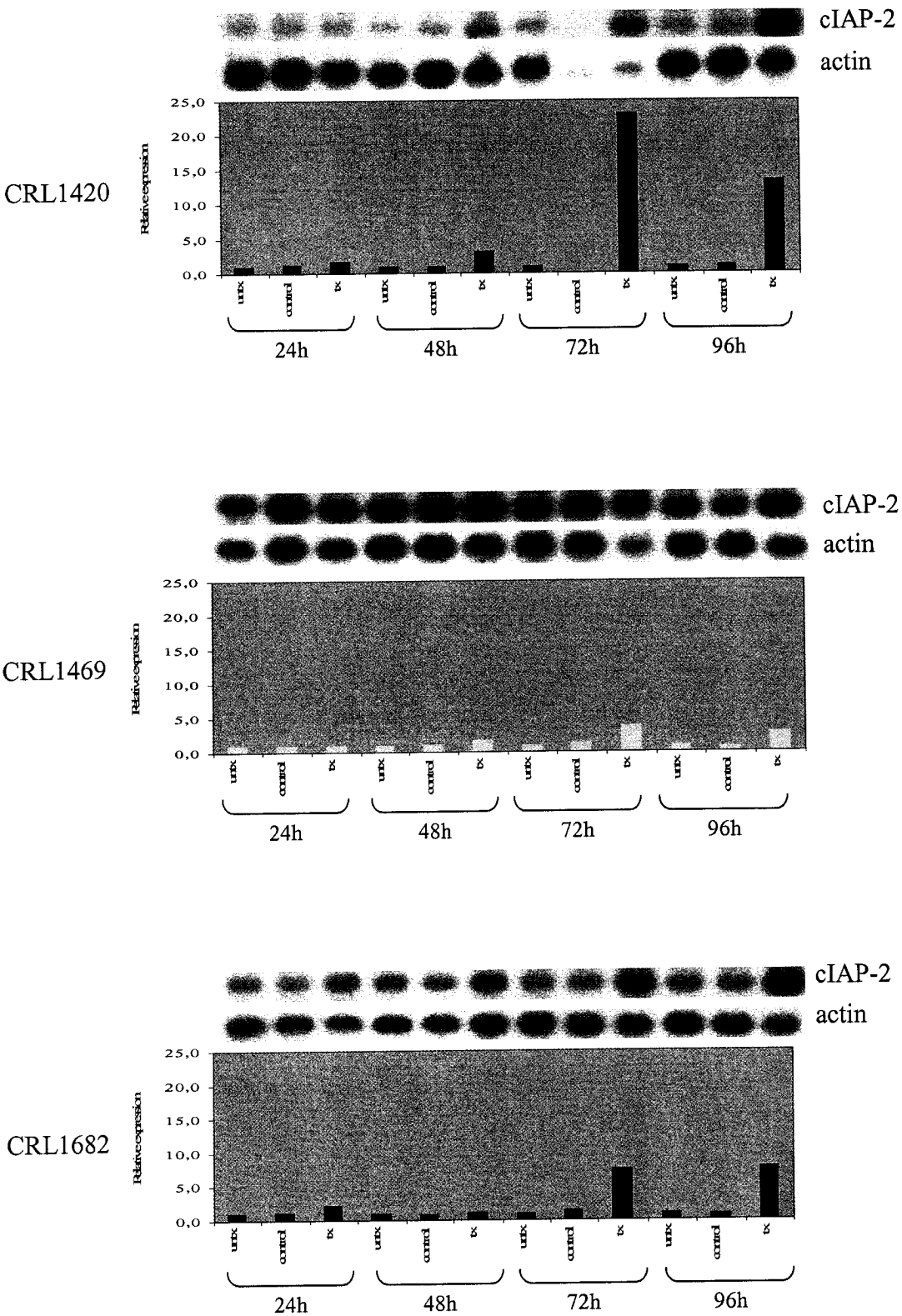
CRL1469



CRL1682

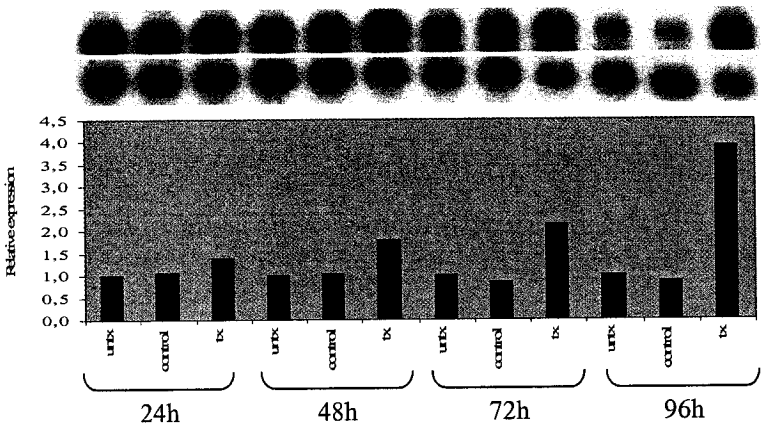


C

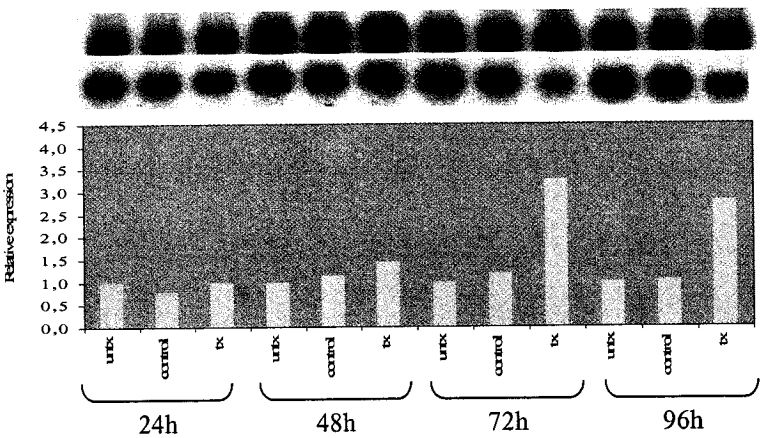

cIAP-2
actin
CRL1420
CRL1469
CRL1682
Rélativeexpression
25,0
20,0
15,0
10,0
5,0
0,0
untx
control
tx
24h
48h
72h
96h

D

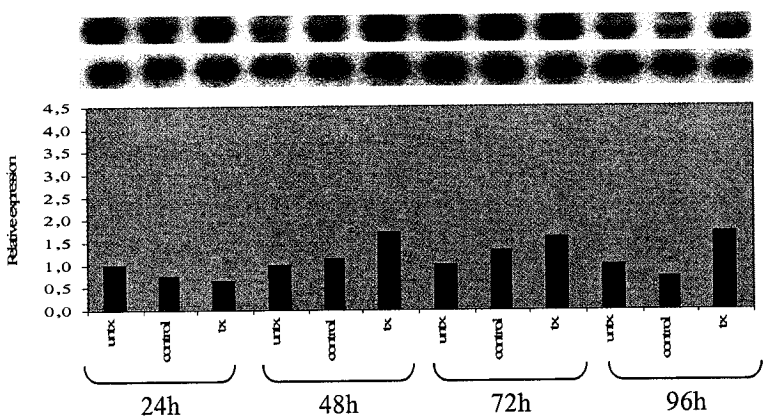
CRL1420



CRL1469



CRL1682



cIAP-2, respectively). The smallest induction was uncovered in CRL1469 (2.8 and 3.9 fold for cIAP-1 and cIAP-2 mRNAs, respectively) while CRL1682 exhibited an intermediate up-regulation of cIAP-1 (3.2 fold) and cIAP-2 (7.6 fold) mRNAs. When reviewing the transcriptional up-regulation of cIAP-2, we must bear in mind that cIAP-2 mRNA expression is barely detectable in CRL1420 and weak in CRL1682 while it is strong in CRL1469 prior to etoposide treatment. Variable cIAP-2 expression in untreated samples may influence the extent of the apparent induction of cIAP-2 mRNA in response to etoposide. As such, low initial levels of cIAP-2 mRNA may be better prognosticators of etoposide-responsiveness.

Lastly, transcriptional up-regulation of survivin peaked 96h post-etoposide challenge in CRL1420 (3.9 fold) and CRL1682 (1.7 fold) cells while survivin induction was maximal at 72h in CRL1469 (3.3 fold induction). As the fold inductions in survivin mRNA between sensitive and resistant cell lines were small, it is unlikely that the transcriptional up-regulation of survivin plays a significant role in the response to etoposide-induced DNA damage. In short, etoposide induced IAP expression in all cell lines tested, albeit to a different extent. In addition, low initial levels of cIAP-2 mRNA appeared to correlate with the rapidity of onset of programmed cell death.

4.2.3. Caspase-dependent cleavage of Xiap and activation of caspase-3 in the human pancreatic adenocarcinoma cell line CRL1420 challenged with etoposide

To verify if the observed etoposide-induced expression of Xiap mRNA correlated with protein expression levels, etoposide-treated cells were lysed in protein extraction buffer and processed for immunoblotting with a polyclonal anti-Xiap antibody. The emergence of a Xiap cleavage product (approximately 29 kDa in size) upon treatment with 20 μM of

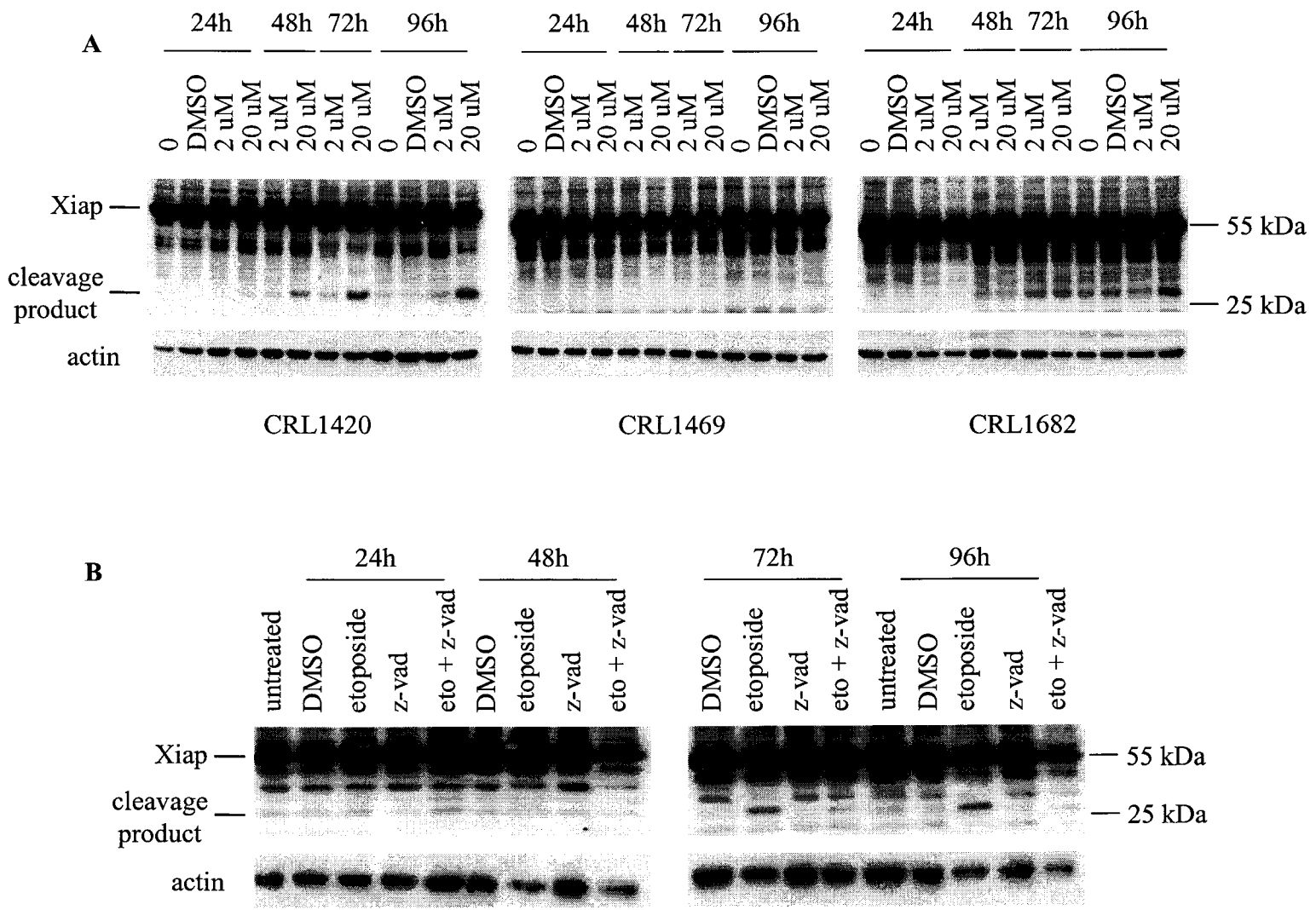
etoposide was detected 48h post-etoposide challenge in CRL1420 while Xiap cleavage occured 96h post-challenge in CRL1682 (Fig.4.4A). No cleavage of Xiap was observed in CRL1469 during the course of the treatment. Finally, exposure to DMSO did not affect the expression of Xiap.

To verify if the cleavage of Xiap was dependent on caspase activity, log phase cultures of CRL1420 cells were challenged with 20 μM of etoposide in the presence or absence of 50 μM z-vad.fmk (carbobenzoxy-val-ala-asp-fluoromethyl ketone), a broad range caspase inhibitor. As clearly demonstrated in Fig.4.4B, challenging CRL1420 cells with etoposide in the presence of 50 μM z-vad.fmk resulted in the abolishment of Xiap cleavage establishing that etoposide-induced cleavage of Xiap is caspase-dependent. Exposing the cells to DMSO or z-vad-fmk alone had no effect on the expression or the cleavage of the Xiap protein.

It has been suggested that cleavage of Xiap at Asp315 allows Xiap BIR3 to bind directly to active caspase-9 and prevent its activity (Sun et al., 2000; Srinivasula et al., 2001), an interaction proposed to prevent the homodimerization of caspase-9 necessary for its activity (Shiozaki et al., 2003). On the other hand, it has been reported that the Xiap BIR1-2 linker region is exclusively needed to achieve inhibition of caspase-3 (Huang et al., 2001; Riedl et al., 2001; C. Sun et al., 1999, 2000). To determine if the observed cleavage of Xiap correlated with reduced levels of activated caspases-3 and –9, etoposide-treated protein lysates were processed for immunoblotting with anti-caspase-3 and anti-caspase-9 antibodies capable of recognizing both the active and inactive forms of the caspases. As can be

Fig.4.4. Etoposide-induced apoptosis involves rapid caspase-dependent cleavage of Xiap in CRL1420 human pancreatic adenocarcinoma cells.

A. Log phase cultures of pancreatic adenocarcinoma cells were treated with 2 or 20 μM of etoposide for 6h and allowed to recover in fresh complete medium up to 96h. Cells (including floaters and residual adherent cells) were lysed in SDS lysis buffer every 24h for up to 96h for protein extraction. Ten μg of proteins were resolved on 12% SDS-PAGE against a size standard (BenchMark™ Pre-Stained Protein Ladder; Invitrogen) and processed for immunoblotting with a polyclonal anti-Xiap antibody. DMSO is the vehicle control in which etoposide is solubilized. A 29 kDa Xiap cleavage product can clearly be observed in CRL1420 at 48h and in CRL1682 at 96h. No cleavage of the 55 kDa Xiap protein was detectable in CRL1469 up to 96h post-etoposide treatment. **B.** Log phase cultures of CRL1420 were grown in a medium supplemented with 50 μM of the pancaspase inhibitor z-vad.fmk (z-vad lane) one hour prior to treatment with 20 μM of etoposide or a combination of both (z-vad + eto lane). Alternatively, cells were challenged with 20 μM of etoposide in the absence of z-vad.fmk (etoposide lane). Etoposide challenges lasted 6h. Every 24h for 96h, cells were lysed in protein extraction buffer, resolved on 12% SDS-PAGE against a size standard and processed for immunoblotting with a polyclonal anti-Xiap antibody. Membranes were stripped and re-hybridized with a polyclonal anti-α-actin antibody to control for lane-to-lane loading. The presence of z-vad.fmk abolished the etoposide-induced cleavage of Xiap.


A
24h
48h
72h
96h
0
DMSO
2 uM
20 uM
Xiap
cleavage product
actin
55 kDa
25 kDa
CRL1420
CRL1469
CRL1682
B
untreated
DMSO
etoposide
z-vad
eto + z-vad

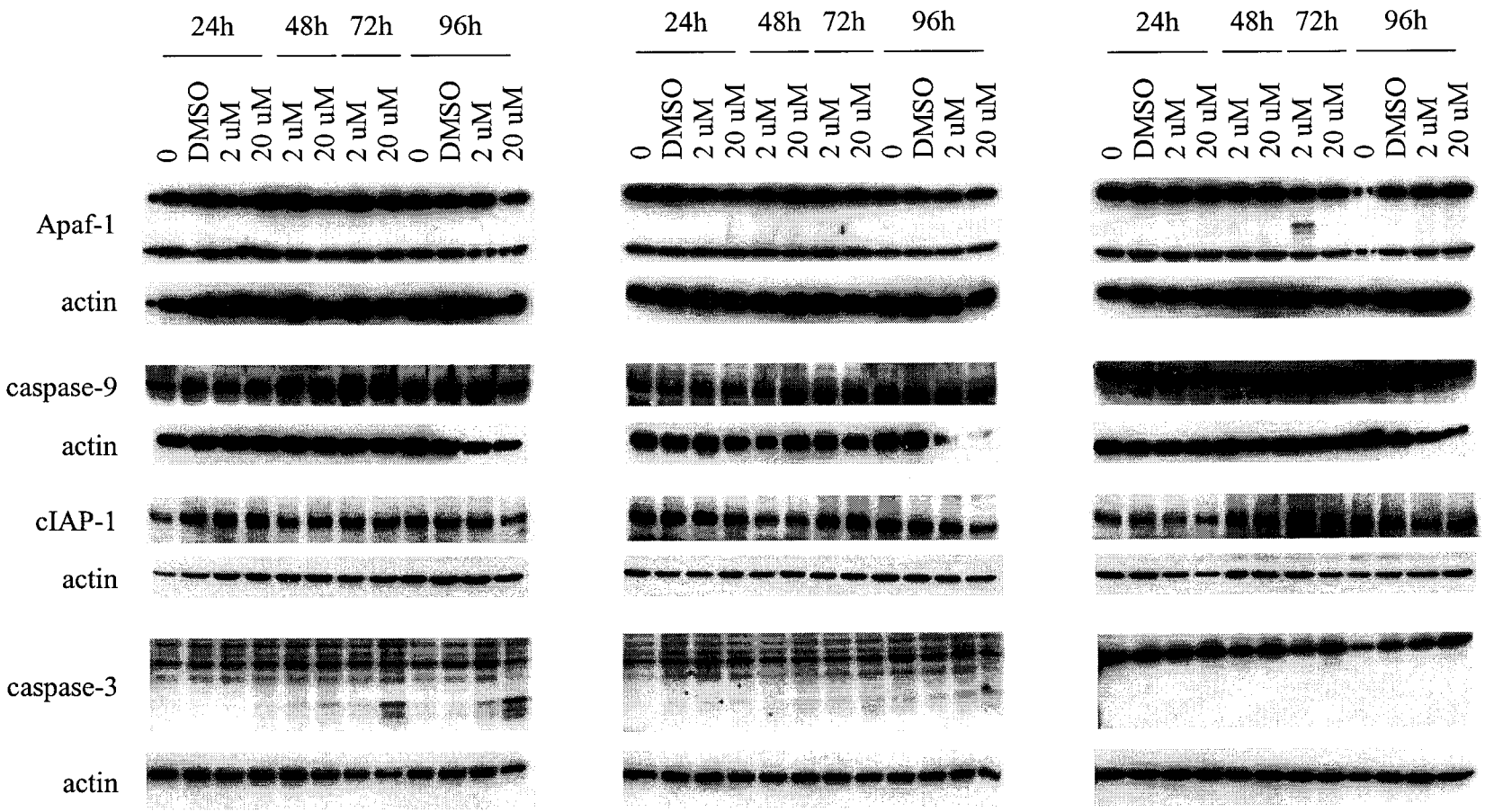
observed in Fig.4.5, no cleavage/activation of caspase-9 was detected in any of the cell lines tested. Furthermore, the levels of Apaf-1 protein (an adaptor protein necessary for the activation of caspase-9) were not altered in any of the etoposide-treated protein lysates. In contrast, proteolytic cleavage of procaspase-3 consistent with activation was observed in etoposide-treated CRL1420 cells as evidenced by the presence of a 17 kDa cleavage product as early as 48h post-treatment (Fig.4.5). Activation of caspase-3 did not occur in CRL1469 and CRL1682 cells which are more resistant to etoposide. Interestingly, proteolytic cleavage of caspase-3 and Xiap cleavage both become apparent 48h post-etoposide challenge. Lastly, contrary to the observed accumulation of cIAP-1 mRNA in CRL1420, cIAP-1 protein expression was not affected by etoposide in these cells as well as in CRL1469 and CRL1682 cells.

4.2.4. Forced Xaf-1 protein expression does not appear to sensitize pancreatic cancer cell lines to etoposide

If IAPs are indeed responsible for the resistance of pancreatic cancer cell lines to chemotherapeutic agents such as etoposide, compromising their function should sensitize cells to etoposide-induced apoptosis. We have observed that, although Xaf-1 mRNA is expressed at variable levels, the protein appears to be repressed in pancreatic cancer cell lines. As Xaf-1 has been proposed to relieve the anti-apoptotic function of Xiap (Liston et al., 2001), we hypothesized that restoring Xaf-1 protein expression in pancreatic cancer cells would sensitize them to apoptosis-inducing agents such as etoposide. To verify this hypothesis, CRL1420 and CRL1469 cells were infected with recombinant adenoXaf-1 and adenolacZ constructs generously provided by Drs. P. Liston and R. Korneluk.

Fig.4.5. Caspase-3 activation during etoposide-induced apoptosis in CRL1420 cells.

Log phase cultures of pancreatic tumor cells were treated with a dose range of etoposide for 6h and then placed in fresh culture medium for 4 days. Cells (including floaters and residual adherent cells) were lysed in SDS lysis buffer every 24h up to 96h for protein extraction. Ten μg of cell lysates were resolved on 12% SDS-PAGE against a size standard (BenchMark™ Pre-Stained Protein Ladder) and transferred to PVDF membranes for immunoblotting with anti-Apaf-1 (130 kDa), anti-caspase-9 (46-48 kDa for the inactive form), anti-cIAP-1 (70 kDa) and anti-caspase-3 (32 kDa for the inactive form and 17 kDa for the active form) antibodies. Membranes were stripped and re-hybridized with an anti-α-actin polyclonal antibody to control for lane-to-lane loading. Cleavage/activation of procaspase-3 was only observed in the chemosensitive cell line CRL1420 while all other proteins tested were unaffected by etoposide.


24h
48h
72h
96h
0
DMSO
2 uM
20 uM
Apaf-1
actin
caspase-9
actin
cIAP-1
actin
caspase-3
actin
CRL1420
CRL1469
CRL1682

Optimal multiplicities of infection (MOI) were determined for both cell lines used by processing adenoXaf-1-infected protein extracts for immunoblotting with a polyclonal anti-Xaf-1 antibody. Infecting CRL1420 and CRL1469 cells with adenoXaf-1 resulted in a MOI-dependent increase in Xaf-1 protein expression (Fig.4.6A).

To determine if Xaf-1 protein expression sensitized cells to etoposide, tumor cells were first infected with adenoXaf-1 (MOI: 25 and 50 for CRL1420 and CRL1469, respectively), allowed to recover in complete growth medium overnight and then challenged or not with 20 μM of etoposide. Cell survival was assessed 48h post-etoposide challenge and expressed relative to that of untreated control cells. Infecting CRL1420 cells with adenoXaf-1 or adenolacZ (control vector) induced a toxicity of 11% and 6%, respectively (Fig.4.6B). Interestingly, infection with recombinant adenoXaf-1 sensitized CRL1420 cells to etoposide (57% versus 90% survival for etoposide treatment only). However, this sensitization was not specific since infection with the control vector (adenolacZ) prior to etoposide treatment had a similar effect (65% survival). On the other hand, infecting CRL1469 cells with either of the recombinant adenoviral constructs had no significant effect on cell survival. In addition, adenoXaf-1-infected CRL1469 cells were not sensitized to etoposide given that cell survival in response to etoposide challenge was 82% in the presence or absence of Xaf-1 protein expression.

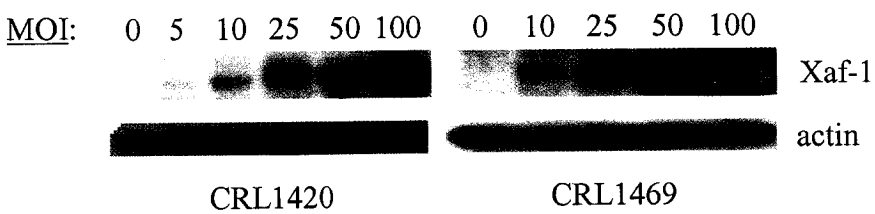
4.2.5. IAPs can be down-regulated with short-interfering siRNAs

RNA silencing has recently emerged as a gene regulatory mechanism capable of limiting transcript levels by eliciting sequence-specific RNA degradation (reviewed in Agrawal et al., 2003). As siRNAs synthesized against different regions of the same target

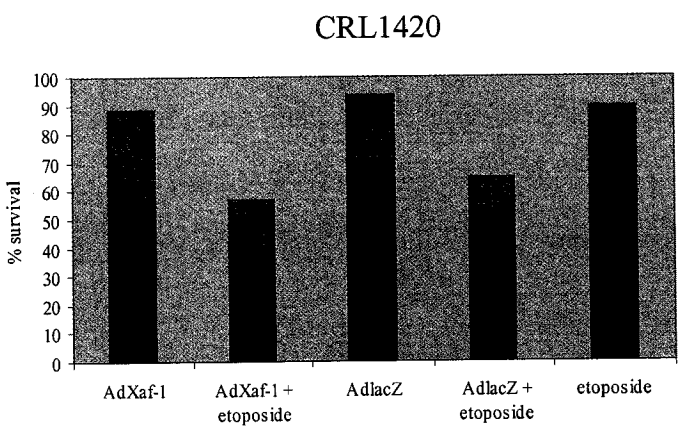
Fig.4.6. Xaf-1 protein expression can be restored in CRL1420 and CRL1469 pancreatic cancer cell lines via infection with an adenoXaf-1 construct.

