From
The Department of Oncology-Pathology
and The Institute of Environmental Medicine,
Karolinska Institutet, Stockholm, Sweden

APOPTOTIC SIGNALING IN LUNG CARCINOMA CELLS

with focus on mechanisms of radioresistance

Jessica Ekedahl

Stockholm 2003

Doctoral thesis
Apoptotic signaling in lung carcinoma cells with focus on mechanisms of radioresistance

ISBN 91-7349-502-6

To my family

"If we knew what it was we were doing, it would not be called research, would it?"
-Albert Einstein

ABSTRACT

In this thesis, IR-induced apoptotic signaling was studied in a panel of lung carcinoma cell lines. The goal was not only to achieve a better understanding of the process, but also to identify possible targets for pharmacological modulation to enhance the efficiency of radiotherapy.

Lung cancer is the most common malignant disease and the leading cause of cancer mortality in the western world. Radiotherapy is a cornerstone in the treatment of lung cancer. However, inherent or acquired radioresistance is a common clinical problem. Many types of anti-cancer treatment, including radiotherapy, work via the induction of apoptosis. The absence of IR-induced apoptosis in NSCLC (non-small cell lung carcinoma) cells was previously described by our group.

The expression of pro-caspases, μ-calpain and the Bcl-2 family proteins Bcl-2, Bcl-X_L and Bax, was analyzed in a panel of lung carcinoma cell lines. No correlation was observed between the expression of the investigated proteins and intrinsic radiosensitivity. Moreover, SCLC (small cell lung carcinoma) cells were characterized by a high Bcl-2/Bax ratio and the lack of pro-caspase-8, -10, -4 and –1 expression.

The functionality of the apoptosis signaling pathways was therefore studied in more detail. Mitochondrial release of cytochrome *c* and subsequent caspase activation was observed in both SCLC and NSCLC cells after IR-treatment. However, only SCLC cells had nuclear localization of active caspase-3 and underwent apoptosis. This led us to analyze the importance of possible inhibitors of apoptotic signaling at this level.

Neither the expression level nor the intracellular localization of IAPs (inhibitor of apoptosis proteins) correlated with radiosensitivity. Moreover, although all cell lines had a strong expression of HSP72 (heat shock protein), known to have versatile and potent anti-apoptotic properties, knockdown of this protein by RNAi did not sensitize NSCLC cells to IR-induced apoptosis.

The role of the pro-apoptotic mitochondrial regulators Bax and Bak in IR-induced apoptosis was investigated in U1810 (NSCLC) and U1285 (SCLC) cells. IR-treatment induced pro-apoptotic conformational changes in Bak, followed by mitochondrial depolarization, caspase-3 activation and appearance of apoptotic nuclear morphology changes, only in the radiosensitive U1285 cells. Moreover, in response to IR, an early and sustained activation of stress-activated protein kinases p38MAPK and JNK was detected in U1285, but not in U1810 cells. Blocking of SAPK activation in U1285 cells by chemical inhibitors totally abrogated IR-induced apoptosis.

The data presented here demonstrate that IR-induced apoptotic signaling in radioresistant NSCLC cells is abrogated at several levels. Interestingly, in response to other agents, initiation and execution of apoptosis can be activated also in radioresistant cells. This suggests that the apoptotic machinery is functional in NSCLC cells, but is not activated vigorously enough to trigger apoptosis in response to IR.

LIST OF PUBLICATIONS

I. **Joseph B*, Ekedahl J*, Sirzén F, Lewensohn R and Zhivotovsky B**
Differences in expression of pro-caspases in small cell and non-small cell lung carcinoma
Biochemical and Biophysical Research Communications. 1999. 262(2): 381-387.

II. **Joseph B, Ekedahl J, Lewensohn R, Marchetti P, Formstecher P and** Zhivotovsky B
Defective caspase-3 relocalization in non-small cell lung carcinoma
Oncogene. 2001. 20(23): 2877-88.

III. **Ekedahl J, Joseph B, Grigoriev M Y, Müller M, Magnusson C, Lewensohn R and Zhivotovsky B**
Expression of Inhibitor of Apoptosis Proteins in small and non-small cell lung carcinoma cells
Experimental Cell Research. 2002. 279(2): 277-290.