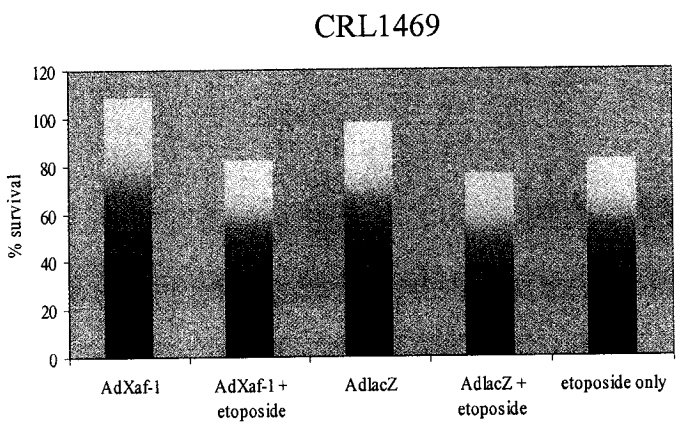
A. Log phase cultures of CRL1420 and CRL1469 human pancreatic cancer cells were infected with a Xaf-1 sense adenoviral construct at different multiplicities of infection (MOI). Twenty-four hours post-infection, cells were lysed in protein extraction buffer and resolved on 12% SDS-PAGE for immunoblotting with an anti-Xaf-1 polyclonal antibody. To assess lane-to-lane loading, membranes were stripped and re-hybridized with a polyclonal anti-α-actin antibody. Xaf-1 protein expression was restored in both cell lines in a dose-dependent manner. **B.** Twenty-four hours post-adenoviral infection, tumor cells were treated with 20 μM of etoposide in order to determine if restoring Xaf-1 protein expression sensitized cells to etoposide. Survival was assessed 48h post-etoposide treatment by incubating the cells in 1X alamarBlue™ for 1h at 37°C/5% CO_2 and establishing absorbance readings on a Cytofluor 2300 automated fluorescence system. Data files were subsequently exported to the SoftMax software for analysis. Readings were corrected for vehicle (DMSO) effect. No sensitization of pancreatic tumor cell lines to etoposide was observed upon Xaf-1 protein expression. Results presented here are preliminary as this experiment was only done once under these exact conditions.

A


MOI: 0 5 10 25 50 100 0 10 25 50 100
Xaf-1
actin
CRL1420
CRL1469

B


CRL1420
% survival
100
90
80
70
60
50
40
30
20
10
0
AdXaf-1
AdXaf-1 + etoposide
AdlacZ
AdlacZ + etoposide
etoposide


CRL1469
% survival
120
100
80
60
40
20
0
AdXaf-1
AdXaf-1 + etoposide
AdlacZ
AdlacZ + etoposide
etoposide only

mRNA exhibit different silencing efficiencies (Holen et al., 2002), pools of siRNA duplexes targeted to cIAP-2, survivin and Xiap were designed by and purchased from Dharmacon RNA Technology. Briefly, the technology developed by this company combines 4 SMART-selected siRNA duplexes in a single pool to ensure more complete silencing of target gene expression.

To determine optimal siRNA concentrations required to abolish at least 50% of target protein expression, CRL1420 and CRL1469 cells were transfected with a dose range (up to 500 nM) of cIAP-2, survivin or Xiap-specific siRNAs. We then analyzed target-specific RNA and protein levels 48h post-transfection (24h and 72h for Xiap expression in CRL1420). To assess the extent of RNA or protein down-regulation, band intensities were analyzed by densitometry, normalized to loading controls (β-actin for RNA and α-actin for protein) and expressed as fold-decreases over that of untransfected cells (Fig.4.7). However, as the data presented here are the result of a single experiment, we cannot conclude that target mRNA and protein expression were down-regulated reproducibly. From this analysis, we observed a reduction of survivin and Xiap protein expression of at least 50% in the presence of 50 nM of the corresponding siRNAs in CRL1420. Intriguingly, while Xiap mRNA levels tend to correlate with protein levels, survivin mRNA and protein profiles do not agree. For example, even though reduction of survivin protein expression was observed upon treatment with 50 nM of survivin siRNA, no reduction in mRNA expression was observed. On the contrary, transfecting CRL1420 cells with survivin-specific siRNA had the opposite effect in that mRNA expression was induced. Furthermore, tranfecting CRL1420 cells with 500 nM of survivin siRNA strongly induced protein expression. Due to the extremely low endogenous expression of cIAP-2 mRNA and protein (Figs.3.4B and 3.7B,

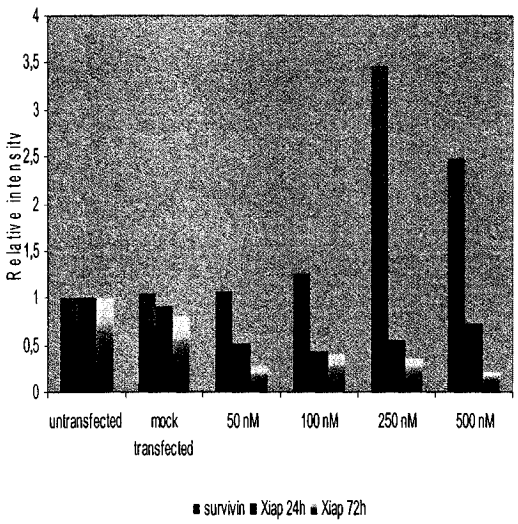
Fig.4.7. Xiap, cIAP-2 and survivin mRNA levels can be down-regulated by siRNA transfection in pancreatic tumor cell lines.

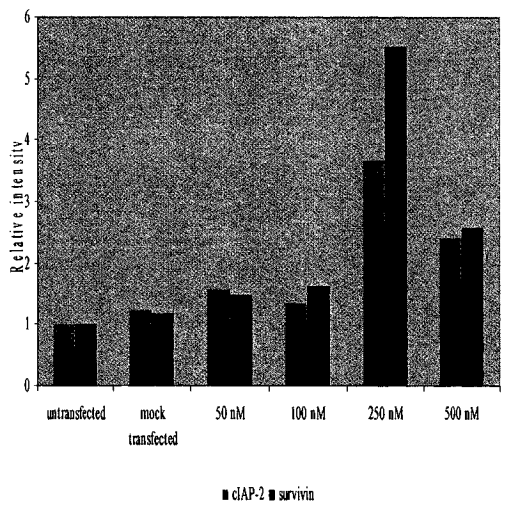
Log phase cultures of CRL1420 and CRL1469 were transfected with pools of siRNA duplexes (SMARTpool™, Dharmacon RNA Technologies) at various concentrations (0-500 nM). Unless otherwise specified, cells were lysed in Trizol or protein extraction buffer 48h post-siRNA transfection for RNA or protein extractions, respectively. **A.** Ten μg of total RNA were resolved on 1% (w/v) denaturing agarose gels and blotted to nylon membranes in 15X SSC. Membranes were hybridized with cIAP-2, survivin or Xiap-specific cDNAs to confirm down-regulation of target mRNAs. To control for lane-to-lane loading, membranes were stripped and re-hybridized with a β-actin probe. Band intensities were analyzed by densitometry, normalized to the internal control and expressed as fold increases over that of the untransfected sample. **B.** Ten μg of protein lysates were resolved on 12% SDS-PAGE gels against a size standard, electro-blotted to PVDF membranes and probed with antibodies against the target genes. Membranes were stripped and re-probed with anti-α-actin to control for lane-to-lane loading. Band intensities were analyzed by densitometry, normalized to the internal control and are expressed as fold increases over that of the untransfected sample. Down-regulation of target protein expression was achieved with 50 nM of siRNA duplexes in CRL1420 while 500 nM (cIAP-2) and 100 nM (survivin) siRNA were necessary to abolish target protein expression in CRL1469. Results presented here are not statistically significant as the experiment was only performed once.

CRL1420

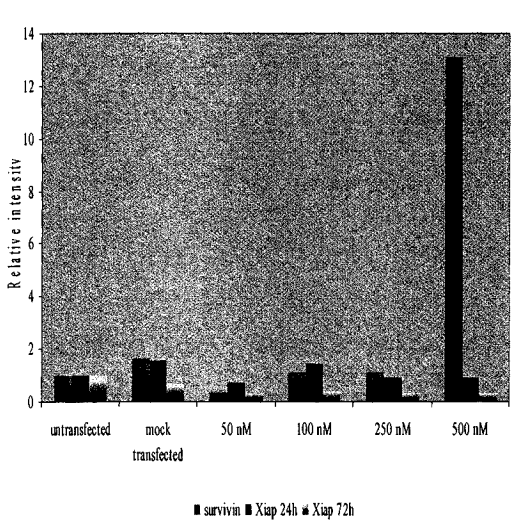
CRL1469

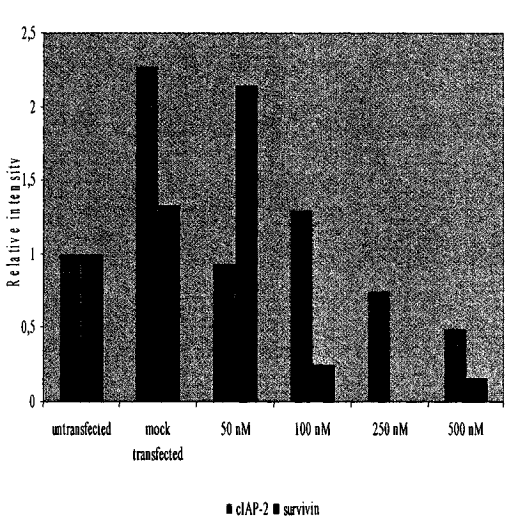
A





B





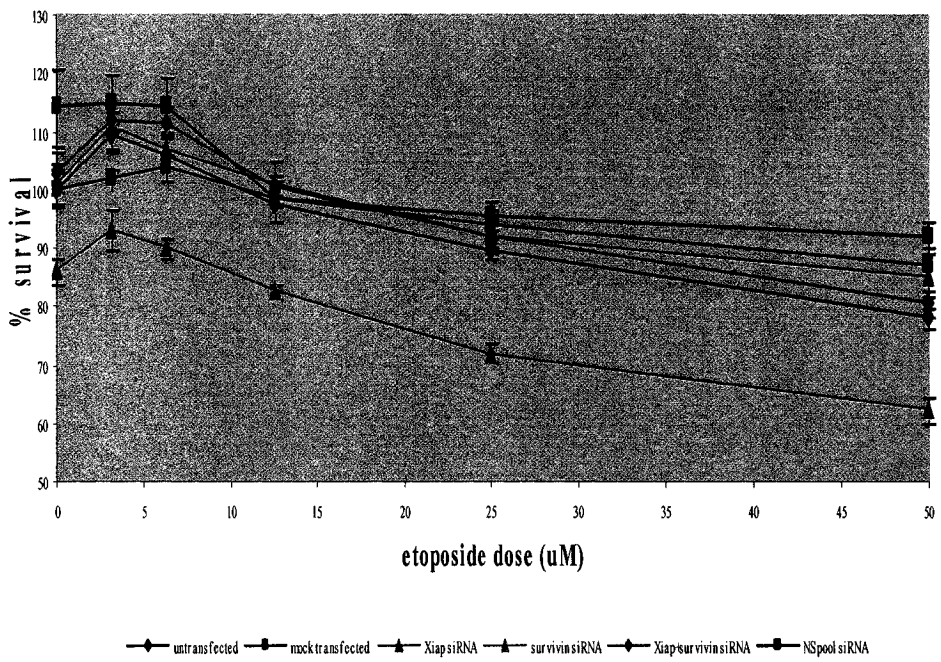
respectively) in CRL1420, we were unable to observe down-regulation of cIAP-2 expression in this cell line. On the other hand, although the expression of target mRNAs did not appear to be affected by siRNA transfection in CRL1469, the expression of cIAP-2 and survivin proteins was reduced by at least 50% in the presence of 100 nM and 500 nM of corresponding siRNA, respectively. In CRL1469, the endogenous expression of Xiap mRNA and protein (Figs.3.4B and 3.7B, respectively) is quite low; therefore, we were unable to show down-regulation of Xiap expression in this cell line.

In an attempt to determine if decreased IAP expression sensitized pancreatic cancer cells to apoptotic stimuli, we challenged CRL1420 and CRL1469 cells with a dose range of etoposide (0-50 μM) 48h post-siRNA transfection and assessed cell viability 48h later. Statistical analyses were performed on data resulting from triplicates of two independent experiments which were considered as n=6 (please refer to section 2.11 for further details). Unfortunately, exposure to siRNAs did not result in potentiation of etoposide sensitivity as there was no statistical difference in the response to etoposide between untransfected and siRNA-transfected cells (Fig.4.8). However, we were surprised to uncover a uniform decrease in the survival of CRL1420 cells following down-regulation of survivin expression. Indeed, even in the absence of etoposide, the intrinsic survival of CRL1420 cells transfected with survivin siRNA (85.96±2.09%) is less than that of untransfected CRL1420 cells (100.00±3.25%), a decrease of approximately 15%. This decrease in the intrinsic survival of CRL1420 cells following down-regulation of survivin expression fits with the current view that survivin has a role in the regulation of cell division (Fraser et al., 1999; Li et al., 1999; Miller, 1999; Reed and Reed, 1999; Uren et al., 1999; Reed and Bischoff, 2000; Fortugno et

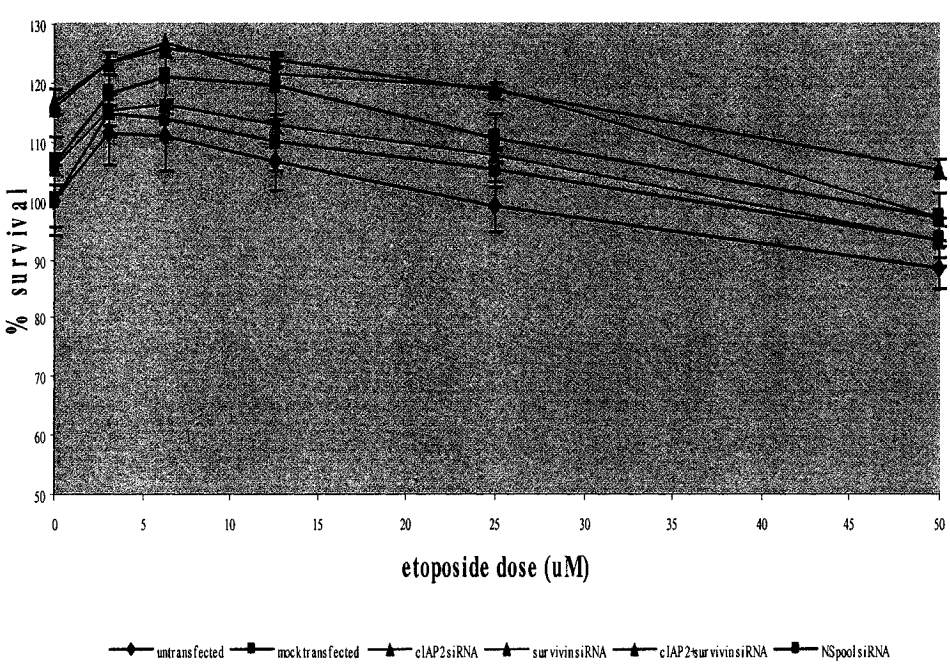
Fig.4.8. Down-regulating survivin expression reduces the intrinsic viability of the human pancreatic cancer cell line CRL1420.

Log phase cultures of CRL1420 and CRL1469 cells were transfected with 50 μM of survivin, Xiap and cIAP-2 siRNA duplexes or combinations thereof for 48h. Cells were then challenged with a 0-50 μM of etoposide for another 48h. Cell viability was assessed by adding 1/10 volume of the cell proliferation reagent WST-1 (Roche), incubating the cells for 1h at 37°C/5% CO_2 and determining absorbance readings spectrophotometrically. Data presented here result from the statistical analysis of triplicates of two independent experiments which were considered as n=6 (please refer to section 2.11 for further details). Following etoposide treatment, survival of mock transfected cells or cells transfected with 50 μM of non-specific (NS) siRNA pools is comparable to that of untransfected cells. In contrast, the intrinsic viability of CRL1420 cells transfected with survivin siRNA was decreased although the down-regulation of survivin did not sensitize the cells to etoposide.

CRL1420



CRL1469



al., 2002). However, although down-regulating survivin expression resulted in a decrease in the intrinsic survival of CRL1420 cells, it did not potentiate the response to etoposide as the difference between the survival curves of untransfected and survivin siRNA transfected cells remained constant (≈16%) at all etoposide doses studied. Down-regulation of survivin expression had no effect in CRL1469 cells (Fig.4.8). The lack of sensitization to etoposide-induced apoptosis following down-regulation of IAP expression may be due to residual expression of IAP as siRNA transfections did not fully repress transcriptional and translational expression of IAPs.

4.3. Summary of results

The specific aim of this chapter was to determine if differential expression of IAPs influenced the sensitivity of cancer cell lines to the chemotherapeutic agent etoposide, a topoisomerase II inhibitor. The experiments performed showed that human pancreatic adenocarcinoma cell lines differ in their *in vitro* sensitivity to etoposide, CRL1420 being the most sensitive of the three cell lines tested. In addition, we observed a good correlation between the caspase-dependent cleavage of Xiap, activation of caspase-3 and sensitivity to etoposide. Furthermore, we observed an up-regulation of IAP mRNAs in response to etoposide. Post-treatment inductions probably identify cells likely to survive the treatment although this hypothesis remains to be verified. Intriguingly, the strongest up-regulation of IAP mRNA expression was uncovered in CRL1420. Lastly, we attempted to sensitize pancreatic cancer cells to etoposide by down-regulating IAP expression or compromising IAP function. In particular, we targeted Xiap activity by restoring Xaf-1 protein expression. Moreover, we achieved siRNA-mediated down-regulation of IAP protein expression in the

pancreatic cancer cell lines CLR1420 and CRL1469. However, neither of these experiments resulted in sensitization to etoposide. Nonetheless, the observed correlation between induction of IAP mRNA expression, caspase-dependent cleavage of Xiap, activation of caspase-3 and the responsiveness of pancreatic cancer cells to etoposide-induced apoptosis suggests that exploiting these or other avenues to modulate IAP expression and sensitize cancer cells to chemotherapeutic agents is warranted.

Chapter 5: Characterization of FLN29/Xaf-2, a candidate IAP-interacting protein, in pancreatic adenocarcinoma cell lines

5.1. Introduction

Allelotyping and karyological analyses have revealed structural and chromosomal abnormalities affecting chromosome 12 in 40-60% of pancreatic tumors (Hahn et al., 1995; Griffin et al., 1995; Kimura et al., 1996). A survey of polymorphic STR loci spanning chromosome 12 performed in our laboratory identified generalized loss of heterozygosity and homozygous microdeletions at 12p12.3 (D12S62), 12q22-23.1 (D12S360) and 12q24.33-qter (D12S324) in pancreatic cancer cell lines (Éthier et al., 1997). These findings agree with other studies reporting deletion within or surrounding the D12S260 region in 60% of pancreatic cancers (Kimura et al., 1998; Klein et al., 2004). The protein encoded by the FLN29 gene bears partial N-terminal amino acid homology to Xaf-1, the latter having been identified as a Xiap-binding partner by yeast-two-hybrid screening suggesting that FLN29 might represent a candidate related family member (referenced in Liston et al., 2001). Interestingly, surveys of integrated map data revealed that FLN29 mapped in proximity to the D12S360 region. We have opted to refer to FLN29 as FLN29/Xaf-2 throughout this thesis although the Xaf nomenclature is purely tentative since there is as yet no evidence for physical interaction with Xiap or any other IAPs.

The potential interaction between FLN29/Xaf-2 and IAPs has never been investigated. Preliminary domain searches uncovered Traf-like ring zinc fingers within the sequence of FLN29/Xaf-2. Intriguingly, Traf1/Traf2 heterocomplexes have been postulated to mediate the interaction between cIAP-1 and cIAP-2 and the TNF receptor complex (Rothe et al., 1995) establishing a link between Traf-like RZF domains and interaction with IAPs.

Furthermore, Trafs play an important role in stress responses via their regulation of stress kinases leading to the activation of transcription factors such as NF-κB, c-Jun and ATF2 (Nishitoh et al., 1998; Baud et al., 1999; Bradley and Pober, 2001). In particular, Traf2 stimulates the activation of NF-κB and mediates anti-apoptotic signals (Lee et al., 1997; Yeh et al., 1997). Moreover, Traf2 has been proposed to protect cells from TNF-α-induced death (Weiss et al., 1998; Erickson et al., 1994) while exposure to TNF-α results in an up-regulation of IAPs (Stehlik et al., 1998a, 1998b). Briefly, binding of TNF-α to TNF-RI leads to the recruitment of TRADD and RIP to the receptor complex (Hsu et al., 1996a, 1996b; Stanger, 1996). In turn, TRADD interacts with FADD to initiate the death pathway and recruits several proteins such as Traf1 and Traf2 to the receptor complex thereby activating NF-κB transcription (Siebenlist et al., 1994; Verma et al., 1995; Baeuerle and Baltimore, 1996; Baldwin, 1996; Maniatis, 1997; May and Ghosh, 1998).

Among other roles, the NF-κB pathway plays a role in inhibition of apoptosis (Beg and Baltimore, 1996; Van Antwerp et al., 1996; Wang et al., 1996). Indeed, NF-κB activates the transcription of target genes such as IAPs and members of the bcl-2 family which block the induction of apoptosis (Karin and Lin, 2002). Constitutive NF-κB activity has also been associated with an increased resistance to cytotoxic therapies (C. Wang et al., 1999). In particular, in pancreatic cancer cells, inhibition of NF-κB activity resulted in increased sensitivity to drug-induced apoptosis while constitutively active NF-κB has been associated with chemoresistance (Arlt et al., 2001; Arlt and Schafer, 2002). Interestingly, the chemotherapeutic inhibition of NF-κB activation reportedly led to an up-regulation of pro-apoptotic genes of the TNF pathway and a down-regulation of IAPs and Traf2 leading to the induction of apoptosis (Biswas et al., 2003). Furthermore, recent reports suggest that, under

stress conditions that stimulate apoptosis, Traf2 is targeted for proteosomal degradation (Habelhah et al., 2002); ubiquitination and degradation of Traf2 has been proposed to be mediated by TNF-RII and cIAP-1 (X. Li, et al., 2002).

The RNase protection assays performed in Chapter 3 revealed that almost all of the pancreatic cancer cell lines tested under-expressed Traf2 mRNA while most pancreatic cancer cell lines over-expressed cIAP-1 or cIAP-2 mRNA. By virtue of its N-terminal Traf-like RING zinc finger, we hypothesized that FLN29/Xaf-2 may possibly substitute for Traf2 in TNF receptor-mediated signaling. The proposed interaction between FLN29/Xaf-2 and the TNF receptor complex would stimulate NF-κB signaling resulting in increased transcription of IAPs to generate a feed forward loop culminating in the establishment of a constitutively elevated apoptotic threshold. If this were so, pancreatic cancer cell lines would gain a survival advantage by over-expressing FLN29/Xaf-2 to compensate for the lack of Traf2 expression. Preliminary domain searches identified a Traf-like RZF, a PEST sequence (indicative of a propensity to serve as a substrate for proteosomal degradation) and two putative caspase cleavage within the genomic sequence of FLN29/Xaf-2. In addition, the human FLN29/Xaf-2 gene sequence was highly conserved among animal species and did not appear to be prone to rearrangements in pancreatic cancer cell lines. Moreover, we uncovered ubiquitous expression of FLN29/Xaf-2 mRNA in non-diseased human tissues and a small tendency towards over-expression of FLN29/Xaf-2 mRNA in pancreatic cancer cell lines and other solid malignancies.

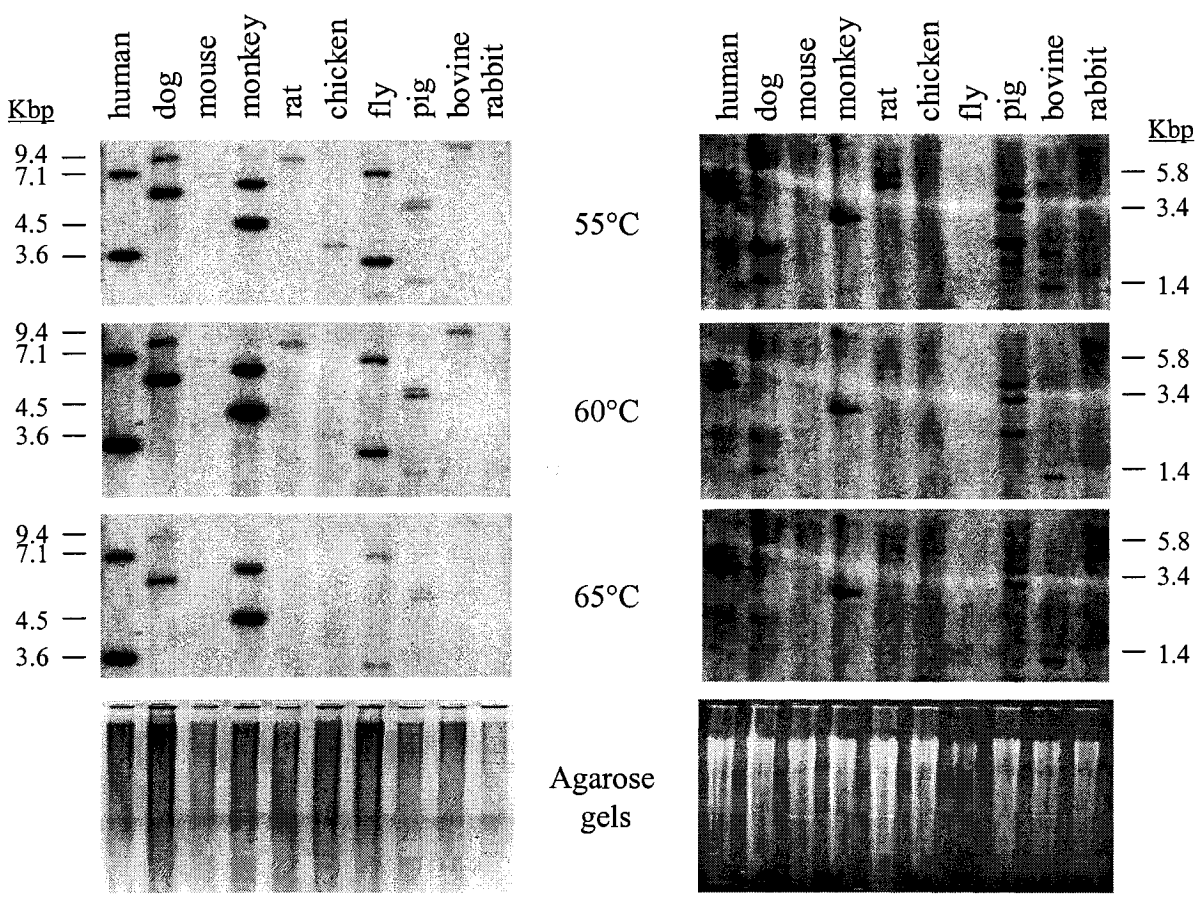
5.2. Results

5.2.1. The FLN29/Xaf-2 gene is highly conserved among animal species

We began by investigating the conservation of the FLN29/Xaf-2 sequence among animal species. *Hind* III- or *Eco* RI-digested genomic DNAs from human, dog, mouse, rhesus monkey, rat, chicken, fly, pig, bovine and rabbit were processed for Southern hybridization with a radio-labelled full-length Xaf-2 cDNA probe. Under lower stringency conditions, the human FLN29/Xaf-2 probe hybridized with all species tested, albeit to varying extent, except for mouse and rabbit (*Hind* III digests) and fly (*Eco* RI digests) (Fig.5.1). The observed lack of hybridization in fly (*Eco* RI digests) can partly be explained by the lower amount of DNA initially loaded on the gel as can be seen in the agarose gel below the autoradiograms. In addition, the hybridization pattern (band sizes) differed between human and other animal species suggesting that non-coding sequences (eg. introns) may differ among species. Increasing the stringency of the washes resulted in the total loss of signal for rat and chicken, partial loss of signals for bovine (*Hind* III digests only) and mouse (*Eco* RI digests only) while signals in dog, rhesus monkey, pig and *Drosophila* were retained. The weak signals in mouse and rat were somewhat unexpected as sequences related to FLN29 were reported in mice and rats (acc.# NM_172275 and XM_341086, respectively). However, while the similarity between human and mouse cDNA sequences is 69%, rat and human only share 30% sequence similarity, primarily because of the truncated version of FLN29/Xaf-2 encoded by the rat cDNA. In summary, this analysis suggested that the FLN29/Xaf-2 locus is strongly conserved through evolution.

Fig.5.1. The FLN29/Xaf-2 gene is highly conserved among animal species.

Three μg of *Hind* III- or *Eco* RI-digested genomic DNAs were separated on a 1% (w/v) agarose gel in 1X TBE buffer, alkali-blotted to a nylon membrane and probed with radiolabelled full-length FLN29/Xaf-2 cDNA. Fragments were sized against a BRL Forensic DNA Analysis Marker (corresponding reference size fragments are indicated beside each autoradiogram). Agarose gel electrophoresis confirmed complete digestion of genomic DNA samples. Blots were successively washed at 55, 60 and 65°C to increase stringency and exposed to X-ray film. Sequences related to human FLN29/Xaf-2 were detected in all species tested.


human
dog
mouse
monkey
rat
chicken
fly
pig
bovine
rabbit
Kbp
9.4
7.1
4.5
3.6
55°C
60°C
65°C
Agarose gels
5.8
3.4
1.4
Hind III
Eco RI

5.2.2. The mRNA encoding FLN29/Xaf-2 is ubiquitously expressed in non-diseased human tissues

Next, we assessed FLN29/Xaf-2 mRNA expression in non-diseased human tissues. Hybridization of Multi Tissue Northern blots with a full-length Xaf-2 cDNA probe revealed ubiquitous expression of a single 2.8 Kbp FLN29/Xaf-2 transcript of varying intensity in all human tissues tested (Fig.5.2). Notably, the strongest FLN29/Xaf-2 expressor appeared to be heart while weak FLN29/Xaf-2 mRNA expression was observed in brain, skeletal muscle, bone marrow and uterus. Interestingly, non-diseased pancreatic tissue was shown to express relatively strong FLN29/Xaf-2 mRNA levels.

5.2.3. The locus coding for Xaf-2 appears to be structurally intact but affected by loss of heterozygosity in pancreatic adenocarcinoma cell lines

Having observed that the FLN29/Xaf-2 coding sequence appeared to be highly conserved in vertebrates and ubiquitously expressed in human tissues, we decided to reconstruct the FLN29/Xaf-2 genomic map and align it against reference transcripts. *In silico* simulations aligned the 2.8 Kbp FLN29/Xaf-2 polyadenylated transcript with 11 exons distributed over approximately 28.8 Kbp of chromosomal DNA provisionally assigned to 12q23-q24 (Fig.5.3A). The FLN29/Xaf-2 protein (582 aa) has a predicted MW of 64.8 kDa, a pI of 5.01 and appears to be a close relative of another IAP-interacting protein, Xaf-1, through conservation of two N-terminal Traf-like zinc finger domains (Fig.5.3B). Preliminary analysis of the FLN29/Xaf-2 genomic region uncovered a PEST sequence (often

Fig.5.2. FLN29/Xaf-2 mRNA is expressed, albeit at varying levels, in most non-diseased human tissues.

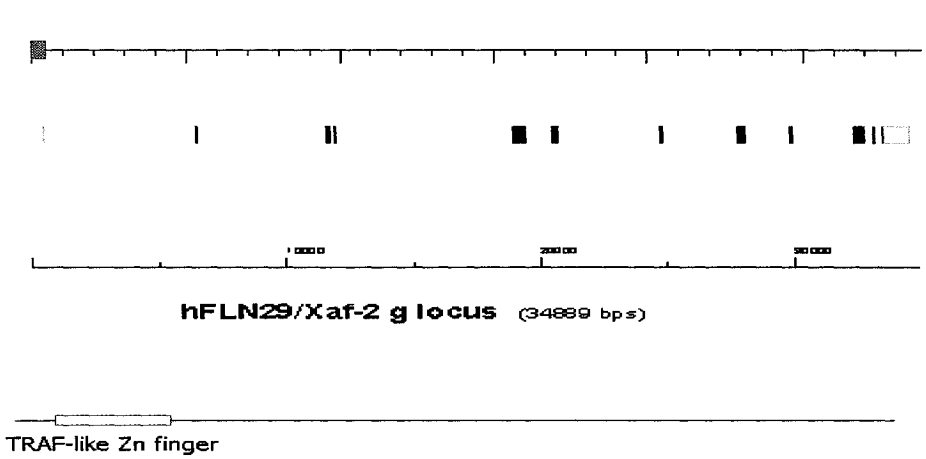
Membranes containing 2 μg of polyA+ RNAs from normal human tissues were obtained from Clontech and probed with radiolabelled full-length FLN29/Xaf-2 cDNAs. Blots were washed to high stringency and exposed to X-ray film. Transcript sizes (indicated at the right of the figure) were determined based on the migration of RNA size markers included on the membranes by the supplier. To control for lane-to-lane loading, blots were hybridized with β-actin cDNA. For actin, a single transcript (2 Kbp) is expected in all tissues tested except in heart and skeletal muscle where a second actin isoform (1.6-1.8 Kbp) should be detected. Tissues known to be prime metastatic sites for pancreatic cancer are highlighted in red. FLN29/Xaf-2 mRNA appears to encode a single transcript, approximately 2.8 Kbp in size.

actin
Xaf-2
heart
brain
placenta
lung
liver
skeletal muscle
kidney
pancreas
stomach
thyroid
spinal cord
lymph node
trachea
adrenal gland
bone marrow
spleen
thymus
prostate
testis
uterus
small intestine
colon
PBLs
2
2.8
Kbp

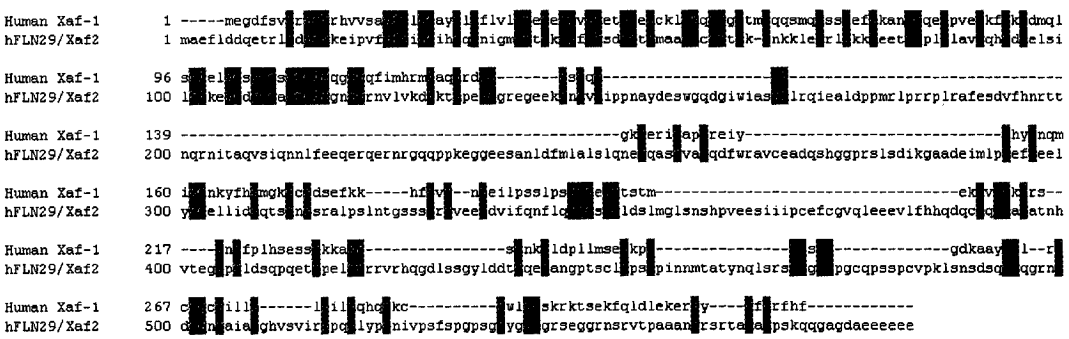
Fig.5.3. Predicted genomic organization of the FLN29/Xaf-2 gene, nucleotide similarity to Xaf-1 and predicted functional domains of the FLN29/Xaf-2 protein.

A. Genomic organization of the human FLN29/Xaf-2 gene (28.8 Kbp). The map was constructed from contig NT_009775 (NCBI Sequence viewer) using the latest version of the Build algorythm. Exon-intron boundaries were verified against cDNA sequences generated for normal tissue and pancreatic cancer cell lines. Major tick marks correspond to 5 Kbp. The blue rectangle identifies part of the candidate promoter. The grey boxes identify the 5' and 3' UTRs, respectively. The FLN29/Xaf-2 gene contains 11 coding exons provisionally mapped to 12q23-q24. Domain searches identified a Traf-like zinc finger in the N-terminal region of FLN29/Xaf-2. **B.** The protein encoded by FLN29/Xaf-2 shares significant N-terminal sequence similarity with Xaf-1. Alignment was performed using AlignPlus software (SciEd Software Inc.) **C.** Predicted domain structure of the FLN29/Xaf-2 protein. A PEST sequence (prldsqpqetspel; typically associated with a propensity to serve as a substrate for proteosomal degradation) also resides between aa 406 and 419. Two putative caspase cleavage sites (dqcd and dsqd) were identified at aa 387 and aa 490 while a tyrosine phosphorylation site (aa 108), an AIPP site (aa 147) and three SH3 ligand sites (aa 178, 182 and 416) were also uncovered.

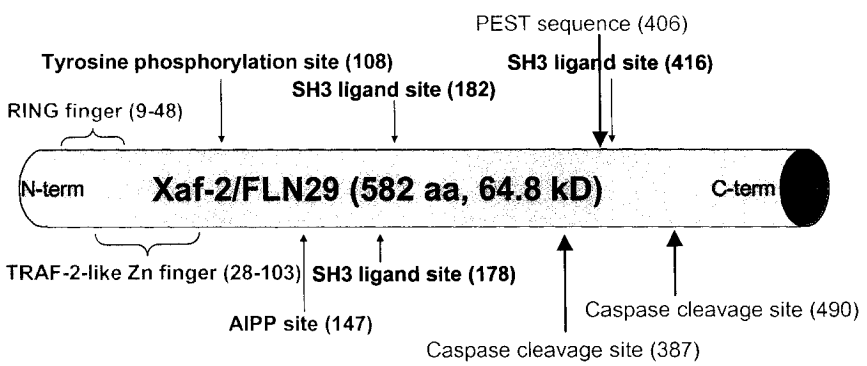
A


hFLN29/Xaf-2 g locus (34889 bps)
TRAF-like Zn finger

B


Human Xaf-1
hFLN29/Xaf2

C

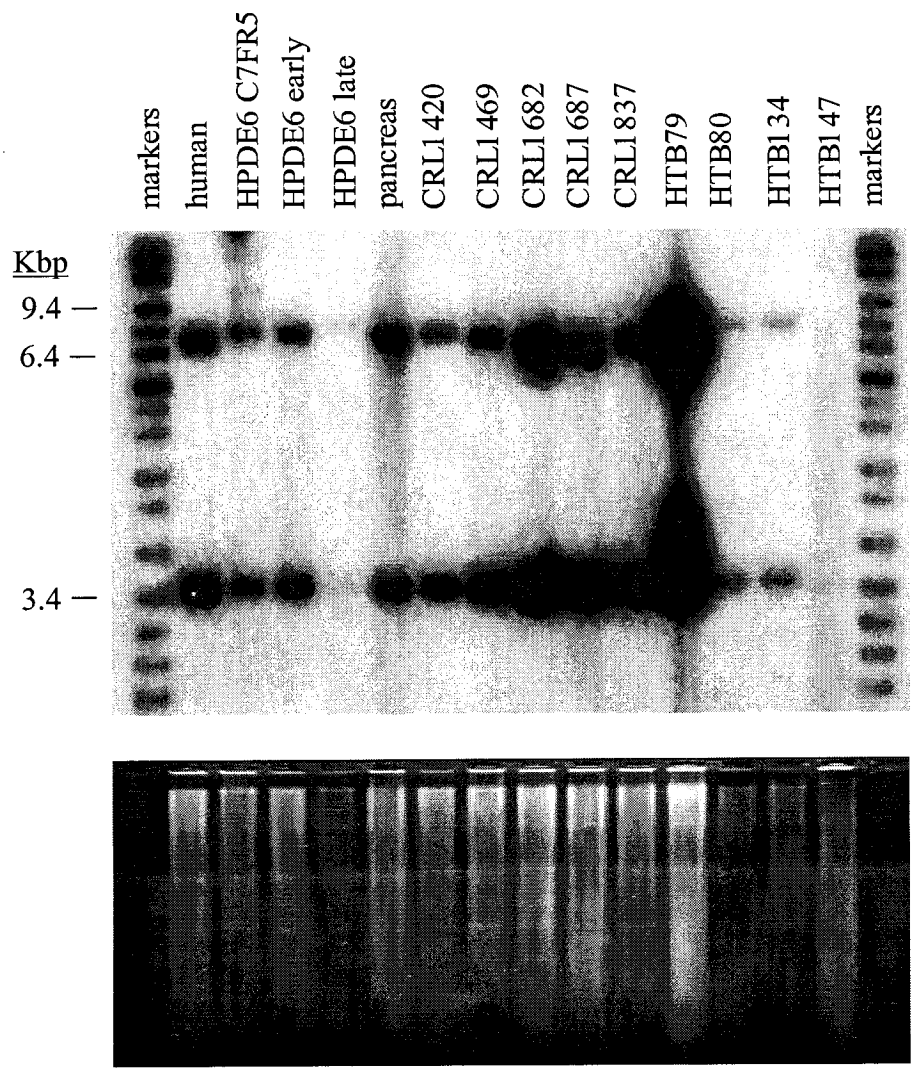

PEST sequence (406)
Tyrosine phosphorylation site (108)
SH3 ligand site (416)
SH3 ligand site (182)
RING finger (9-48)
N-term
Xaf-2/FLN29 (582 aa, 64.8 kD)
C-term
TRAF-2-like Zn finger (28-103)
SH3 ligand site (178)
AIPP site (147)
Caspase cleavage site (490)
Caspase cleavage site (387)

associated with a propensity to serve as a substrate for proteosomal degradation via the ubiquitin pathway) between aa 406 and 419 (Fig.5.3C; T. Ragheb and R. Aubin, unpublished observations). In addition, two putative caspase-cleavage sites (dqcd and dsqd) were identified at amino acids 387 and 490. Finally, a tyrosine phosphorylation site (aa 108), an AIPP site (aa 147) and three SH3 ligand sites (aa178, 182 and 416) were also uncovered within the FLN29/Xaf-2 sequence.

We then proceeded to evaluate FLN29/Xaf-2 gene structure and expression in pancreatic cancer cell lines. As such, *Hind* III-digested genomic DNAs from normal pancreatic tissue, immortalized ductal cell lines (HPDE series) and a panel of pancreatic cancer cell lines (CRL and HTB series) were processed for Southern hybridization with a radiolabelled full-length FLN29/Xaf-2 cDNA probe. Number and sizes of restriction fragments (3 fragments of 7.8, 3.4 and 1.4 Kbp) were predicted by aligning the probe against the *Hind* III restriction map of human FLN29/Xaf-2 as detailed previously (Chapter 3). Two major bands (7.8, and 3.4 Kbp) were detected in normal pancreatic tissue and all cancer cell lines tested except HTB147 (Fig.5.4). The predicted 1.4 Kbp band was absent from all samples; a possible explanation being that it ran off the gel. Lack of signal in HTB147 was somewhat surprising as agarose gel electrophoresis clearly showed the presence of DNA (albeit partly degraded) on the gel. In fact, there was more DNA for HTB147 than for HPDE6 late, HTB80 and HTB134, three cell lines whose signals were weak but detectable on the FLN29/Xaf-2 autoradiogram. Of the two FLN29/Xaf-2 bands detected, the 3.4 Kbp band appeared to be slightly more intense than the 7.8 Kbp band in all cell lines studied. In short, the FLN29/Xaf-2 locus does not appear to be rearranged, at least in *Hind* III restriction fragments nor does it appear to vary in copy number among pancreatic cancer cell lines.

Fig.5.4. The FLN29/Xaf-2 gene is structurally intact in most pancreatic cancer cell lines.

Three μg of *Hind* III-digested genomic DNAs were separated on a 1% (w/v) agarose gel in 1X TBE buffer, alkali-blotted to a nylon membrane and hybridized with a FLN29/Xaf-2-specific full length cDNA probe. Fragments were sized against a BRL Forensic DNA Analysis Marker (corresponding reference size fragments are indicated beside each autoradiogram). Agarose gel electrophoresis confirmed complete digestion of genomic DNA samples. Blots were washed to high stringency and exposed to X-ray film. No gross rearrangements of the FLN29/Xaf-2 gene were observed in pancreatic cancer cell line as evidenced by the presence of two bands of equal size in all cell lines tested.


markers
human
HPDE6 C7FR5
HPDE6 early
HPDE6 late
pancreas
CRL1 420
CRL1 469
CRL1 682
CRL1 687
CRL1 837
HTB79
HTB80
HTB134
HTB147
markers
Kbp
9.4
6.4
3.4

Hind III

Allelotyping studies having identified the 12q22-23.1 and 12q24.33-qter regions as prone to LOH and homozygous microdeletions, we wished to determine if the genomic region encompassing the FLN29/Xaf-2 locus was affected by LOH. To do so, two fluorescently-labelled microsatellite markers (D12S105 and D12S369) flanking the 12q23 region were used for allelotyping. Both markers used were informative, consistent with their reported heterozygosity indices in the normal population of 72% and 80% for D12S105 and D12S369, respectively (www.resgen.com). Control DNAs were the same as those described for allelotyping of the Xaf-1 genomic region (section 3.2.3) while pancreatic cancer cell lines served as test DNA samples. As can be observed in Table 5.1, none of the pancreatic adenocarcinoma cell lines tested (CRL1420 to PK8) were heterozygous at D12S105 while 4 of the 10 informative cell lines tested (40%) were heterozygous at D12S369. In contrast, 67% and 50% of the control cell lines tested were heterozygous at D12S105 and D12S369, respectively. As explained in Chapter 3, the limited sample size in our analysis can account for the discrepancy with the reported heterozygosity indices.

Overall, these observations suggest that the genomic region encoding the FLN29/Xaf-2 locus, although structurally intact, may be prone to loss of heterozygosity in pancreatic cancer cell lines.

5.2.4. The mRNA encoding FLN29/Xaf-2 mRNA appears to be over-expressed in pancreatic adenocarcinoma cell lines

We next opted to assess FLN29/Xaf-2 mRNA expression in pancreatic cancer cell lines. RT-PCR using FLN29/Xaf-2-specific primers revealed that the FLN29/Xaf-2 mRNA was expressed in normal pancreatic tissue and in pancreatic cancer cell lines although these

Table 5.1. General trend towards loss of heterozygosity at the FLN29/Xaf-2 locus in pancreatic cancer cell lines.

Forty ng of genomic DNA were amplified using fluorescently-labelled D12S105 and D12S369 microsatellite markers flanking the FLN29/Xaf-2 region. Amplicons were resolved on an ABI 310 gene sequencer, detected by charge-coupled device capture of laser-induced fluorescence and analysed using GeneTyper 3.7. Peaks whose height was less than 32% of the highest peak were considered background and disregarded. Y indicates heterozygous cell lines while N applies to homozygous cell lines. N/A = non-available

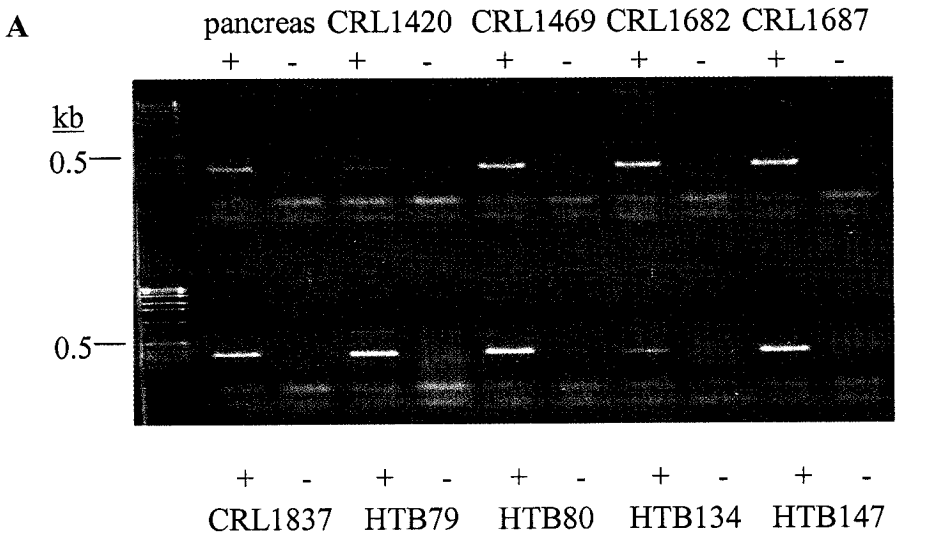
	Cell line	D12S105 (0.72)	D12S369 (0.80)
Normal cell lines	Human	Y	Y
	GM38	N	N
	GM9948	Y	N
	Hpan	Y	N
	HPDE4	Y	Y
	HPDE6	N	Y
Tumor cell lines	CRL1420	N	N
	CRL1469	N	Y
	CRL1682	N	Y
	CRL1687	N	N
	CRL1837	N	Y
	HTB79	N	N/A
	HTB80	N	N
	HTB134	N	N
	HTB147	N	N
	PK1	N	Y
	PK8	N	N
	% Het	0	0,4

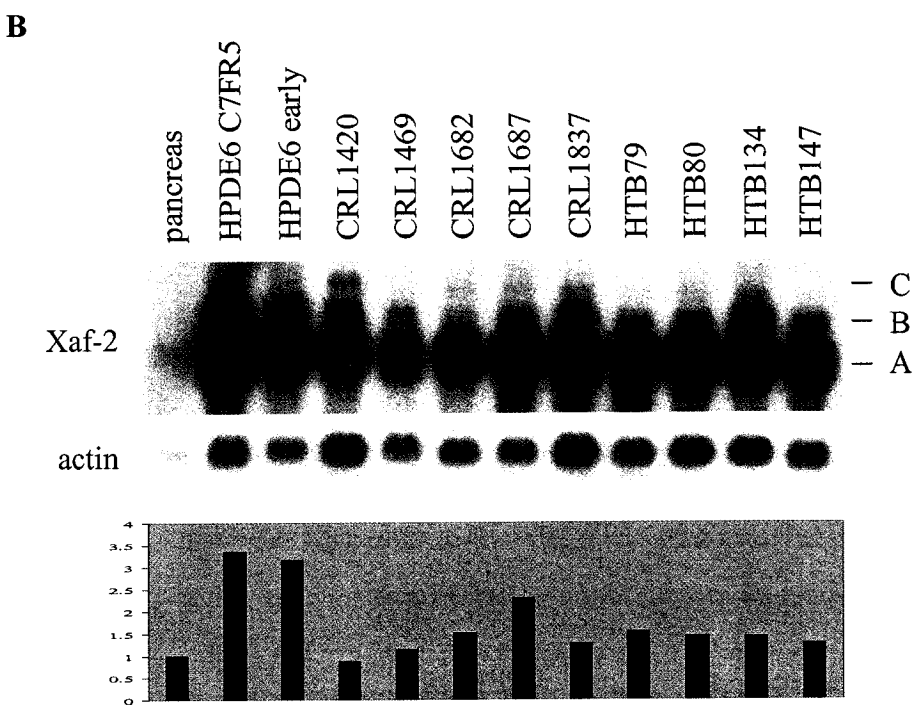
observations are not quantitative (Fig.5.5A). Semi-quantitative assessment of FLN29/Xaf-2 steady-state mRNA levels was subsequently performed by processing mRNAs isolated from non-diseased human pancreatic tissue, immortalized ductal cell lines and pancreatic tumor cell lines for hybridization with a FLN29/Xaf-2-specific probe (Fig.5.5B). Contrary to previous observations in non-diseased tissues where a single 2.8 Kbp FLN29/Xaf-2 transcript was observed, two major FLN29/Xaf-2 transcripts (arbitrarily termed A and B) were detected in immortalized ductal cell lines and pancreatic cancer cell lines, the lower molecular weight transcript (A) being of greater intensity than the higher molecular weight one (B). In addition, the intensity of transcript variant A was relatively constant while the intensity of transcript variant B varied greatly among cell lines ranging from barely detectable (eg. CRL1682) to very strong (eg. CRL1837 and HTB134). A possible third transcript (arbitrarily labelled C) was detected in three pancreatic cancer cell lines (CRL1420, CRL1837 and HTB134). Given the intensity of transcript variant A, we propose that this transcript corresponds to the 2.8 Kbp transcript observed in non-diseased human tissues.