IV. **Ekedahl J, Joseph B, Marchetti P, Fauvel H, Formstecher P, Lewensohn R and Zhivotovsky B**
The role for Heat Shock Protein 72 in the resistance of non-small cell lung carcinoma to ionizing radiation
(submitted for publication)

V. **Viktorsson K*, Ekedahl J*, Lindebro M.C, Lewensohn R, Zhivotovsky B, Linder S and Shoshan M C**
Defective stress kinase and Bak activation in response to ionizing radiation but not cisplatin in a non-small lung carcinoma cell line
(submitted for publication)

*****Authors contributed equally to this work
Previously published papers were reproduced with permission from the respective copyright holders

Additional papers
Zhang A, Zheng C, Lindvall C, Hou M, Ekedahl J, Lewensohn R, Yan Z, Yang X, Henriksson M, Blennow E, Nordenskjöld M, Zetterberg A, Björkholm M, Gruber A and Xu D
Frequent amplification of the telomerase reverse transcriptase gene in human tumors
Cancer Research. 2000. 60(22): 6230-5.

Meijer A E, Ekedahl J, Joseph B, Castro J, Harms-Ringdahl M, Zhivotovsky B and Lewensohn R
High-LET radiation induces apoptosis in lymfoblastoid cell lines derived from ataxia-telangiectasia patients
International Journal of Radiation Biology. 2001. 77(3): 309-17.

CONTENTS

LIST OF ABBREVIATIONS

Δψm	mitochondrial membrane potential
Acinus	apoptotic chromatin condensation inducer in the nucleus
ADP	adenosine di-phosphate
AI	apoptotic index
AIF	apoptosis inducing factor
ANT	adenine nucleotide translocator
AP	activator protein
Apaf	apoptosis protease activating factor
ARTS	apoptosis-related protein in the TGFβ signaling pathway
ASK	apoptosis stimulating kinase
ATF	activating transcription factor
ATM	ataxia telangiectasia mutated
ATP	adenosine tri-phosphate
ATR	ATM and Rad3-related
Bak	Bcl-2 homologous antagonist/killer
Bax	Bcl-2 associated X protein
Bcl	B-cell lymphoma
BH	Bcl-2 homology
Bid	BH3-interacting-domain death agonist
BRCA	breast cancer
CAD	caspase-activated DNase/DFF40
caspase	cysteinyl aspartate protease
CDK	cyclin dependent kinase
Ced	cell death abnormal
CHOP	C/EBP homologous protein/GADD153
CP	cis-diamminedichloroplatinum(II), cisplatin
DAPI	4',6-diamidino-2-phenylindole dihydrochloride
DED	death effector domain
DFF40	DNA fragmentation factor of 40 kDa/CAD
DFF45	DNA fragmentation factor of 45 kDa/ICAD
DIABLO	direct IAP-binding protein with low pI
DISC	death-inducing signaling complex
DNA-PKcs	DNA-dependent protein kinase catalytic subunit
DSB	DNA double-strand break
EGF-R	epidermal growth factor receptor
Egl	egg-laying abnormal
ER	endoplasmic reticulum
ERK	extracellular regulated kinase
FADD	Fas-associated death domain
FAP	Fas-associated phosphatase
FLIP	flice (caspase-8) like inhibitor protein (Casper/I-FLICE/FLAME-1/CASH/CLARP/MRIT/Usurpin)
GADD 153	growth arrest and DNA damage gene 153 /CHOP
Her2/Neu	heregulin/neuregulin receptor/ErbB2
HR	homologous recombination