As previously indicated, RNA isolated from human pancreatic tissue is often of poor quality. In fact, even though equivalent amounts (100 μg) of total RNA from pancreatic tissue and all the other cell lines were used as starting material for mRNA isolation, the intensity of the actin signal clearly indicated that there was much less mRNA from normal pancreatic tissue on the blot than for any other cell line tested. Nonetheless, densitometric analyses suggest that the mRNA encoding FLN29/Xaf-2 tends to be over-expressed in pancreatic adenocarcinoma cell lines.

Fig.5.5. The mRNA encoding FLN29/Xaf-2 is expressed in the normal pancreas as well as in pancreatic cancer cell lines.

A. First-strand cDNAs were synthesized from 5 μg of total RNA using oligo(dT) and SuperScript II RNase H^- reverse transcriptase. Two μL aliquots of these cDNAs (+) along with no RT controls (-) were amplified using FLN29/Xaf-2-specific primers. Amplicons were resolved on a 2% (w/v) agarose gel in 1X TBE buffer and sized against a DNA ladder (1 Kbp, Invitrogen). Amplicons corresponding to the expected size (350 bp) were detected in all cell lines tested. No amplification was observed in no RT controls thereby confirming that RNA was isolated free of contaminating genomic DNA. **B**. mRNAs from normal pancreatic tissue, immortalized ductal and pancreatic cancer cell lines were isolated from 100 μg of total RNA, separated by 1% (w/v) denaturing gel electrophoresis and blotted to a nylon membrane. Membrane was hybridized with a FLN29/Xaf-2-specific cDNA probe, washed to high stringency and exposed to X-ray film. To control for lane-to-lane loading, membrane was stripped and re-hybridized with β-actin. Densitometric analysis (bottom panel) revealed that pancreatic cancer cell lines tend to over-express FLN29/Xaf-2 mRNA.


A
pancreas CRL1420 CRL1469 CRL1682 CRL1687
+ - + - + - + - + -
kb
0.5
0.5
+ - + - + - + - + -
CRL1837 HTB79 HTB80 HTB134 HTB147


B
pancreas
HPDE6 C7FR5
HPDE6 early
CRL1420
CRL1469
CRL1682
CRL1687
CRL1837
HTB79
HTB80
HTB134
HTB147
Xaf-2
C
B
A
actin
4
3.5
3
2.5
2
1.5
1
0.5
0

5.2.5. The FLN29/Xaf-2 coding sequence is not mutated in pancreatic adenocarcinoma cell lines

Having observed expression of FLN29/Xaf-2 mRNA in pancreatic cancer cell lines, we wanted to determine if this expression was coupled to a mutational event which could affect the functional properties of the FLN29/Xaf-2 protein. To verify this, the full-length FLN29/Xaf-2 cDNAs were sequenced in normal pancreatic tissue, immortalized ductal cell lines and pancreatic adenocarcinoma cell lines and compared to the FLN29/Xaf-2 sequence deposited in GenBank (accession # NM_006700). This analysis failed to uncover any mutations. Thus, we conclude that the FLN29/Xaf-2 coding sequence is not a mutational target during pancreatic carcinogenesis.

5.2.6. The FLN29/Xaf-2 gene is over-expressed in human solid tumors

Thus far, we have shown that the mRNA encoding FLN29/Xaf-2 appears to be over-expressed in pancreatic tumor cell lines. To expand these observations to other solid malignancies and investigate the generality of this phenomenom, matched normal/tumor RNA sets from a variety of human malignancies were probed with full-length FLN29/Xaf-2 cDNA. Unfortunately, the presence of a low number of samples for pancreas (n=1), cervix (n=1) and small intestine (n=2) prevented their inclusion in our analysis. Tumors were deemed to over-express FLN29/Xaf-2 mRNA when expression in the tumor sample was at least twice that of the matched normal sample. In contrast, the opposite observation, i.e. tumors expressing half the amount of FLN29/Xaf-2 compared to their matched normal counterparts were considered to be FLN29/Xaf-2 under-expressors.

We observed over-expression of FLN29/Xaf-2 mRNA in 36% of the tumors analyzed (all sites combined) while 19% of tumors under-expressed this mRNA (Fig.5.6). It is interesting to note that in sites such as ovary, stomach, breast and kidney, FLN29/Xaf-2 over-expression occured in 43-50% of these samples while the proportion of tumors under-expressing FLN29/Xaf-2 was quite low (5-15%) in these tissues. In short, this analysis suggested that the expression of FLN29/Xaf-2 mRNA in solid tumors appears to be biased towards over-expression. Nonetheless, a significant proportion of tumors (45%) exhibited no clear difference in FLN29/Xaf-2 expression between normal and tumor RNA. As no ubiquitin control was available for this particular Cancer Profiling Array, this analysis will be repeated to include Traf2 and ubiquitin, perform densitometric analyses and confirm these findings.

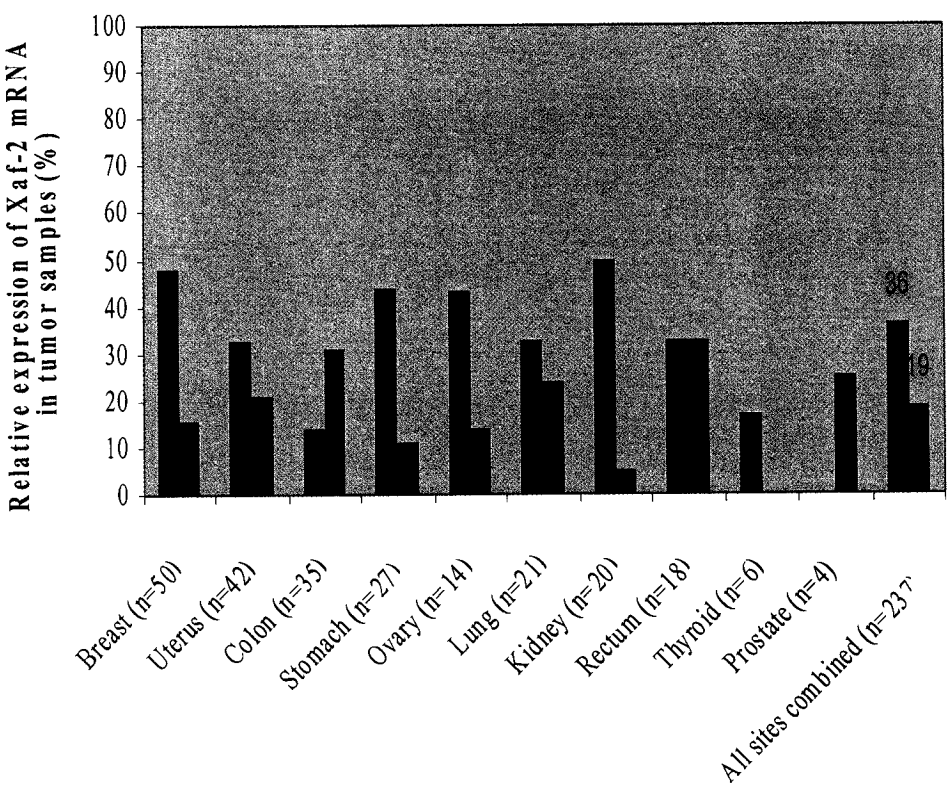
5.3. Summary of results

The analysis of a candidate IAP-interacting protein, FLN29/Xaf-2 performed in this chapter revealed that FLN29/Xaf-2 is highly conserved among animal species. In addition, a 2.8 Kbp FLN29/Xaf-2 transcript encoding a 582 amino acid protein predicted to be 64.8 kDa and have a pI of 5.01 was found to be ubiquitously expressed in non-diseased human tissues.

Analysis of FLN29/Xaf-2 in pancreatic adenocarcinoma cell lines revealed that the genomic locus encoding FLN29/Xaf-2 was not subject to gross structural rearrangement, at least at *Hind* III restriction sites. In addition, copy numbers of the FLN29/Xaf-2 gene did not appear to vary significantly among cell lines while the coding sequence was not subject to mutagenesis. On the other hand, the FLN29/Xaf-2 genomic region was shown to be prone to

Fig.5.6. FLN29/Xaf-2 mRNA appears to be over-expressed in human solid tumors.

Normalized SMART™-amplified cDNA from tumors and corresponding normal tissues (Cancer Profiling Array, Clontech) were probed with FLN29/Xaf-2-specific cDNA. Band intensities were analysed visually due to the lack of a loading control. Tumor samples over-expressing (red bars) and under-expressing (blue bars) FLN29/Xaf-2 are expressed as a percentage of total sample numbers.


Relative expression of Xaf-2 mRNA in tumor samples (%)
100
90
80
70
60
50
40
30
20
10
0
36
19
Breast (n=50)
Uterus (n=42)
Colon (n=35)
Stomach (n=27)
Ovary (n=14)
Lung (n=21)
Kidney (n=20)
Rectum (n=18)
Thyroid (n=6)
Prostate (n=4)
All sites combined (n=237

loss of heterozygosity. Steady-state levels of FLN29/Xaf-2 mRNA appeared to be over-expressed in pancreatic cancer cell lines. Finally, analysis of FLN29/Xaf-2 mRNA expression in matched normal/tumor RNA sets of solid tumors uncovered an apparent trend towards over-expression of FLN29/Xaf-2 in human solid malignancies.

Chapter 6: General Discussion

Late-stage diagnosis and poor responses to anti-cancer therapies contribute to the dismal prognosis of pancreatic cancer. The efficacy of many chemotherapeutic agents relies on their ability to trigger apoptosis (Kaufmann and Earnshaw, 2000; Dive, 1997; Makin and Hickman, 2000; Haq and Zanke, 1998). However, accumulating evidence suggests that resistance to apoptosis is a hallmark of cancer cells (reviewed in Hanahan and Weinberg, 2000). In fact, animal models of pancreatic carcinogenesis have revealed that tumor cells utilize multiple pathways to down-modulate apoptosis (Hager and Hanahan, 1999). As such, rationalized drug designs require the identification of molecular targets in the apoptotic pathway that are differentially regulated in normal and neoplastic cells (L. Yang et al., 2003).

Several reports now document the up-regulation of IAPs (eg. survivin, Xiap, cIAP-1 and cIAP-2) in many human malignancies (Satoh et al., 2001, Ambrosini et al., 1997; Sarela et al., 2001, Tanaka et al., 2000, Tamm et al., 2000, Jonsson et al., 2003, Gagnon et al., 2003). In addition, Xiap over-expression has been correlated with decreased survival of acute myelogenous leukemia patients (Tamm et al., 2000) while a postulated causal chromosomal translocation involving cIAP-2 (leading to its over-expression) has been uncovered in MALT lymphomas (Dierlamm et al., 1999). Finally, recent studies suggest that over-expression of IAPs confers resistance to several apoptotic triggers (Jonsson et al., 2003; Holcik et al., 2000; Crul et al., 1997) while down-regulation of Xiap was shown to induce apoptosis in chemoresistant human ovarian cancer cells following exposure to cisplatin (Sasaki et al., 2000).

One proposed mechanism for resistance to cytotoxic therapies involves elevating the apoptotic threshold. We hypothesized that high levels of anti-apoptotic proteins and low levels of pro-apoptotic factors might result in decreased sensitivity to apoptotic triggers; in

other words, a shift towards an elevated apoptotic threshold might explain, at least in part, the apparent intrinsic resistance of pancreatic tumors to cytotoxic therapies.

6.1. Inhibitor of apoptosis proteins and their contribution to chemoresistance

Several studies have reported over-expression of IAPs in human malignancies (Ambrosini et al., 1997; Satoh et al., 2001; Imoto et al., 2001, 2002; L. Yang et al., 2003). In particular, while survivin is not expressed in differentiated adult tissues, it is expressed in neoplastic samples (Ambrosini et al., 1997). Moreover, survivin over-expression was reported to correlate with pancreatic cancer cell apoptosis (Satoh et al., 2001). Finally, a recent study revealed that survivin expression could be up-regulated by oncogenic H-ras (Sommer et al., 2003), an observation that is quite interesting given that up to 95% of pancreatic tumors show constitutive activation of Ki-ras (Almoguera et al., 1988; Smit et al., 1988; Scarpa et al., 1994; Hruban et al., 1993). Published studies regarding the expression of apoptotic regulators in pancreatic cancer have uncovered that: 1. the high expression of pro-apoptotic bax mRNA correlates with longer survival times (Friess et al., 1998), 2. caspase-1 over-expression is associated with the expression of cyclin D1, EGF and EGF-R, all of which contribute to a poor prognosis (Gansauge et al., 1998), 3. cytoplasmic localization of over-expressed caspase-3 mRNA may contribute to the invasiveness of pancreatic tumors (Satoh et al., 2000) and 4. the up-regulation of survivin may be an early event in the development of pancreatic cancer (Satoh et al., 2001).

To our knowledge, our study represents the first systematic analysis of steady-state levels of apoptotic modulator/effector gene expression in pancreatic adenocarcinoma cell lines and of the IAPs in cell lines and clinical samples. We opted to characterize the

expression of IAPs and their regulators in pancreatic tumors for several reasons. First, IAPs are currently believed to be the only direct antagonists of caspase activity; caspases being crucial to the induction and execution of programmed cell death (Deveraux et al., 1997; Roy et al., 1997, Maier et al., 2002). Second, the identification of IAP antagonists in mammalian systems suggests that IAP expression is itself submitted to strict control and implies that deregulating interactions between caspases, IAPs and IAP antagonists should influence cancer cell survival and impact on therapeutic outcome. For instance, it has been proposed that an interaction between Xaf-1 and Xiap sequesters the latter away from the apoptosome allowing for unrestricted caspase activity thus favoring cell death (Liston et al., 2001). In addition, as Smac/DIABLO and Omi/HtrA2 compete with Xiap for binding to homologous residues on caspase-9, it has been suggested that these IAP antagonists function by displacing Xiap from caspase-9 thereby preventing Xiap's inhibition of caspase-9 activity once again favoring the initiation of cell death (Sun et al., 2000; Srinivasula et al., 2001). Lastly, preliminary experiments in our laboratory had revealed that the expression of IAPs and Xaf-1 appeared to be altered in pancreatic cancer cell lines. Based on this, our first objective was to establish steady-state levels of apoptotic modulator/effector genes in non-diseased and neoplastic pancreatic tissues as well as in immortalized ductal and pancreatic adenocarcinoma cell lines.

When this project was undertaken, Xaf-1 was the only Xiap-interacting factor that we knew about (publications regarding Smac/DIABLO and Omi/HtrA2 came at a later date). In *Drosophila*, five pro-apoptotic genes (hid, grim, rpr, skl and Jafrac2) were shown to inhibit the activity of DIAP1 by blocking caspase-binding sites in DRONC, the *Drosophila* homolog of human caspase-9 and promoting caspase activation (Goyal et al., 2001; Song et al., 2000; Tenev et al., 2002). In addition, it has been shown that hid, grim and rpr mRNAs

are negatively regulated by an activated ras oncogene (Bergmann et al., 1998; McNeill and Downward, 1999; Kurada and White, 1998; Sawamoto et al., 1998), an observation that is particularly relevant as the ras/MAPK signaling pathway is constitutively active in most pancreatic tumors. These observations raised the possibility that lack of Xaf-1 expression could be due to constitutively active ras signaling as is the case in *Drosophila*. On the premise that functional homologs often share consensus sequences, we assessed the conservation of the Xaf-1 locus across species by generating a zooblot (containing DNA from 10 different species) and hybridizing it with a partial human Xaf-1 cDNA probe. Based on *Hind* III and *Eco* RI restriction patterns, this experiment failed to detect sequences bearing significant similarity to a *Drosophila* equivalent; it did, however, reveal hybridization of the human Xaf-1 probe with rhesus monkey and pig suggesting evolutionary divergence of the Xaf-1 locus. Lack of signal in mouse was surprising as a murine sequence similar to human Xaf-1 was deposited in GenBank (acc.# NM_354621). However, to significantly conclude that sequences related to human Xaf-1 are not found in *Drosophila* or mouse, we would need to repeat this experiment using a wider array of restriction enzymes as one cannot exclude the possibility that the *Hind* III/*Eco* RI fragments containing Xaf-1-related sequences were too large to transfer to the membrane. Additionally, a comparative genome blast could be attempted to support the findings. In short, as *Drosophila* death genes differ in sequence among each other but share structural and functional domains (White et al., 1994), it is still possible that human Xaf-1 is a functional homolog of the aforementioned *Drosophila* death genes. We could verify this by transfecting *Drosophila* cells lacking death genes with human Xaf-1 and evaluating the effect on caspase activity. If Xaf-1 is a functional homolog of the *Drosophila* death genes, we would expect *Drosophila* embryos expressing Xaf-1 to be

sensitive to apoptotic triggers. Alternatively, genetic complementation studies could provide insight into the function of Xaf-1.

Previous reports revealed that the genomic region encompassing Xaf-1 is prone to loss of heterozygosity in a majority of cell lines from the NCI 60 cancer cell line panel (Fong et al., 2000). In addition, Xaf-1 lies proximal to p53 (Fong et al., 2000) in a genomic region known to be affected by LOH in pancreatic cancer (Redston et al., 1994; Johansson et al., 1992). Microsatellite allelotyping using markers flanking the Xaf-1 locus revealed a marked reduction to homozygosity. In fact, although our sample size was limited, only one of 11 pancreatic cancer cell lines tested (CRL1837) was heterozygous at both microsatellite markers studied (het. index ~80%). Unfortunately, the unavailability of matched clinical samples precluded the analysis of Xaf-1 heterozygosity in diseased versus control tissues from the same individuals. Given the proximity of the Xaf-1 and p53 loci, it is tempting to speculate that loss of Xaf-1 coupled with an inactivation of p53 could act in synergy during tumorigenesis. Indeed, loss of p53 would allow the accumulation of unrepaired DNA lesions and genomic instability while Xaf-1 loss would raise the apoptotic threshold and facilitate mutagenesis by allowing Xiap to exert its anti-apoptotic function. As several apoptotic pathways have been reported to be p53-dependent (Jin et al., 2003; Su et al., 2002; Blandino et al., 1999; Lutzker and Levine, 1996; Sabbatini et al., 1995; Wu and Levine, 1994), inactivation of p53 would also likely result in resistance to apoptogenic signals. Targeting this particular DNA region for deletion would therefore hinder two mechanisms intimately involved in malignant transformation and tumor progression.

The reported absence of Xaf-1 mRNA expression along with the demonstration of LOH in human cancer cell lines suggested that Xaf-1 may function as a tumor suppressor gene (Fong et al., 2000). By definition, inactivation of both alleles of tumor suppressor

genes is required to effectively suppress their function (Knudson, 1971). While the observed LOH at the Xaf-1 locus can account for the inactivation of one Xaf-1 allele, it fails to explain inactivation of the second allele. Epigenetic events such as methylation of cytosine residues within the context of CpG islands and histone deacetylation have recently been proposed to play a role in gene silencing (Worm and Guldberg, 2002; Garinis et al., 2002, Baylin, 2002; Nephew and Huang, 2003). In light of this, we hypothesized that methylation of Xaf-1 could account for its previously reported lack of expression (Fong et al., 2000). Indeed, treatment with the DNA methyl transferase inhibitor 5'-aza-2'-deoxycytidine resulted in increased Xaf-1 mRNA expression suggesting that Xaf-1 is methylated. To confirm this observation, DNA from three pancreatic cancer cell lines were digested with *Hpa* II and *Msp* I and processed for hybridization with a Xaf-1-specific cDNA probe. The isoschizomers *Hpa* II and *Msp* I recognize the same consensus sequence except that, while *Hpa* II cleavage is blocked by CpG methylation, cleavage by *Msp* I is insensitive to methylation. This analysis revealed that Xaf-1 is, in fact, methylated as differential restriction patterns were observed in *Hpa* II versus *Msp* I-digested samples. Indeed, whereas a strong 1.2 Kbp *Msp* I band was detected in the three pancreatic cancer cell lines tested, this band was absent in *Hpa* II-digested samples from the same cell lines. In addition, treatment with 5'-aza-2'-deoxycytidine resulted in the appearance of a 1.2 Kbp band in *Hpa* II-digested samples further supporting reversible Xaf-1 methylation in pancreatic cancer cell lines. Taken together, these observations support methylation of CpG islands as a means of silencing Xaf-1 mRNA expression as was recently reported in human gastric adenocarcinomas (Byun et al., 2003). However, while hypermethylation of the Xaf-1 promoter region was analyzed in human gastric cancers, our study suggests intragenic methylation of the Xaf-1 gene as we used a Xaf-1-specific probe derived from the coding region of the gene.

Having hypothesized that deregulated expression of apoptotic modulator/effector genes contributes to the intrinsic resistance of pancreatic tumors to cytotoxic therapies, we expected our systematic profiling of apoptotic regulators to uncover up-regulation of IAPs and down-regulation of pro-apoptotic factors such as caspases and IAP antagonists. We chose to express mRNA and protein expression levels in pancreatic cancer cell lines relative to those of the immortalized ductal cell line HPDE6 late for several reasons. First, RNA isolated from total pancreatic tissue is often of poor quality. Second, the pancreatic adenocarcinoma cell lines studied were of ductal origin. Lastly, we chose HPDE6 late rather than HPDE6 early as the former cell line has emerged from the immortalization crisis. We assessed the expression of apoptotic modulator/effector genes via Northern hybridization, RPA and immunoblotting. However, we did observe a few contradictions among these profiles. Indeed, while Xiap mRNA levels were shown to be down-regulated by Northern hybridization, RPA analyses uncovered a slight tendency towards over-expression of Xiap mRNA in pancreatic cancer cell lines. Western profiles for Xiap agreed with Northern profiles in that all cell lines studied showed reduced expression of Xiap but disagreed with RPA profiles. The discrepancy between Xiap mRNA and protein levels is possibly due to the reported IRES element located upstream of the initiation codon of Xiap (Holcik et al., 1999). It was suggested that regulation of Xiap gene expression may well be translational rather than transcriptional (Holcik et al., 2000). This proposed mechanism of Xiap translation would be beneficial to cancer cells. Under stress conditions, for instance, exposure to chemotherapeutic agents, cap-dependent translation usually shuts down. By allowing cap-independent translation of Xiap, cancer cells would be able to express high levels of Xiap protein and effectively prevent caspase activity resulting in resistance to apoptotic triggers. It was also reported that IRES-mediated translation of Xiap protected

cells against serum withdrawal-induced apoptosis (Holcik et al., 1999). On the other hand, IRES-mediated translational up-regulation of Xiap was observed upon treatment with low doses of γ-irradiation; Xiap up-regulation correlated with enhanced survival of non small cell lung carcinoma cell lines (Holcik et al., 2000).

Next, cIAP-1 Northern and RPA profiles mostly agreed; a good proportion of pancreatic cancer cell lines showing over-expression (up to 5 fold) of cIAP-1 mRNA. Although non quantitative, the expression of cIAP-1 protein also appeared to be up-regulated in pancreatic cancer cell lines, in agreement with mRNA levels. In contrast, while RPA analysis revealed that all but two cancer cell lines under-expressed cIAP-2 mRNA, Northern blots revealed cIAP-2 mRNA to be significantly (more than 2-fold) up-regulated in 4 cancer cell lines. Western blots, though not quantitative, revealed variable expression of cIAP-2 protein in pancreatic tumor cell lines. Finally, while survivin was shown to be up-regulated in malignant versus benign pancreatic tumors by immunohistochemistry (Satoh et al., 2001), our analysis uncovered a down-regulation of both survivin mRNA and protein in most pancreatic cancer cell lines studied.

Xaf-1 and Smac/DIABLO having been identified as antagonists of Xiap activity (Liston et al., 2001; Du et al., 2000; Verhagen et al., 2000), we predicted the expression of their respective gene products to be significantly down-regulated in pancreatic cancer cell lines thereby facilitating Xiap-mediated caspase inhibition. In agreement with earlier reports (Fong et al., 2000), our Northern analysis showed down-regulation of Xaf-1 mRNA in a majority (5/9) of pancreatic cancer cell lines while expression of Xaf-1 mRNA in the remaining cell lines was similar to that of the control cell line. However, the Xaf-1 protein was not detected in any of the cancer cell lines tested. Lack of concordance between Xaf-1

mRNA and protein profiles may be due to the poor quality of the Xaf-1 antibody. Alternatively, it may be that Xaf-1 expression is regulated at the translational level or by post-translational modifications or that Xaf-1 is repressed by constitutively active ras signaling as was reported for hid in *Drosophila* (Bergmann et al., 1998; Kurada and White, 1998). Next, expression of Smac/DIABLO mRNA and protein were not significantly altered in most pancreatic cancer cell lines although mRNA profiles revealed a mild (maximum 2-fold) induction in Smac/DIABLO expression while protein profiles revealed a mild repression (maximum 50% of control expression) of Smac/DIABLO.

Caspases being crucial for the induction and execution of the apoptotic program, we hypothesized that down-regulating caspase expression would confer a survival advantage to tumor cells. Indeed, a trend towards down-regulation (although probably not statistically significant) of both initiator and effector caspases in most pancreatic tumor cell lines studied was revealed by RPA. In addition, we observed decreased caspase-3 and -9 protein expression in all pancreatic cancer cell lines tested compared to normal pancreatic tissue by immunoblotting. It is important to point out that caspase expression was not completely repressed in cancer cell lines in agreement with a recent study reporting the coexistence of high levels of apoptotic signaling factors and IAPs (indicative of intact apoptotic machinery) in human cancer cell lines (L. Yang et al., 2003). It was also proposed that the molecular changes associated with malignant transformation activated apoptosis; however, up-regulation of IAPs blocked the caspase cascade further supporting a role for IAP up-regulation in the resistance of tumors to apoptosis (L. Yang et al., 2003). On the other hand, protein expression of the adaptor protein Apaf-1 was not significantly altered in pancreatic cancer cell lines.

The relative abundance of pro- versus anti-apoptotic members of the bcl-2 family has been postulated to regulate the onset of apoptosis (reviewed in Adams and Cory, 1998). Among other effects, bcl-2 has been shown to exert its anti-apoptotic function by preventing the mitochondrial release of cytochrome c (Yang et al., 1997; Kluck et al., 1997) while bax can induce cytochrome c release (Gross et al., 1998; Rosse et al., 1998) leading to activation of procaspase-9 and induction of apoptosis. Over-expression of bcl-2 has been correlated with apoptotic resistance and increased metastatic potential in pancreatic cancer cell lines (Bold et al., 2001). In addition, enhanced sensitivity to ionizing radiation was associated with the induction of bax expression in the pancreatic cancer cell line CRL1420 (Ahmed et al., 2002). Our analysis of steady-state levels of apoptotic regulatory proteins uncovered a pronounced down-regulation of bcl-2 and ubiquitous expression of bax in pancreatic cancer cell lines. These findings suggest that bcl-2 might not play a determining role in increasing the apoptotic threshold of pancreatic tumors. Furthermore, the combined expression of pro-apoptotic bax and apparent repression of bcl-2 would likely favor an increased sensitivity to apoptotic triggers. As pancreatic tumors are known to be refractory to apoptotic stimuli, we propose that IAPs are key regulators of apoptosis in these cell lines as they appear to compensate for varying levels of bcl-2. Interestingly, the recent report that hyperplastic pancreatic ductal lesions do not express bcl-2 suggests that bcl-2 is not likely involved in the progression of pancreatic malignancy (Campani et al., 2001). Even so, in cases where cells show elevated expression of bcl-2 and IAPs, both might synergize to elevate the apoptotic threshold.

We then sought to expand our analysis to other solid human malignancies in order to correlate these observations with our findings in pancreatic cancer cell lines. Analysis of mRNA levels in tumors versus matched non-tumorigenic controls revealed that 15, 5 and

24% of tumors analyzed over-expressed Xiap, cIAP-1 or cIAP-2 mRNA, respectively while 32, 24 and 18% of said tumors showed decreased levels of Xiap, cIAP-1 and cIAP-2 mRNA, respectively. It is interesting to note a bias towards down-regulation of Xiap and cIAP-1 mRNA and up-regulation of cIAP-2 mRNA. The down-regulation of Xiap mRNA and up-regulation of cIAP-2 mRNA are in agreement with our Northern profiles in pancreatic cancer cell lines. However, there is a discrepancy between the up-regulation of cIAP-1 mRNA encountered in pancreatic cancer cell lines and the tendency towards down-regulation in human solid tumors. In contrast, analysis of survivin mRNA expression in a similar array of matched normal/tumor samples revealed that 44% of the tumors surveyed over-expressed survivin mRNA while none of the tumors surveyed under-expressed survivin mRNA. These observations agree with previous reports regarding survivin over-expression in human malignancies (Ambrosini et al., 1997; Adida et al., 1998; Lu et al., 1998; Kawasaki et al., 1998, Tanaka et al., 2000; Sarela et al., 2001; Satoh et al., 2001). Of particular interest, analysis of survivin expression in pancreatic tumor samples revealed that 43% (3/7) of tumors analyzed significantly (more than twice) over-expressed survivin, which is contrary to what we observed in pancreatic cancer cell lines. The discrepancy can best be explained by the small number of samples available and by the fact that survivin expression in the CPA was assessed in matched normal/tumor RNA sets while our Northern analysis compared survivin expression in tumor cell lines to that of an immortalized ductal cell line control.

This study also revealed that alterations in IAP mRNA expression differ depending on the origin of the tumor. For instance, we observed an up-regulation of cIAP-2 mRNA in 65% of kidney tumors whereas Xiap and cIAP-1 mRNAs were down-regulated 30 and 65%, respectively of the same kidney tumors. As such, it would be very interesting to correlate IAP expression with prognosis in malignancies which show bias towards up- or down-

regulation of specific IAP mNRAs to determine if up-regulation of Xiap, cIAP-1 or cIAP-2 is associated with shorter or longer survival. Interestingly, previous reports have already documented a correlation between IAP expression and cancer survival. For instance, elevated Xiap levels correlated with decreased survival of AML patients (Tamm et al., 2000) while high levels of survivin were associated with decreased survival of breast and colorectal cancer patients (Tanaka et al., 2000; Sarela et al., 2001). Overall, the over-expression of IAPs, namely cIAP-2 and survivin observed in a significant proportion of human cancers suggests that targeting IAPs for down-modulation may prove beneficial to patients by enhancing sensititivity to current cytotoxic therapies. In fact, recent studies have reported that down-regulation of Xiap expression enhances the response of lung and ovarian cancer cells to chemotherapeutic agents (Hu et al., 2003; Sasaki et al., 2000).

We also addressed the issue of whether the expression of mRNAs encoding pro-apoptotic factors such as IAP antagonists (namely Xaf-1 and Smac/DIABLO) and caspases were differentially expressed in tumor samples compared to matched non-tumorigenic reference tissues with the premise that down-regulation of these factors would confer a survival advantage to tumor populations. Xaf-1 mRNA was found to be down-regulated in 22% of tumors while it was-up-regulated in 17% of tumors. The small bias towards under-expression of Xaf-1 mRNA in tumor samples agrees with earlier findings reporting lack of Xaf-1 mRNA expression in a majority of cancer cell lines (Fong et al., 2000) although we uncovered both Xaf-1 over- and under-expression in a similar proportion of tumor samples. Next, contrary to our predictions, we uncovered over-expression of Smac/DIABLO in 29% of tumors analyzed while 4% of tumors tested under-expressed Smac/DIABLO suggesting that down-regulation of Smac/DIABLO is not a common feature of human neoplasms. Having observed decreased expression of caspase-3 and caspase-9 mRNAs in pancreatic

cancer cell lines, we sought to determine their relative expression in a series of human tumors. We found that 21% and 16% of tumors over- and under-expressed caspase-3 mRNA, respectively while up- and down-regulation of caspase-9 mRNA was observed in 14% and 8% of tumors tested, respectively suggesting that caspase expression is not altered in a significant proportion of tumors.

Overall, the expression of pro-apoptotic factors does not appear to be targeted for deregulation in a significant proportion of tumors. In contrast, we observed a moderate tendency towards up-regulation of survivin and cIAP-2 expression in human malignancies suggesting a role for the up-regulation of these particular IAPs not only in pancreatic tumorigenesis but in malignant transformation of solid tumors in general.

Late-stage diagnosis is a major problem for the treatment of pancreatic cancer since tumors are usually identified after the dissemination of cancer cells to other organs (Eriksson et al., 1990; Gonzalez-Campora et al., 1995; Staley et al., 1996). In fact, lung, liver and spleen are prime metastatic sites for pancreatic tumors, a factor that significantly hampers satisfactory therapeutic outcomes since successful eradication of pancreatic cancer must target both the primary mass and its metastases. As a result, it is important to evaluate the intrinsic apoptogenic potential of metastatic host tissues in a non-diseased state. Analysis of non-diseased human tissues revealed ubiquitous expression of Xaf-1, cIAP-1, cIAP-2 and Xiap mRNAs and/or proteins, in agreement with previous findings (Liston et al., 1997, 2001; Lagacé et al., 2001; Young et al., 1999). However, mRNA profiles did not always correlate with protein expression. For instance, in spite of a strong expression of Xaf-1 mRNA in heart, spleen and liver, we were unable to detect a protein of the predicted size (33 kDa) in any of the tissues tested, possibly due to the poor quality of the antibody. In addition, while Xiap mRNA was strongly expressed in all tissues surveyed except for lung, brain, spinal

cord and bone marrow (as was reported in a prior study (Lagacé et al., 2001)), lack of Xiap protein expression in spleen, heart and liver was surprising. The reason for this remains to be determined.

We also investigated the expression of Xiap, cIAP-1, cIAP-2 and survivin in *in vivo* samples of evolving intraductal neoplastic lesions and adenocarcinomas of the pancreas via immunohistological assays. This survey uncovered over-expression of cIAP-1, cIAP-2 and survivin coupled with redistribution of these IAPs to the nuclei of cancerous cells. On the contrary, the expression and subcellular localization of Xiap appeared to be unaffected in pancreatic adenocarcinomas. The mechanism behind the unexpected redistribution of IAPs in high grade adenocarcinomas remains to be determined. Nonetheless, nuclear redistribution could prove beneficial to cancer cells if the phenomenon serves to sequester an IAP coupled to a caspase (eg. caspase-3) away from the cytoplasm where initiation of the apoptotic cascade transpires. Whether this redistribution is mediated by Xaf-1 (Liston et al., 2001) or by the mitochondrial Xiap-binding protein ARTS (Larisch et al., 2000), two proteins which are capable of translocating to the nucleus remains to be investigated.

6.2. IAPs in etoposide-induced apoptosis

Given that over-expression of IAPs has been reported to suppress etoposide-induced apoptosis (Uren et al., 1996; Deveraux et al., 1997, 1998), our second objective was to verify if differential IAP expression influenced the response of pancreatic cancer cell lines to etoposide. Treating cancer cells with a dose range of etoposide clearly established that cancer cells die in response to increasing doses of the DNA-damaging agent. Nonetheless, the cancer cell lines studied differed in their sensitivity to etoposide as evidenced by the

magnitude of the response to the drug. Indeed, statistical analyses revealed that, of the three cell lines tested, CRL1420 was more sensitive than CRL1469 and CRL1682 to etoposide as evidenced by a more pronounced cell death in response to equivalent doses of the drug. However, we did not find any statistical difference between CRL1469 and CRL1682 cells in terms of response to etoposide. Etoposide-induced cell death was established as apoptotic due to the presence of apoptotic bodies and PARP cleavage in CRL1420.

The response of CRL1420 cells to etoposide suggested that the cell population is not homogeneous in terms of sensitivity to etoposide. The shape of the survival curve suggests that a subpopulation of cells within the total cell population is more sensitive to etoposide than the rest of the cells as we observed a rapid induction of cell death in CRL1420 which levelled off after 10 μM of etoposide. To investigate this possibility, we could treat cancer cells with etoposide, harvest the surviving cells, treat them again with etoposide, harvest the surviving cells and so on to obtain cells which are increasingly more resistant to etoposide. Following this, we could determine if the more etoposide-resistant cells express higher levels of IAPs. In addition, we could then treat all of these different cell populations with a dose range of etoposide to ascertain whether or not they differ in terms of sensitivity to etoposide. If they do, rational designs of oncotherapies need to take this reality into account as anti-cancer therapies must eliminate all cancer cells in order to be successful.

Next, we addressed the possibility that transcriptional up- or down-regulation of IAPs accounted for the observed differences in etoposide sensitivity. As IAPs are endogenous inhibitors of caspase activity, we hypothesized that IAP expression would decrease with time in cells responding to etoposide to allow for unrestricted initiation of the caspase cascade. Surprisingly, our analysis revealed that, in response to etoposide, Xiap, cIAP-1, cIAP-2 and

survivin RNA levels were up-regulated in all three cell lines tested; the transcriptional induction being maximal 72h post-etoposide challenge. Surprisingly, fold increases in cIAP-1, cIAP-2 and, to a lesser extent, Xiap and survivin mRNAs were greater in the more etoposide-sensitive CRL1420 cells, contrary to our hypothesis. Indeed, it was counter-intuitive for us to suggest that IAP mRNA levels be more elevated in cells which are actively dying (CRL1420) than in cells which are more resistant to etoposide-induced cell death (CRL1469 and CRL1682). However, our findings indicate a correlation between the early transcriptional up-regulation of IAPs (mostly for Xiap) and cell death. Given that exposure to etoposide leads to extensive DNA damage which, if unrepairable, initiates the apoptotic response (namely through the activity of caspases), it is possible that IAPs are up-regulated in an attempt to limit the activity of caspases and prevent cell death.

To further characterize IAP expression in etoposide-induced cell death, we surveyed the expression of key apoptotic proteins in etoposide-treated cell lysates and uncovered caspase-dependent cleavage of Xiap protein and activation of caspase-3 in CRL1420 cells. In contrast, cleavage of Xiap and activation of caspase-3 were not observed in the chemoresistant cell line CRL1469. The observed cleavage of Xiap is consistent with previous findings showing that Xiap is cleaved into two distinct fragments in Fas-induced apoptosis (Deveraux et al., 1999). Cleavage of the Xiap protein could thus counteract the observed increase in Xiap mRNA expression in CRL1420 and explain the etoposide-induced cell death observed in this cell line. In contrast, the early up-regulation of Xiap mRNA coupled with the absence of Xiap cleavage in CRL1469 could account for its resistance to etoposide-induced apoptosis. It is interesting to note that cleavage of Xiap occurred later in CRL1682 than in CRL1420 cells possibly accounting for the relative etoposide-resistance of this cell line. However, cleavage of Xiap protein in CRL1682 was not associated with a

significant change in sensitivity to etoposide as survival curves for CRL1682 48 and 96h post-etoposide challenge were similar in terms of slope and magnitude of response. On the other hand, Xiap cleavage has also been reported to lower the threshold of caspase activity necessary for induction of apoptosis (Deveraux et al., 1999). Interestingly, we observed proteolytic cleavage of caspase-3 consistent with activation in CRL1420 48h post-etoposide challenge suggesting a correlation between cleavage of Xiap and unrestricted caspase activity although a direct link between Xiap cleavage and activation of procaspase-3 was not tested. Cleavage of procaspase-3 was not detected in the more resistant cell lines CRL1469 and CRL1682 consistent with their apparent resistance to etoposide-induced apoptosis.

The observed correlation between IAP up-regulation, caspase activity and cell death warrants discussing the so-called "point of no return" in the apoptotic cascade. It was long believed that initiation of the caspase cascade resulted in the absolute commitment of the cell to apoptosis. However, recent findings have established that caspase activity can be controlled. As IAPs are the only known direct regulators of effector caspases, they are prime candidates for prohibiting the uncontrolled activity of caspases. Interestingly, several anti-cancer agents stimulate the release of cytochrome c and Smac/DIABLO from the mitochondria leading to the activation of caspases and induction of apoptosis (Du et al., 2000; Verhagen et al., 2000; Halestrap et al., 2000; Gross et al., 1999). In addition, Smac/DIABLO and Omi/HtrA2 have been shown to antagonize IAP activity (Du et al., 2000; Verhagen et al., 2000; Martins et al., 2002), supporting a model in which the balance between pro- and anti-apoptotic factors is what ultimately determines the fate of the cell (Oltvai et al., 1993). Indeed, Smac/DIABLO peptides were documented to enhance the effects of chemotherapeutic agents by binding Xiap and cIAP-1 *in situ* (Arnt et al., 2002). Further complicating this delicate balance is the recent discovery that both cIAP-1 and cIAP-

2 function as ubiquitin ligases for Smac/DIABLO (Hu and Yang, 2003). By forcing proteosomal degradation of Smac/DIABLO, cellular IAPs would facilitate the anti-apoptotic properties of Xiap. In addition, a recent study has demonstrated that an interaction between Smac and survivin is essential for the anti-apoptotic properties of survivin during TRAIL-induced apoptosis (Song et al., 2003). Moreover, Omi/HtrA2 can catalytically cleave IAPs thereby inactivating them and promoting apoptosis (Q. Yang et al., 2003). Finally, the etoposide-induced activation of p53 increases Omi/HtrA2 transcription (Jin et al., 2003). All of these findings clearly underline the need for intricate control of caspase activity and suggest a predominant role for IAPs in the "point of no return" of the self-amplifying apoptotic cascade. Given our preliminary observations regarding IAP expression in etoposide-induced cell death, we need to extend these studies to include other regulators such as Smac/DIABLO and Omi/HtrA2.

It has been documented that cytotoxic drugs such as etoposide elicit a p53-dependent apoptotic response (Lowe et al., 1993a, 1993b; Perego et al., 1996) although p53-independent apoptosis has also been reported in certain cell types (Bertrand et al., 1993). In fact, p53 can activate the transcription of pro-apoptotic genes such as bax and Fas and trigger apoptosis (Zhan et al., 1994; Miyashita and Reed, 1995, Owen-Schaub et al., 1995). It was recently demonstrated that the mitochondrial release of pro-apoptotic proteins is differentially regulated by members of the bcl-2 family. While bak was proposed to mediate the release of cytochrome c in TRAIL-induced apoptosis, a pivotal role for bax in the release of Smac/DIABLO and Omi/HtrA2 in TRAIL- and drug-induced apoptosis was suggested (Kandasamy et al., 2003; Yamaguchi et al., 2003). As a result, p53-induced activation of bax transcription would trigger the mitochondrial release of Smac/DIABLO and Omi/HtrA2 which would, in turn, prevent Xiap-mediated inhibition of caspase-9 activity. In addition,

the reintroduction of p53 in $p53^{null}$ Saos-2 cells has previously established a link between p53 and the bax-dependent release of cytochrome c from the mitochondrial intermembrane space (Schuler et al., 2000). In the presence of dATP, cytochrome c facilitates oligomerization and activation of Apaf-1 and caspase-9 and, ultimately, the activation of procaspase-3 (Green and Reed, 1998). The pancreatic cancer cell lines studied differ in regard to p53 status. While CRL1682 is $p53^{null}$, CRL1420 contains a partially reactivatable R248W mutant and CRL1469 contains a non-reactivatable R273H mutant (G. Muradia, R. Aubin, unpublished observation). It is tempting to propose that, by some as yet undetermined mechanism, etoposide reactivates p53 in CRL1420 thereby enhancing the ability of this cell line to respond to DNA damage possibly via an up-regulation of bax transcription. Interestingly, preliminary studies in our laboratory have revealed that reactivation of p53 involves phosphorylation on Ser46 and up-regulation of p53-responsive targets such as bax and p21 (G. Muradia and R. Aubin, unpublished observations). In turn, elevated bax levels could trigger the release of Smac/DIABLO and Omi/HtrA2 from the mitochondria which would effectively antagonize Xiap and allow for the induction of programmed cell death. In contrast, the $p53^{R273H}$ mutant in CRL1469 cannot be reactivated, consistent with the decreased response to etoposide observed in this cell line. Alternatively, etoposide-induced apoptosis could proceed via a p53-independent mechanism or result from a combination of both. These are other avenues of research which should be explored to complement the current studies.

Our findings having suggested that IAPs may be important regulators of the apoptotic threshold in pancreatic adenocarcinoma cell lines, we attempted to re-sensitize pancreatic cancer cells to apoptotic stimuli by down-regulating the expression of IAPs, in particular Xiap. In fact, several studies have reported that down-regulation of Xiap expression leads to

enhanced chemotherapeutic activity (Hu et al., 2003; Sasaki et al., 2000; Ferreira et al., 2001; Li et al., 2001; Chow et al., 2003; Notarbartolo et al., 2002). However, while we were successful in restoring Xaf-1 protein expression by transfection with recombinant adenoviral constructs, we were unable to show any sensitization to etoposide-induced cell death, in contrast to previous findings (Liston et al., 2001). Likewise, RNA-mediated interference (in the form of transfection with siRNAs) effectively lowered target protein expression by at least 50% in pancreatic cancer cell lines but did not potentiate etoposide sensitivity. It is important to note that, in the latter case, complete repression of IAP expression was not achieved. It is possible that residual IAP activity is sufficient to effectively prevent apoptosis. Alternatively, the recent report that p53 is required for the chemosensitizing effects of Xiap down-regulation (Fraser et al., 2003) suggests that combining p53 reactivation with Xiap down-regulation may prove more effective for chemosensitization. Then again, it is not unconceivable to think that inactivation of Xaf-1 protein function may be attributable to constitutive ras/MAPK signaling in pancreatic adenocarcinoma cells.

We also observed a uniform decrease in cell survival following down-regulation of survivin expression in CRL1420 cells suggesting that survivin expression is required for the intrinsic survival of these cells. However, this effect was not observed in CRL1469. Determining whether or not survivin is essential to the intrinsic survival of cells (possibly due to its role as regulator of the cell cycle) will require further experimentation. The reason for the lack of concordance between CRL1420 and CRL1469 cells also remains to be determined.

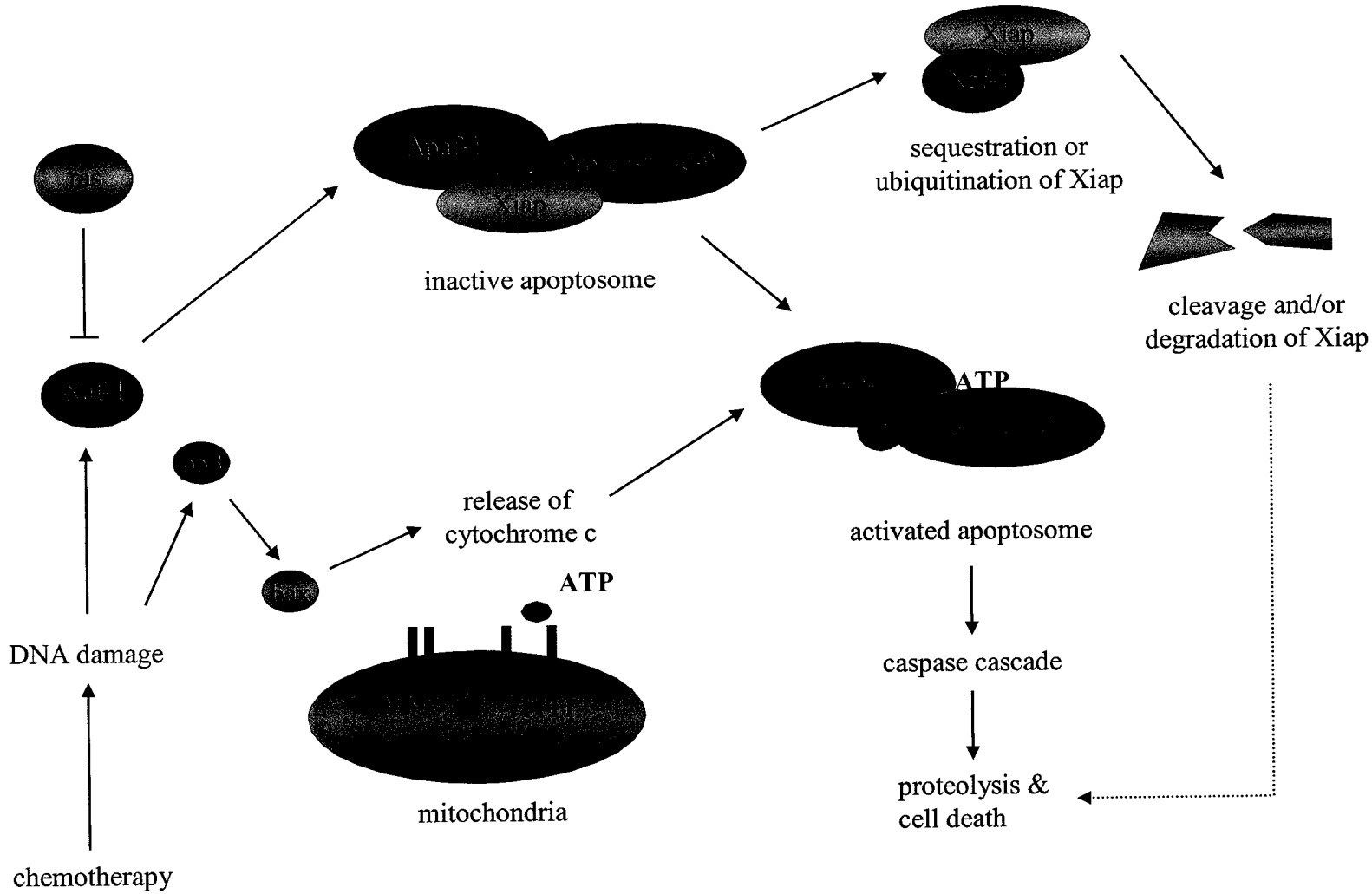
Overall, this part of the project suggested tha differential expression of IAPs in pancreatic adenocarcinoma cell lines may contribute to the intrinsic resistance of pancreatic tumors to cytotoxic therapies via an elevation of the apoptotic threshold. Furthermore, the

data suggested that IAPs appear to be important anti-apoptotic regulators even compensating for varying levels of bcl-2 in pancreatic cancer cell lines. Our data also indicated that Xaf-1 expression is down-regulated in pancreatic adenocarcinoma cell lines. In light of this, we propose that the relative expression of Xiap and Xaf-1 partly accounts for the resistance of pancreatic tumors to cytotoxic therapies. The ratio between Xiap and Xaf-1 was very recently shown to correlate with the resistance of motoneurons to apoptosis (Perrelet et al., 2004). Fig.6.1 depicts a model in which Xiap holds the Apaf-1/procaspase-9 complex inactive and Xaf-1 acts to relieve this repression. If the interaction of Xiap with the apoptosome (through an interaction with procaspase-9) renders the latter inactive, the interaction between Xaf-1 and Xiap may displace/sequester Xiap or could activate the E3 ubiquitin ligase activity of Xiap targeting it or some other protein for degradation. Elevated levels of Xiap could thus contribute to the intrinsic resistance of pancreatic cancer cell lines to cytotoxic therapies by desensitizing them to apoptotic stimuli. The transcriptional and/or translational repression of Xaf-1, possibly via a constitutively active ras oncogene, could free Xiap to interact with the apoptosome and compete with Apaf-1 for binding to the CARD domain of caspase-9 thereby inactivating the intrinsic apoptotic pathway. To investigate this possibility, we could inhibit ras activity using dominant negative ras mutants or pharmacological inhibitors of ras activity such as farnesyl transferase inhibitors and investigate the induction of Xaf-1 expression by immunoblotting.