HSP	heat shock protein
IAP	inhibitor of apoptosis protein
ICAD	inhibitor of caspase-activated DNase/DFF45
IGF-R	insulin-like growth factor receptor
IR	ionizing radiation
JNK	c-Jun NH_2-terminal protein kinase
MAPK	mitogen-activated protein kinase/ERK
MAPKK	MAPK kinase
MAPKKK	MAPKK kinase
Mdm-2	mouse double minute-2
MEK	MAP/ERK kinase
MEKK	MEK kinase
MPT	mitochondrial permeability transition
NBS	Nijmegen breakage syndrome
NEHJ	non-homologous end joining
NES	nuclear exit signal
NFκB	nuclear factor kappa B
NLS	nuclear localization signal
NSCLC	non-small cell lung carcinoma
Nuc	nuclease abnormal
OMP	outer mitochondrial membrane permeabilization
PAK	p21-activated kinase
PARP	poly (ADP-ribose) polymerase
PBR	peripheral benzodiazepine receptor
PI	propidium iodide
PRIMA	p53 reactivaton and induction of massive apoptosis
PT	permeability transition
RNAi	RNA interference
SAPK	stress-activated protein kinase
SCID	severe combined immunodeficiency
SCLC	small cell lung carcinoma
SEK	SAPK/ERK kinase
SF	surviving fraction
siRNA	short interfering RNA
Smac	second mitochondria-derived activator of caspases/DIABLO
SSB	single-strand break
TNF	tumor necrosis factor
TRAIL	TNF-related apoptosis inducing ligand
VDAC	voltage dependent anion channel
VP16	etoposide
XRCC	X-ray cross complementation

BACKGROUND

1.1 LUNG CANCER

Lung cancer is the most common malignant disease and the leading cause of cancer mortality in the western world (Parkin et al., 2001). In Sweden, primary tumors of the bronchus and lung are the third and fourth most common cancer diagnose in men and women, respectively (statistics for year 2000 from the Swedish National Board of Health and Welfare). A decreasing incidence of lung cancer in Swedish men was observed around 1980 and this trend is continuing. However, since the 1960's, the lung cancer incidence among Swedish women has steadily increased and is now surpassing the male incidence in the age group 0-54 years.

Up to 15% of the total lung cancer incidence occur among non-smokers. Causes/contributing factors other than smoking have been identified and includes genetic predisposition, e.g. by allelic variants of genes important for detoxification, loss of tumor suppressor genes and exposure to environmental factors such as passive smoking, chemicals and exposure to radon (Williams & Sandler, 2001). However, it is well established that the major cause of lung cancer is tobacco smoking, responsible for approximately 85-90% of bronchogenic carcinoma (Williams & Sandler, 2001). Tobacco smoke contains hundreds of known human carcinogens and smoking is associated with increased cancer rates in virtually every organ of the body including the lung, oral cavity, esophagus, colon, pancreas, bladder, bone marrow, cervix and kidney (Shields, 2002). Lung cancer incidence faithfully mirrors the socio-geographical and gender differences in smoking habits. In Sweden, one million people are daily smokers, and approximately 8000 persons/year will die in diseases caused by smoking (source: The Swedish Council for Information on Alcohol and other Drugs). The majority of new smokers in Sweden are young women in their early adolescence, however, a decrease in the total smoking prevalence in Sweden has been observed during the last two decades (source: Statistics Sweden). Prevention is the obvious and only measure to take against lung cancer. More information together with new legislation is needed to deter people from tobacco smoking.

1.1.1 Lung cancer classification

Lung carcinomas are classified into four histological types: small cell lung carcinoma (SCLC), squamous cell lung carcinoma (SCC), adenocarcinoma (AC), and the more undifferentiated large cell lung carcinoma (LC). The histological features and clinical course makes SCLC a separate entity. The other three types are referred to as non-small cell lung cancer (NSCLC, WHO-classification 1977). The characteristics of the different histological types of lung cancer are summarized in **Figure 1**.

It is common to find tumors with mixed histology. The cell of origin in lung cancer has not been identified but is thought to be a pluripotent epithelial stem cell that would differentiate into the cell types present in the lung, namely the mucus producing cylindrical cells, neuroendocrine cells and type I and II pneumocytes.

Squamous cell carcinoma (~30%)

* Closely correlated with smoking (dose-dependent)

* Tends to spread locally

* Highly expressed genes encoding proteins with detoxification/antioxidant properties

Adenocarcinoma (~30%)

* Most common type of lung cancer in women and non-smokers

* Worldwide incidence increasing

* Highly expressed genes encoding small-airway-associated and immunologically related proteins

* *K-RAS* mutations frequently reported

Large cell carcinoma (5-10%)

*Very primitive, undifferentiated cells

* High tendency to metastasize

Small cell lung carcinoma (15-20%)

* Occurs almost exclusively in smokers, more prevalent in women than men

*Tendency to disseminate early

* Initially chemo- and radiosensitive, becoming resistant

* *TP53* is mutated in almost all tumors

Figure 1

Histopatological classification of lung carcinoma and some characteristic traits.