Xaf-1 was recently identified as an IFN-stimulated gene that augments TRAIL-induced apoptosis (Leaman et al., 2002) further suggesting a role for Xaf-1 in potentiating the apoptotic response. Moreover, a very recent search to identify genes useful for predicting or targeting apoptosis induction revealed that Xaf-1 expression was induced upon

Fig.6.1. Proposed model for Xaf-1 facilitation of the intrinsic apoptotic pathway.

We propose that, during apoptosis, Xaf-1 acts to sequester Xiap away from the apoptosome. Alternatively, binding of Xaf-1 to Xiap could trigger the ubiquitination and degradation of Xiap. Down-regulation of Xaf-1, possibly via constitutive activation of the ras oncogene, would prove beneficial to cancer cells as it would allow unrestricted Xiap activity. In this model, Xiap competes with Apaf-1 for binding to the CARD domain of caspase-9. In contrast, in the absence of Xiap, the cytochrome c and ATP-dependent oligomerization of Apaf-1 and pro-caspase-9 leads to activation of caspase-9 and the initiation of the self-amplifying caspase cascade.


Xiap
sequestration or
ubiquitination of Xiap
inactive apoptosome
cleavage and/or
degradation of Xiap
ATP
activated apoptosome
release of
cytochrome c
ATP
DNA damage
caspase cascade
mitochondria
proteolysis &
cell death
chemotherapy

stimulation with IFN-β in melanoma cells (Leaman et al., 2003). Taken together, these observations support a role for Xaf-1 in facilitating induction of apoptosis. In fact, over-expression of IAPs in neonatal axotomized motoneurons was previously shown to delay cell death (Perrelet et al., 2000, 2002) while over-expression of exogenous Xaf-1 abrogates the natural resistance to axotomy (Perrelet et al., 2004). Our data indicate, at least in pancreatic adenocarcinoma cell lines, that the up-regulation of IAPs together with the apparent silencing of Xaf-1 may contribute to an elevation in the apoptotic threshold which, in turn, leads to an increased resistance to apoptotic stimuli. The interaction between the differential expression of IAPs, IAP antagonists and constitutively active ras signaling clearly warrants further investigation in pancreatic cancer cell lines.

6.3. Characterization of FLN29/Xaf-2, a candidate IAP-interacting protein in pancreatic adenocarcinoma cell lines

Our last objective was to investigate the potential involvement of a novel IAP-interacting factor, FLN29/Xaf-2, in mediating cell survival. Allelotyping of chromosome 12 performed in our laboratory had uncovered generalized loss of heterozygosity and homozygous microdeletions at 12q22-23.1 in pancreatic cancer cell lines (Ethier et al., 1997). Surveys of integrated map data identified FLN29/Xaf-2 as a potential growth regulator in this genomic region. Interestingly, FLN29/Xaf-2 was reported to share partial homology to Xaf-1 and postulated to be an IAP-interacting partner (referenced in Liston et al., 2001 as unpublished observation). In addition, domain searches identified a Traf-like RING zinc finger, a PEST sequence (associated with a propensity to serve as a substrate for proteosomal degradation) and two putative caspase cleavage sites within FLN29/Xaf-2.

Traf1/Traf2 heterocomplexes are postulated to mediate the association of cIAP-1 and cIAP-2 with the TNF-R complex (Rothe et al., 1995). Signaling via the TNF-R has been shown to stimulate both proliferative and anti-apoptotic pathways (Tartaglia et al., 1991, 1993; Wang et al., 2001). It was recently demonstrated that the rapid formation of the membrane-bound complex I (composed of TNF-RI, TRADD, RIP, Traf2 and cIAP-1) stimulates a NF-κB response but fails to trigger apoptosis while the cytoplasmic formation of a secondary complex (complex II) which includes FADD and procaspases-8 and 10 but lacks TNF-RI initiates apoptosis as long as the expression of anti-apoptotic proteins is not induced by NF-κB signaling (Micheau and Tschopp, 2003). Therefore, this model suggest that cIAP-1 might contribute to anti-apoptotic or proliferative outcomes as it can be part of both complexes. In addition, the RZF of Traf2 has been implicated in signal transduction pathways which reportedly activate NF-κB (Rothe et al., 1994, 1995; Berberich et al., 1994); a function proposed to mediate anti-apoptotic signals (Lee et al., 1997; Yeh et al., 1997). In fact, a protective role has been attributed to Traf2 in TNF-α-induced apoptosis (Weiss et al., 1998; Erickson et al., 1994). Furthermore, recruitment of cIAP-1 and cIAP-2 to the TNF-R complex results in inhibition of TNF-α-induced apoptosis (Wang et al., 2001).

Activation of NF-κB signaling can reportedly suppress apoptosis via up-regulation of IAPs and Trafs providing for amplification of anti-death signals (reviewed in Barkett and Gilmore, 1999). In addition, elevated levels of active NF-κB lead to increased cell proliferation and resistance to apoptosis (Biswas et al., 2001; Sun et al., 1998; Ghosh and Karin, 2002) while the chemotherapeutic inhibition of NF-κB activation induces apoptosis (Biswas et al., 2003). In particular, inhibition of NF-κB sensitizes human pancreatic cancer cells to etoposide-induced apoptosis (Arlt et al., 2001). Finally, ras-dependent signaling can

activate NF-κB (Finco and Baldwin, 1993; Norris and Baldwin, 1999; Finco et al., 1997) possibly serving to up-regulate IAP expression.

The N-terminal domain similarities between Xaf-1 and FLN29/Xaf-2 suggested that they may share functional properties. If this were so, Xaf-2, like Xaf-1, may function to negatively regulate IAP expression and cancer cells would therefore gain a survival advantage by repressing FLN29/Xaf-2 expression to allow for unrestricted Xiap activity. Alternatively, the Traf-like RZF within the FLN29/Xaf-2 protein may mediate an interaction with cIAPs and possibly function in NF-κB signaling. In this case, up-regulation of FLN29/Xaf-2 expression would confer a survival advantage to cancer cells as it would facilitate the anti-apoptotic function of NF-κB. In light of this, we hypothesized that FLN29/Xaf-2 may participate in an NF-κB-dependent cell survival pathway.

To provide insight into the possible role of FLN29/Xaf-2 in mediating cell survival, we began by characterizing FLN29/Xaf-2 expression. Due to the novelty of the FLN29/Xaf-2 gene, most of our experiments were primarily designed to characterize its expression in non-diseased tissues and pancreatic cancer cell lines. We first investigated the conservation of the FLN29/Xaf-2 locus among animal species. Interestingly, sequences related to human FLN29/Xaf-2 were uncovered in several animal species, in particular dog, rhesus monkey, *Drosophila* and pig suggesting strong phylogenetic conservation of the FLN29/Xaf-2 sequence. We also assessed FLN29/Xaf-2 mRNA expression in non-diseased human tissues and discovered ubiquitous expression of a single 2.8 Kbp transcript in all tissues tested.

Subsequently, Southern hybridization revealed that the FLN29/Xaf-2 genomic locus was not prone to gene rearrangements, at least in *Hind* III restriction fragments in pancreatic cancer cell lines. Further analyses (with other restriction enzymes) will be necessary to

validate this claim. In addition, no variations in FLN29/Xaf-2 copy numbers were observed in pancreatic tumor cell lines. Next, allelotyping confirmed earlier observations regarding LOH in the FLN29/Xaf-2 region. Indeed, the locus coding for FLN29/Xaf-2 lies within a genomic region that appears to be prone to LOH suggesting that FLN29/Xaf-2 expression may be down-regulated in pancreatic cancer cell lines. However, the presence of at least two FLN29/Xaf-2 transcripts in all pancreatic cell lines tested was detected by Northern blot analysis. Moreover, FLN29/Xaf-2 mRNA appeared to be moderately over-expressed in pancreatic cancer cell lines compared to unadulterated pancreatic tissue. Interestingly, the higher molecular weight transcript was absent in human pancreatic tissue, in agreement with our survey of FLN29/Xaf-2 expression in non-diseased tissues.

We cannot exclude the possibility that the expression of the higher molecular weight FLN29/Xaf-2 transcript could be restricted to duct cells as its expression was only encountered in duct-enriched cell populations. Indeed, HPDE6 cell lines represent normal pancreatic ductal cells infected with a retroviral vector expressing the E6E7 genes of HPV-16 (Furukawa et al., 1996). As epithelial cells of ductal origin constitute a small proportion (typically 4%) of the pancreatic volume (Githens, 1988; Tasso et al., 1973), detection of the higher molecular weight FLN29/Xaf-2 transcript in whole pancreatic tissue would be difficult due to the frequent poor quality of the total RNA obtained from this tissue. In contrast, as the cancer cell lines tested were derived from ductal pancreatic adenocarcinomas, they can be considered duct-enriched thereby facilitating detection of the larger FLN29/Xaf-2 transcript.

Having observed a modest tendency towards over-expression of FLN29/Xaf-2 transcripts in pancreatic cancer cell lines, we asked if solid tumors in general might also over-express the gene at the mRNA level. Studies of matched normal/tumor RNA sets

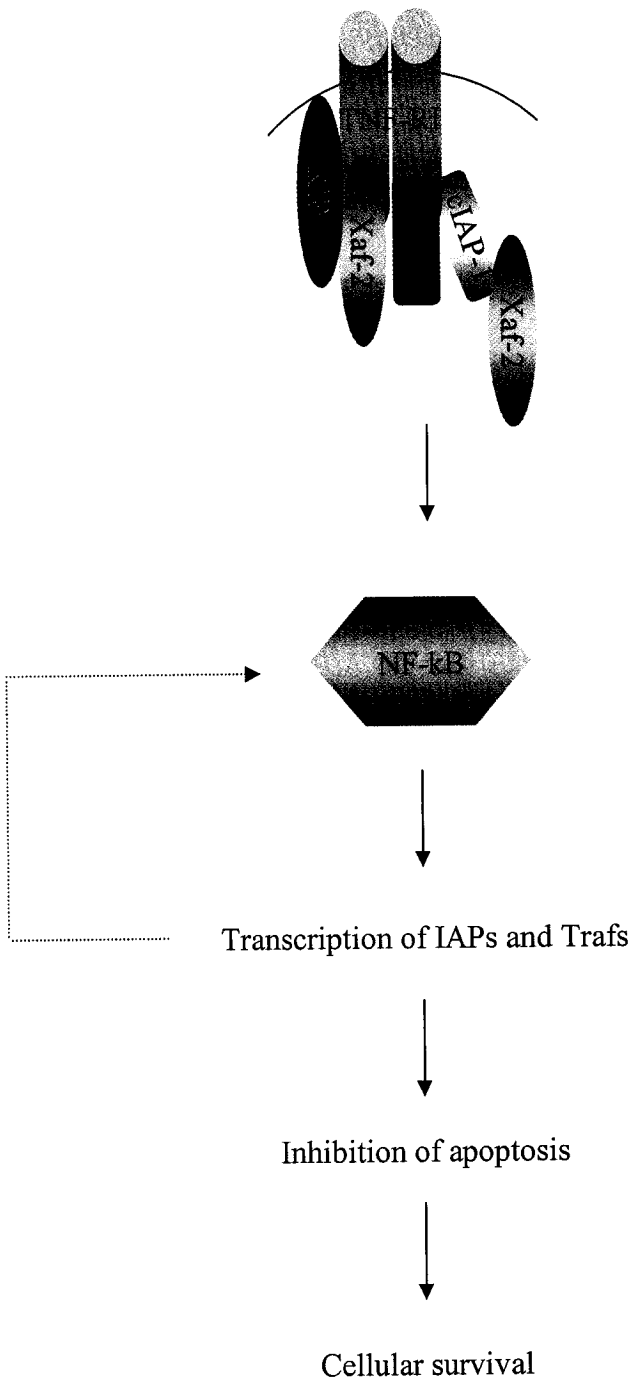
revealed that 36% of the tumors analyzed over-expressed FLN29/Xaf-2 when compared to their normal counterparts while 19% of tumors under-expressed this mRNA. It is interesting to note that FLN29/Xaf-2 was over-expressed in 43-50% of ovarian, renal, stomach and breast tumors while under-expression of FLN29/Xaf-2 occurred in 5-15% of these tumors. In light of the technical difficulties encountered with this particular CPA membrane which prevented re-hybridizing with a ubiquitin probe to control for loading, this experiment will be repeated to include ubiquitin and confirm the findings. Nonetheless, preliminary analysis of the FLN29/Xaf-2-probed CPA suggested a possible bias towards up-regulation of FLN29/Xaf-2 in some solid tumor types.

The split personality of TNF-RI (induction of apoptosis and activation of NF-κB which stimulates the expression of anti-apoptotic proteins) has puzzled researchers for a long time. However, the recent finding that TNF-RI-mediated apoptosis is induced via two sequential complexes (Micheau and Tschopp, 2003) demonstrated that assembly of the first complex (non-apoptotic) can activate survival signals and influence the activity of the second complex (pro-apoptotic). This model provides a cellular checkpoint to control induction of the apoptotic pathway (Barnhart and Peter, 2003). Having observed down-regulation of Traf2 and ubiquitous expression of FLN29/Xaf-2 in pancreatic cancer cell lines, we propose a model in which FLN29/Xaf-2 substitutes for Traf2 during assembly of the TNF-RI complex (Fig.6.2). If FLN29/Xaf-2 can indeed substitute for Traf2 and associate with TNF-RI by virtue of its Traf-like RZF, it may play a role in the regulation of IAP expression by favoring the activation of NF-κB which, in turn would stimulate the transcription of IAPs. Interestingly, cIAP-2 has been reported to activate NF-κB, possibly by forming a positive feedback loop which may mediate the anti-apoptotic effects of NF-κB (Chu et al., 1997).

Fig.6.2. Proposed role of FLN29/Xaf-2 in NF-κB signaling.

In light of the moderate tendency towards over-expression of FLN29/Xaf-2 in human solid malignancies, we hypothesize that FLN29/Xaf-2 plays a role in regulating cell growth and/or proliferation. We propose that FLN29/Xaf-2 acts to stimulate NF-κB signaling and may therefore be a positive regulator of IAP expression. In this model, FLN29/Xaf-2 would substitute for Traf2 in the TNF-RI receptor complex; FLN29/Xaf-2 would contact the receptor complex either directly or indirectly via an association with cellular IAPs (cIAPs). This association would trigger a signaling cascade ultimately resulting in enhanced NF-κB transcription. In turn, activated NF-κB would favor transcription of anti-apoptotic genes such as IAPs and Trafs resulting in successful inhibition of apoptosis. Modified from Micheau and Tschopp (2003).

TNF signaling complex I



However, we must stress that our current data does not support this model; however, it would be interesting to investigate this model. Nonetheless, the small up-regulation of FLN29/Xaf-2 observed in pancreatic and other tumors warrants further investigation into its possible role in mediating cell survival. Therefore, we plan to determine if FLN29/Xaf-2 can associate with TNF-RI and participate in NF-κB signaling. Stimulation of the NF-κB pathway would increase transcription of anti-apoptotic factors such as IAPs and effectively suppress programmed cell death. To validate this model, we need to show convincing proof that FLN29/Xaf-2 is part of the TNF-RI complex (for instance by immunoprecipitation) and that its over-expression (via transfection studies) stimulates NF-κB signaling and/or influences the expression of IAPs. Determining if decreased Traf2 expression correlates with increased FLN29/Xaf-2 in individual tumors would also be interesting. Lastly, an anti-FLN29/Xaf-2 antibody would be a great asset to these studies but attempts at generating one have been unsuccessful to date.

6.4. Concluding Remarks

In conclusion, the profiling of apoptotic regulators in pancreatic cancer cell lines presented in this study favors an implication of IAPs in the documented resistance of pancreatic tumors to cytotoxic therapies. In fact, the delicate balance between pro- and anti-apoptotic factors appears to be shifted towards an over-expression of anti-apoptotic genes (IAPs) not only in pancreatic cancer cell lines but also in several human malignancies. This shift is proposed to elevate the apoptotic threshold of cancer cells thereby decreasing their sensitivity and/or responsiveness to apoptogenic signals. We also found Xaf-1 mRNA to be down-regulated in pancreatic cancer cell lines possibly due to intragenic methylation of the

Xaf-1 locus. In addition, a nuclear redistribution of IAPs in late stage intraductal neoplasias and adenocarcinomas was revealed in an immunohistological survey. Our study on etoposide-induced cell death in three human pancreatic adenocarcinoma cell lines uncovered a good correlation between the transcriptional up-regulation of IAPs, the caspase-dependent cleavage of Xiap, the activation of caspase-3 and the rapidity of onset of apoptosis. Lastly, analysis of the candidate IAP-interacting protein, FLN29/Xaf-2 revealed this gene to be highly conserved across species, ubiquitously expressed in non-diseased tissues and slightly over-expressed in pancreatic cancer cell lines and other solid malignancies.

Taken together, our results suggest that IAPs are important regulators of the so-called "point of no return" in the caspase cascade as their expression appears to be sufficient to prevent apoptosis. Indeed, over-expression of IAPs coupled with repression of IAP antagonists such as Xaf-1 would provide pancreatic cancer cells with a survival advantage and ensure resistance to apoptotic stimuli such as those induced by chemotherapeutic agents. Unravelling the particular mechanisms by which this occurs clearly warrants further investigation and should prove beneficial to the rational design of oncotherapies targeted to this particularly dreadful disease.

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Contributions of Collaborators

1. The Cancer Profiling Arrays probed with Xaf-1, cIAP-1, cIAP-2 and Xiap along with the ubiquitin control were provided for analysis by Charles Lefebvre and Dr. Eric LaCasse, Aegera Technologies, Inc., Ottawa, ON.

2. The western blot probed with Riap1 (for cIAP-1 and cIAP-2 expression) was provided for analysis by Charles Lefebvre and Dr. Eric LaCasse, Aegera Technologies, Inc., Ottawa, ON.

3. Immunohistological surveys were performed by Dr. Ming S. Tsao, Department of Pathology and Laboratory Medicine, Princess Margaret Hospital, University of Toronto, Toronto, ON.

Appendix I: Stock solutions

Hybsol

22.5 mM NaCl (VWR)
15 mM NaH_2PO_4 (Sigma)
1.5 mM EDTA (Sigma)
7% SDS (Sigma)
10% (w/v) PEG (Sigma)

10X MOPS buffer

0.2 M MOPS (Sigma)
50 mM sodium acetate (VWR)
10 mM EDTA, pH 7.0 (Sigma)

Phosphate buffered saline

137 mM NaCl (Sigma)
2.7 mM KCl (KCl)
6.6 mM Na_2HPO_4 (BDH)
1.5 mM KH_2PO_4 (BDH)

Protein extraction buffer

62.5 mM Tris HCl pH 6.8 (Boehringer Mannheim Canada)
2% SDS (Sigma)
10% glycerol (Invitrogen)
4.9% β-mercaptoethanol (BDH)
1 mM PMSF (Sigma)
1X protease inhibitor (Boehringer Mannheim Canada)
1 mM sodium ortho-vanadate (Sigma)
20 mM β-glycerophosphate (Sigma)

Protein extraction buffer containing 6M urea

62.5 mM Tris HCl pH 6.8 (Boehringer Mannheim Canada)
36.04% urea (Invitrogen)
10% glycerol (Invitrogen)
2% SDS (Sigma)
1 mM PMSF (Sigma)
1X protease inhibitor (Boehringer Mannheim Canada)
1 mM sodium ortho-vanadate (Sigma)
20 mM β-glycerophosphate (Sigma)
4.9% β-mercaptoethanol (BDH)
0.003% bromophenol blue (Biorad)

Rapid extraction buffer (REX)

1% (w/v) N-lauryl sarcosine sodium salt (Sigma)
10 mM CDTA pH 8.0 (Sigma)
0.1 M Tris HCl pH 8.0 (Invitrogen)
0.2 M NaCl (VWR)
0.4 M urea (Invitrogen)

SOB medium

2% (w/v) Bacto tryptone (Becton Dickinson)
0.5% (w/v) yeast extract (Becton Dickinson)
8.5 mM NaCl (VWR)
2.5 mM KCl (BDH)

Solution D

4 M guanidium isothiocyanate (Invitrogen)
25 mM sodium citrate (Sigma)
0.5% (w/v) N-lauryl sarcosine sodium salt (Sigma)
0.1 M β-mercaptoethanol (BDH)

1X TBE buffer

100 mM Tris-borate (Sigma)
2 mM EDTA (Sigma)

1X TBST

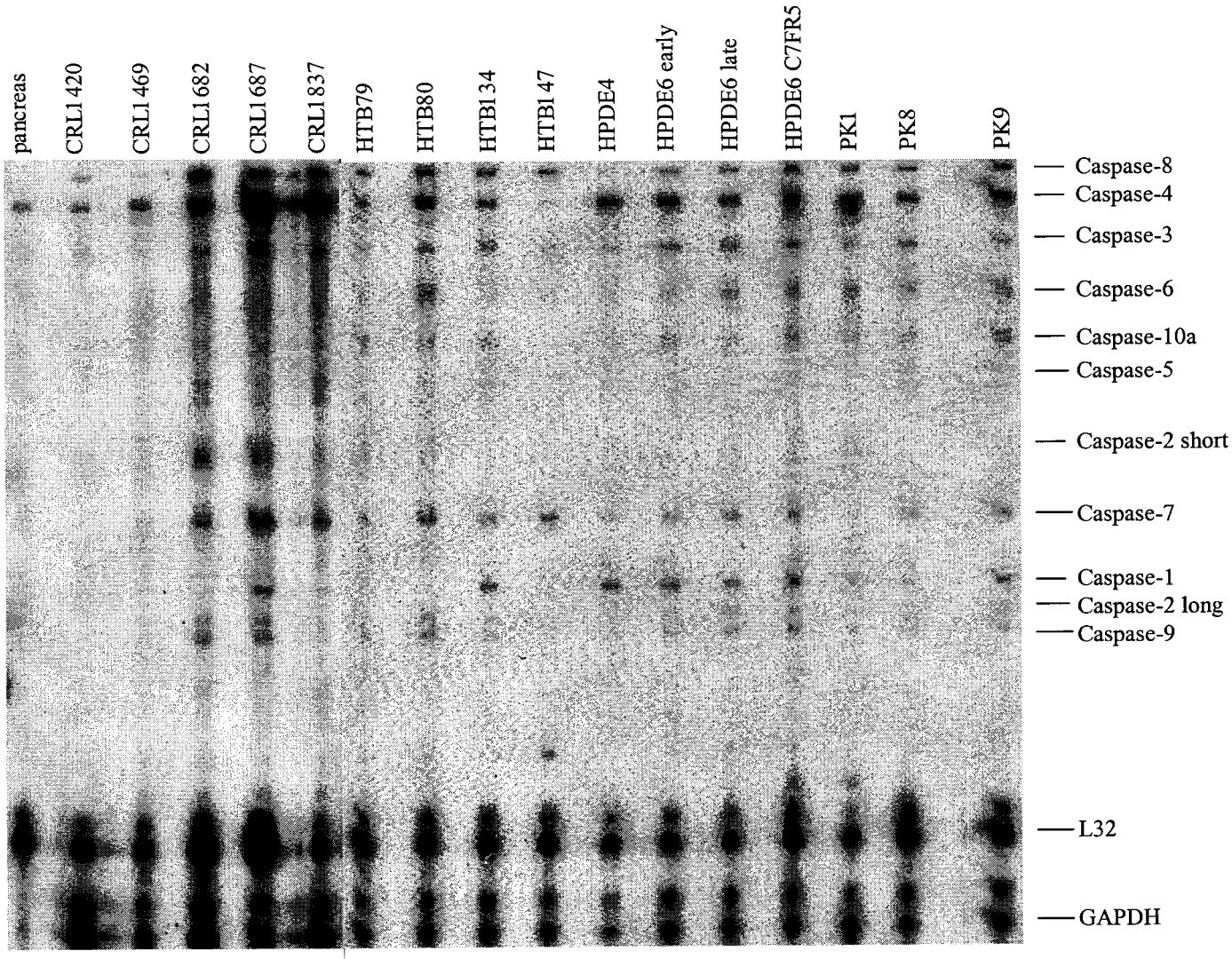
50 mM Tris HCl pH 7.5 (Boehringer Mannheim Canada)
30 mM NaCl (VWR)
0.1% (v/v) Tween 20 (Sigma)

Appendix II

Raw data including autoradiograms and densitometric analyses which support data presented in the results chapter are included in this section.

Fig.AII.1. Schematic representation of RNase protection assays.

RNase protection assays were performed on two human apoptotic templates (hAPO1C and hAPO5) using pancreatic ductal and tumor RNAs as targets. RNase protected fragments were resolved on 5% polyacrylamide gels and exposed to X-ray films. Autoradiograms of the data that was analysed by densitometry are presented in this figure.
(Figure continues on next page)


pancreas
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CRL1469
CRL1682
CRL1687
CRL1837
HTB79
HTB80
HTB134
HTB147
HPDE4
HPDE6 early
HPDE6 late
HPDE6 C7FR5
PK1
PK8
PK9
Caspase-8
Caspase-4
Caspase-3
Caspase-6
Caspase-10a
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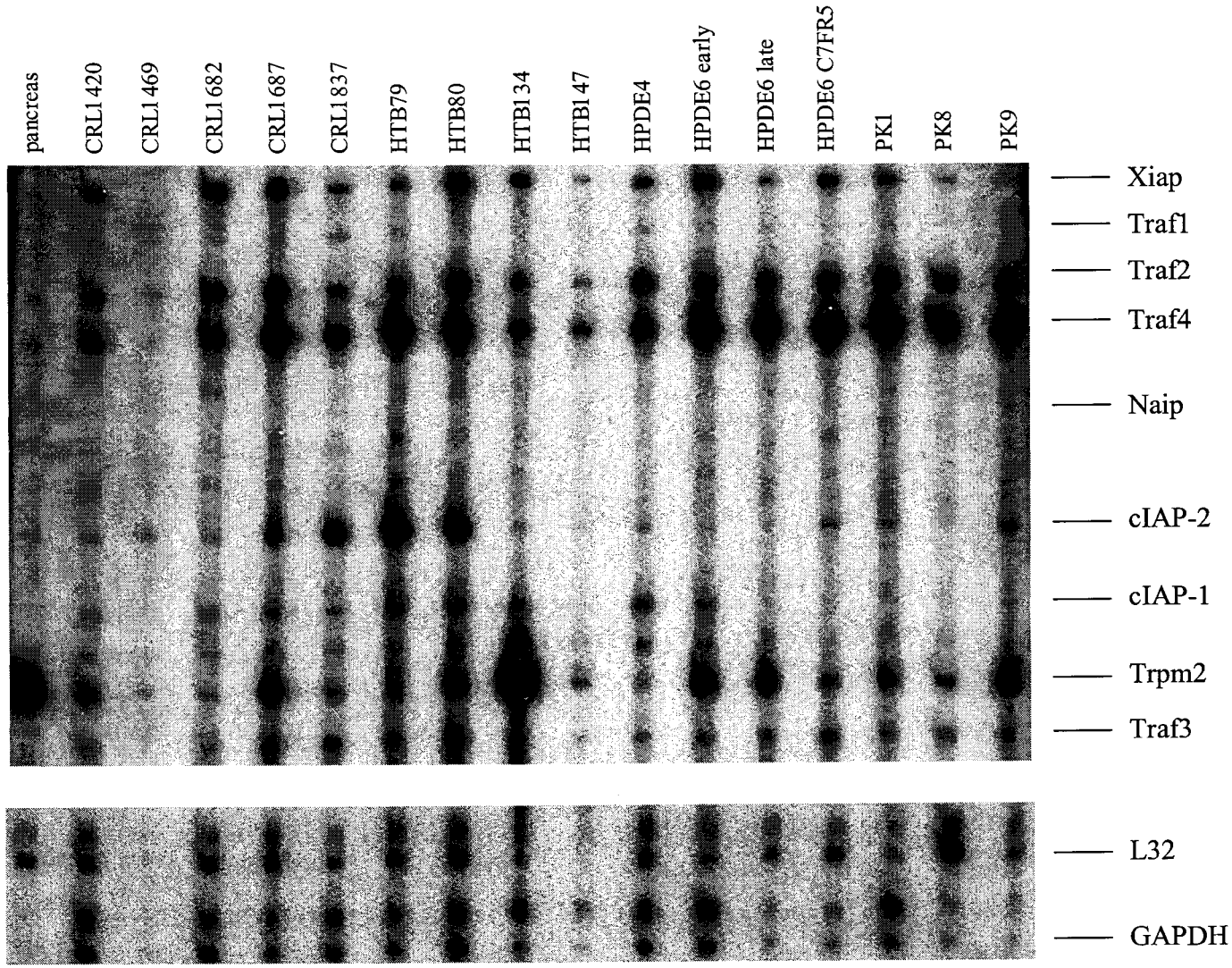
Caspase-2 long
Caspase-9
L32
GAPDH

hAPO1C


pancreas
CRL1420
CRL1469
CRL1682
CRL1687
CRL1837
HTB79
HTB80
HTB134
HTB147
HPDE4
HPDE6 early
HPDE6 late
HPDE6 C7FR5
PK1
PK8
PK9
Xiap
Traf1
Traf2
Traf4
Naip
cIAP-2
cIAP-1
Trpm2
Traf3
L32
GAPDH

hAPO5

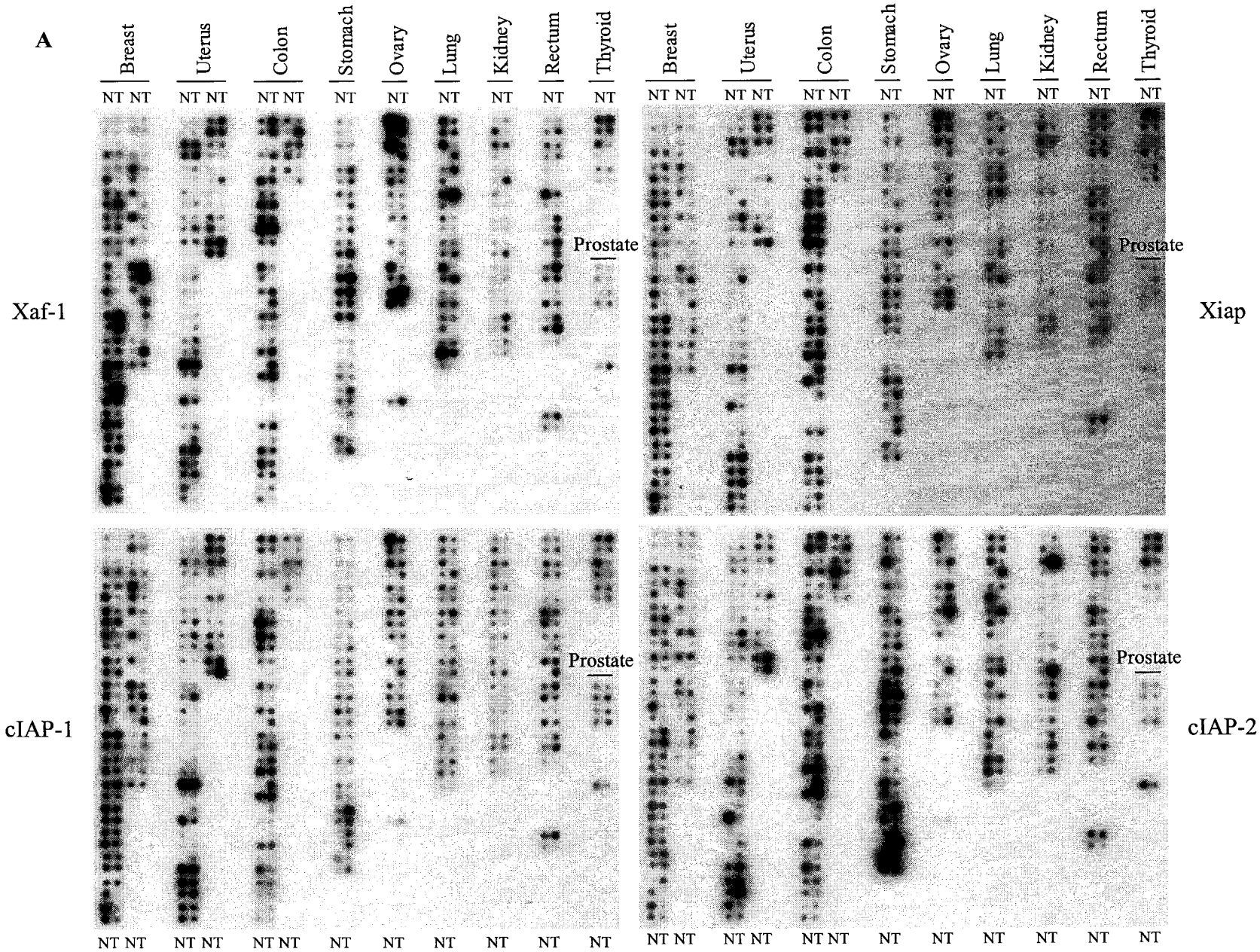
Table AII.1. Relative mRNA expression of apoptotic modulator/effector gene as revealed by RNase protection assays.

RNase protection assays were performed on two human apoptotic templates (hAPO1C and hAPO5) using pancreatic ductal and tumor RNAs as targets. RNase protected fragments were resolved on 5% polyacrylamide gels and exposed to X-ray films. Band intensities were determined using a personal densitometer. Target RNA expression intensities were normalized to that of the internal standard L32 and are expressed relative to the expression of HPDE6 late.

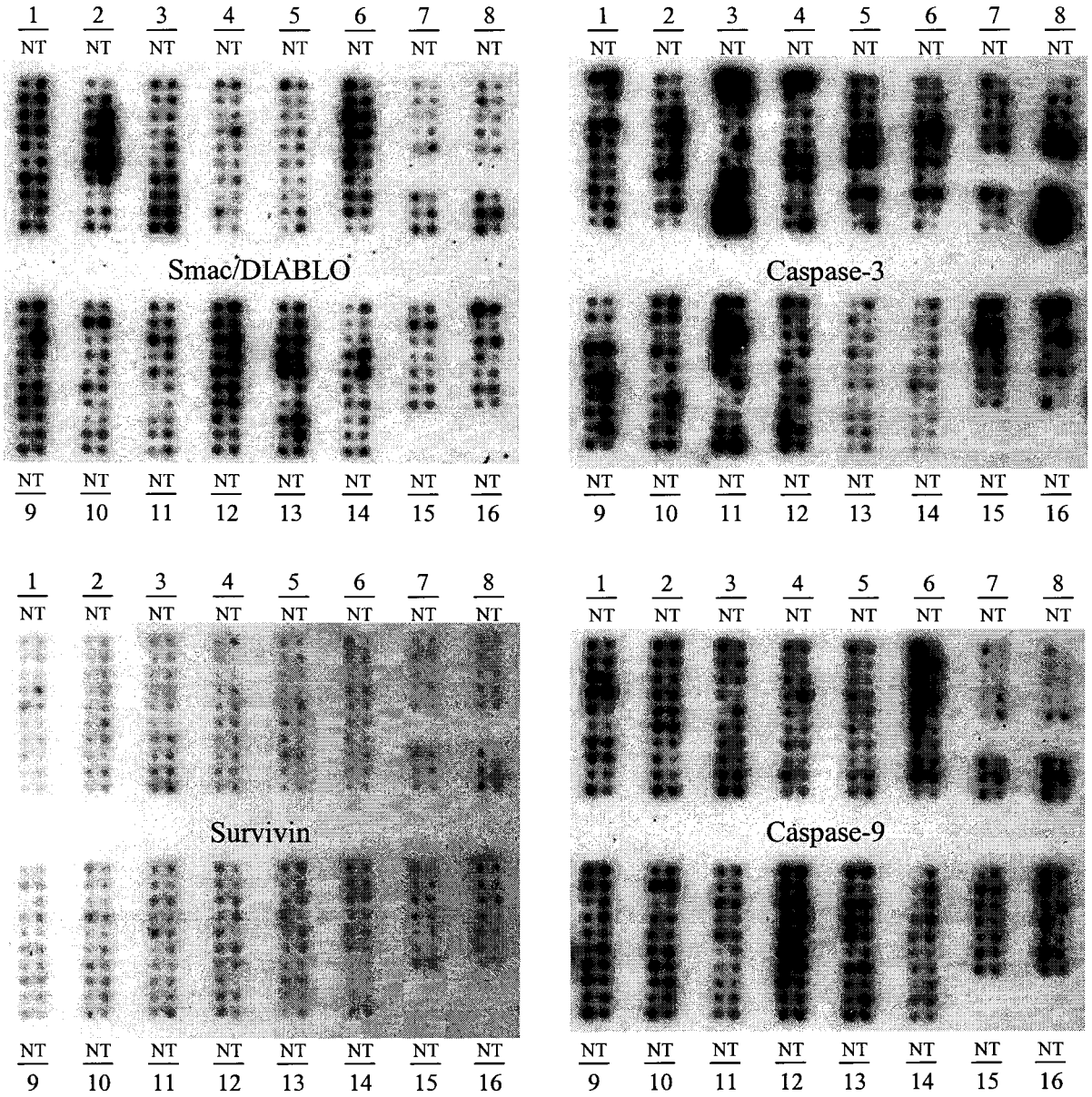
	Xiap	cIAP-1	cIAP-2	Naip	Traf1	Traf2	Traf3	Traf4
HPDE6 late	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
PK1	1.27	1.17	0.99	1.06	0.96	1.06	0.52	1.02
PK8	0.47	0.43	0.42	0.45	0.43	0.40	0.20	0.42
CRL1420	2.36	1.35	1.32	1.38	2.07	0.79	0.70	0.46
CRL1469	0.77	0.60	0.61	0.65	0.92	0.26	0.14	0.11
CRL1682	2.61	1.06	0.96	1.22	1.26	0.95	0.32	0.57
CRL1687	1.42	1.23	0.64	0.65	0.71	0.66	0.61	0.53
CRL1837	0.76	2.01	0.60	0.53	0.66	0.39	0.25	0.36
HTB79	0.68	2.97	0.90	0.60	0.61	0.55	0.31	0.54
HTB80	1.42	1.87	0.57	0.53	0.57	0.62	0.50	0.42
HTB134	1.28	0.79	0.75	0.75	0.81	0.55	2.11	0.36
HTB147	1.27	1.30	1.25	1.19	1.39	0.60	0.57	0.51
	caspase-2	caspase-8	caspase-9	caspase-10a	caspase-1	caspase-3	caspase-6	caspase-7
HPDE6 late	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
PK1	1.38	1.17	0.92	1.17	0.71	1.29	1.21	0.64
PK8	0.26	0.30	0.28	0.27	0.13	0.05	0.31	0.21
CRL1420	0.61	0.45	0.56	0.39	0.29	0.51	0.28	0.30
CRL1469	0.83	0.28	0.53	0.48	0.27	1.20	0.43	0.44
CRL1682	1.03	0.72	0.66	0.71	0.28	1.65	0.53	0.58
CRL1687	1.67	0.94	0.78	1.24	0.95	1.36	1.13	1.24
CRL1837	1.69	0.86	0.80	1.30	0.71	1.28	1.00	0.86
HTB79	0.50	0.60	0.44	0.50	0.29	0.75	0.27	0.37
HTB80	0.76	1.37	0.74	0.70	0.29	1.12	0.97	0.85
HTB134	0.38	0.55	0.42	0.43	0.48	0.11	0.20	0.39
HTB147	0.18	0.38	0.14	0.08	0.05	0.00	0.14	0.43

Fig.AII.2. Schematic depiction of Cancer Profiling Arrays.

Cancer Profiling Arrays (Clontech) containing SMART™-amplified cDNA from tumors and corresponding normal tissues were hybridized with a series of probes. **A.** A first array (CPAI) containing 241 matched samples was probed with cDNA specific to Xaf-1 and Xiap and partial 3' cIAP-1 and cIAP-2 sequences in succession. **B.** A similar array (CPAII) containing 154 matched samples was probed with survivin, caspase-3, caspase-9 and Smac/DIABLO. **C.** Ubiquitin served as an internal loading control.
(Figure continues on next pages)

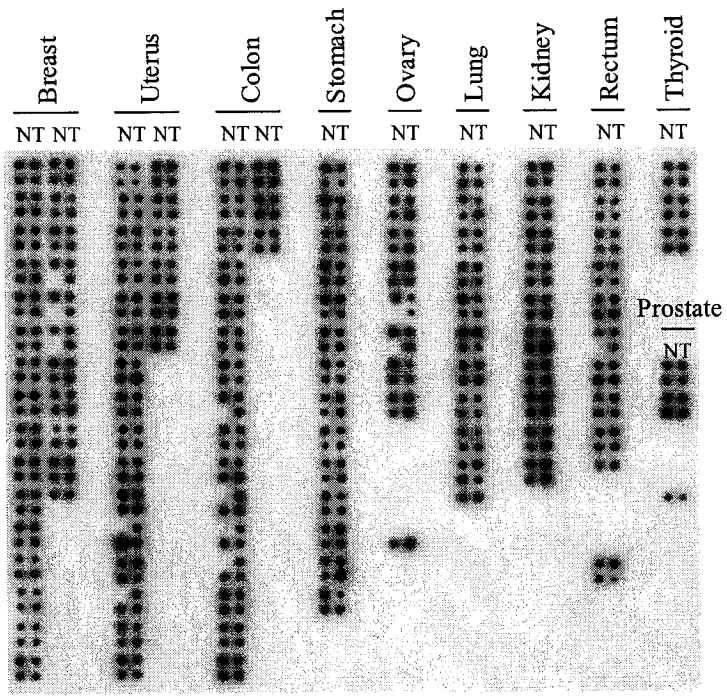

A
Breast
Uterus
Colon
Stomach
Ovary
Lung
Kidney
Rectum
Thyroid
NT NT
NT
Prostate
Xaf-1
Xiap
cIAP-1
cIAP-2

B



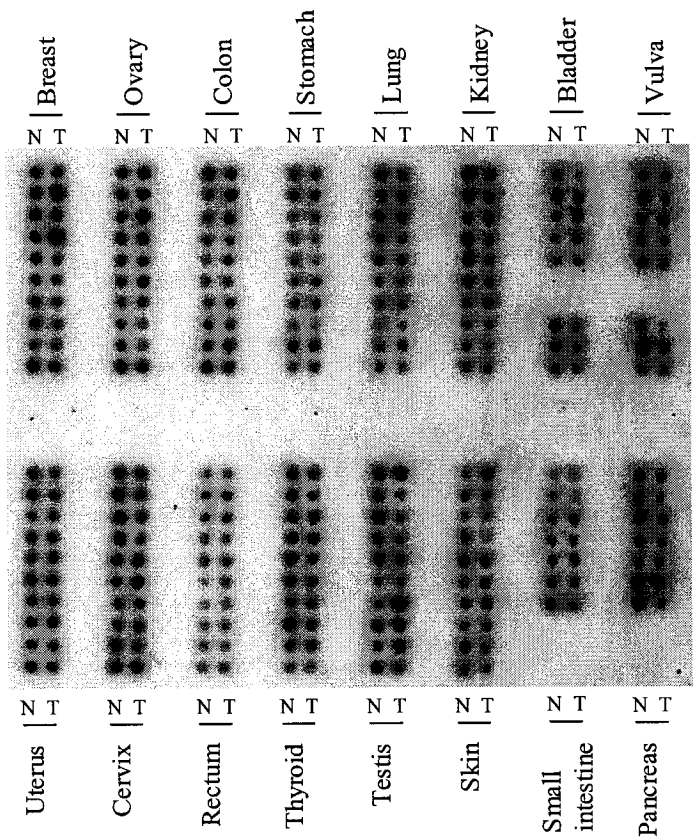
1	Breast
2	Ovary
3	Colon
4	Stomach
5	Lung
6	Kidney
7	Bladder
8	Vulva
9	Uterus
10	Cervix
11	Rectum
12	Thyroid
13	Testis
14	Skin
15	Small intestine
16	Pancreas

C



Cancer Profiling Array I

Ubiquitin



Cancer Profiling Array II

Ubiquitin

Table AII.2. Relative mRNA expressions of apoptotic regulators in human solid tumors.

Cancer Profiling Arrays depicted in Fig.AII.2 were analysed using a personal densitometer. Band intensities were first normalized to those of control ubiquitin. Tumor samples were deemed to over-express target RNA when the intensity of the signal was twice that of the normal control (highlighted in red). In contrast, tumors were deemed to down-regulate target RNA expression when the intensity of the signal was half that of the normal counterpart (highlighted in blue).

Note: all sites combined refers to the total number of matched normal/non-tumorigenic RNA sets analysed on each CPA.

(Table continues on next pages)

Analyzed data Xaf-1

	Breast	Uterus	Colon	Stomach	Ovary	Lung	Kidney	Rectum	Thyroid	Prostate
1	0.53	0.42	0.33	0.93	0.34	0.19	1.30	1.14	0.56	1.02
2	0.42	0.58	1.01	1.68	1.86	0.53	1.74	4.00	0.73	1.93
3	0.22	0.78	0.72	0.14	1.33	0.53	1.79	2.96	0.62	0.55
4	0.19	0.51	0.37	2.03	0.24	0.28	4.28	0.61	0.39	0.54
5	0.73	1.34	0.69	1.69	0.77	0.70	0.97	0.97	1.30	
6	0.43	1.91	1.32	1.48	1.21	1.02	0.72	1.57	1.68	
7	0.43	0.65	1.62	1.26	0.85	0.94	0.78	3.71		
8	0.51	0.71	1.30	0.56	0.65	1.33	0.94	3.37		
9	0.89	0.82	0.68	0.48	0.31	0.21	1.62	3.87		
10	1.91	0.72	1.42	0.82	1.04	1.66	0.42	3.37		
11	0.61	1.40	1.87	1.15	0.86	0.90	1.12	0.63		
12	0.70	1.57	4.33	7.12	0.23	0.56	0.76	0.43		
13	0.69	1.76	2.02	0.42	0.51	0.58	0.40	0.34		
14	1.07	1.01	1.86	2.23	0.36	1.49	0.34	0.67		
15	1.85	0.68	1.04	2.45		2.46	7.12	1.68		
16	2.12	0.49	1.58	0.71		0.39	1.39	1.20		
17	0.61	0.71	4.78	4.73		2.91	0.46	2.68		
18	0.31	1.04	1.56	0.85		1.18	0.73	1.61		
19	0.45	1.13	0.34	1.36		0.31	0.13			
20	0.34	0.97	0.21	1.64		0.70	1.18			
21	0.73	1.92	1.48	2.92		0.46				
22	0.64	0.76	1.15	4.40						
23	0.55	1.05	0.94	1.54						
24	0.96	0.77	0.55	3.44						
25	1.51	1.24	0.72	0.44						
26	0.91	1.30	0.57	2.36						
27	0.42	1.35	0.36	0.56						
28	0.86	1.04	1.59							
29	0.76	1.42	2.14							
30	0.80	0.99	1.42							
31	0.64	1.31	2.91							
32	0.76	2.01	3.26							
33	0.66	0.37	1.52							
34	4.20	0.53	0.41							
35	1.37	0.81	0.61							
36	0.83	0.78								
37	1.95	2.92								
38	2.31	0.74								
39	0.95	0.56								
40	3.05	1.06								
41	1.38	0.99								
42	0.32	1.92								
43	0.35									
44	0.26									
45	0.21									
46	0.38									
47	0.47									
48	0.78									
49	0.51									
50	0.67									
Up-regulated (%)	14.00	9.50	22.90	33.30	7.10	9.50	10.00	38.90	0.00	25.00
Down-regulated (%)	34.00	9.50	17.10	14.80	42.90	28.50	25.00	11.10	16.70	0.00

Analyzed data cIAP-1

	Breast	Uterus	Colon	Stomach	Ovary	Lung	Kidney	Rectum	Thyroid	Prostate
1	0.40	0.61	0.58	1.24	1.01	0.60	0.31	0.57	1.05	1.19
2	0.43	0.97	1.16	1.05	1.04	0.84	0.48	1.57	0.87	1.59
3	0.33	0.91	0.70	1.29	1.03	0.70	0.49	1.88	0.74	1.23
4	0.77	0.69	1.08	1.80	1.12	0.70	0.89	0.67	0.44	0.68
5	0.49	0.82	0.68	1.09	1.03	0.47	0.47	0.66	0.85	
6	0.55	1.58	0.96	1.58	0.98	0.91	0.68	0.62	1.32	
7	0.46	0.65	1.01	0.86	0.63	0.79	0.50	1.48		
8	0.67	0.67	1.73	0.84	0.80	0.50	0.42	0.84		
9	0.64	0.85	0.92	0.39	1.31	0.52	0.67	1.18		
10	0.53	1.00	1.97	0.60	0.47	1.69	0.15	0.76		
11	0.82	0.98	1.30	0.76	1.18	1.13	0.59	0.41		
12	1.27	1.12	3.43	0.47	0.47	1.39	0.80	0.75		
13	0.59	1.28	1.12	0.37	0.22	0.68	0.35	0.86		
14	0.79	0.97	2.07	0.37	0.19	0.79	0.40	0.69		
15	0.73	0.64	1.60	0.83		2.14	0.77	1.06		
16	1.12	0.46	1.00	1.23		0.63	0.64	0.90		
17	0.36	1.33	3.72	1.16		0.57	0.34	1.29		
18	0.19	1.12	0.71	0.82		0.90	0.72	0.83		
19	0.27	1.06	0.69	1.05		0.64	0.16			
20	0.34	0.92	0.77	0.77		0.73	0.38			
21	0.58	1.61	2.12	1.24		0.68				
22	0.29	1.62	1.03	0.85						
23	0.35	1.47	0.51	0.85						
24	0.51	1.30	0.31	0.69						
25	0.55	0.88	0.21	0.81						
26	0.40	0.57	0.42	0.93						
27	0.28	1.01	0.56	0.79						
28	1.03	0.94	1.27							
29	0.97	1.72	1.38							
30	1.13	1.52	1.33							
31	0.82	6.26	1.07							
32	0.42	1.71	1.44							
33	0.52	0.45	0.77							
34	0.91	0.61	0.47							
35	0.96	0.90	0.67							
36	1.48	1.05								
37	2.17	1.22								
38	1.83	0.73								
39	0.70	0.57								
40	1.31	0.98								
41	0.36	0.60								
42	0.17	0.39								
43	0.39									
44	0.32									
45	0.22									
46	0.19									
47	0.46									
48	0.63									
49	0.31									
50	0.43									
Up-regulated (%)	4.00	2.40	14.30	3.70	0.00	4.80	0.00	5.60	0.00	0.00
Down-regulated (%)	48.00	7.10	14.30	14.80	28.60	14.20	60.00	5.60	16.70	0.00