1.1.2 Genetic and molecular changes in lung cancer

The hallmarks of cancer, as described by Hanahan and Weinberg, include growth signal self-sufficiency, insensitivity to growth-inhibitory signals, evasion of programmed cell death (apoptosis), limitless replicative potential, sustained angiogenesis, and tissue invasion and metastasis (Hanahan & Weinberg, 2000). Genetic and molecular alterations in lung cancer are described that enable all these features.

1.1.2.1 Growth factor independence

Overexpression of growth-receptors, excretion of growth factors, and dysregulation of the downstream signaling pathways all represent ways to obtain independence of growth factor stimulation.

Insulin-like growth factor I (IGF-I) expression is elevated in more than 95% of SCLCs and 80% of NSCLCs (Minuto et al., 1986; Reeve et al., 1990). Moreover, a causal role for IGF-II in lung adenocarcinoma was recently demonstrated in a mouse model (Moorehead et al., 2003).

The IGF-I receptor is present in most lung cancers (Kaiser et al., 1993). Up to 80% of SCCs overexpress EGF-R, and 20% of ACs overexpress Her2/neu (Hirsch et

al., 2002). These growth factor receptor tyrosine kinases normally transduce proliferative extracellular signals. In addition, they are now therapeutic targets since the recent development of tyrosine kinase inhibitors.

Virtually all SCLC and up to 70% of NSCLC tumors exhibit neuroendocrine features with production of neuroendocrine peptides, such as gastrin releasing peptide, bradykinin, gastrin, insulin-like growth factor I, and vasopressin, of importance for growth stimulation by para- and autocrine loops (Hirsch et al., 2002).

The small GTPase p21-Ras, encoded by the *RAS* oncogene, is important for the transduction of receptor tyrosine kinase signaling and subsequent activation of the mitogen-activated protein kinase (MAPK)-cascade, or the phosphatidylinositol-3-kinase pathway (Macaluso et al., 2002). Mutated Ras is locked in a permanent active state and is thus independent of upstream signals. The main members of the *RAS* gene family are *H-RAS*, *K-RAS* and *N-RAS*. *RAS* mutations, especially in *K-RAS*, were found in ~30% of NSCLC (Macaluso et al., 2002), mainly ACs, and is a marker for poor prognosis (Sekido et al., 1998).

1.1.2.2 Cell cycle dysregulation

Cell cycle progression is tightly controlled via the family of cyclins and their partners, the cyclin-dependent kinases (CDKs). Loss of negative control functions, due to mutation of key regulators, is commonly found in cancer and cause insensitivity to growth-inhibitory signals.

The Rb protein, encoded by the retinoblastoma tumor suppressor gene, is responsible for the timely release of transcription factors of the E2F-family to initiate the entry into the G1-phase of the cell cycle. Inactivating mutations of *RB* is found in >90% of SCLC but only in 15% of NSCLC (Kelley et al., 1995). However, the *p16INK4A* gene, encoding an inhibitor of CDK4 and thus negatively regulating the Rb-signaling pathway, is inactivated in >50% of NSCLC (Sekido et al., 1998). Moreover, cyclin D1, the partner of CDK4, is overexpressed in NSCLC (Reissmann et al., 1999). All these changes contribute to the loss of negative cell cycle regulation.

MYC is a proto-oncogene encoding a nuclear transcription factor that is essential for cell proliferation and prevention of differentiation. The function of Myc is regulated through heterodimerization. The Myc/Max heterodimer recognizes the consensus sequence with high affinity, resulting in transcription, while formation of the Max/Mad complex is thought to antagonize Myc function. Abnormal expression of all three members of the Myc family (n-Myc. l-Myc and Myc) is commonly found in both SCLC and NSCLC (Richardson & Johnson, 1993). In SCLC, Myc and n-Myc overexpression is associated with poor prognosis (Viallet & Minna, 1990).

1.1.2.3 Evasion of apoptosis

Evasion of cell death can be conferred by mutation of the tumor suppressor gene *TP53*, located at chromosome 17p13.1. This is one of the most conspicuous genetic alterations in cancer and was found in >90% of SCLC and >50% of NSCLC (Greenblatt et al., 1994). *TP53* encodes the "Guardian of the genome", p53. Upon exposure to cellular stress, such as hypoxia or DNA damage, the p53 protein is activated by phosphorylation, resulting in accumulation in the cell nucleus. The induction of p53 can result in either cell cycle arrest or apoptosis through transactivation/transrepression of a number of target genes. An intact p53-response

is of paramount importance for the integrity of the genome (Gudkov & Komarova, 2003).

Bcl-2 is a potent anti-apoptotic protein that was found highly expressed in 75% of SCLC, but not in NSCLC, biopsies (Jiang et al., 1995; Kaiser et al., 1996). However, it appears that Bcl-2 expression does not correlate with disease progression. The higher sensitivity of SCLC to apoptosis-inducing agents, such as most anti-cancer drugs, makes this an apparent paradox. The Bcl-2 family consists of both pro- and anti-apoptotic proteins, and the intricate balance between them determined the final influence on apoptotic signaling. The mechanisms of apoptosis inhibition by Bcl-2 family proteins are discussed in more detail in section 1.2.3.3.

1.1.2.4 Immortalization

Differentiated cells normally undergo a limited number of possible cell divisions before they become senescent. Telomers are structures of repetitive DNA sequences that cap the ends of chromosomes and protect against loss of chromosomal information, stabilizing chromosomes from degradation and illegitimate recombination. Due to the "end replication problem", the telomers are shortened at each mitotic cycle and this telomeric shortening is considered to act like a biological clock, ticking towards cellular senescence. The DNA-polymerase telomerase is capable of restoring telomeric length and is therefore a potential oncogene. Activation of human telomerase and amplification of the telomerase reverse transcriptase gene (*hTERT*), encoding the rate-limiting component of telomerase activity, was found in both SCLC and NSCLC (Sekido et al., 1998; Zhang et al., 2000).

1.1.2.5 Angiogenesis

Both primary and metastatic tumor growth require blood supply via the recruitment of vessels from the surrounding tissue. Vascular endothelial factor (VEGF) and basic fibroblast factor (bFGF) are the two most important inducers of tumor angiogenesis. VEGF-expression was found to negatively correlate with overall survival, and the receptor for VEGF, Flt-1, is frequently expressed in SCC tumors (Volm et al., 1997). bFGF expression was detected in ~70% of NSCLCs (Takanami et al., 1996) but the prognostic use of this finding is not so clear.

1.1.2.6 Metastasis

Metastatic potential is determined by several factors; the ability to detach from the basal membrane and survive without adhesion-dependent stimulation, the expression of proteases necessary for invasion of the blood- or lymphatic circulation and other tissues, and finally self sufficiency in growth stimulation and the capability of angiogenesis (Hanahan & Weinberg, 2000). For tissue invasion, the tumor needs to activate proteolytic enzymes to breech the barrier of the basal membrane and to dissolve the extra-cellular matrix. The family of matrix metalloproteinases (MMPs) is important for tumor invasion, metastatic potential and tumor-related angiogenesis. However, not all lung cancers express the MMPs believed to be most important in promoting the neoplastic process, and there are conflicting reports regarding the prognostic significance of MMPs in lung cancer (Bonomi, 2002).

A possible sequence of transforming events taking place in the development of lung cancer is outlined in **Figure 2**. Smokers were found to have multiple pre-neoplastic alterations in the mucous membranes outlining the major bronchi. These lesions harbored abnormalities identical to some of those found in invasive carcinoma, including *TP53* mutations, upregulation of Myc and Ras proteins, cyclin D1 overexpression, Bcl-2 overexpression, allele loss at several loci (3p, 9p, 8p and 17p) and aneuploidy (Hirsch et al., 2001).

The loss of alleles at 3p is observed in more than 90% of SCLC tumors and approximately 80% of NSCLC tumors (Kok et al., 1987). The *FHIT* gene, localized at 3p14.2, may represent one of several potential tumor-suppressor genes located on chromosome 3p. Eighty per cent of SCLC tumors show abnormalities of this gene (Sozzi et al., 1996). Loss of the *FHIT* gene results in the accumulation of diadenosine tetraphosphate that could lead to stimulation of DNA synthesis and cell proliferation (Croce et al., 1999).