Analyzed data cIAP-2

	Breast	Uterus	Colon	Stomach	Ovary	Liver	Kidney	Rectum	Thyroid	Prostate
1	0.66	1.10	0.87	1.27	1.89	0.51	2.39	0.76	1.29	2.26
2	0.84	1.76	0.29	1.76	1.51	0.89	4.86	0.95	1.30	2.09
3	1.01	3.09	0.10	2.51	1.17	0.53	4.45	1.40	1.41	1.18
4	2.01	1.96	0.44	2.04	1.82	0.34	4.29	0.23	0.60	1.11
5	1.15	2.40	1.61	0.71	1.47	0.59	4.08	0.89	1.26	
6	1.28	2.62	0.81	1.58	0.86	2.85	1.65	0.95	1.60	
7	0.54	0.47	0.68	0.44	2.48	1.32	3.58	1.36		
8	0.82	0.34	1.15	0.42	5.67	1.10	3.08	1.53		
9	0.52	1.35	0.69	0.48	1.89	0.52	4.75	1.58		
10	0.67	0.98	1.06	0.41	2.86	3.19	1.54	1.36		
11	0.91	1.99	0.60	0.39	1.07	1.28	1.52	0.62		
12	1.00	2.62	0.55	0.25	1.64	1.49	0.96	0.44		
13	0.70	2.81	1.75	0.23	0.28	1.09	0.67	0.80		
14	1.02	1.58	2.69	0.44	0.21	0.98	0.72	2.44		
15	1.53	1.06	2.60	1.29		2.79	1.96	1.42		
16	1.91	0.71	7.25	0.89		0.21	2.62	0.84		
17	0.38	0.90	2.19	2.38		1.31	2.56	0.81		
18	0.26	1.15	2.30	1.00		2.65	8.05	0.94		
19	0.75	1.28	1.18	1.18		1.01	1.01			
20	0.50	1.23	0.92	0.98		1.26	2.38			
21	0.73	2.70	6.27	1.40		1.20				
22	0.81	2.46	3.10	0.51						
23	0.84	1.47	0.70	0.62						
24	0.84	1.12	0.26	0.49						
25	1.22	0.93	0.90	0.51						
26	0.78	1.19	0.93	0.33						
27	0.63	0.90	0.41	0.44						
28	1.81	1.14	2.37							
29	0.92	2.19	0.67							
30	1.12	1.73	0.43							
31	0.93	3.63	0.82							
32	0.78	2.22	2.14							
33	0.65	0.52	0.93							
34	0.91	0.67	1.28							
35	1.00	1.54	0.64							
36	2.20	1.72								
37	2.48	2.51								
38	1.67	1.76								
39	0.89	1.00								
40	0.52	1.32								
41	0.36	0.71								
42	0.47	0.67								
43	0.85									
44	0.36									
45	0.20									
46	0.19									
47	0.42									
48	0.93									
49	0.31									
50	0.58									
Up-regulated (%)	10.00	31.00	25.70	11.10	42.90	19.00	65.00	5.60	0.00	50.00
Down-regulated (%)	24.00	7.10	17.10	48.10	14.30	19.00	0.00	11.10	0.00	0.00

Analyzed data Xiap

	Breast	Uterine	Colon	Stomach	Ovary	Lung	Kidney	Rectum	Thyroid	Prostate
1	0.36	0.52	0.67	1.13	1.08	0.63	0.53	0.40	1.26	0.77
2	0.35	0.87	1.68	1.06	1.30	1.13	0.56	0.92	1.35	1.46
3	0.41	0.78	0.98	2.81	2.23	1.00	0.74	1.34	0.91	1.36
4	0.79	1.02	1.00	3.44	0.41	1.06	0.99	0.85	1.19	0.65
5	0.50	0.89	0.66	0.88	1.84	0.86	0.56	0.51	0.92	
6	0.57	5.13	1.03	1.59	1.08	1.71	1.31	0.47	1.13	
7	0.42	0.72	1.07	0.95	0.92	1.09	0.49	0.85		
8	0.62	0.44	1.33	0.54	2.02	1.15	0.40	0.82		
9	0.78	1.85	0.50	0.32	2.02	0.42	0.49	1.51		
10	1.04	1.06	0.97	1.04	0.93	2.27	0.30	0.80		
11	0.71	1.55	1.91	0.87	2.06	2.19	0.57	0.56		
12	0.90	4.86	1.35	0.59	0.80	2.12	0.54	0.80		
13	0.86	12.96	1.45	0.40	0.64	1.25	0.54	0.55		
14	0.83	5.56	1.18	0.64	0.63	0.98	0.70	1.13		
15	0.95	0.36	1.43	0.97		1.64	0.61	1.42		
16	1.17	0.63	1.20	1.63		1.02	0.69	1.73		
17	0.42	2.34	1.60	0.85		1.51	0.41	1.27		
18	0.23	2.50	0.92	1.16		0.78	0.80	1.40		
19	0.37	0.83	1.16	1.22		0.98	0.41			
20	0.58	0.78	1.58	1.17		2.48	0.58			
21	0.40	3.18	1.54	4.67		0.85				
22	0.56	2.78	1.88	0.95						
23	0.73	4.38	1.25	0.96						
24	0.66	0.82	2.86	0.59						
25	0.70	1.75	1.66	1.21						
26	0.84	1.25	1.70	2.00						
27	0.74	1.69	0.80	0.85						
28	1.87	1.15	1.33							
29	1.08	2.42	0.73							
30	0.48	49.98	0.34							
31	0.79	2.30	0.52							
32	1.00	2.12	1.02							
33	1.04	0.34	0.87							
34	1.48	0.45	0.97							
35	1.65	1.00	1.00							
36	2.92	0.74								
37	1.78	2.38								
38	4.10	0.85								
39	0.91	0.52								
40	1.59	0.90								
41	0.47	1.00								
42	0.40	0.62								
43	0.57									
44	0.31									
45	0.21									
46	0.35									
47	0.24									
48	0.15									
49	0.37									
50	0.50									
Up-regulated (%)	8.00	35.70	8.60	14.80	35.60	19.00	0.00	0.00	0.00	0.00
Down-regulated (%)	38.00	14.30	8.60	7.40	7.10	4.80	30.00	16.70	0.00	0.00

Analyzed data survivin

	Bladder	Vulva	Breast	Cervix	Colon	Kidney	Liver	Lung	Ovary	Pancreas	Prostate	Rectum	Skin	Sm. Intestine	Stomach	Testis	Thyroid	Trachea	Uterus
1	1.51	1.16	3.33	2.05	1.74	0.61	1.86	1.46	1.30	1.26	0.87	1.12	0.62	0.87	1.71	2.73	0.87	1.12	2.78
2	0.93	1.45	5.74	3.16	2.71	1.70	1.71	2.32	2.83	1.64	1.64	0.83	1.06	1.96	1.37	17.86	4.91	2.08	2.93
3	0.80	1.32	3.88	3.25	2.76	0.79	2.11	1.82	2.08	2.32	1.16	1.10	2.00	1.06	1.38	7.31	1.52	0.88	4.39
4	1.78	1.35	2.65	1.46	4.39	1.59		2.50	3.18	0.83	2.74	1.26	9.88	1.33	2.37	0.93	1.87		3.08
5	1.36	1.57	2.81	1.47	2.08	1.13		2.16	2.42	1.04		0.78	4.16	1.64	2.65	1.80	1.06		1.25
6			2.16	2.75	5.08	1.05		1.60	3.45	1.79		1.30	1.27	1.83	1.74	3.66	1.42		2.43
7			1.99	1.33	2.07	1.11		1.27	1.48	2.92		1.81	1.48	1.54	1.82	1.04	1.36		1.54
8			2.02	1.86	2.55	1.43		1.98	1.73			1.26	3.44		3.20	1.91	0.74		2.18
9			3.90	3.04	1.51	1.07		1.95	1.94			2.26	1.72		1.33	3.25	0.71		2.70
10			3.63	1.72	2.63	0.65		9.36	1.52			1.98	2.63		2.93	4.34	1.23		1.00
Up-regulated (%)	0	0	100	50	80	0	33	60	60	29	25	20	50	14	40	70	10	33	70
Down-regulated (%)	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0

Analyzed data Smac/DIABLO

	Bladder	Vulva	Breast	Cervix	Colon	Kidney	Liver	Lung	Ovary	Pancreas	Prostate	Rectum	Skin	Sm. Intestine	Stomach	Testis	Thyroid	Trachea	Uterus
1	0.80	0.82	2.07	1.49	1.65	0.30	0.90	0.65	0.85	0.58	0.94	0.84	1.95	1.18	1.84	1.58	2.10	0.93	3.35
2	1.19	0.88	1.11	1.45	10.24	0.72	1.45	0.91	2.04	1.12	1.24	1.06	1.57	1.01	1.50	3.48	1.51	2.08	2.57
3	1.17	1.51	0.90	1.78	7.42	0.51	0.37	0.99	2.11	1.74	1.09	0.61	2.87	1.21	1.66	2.47	1.69	1.38	4.77
4	1.84	1.11	0.58	2.00	14.29	0.95		2.13	3.14	0.56	1.53	1.78	4.32	1.73	5.49	0.76	2.07		2.45
5	1.63	1.82	0.76	1.54	38.43	0.64		2.34	2.58	1.26		0.71	3.52	0.97	2.15	0.93	1.47		0.87
6			1.84	0.40	9.49	0.34		1.14	1.91	1.10		1.21	0.81	1.70	1.65	1.84	1.45		1.70
7			1.01	0.86	3.66	0.37		1.30	1.70	2.06		3.49	1.31	1.23	1.55	1.25	1.48		1.13
8			1.43	1.01	4.30	0.82		1.30	1.45			1.19	1.86		4.89	2.39	1.04		1.17
9			2.05	1.37	2.80	0.88		2.06	2.07			1.77	1.31		0.72	2.17	1.06		2.48
10			1.09	0.79	3.54	0.91		4.46	0.83			1.70	2.11		9.73	1.55	1.25		1.33
Upregulated (%)	0	0	20	10	90	0	0	40	60	14	0	10	50	0	40	40	20	33	50
Downregulated (%)	0	0	0	10	0	40	33	0	0	0	0	0	0	0	0	0	0	0	0

Analyzed data caspase-3

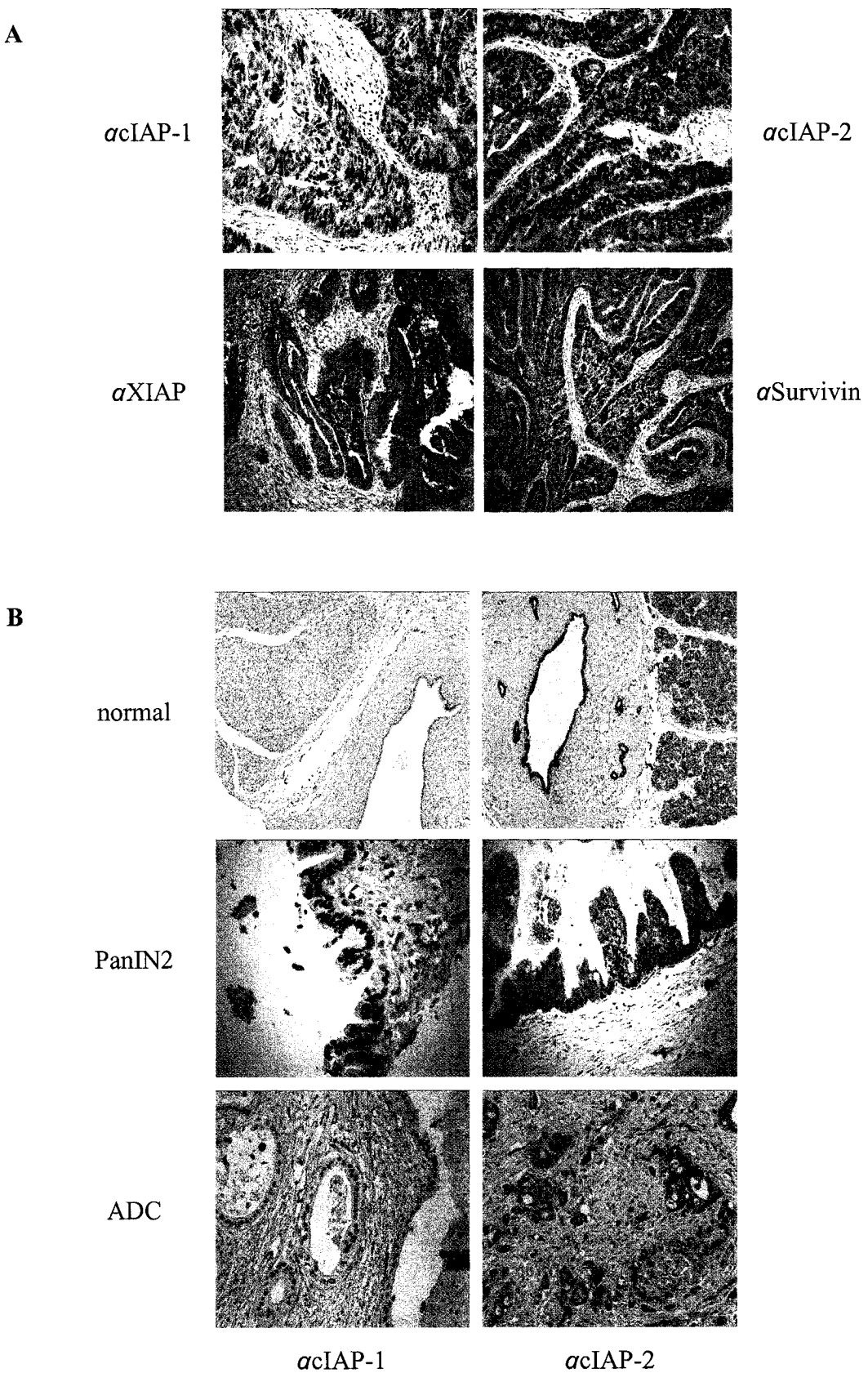
	Bladder	Vulva	Breast	Cervix	Colon	Kidney	Liver	Lung	Ovary	Pancreas	Prostate	Rectum	Skin	Sm. Intestine	Stomach	Testis	Thyroid	Trachea	Uterus
1	0.55	4.68	1.63	1.46	0.53	0.78	0.49	0.56	0.44	0.97	0.34	0.35	1.02	0.73	1.38	2.21	0.42	0.70	0.99
2	1.93	1.34	2.63	1.21	0.84	1.55	0.70	0.45	1.05	1.63	1.42	0.40	1.17	0.69	0.46	0.77	0.84	1.81	0.96
3	0.75	0.97	1.55	1.40	2.07	1.07	0.27	0.50	1.75	3.76	1.42	0.57	1.60	0.57	1.65	2.11	1.28	1.42	1.08
4	1.28	1.17	0.80	0.91	5.10	1.87		1.85	4.30	0.65	1.88	0.29	3.24	1.33	4.14	0.58	1.51		1.38
5	1.73	2.04	0.62	2.09	6.44	1.24		1.74	2.51	0.89		0.25	1.01	1.30	0.99	0.94	0.38		1.06
6			1.19	1.72	3.19	0.85		0.93	2.95	0.39		1.56	0.43	1.12	1.08	0.81	1.48		2.80
7			0.82	4.95	1.60	0.65		0.69	1.28	-0.05		1.08	0.80	2.31	2.04	0.30	1.26		2.33
8			0.84	3.01	0.96	0.93		1.61	3.14			0.58	1.25		28.68	0.88	0.58		2.07
9			2.05	2.18	0.50	0.93		1.07	4.14			1.14	1.22		1.84	0.91	0.56		4.77
10			7.92	1.53	1.00	0.26		8.87	1.56			1.12	1.16		10.78	1.67	0.69		1.21
Up-regulated (%)	10	20	30	40	40	0	0	10	50	14	0	0	10	14	40	20	0	0	40
Down-regulated (%)	10	0	0	0	20	10	67	30	10	29	25	40	10	14	10	10	30	0	0

Analyzed data caspase-9

	Bladder	Vulva	Breast	Cervix	Colon	Kidney	Liver	Lung	Ovary	Pancreas	Prostate	Rectum	Skin	Sm. Intestine	Stomach	Testis	Thyroid	Trachea	Uterus
1	1.02	0.47	0.70	2.03	0.73	0.26	0.91	0.89	1.16	0.61	0.64	0.88	3.25	1.34	0.90	1.09	1.24	1.05	1.27
2	0.78	1.46	0.67	1.67	2.04	0.79	1.25	0.73	1.23	0.59	0.79	0.98	3.64	1.10	0.52	1.07	1.07	1.55	0.96
3	0.92	0.91	0.83	1.99	2.69	0.45	0.35	0.73	0.68	1.26	0.91	0.65	3.12	0.92	0.81	1.59	1.11	0.91	1.66
4	1.57	1.32	0.73	1.65	3.96	0.81		0.87	1.18	0.33	1.16	0.94	4.32	1.08	2.61	0.75	1.84		2.06
5	2.14	1.49	0.66	0.82	8.08	0.46		1.72	1.25	0.73		0.78	1.70	1.42	1.04	0.49	1.21		1.05
6			1.04	0.97	3.50	0.32		0.78	0.81	0.46		1.24	0.90	0.84	0.99	0.81	1.27		1.68
7			1.14	0.86	2.45	0.52		1.46	0.98	1.23		1.11	1.01	1.03	0.68	0.59	1.04		0.81
8			1.17	1.58	2.00	0.59		1.97	0.52			0.74	1.92		0.70	0.93	0.96		0.80
9			0.93	2.32	1.52	0.86		1.70	0.76			1.28	1.71		0.44	1.30	0.95		1.01
10			0.62	0.75	2.39	0.82		2.51	0.99			1.26	2.38		0.77	0.76	1.35		0.69
Up-regulated (%)	10	0	0	30	80	0	0	20	0	0	0	0	50	0	10	0	0	0	10
Down-regulated (%)	0	10	0	0	0	50	33	0	10	29	0	0	0	0	20	10	0	0	0

Fig.AII.3. cIAP-1, cIAP-2, Xiap and survivin in tissue micro-arrays of normal pancreatic tissue and evolving intraductal pancreatic lesions.

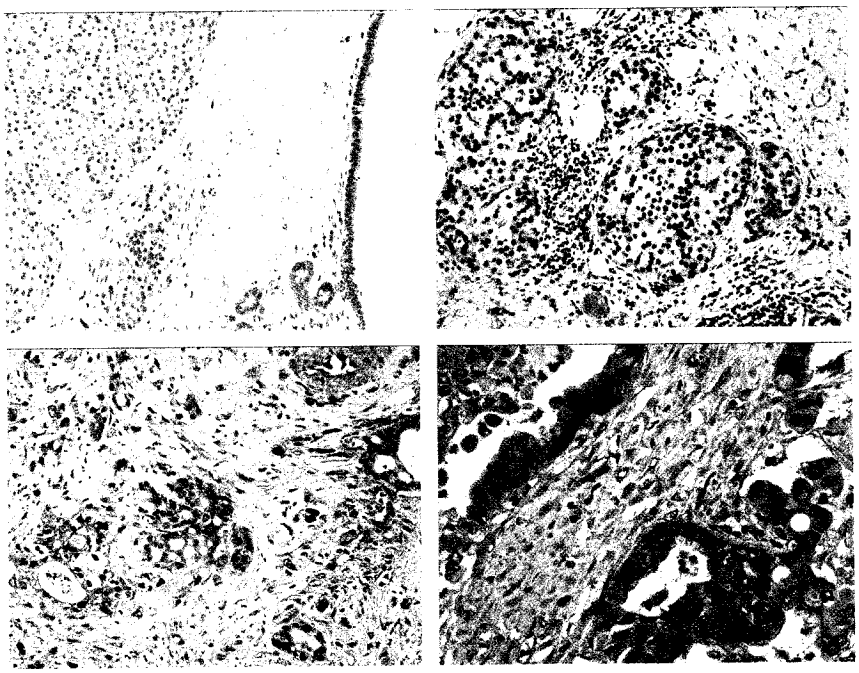
Immunohistochemistry was carried out on tissue micro-arrays using 1:200 dilutions of cIAP-1, cIAP-2, Xiap and survivin antibodies. After blocking endogenous peroxidase activity and washing in PBS, secondary antibody incubation (using the multi-species link reagent) was followed by incubation with streptavidin-horseradish peroxidase. Immuno-reactivities were revealed by incubation in Nova Red substrate for 5 min. Slides were counterstained in Mayer's hematoxylin and mounted in Permount. **A.** Immunohistochemical staining of cIAP-1, cIAP-2, Xiap and survivin in a human colon cancer positive reference sample showing intense nuclear staining for cIAP-1, cIAP-2 and survivin in the majority of cancer cells. Original magnification was 100x. **B.** Immunohistochemical staining for cIAP-1 and cIAP-2 in normal (40x), PanIN2 (100x) and adenocarcinoma (100x) of the pancreas. **C.** Immunohistochemical staining for Xiap in normal pancreas (40x, islets at 100x) and adenocarcinoma (left panel, 100x; right panel 200x). **D.** Immunohistochemical staining for survivin in normal pancreas (20x), PanIN3 (100x) and adenocarcinoma (100x).
ADC refers to adenocarcinoma.


A
αcIAP-1
αcIAP-2
αXIAP
αSurvivin
B
normal
PanIN2
ADC
αcIAP-1
αcIAP-2

C

Normal (acini + ducts)

Normal (islets)



ADC

ADC

*α*XIAP

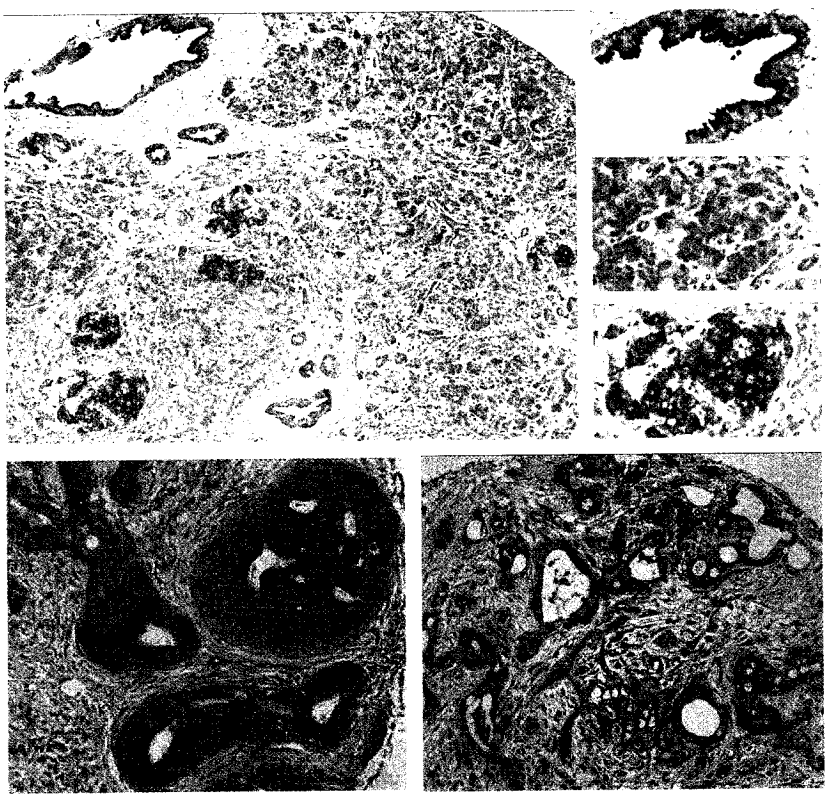
D

normal

duct

acini

islet



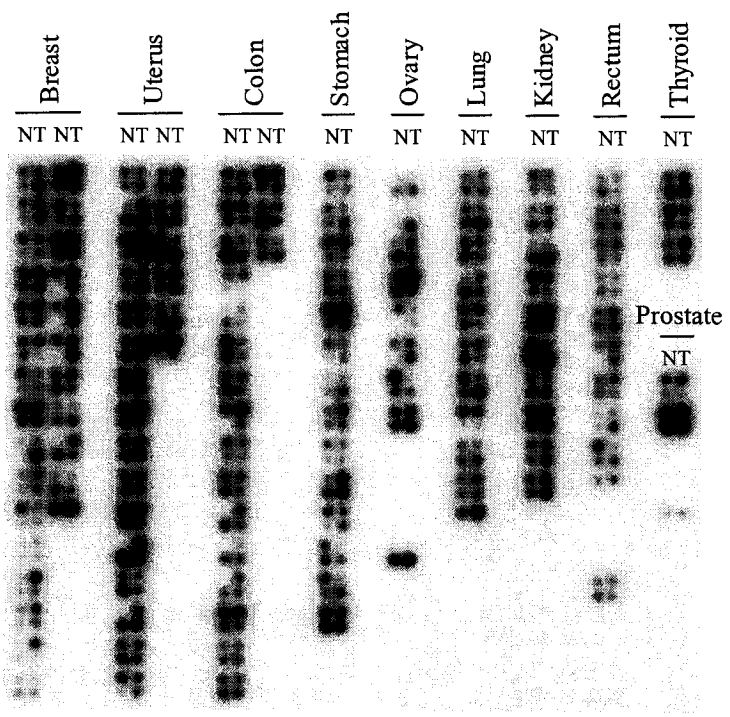
PanIN3

ADC

*α*Survivin

Fig.AII.4. Schematic depiction of FLN29/Xaf-2 Cancer Profiling Array.

A Cancer Profiling Array (Clontech) containing 241 SMART™-amplified cDNAs from tumors and corresponding normal tissues was hybridized with a radiolabelled FLN29/Xaf-2 probe.


Breast
NT NT
Uterus
NT NT
Colon
NT NT
Stomach
NT
Ovary
NT
Lung
NT
Kidney
NT
Rectum
NT
Thyroid
NT
Prostate
NT

Xaf-2

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Abstracts

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