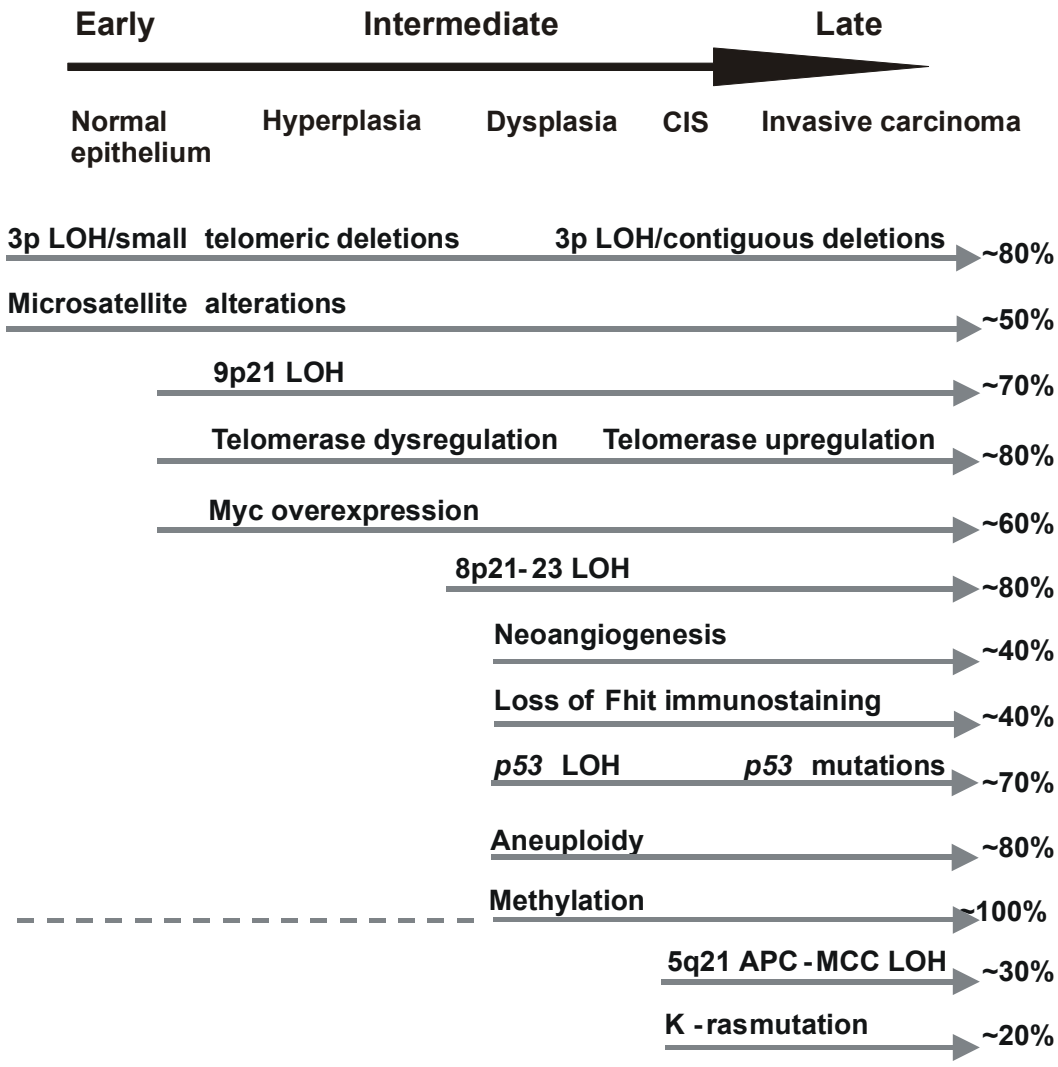


Figure 2

Sequential changes during lung cancer pathogenesis. Modified after Hirsch et al. 2001. Abbreviations; CIS, cancer in situ, LOH, loss of homozygosity; Fhit, fragile histidine triad; APC-MCC, adenomatous polyposis coli-mutated in colon cancer.

1.1.3 Lung cancer therapy - the problem of resistance

The clinical treatment of lung cancer, summarized in **Figure 3**, is determined by the histological type and tumor stage (source: Swedish National Therapy Guidelines for lung cancer, 2001).

The prognosis for NSCLC correlates closely to the tumor stage. For apparently local disease classified as stage IA (i.e. a tumor less than 3 cm in diameter and no evidence for local lymph node engagement) the 5-year survival after surgery is ~75% thus underscoring the problem of early occult dissemination (Porrello et al., 2002). NSCLC is frequently resistant to both drug- and radiotherapy and complete response to therapy is rare. Therefore, resistance to treatment presents a major problem. The resistance mechanisms are numerous and diverse and depend on the detoxifying capacity of the cells, DNA repair capacity, drug uptake/excretion and many other factors (reviewed in Volm & Rittgen, 2000).

SCLC is initially among the most drug- and radiosensitive tumors. If the disease is local, i.e. is confined to one side of the thorax and can be covered within an irradiation-field, concomitant chemo- and radiotherapy can be curative. The probability of complete clinical remission is even higher for patients with limited-stage SCLC than for patients with stage III NSCLC after combined radio- and chemotherapy (Erridge & Murray, 2003). However, only approximately 10% of SCLC patients presents with limited disease. SCLC is associated with early metastasis and relapse within 2 years is typical. Relapse is associated with a quick development of resistance to both drug- and radiotherapy, making the overall long-term prognosis for SCLC very poor (less than 10%).

NSCLC

Stage IA-B (local disease) and IIA-B (local lymph node engagement)
* Surgery
* Curative radiotherapy if surgery is contraindicated
* Adjuvant or neo-adjuvant chemo- or radiotherapy

Stage IIIA (local lymph node engagement)
* Surgery in selected cases
* Radiotherapy alone, benefits 5-10% of patients
* Chemotherapy + radiotherapy / neoadjuvant therapy
* Post-operative adjuvant chemotherapy

Stage IIIB (regional lymph node engagement)
* Chemo- or radiotherapy alone
* Chemotherapy + radiotherapy

Stage IV (distant metastasis)
* Palliative chemotherapy (platinum-based)
* New chemotherapy agents
* Palliative radiotherapy

SCLC

Limited disease (i.e within one side of the thorax)
* Concomitant platinum-based chemo-radiotherapy with PCI for responders

Extensive disease
* Combined chemotherapy +/- PCI
* Palliative radio- or chemotherapy

Figure 3.

Treatment options for lung cancer. Abbreviations; PCI, prophylactic cranial irradiation

1.2 APOPTOSIS

In the seminal paper by Kerr, Wyllie and Currie, a distinct form of cell death was described (Kerr et al., 1972). This cell death was different from necrosis and characterized by specific morphological changes including cell shrinkage, chromatin condensation and nuclear fragmentation. Moreover, this specific type of cell death was found to occur during both physiological and pathological conditions. The term apoptosis, put together from the Greek words apo (από =off, from) and ptosis (πτώσίσ =falling, drooping), was coined by Kerr et al. to denote this form of cell death. Although this finding may be considered the take off point for apoptosis research, observations of physiological cell death dates back more than a hundred years. One of the first findings consistent with apoptosis was reported in 1885 by Walther Fleming who observed a morphologically distinct form of cell death in regressing ovarian follicles (Clarke & Clarke, 1996).

During the last two decades, the genetic and biochemical events responsible for the regulation and execution of apoptosis have been studied in detail. Apoptosis is an active, energy-demanding process that can be switched towards necrosis should the ATP level be insufficient (Leist et al., 1997). What distinguishes apoptotic cell death is the intactness of organelles, membranes and overall ultrastructure during the process. This is in contrast to necrotic cell death, where the release of intracellular material causes an inflammatory response in the surrounding tissue. The resolution of apoptosis is that the cell remnants, the apoptotic bodies, are phagocytosed by neighboring cells or macrophages (reviewed by Henson et al., 2001).

Timely regulated cell death is of paramount importance for embryogenesis, tissue homeostasis and for the development and functional regulation of the immune system (Thompson, 1995). Dysregulation of apoptosis is associated with a number of diseases. Excessive triggering of apoptosis occurs in HIV, Parkinson's disease and the secondary cell death triggered in the healthy tissue surrounding a myocardial infarction or a stroke (Kam & Ferch, 2000). Failure to induce apoptosis can result in lymphoproliferative diseases or cancer (Fadeel et al., 1999). In tumorigenesis, dysregulation of apoptosis together with deregulation of the cell cycle are considered necessary events for tumor progression (Hanahan & Weinberg, 2000).

The terms programmed cell death (PCD) and apoptosis are often used interchangeably. However, while cell death during development often displays apoptotic morphology, other types of programmed cell death, exhibiting only some features of apoptosis or even necrotic morphology, have been described (reviewed in Kitanaka & Kuchino, 1999). PCD may thus be a more proper term for physiological cell death, e.g. during embryogenesis and morphogenesis, with or without apoptotic morphology. In the present thesis, apoptosis denotes cell death that exhibits both the typical morphology described above as well as the stereotypic biochemical events involving caspase activation (Samali et al., 1999b).

1.2.1 Genetic control of cell death

The genetic regulation of programmed cell death was first elucidated in the roundworm *Caenorhabditis elegans*. In this organism, a set of evolutionary conserved

genes is activated that specifically regulate the induction and execution of cell death during its development

(Ellis & Horvitz, 1986; Ellis et al., 1991; Hengartner et al., 1992; Yuan et al., 1993). The implication of this paradigm was the existence of a limited set of genes that are specialized in the regulation of cell death, and that are both necessary and sufficient to drive the process. The genes discovered in *C. elegans* served as a guide in the exploration of the mammalian homologues. A schematic comparison of the most important gene products of *C. elegans* and their mammalian homologues is presented in **Figure 4**.

The *CED-3* gene encodes a cysteine protease, in other words a caspase-like protease, which needs an adaptor protein, Ced-4, to be auto-catalytically cleaved and functional. Ced-9 is a regulatory protein that can bind to Ced-4 and thus abrogates the activation of Ced-3. Finally, Egl-1 is a Ced-9 antagonist that promotes Ced-3 activation by binding to Ced-9 (Conradt & Horvitz, 1998).

C. elegans	**mammalian homologue**	**function**	***C. elegans* Refs.**
Egl-1	BH3-only proteins	activator of Ced-4	Conradt et al. *Cell.* 1998
Ced-9	Bcl-2	binds Ced-3	Hengartner et al. *Nature.* 1992
Ced-4	Apaf-1	adaptor protein	Ellis et al. *Cell.* 1986
Ced-3	caspases	proteases	Ellis et al. *Cell.* 1986
Nuc-1	DNase II	DNA degradation	Wu et al. *Genes Dev*. 2000
			Lyon et al. *Gene*. 2000
Wah-1	AIF	DNA degradation	Wang et al. *Science* 2002
CPS-6	endonuclease G	DNA degradation	Parish et al. *Nature* 2001

Figure 4

An overview of the proteins participating in regulation and execution of programmed cell death in *C. elegans* and their mammalian homologues.

1.2.2 Initiation and execution of apoptosis

1.2.2.1 Apoptotic signaling pathways

Two major apoptotic signaling pathways are recognized in mammalian cells. They are the extrinsic, receptor-mediated (reviewed in Schmitz et al., 2000), and the intrinsic, mitochondria-mediated (reviewed in Debatin et al., 2002) pathways. The main participants and their place in these signaling pathways are outlined in **Figure 5**.

The extrinsic pathway is activated upon the binding of an appropriate ligand to members of the TNF-receptor superfamily that contain an intracellular death domain, e.g. Fas-R/CD95, TNF-R and TRAIL-R. This results in receptor trimerization and recruitment of intracellular adaptor proteins, TRADD or FADD, necessary for the further recruitment of initiator pro-caspases-8 and -10. The assembled receptor/adaptor-protein/pro-caspase aggregate forms the so-called death inducing

signaling complex (DISC) in which the initiator pro-caspases can be activated by auto-proteolytic cleavage (Medema et al., 1997). The subsequent events are cell type specific (Scaffidi et al., 1998). In "type I" cells, the initiator caspases directly activate executioner caspases-3 and -7. In "type II" cells, active caspase-8 cleaves the pro-apoptotic Bcl-2-family member Bid. Truncated Bid (tBid) induces translocation and oligomerization of pro-apoptotic Bax-like proteins, resulting in mitochondrial permeabilization and subsequent activation of executioner caspases (Li et al., 1998b). The mitochondria can also function as amplifiers for the extrinsic pathway since caspase-3 can initiate "type II" signaling by cleaving pro-caspase-8 (Kuwana et al., 1998).

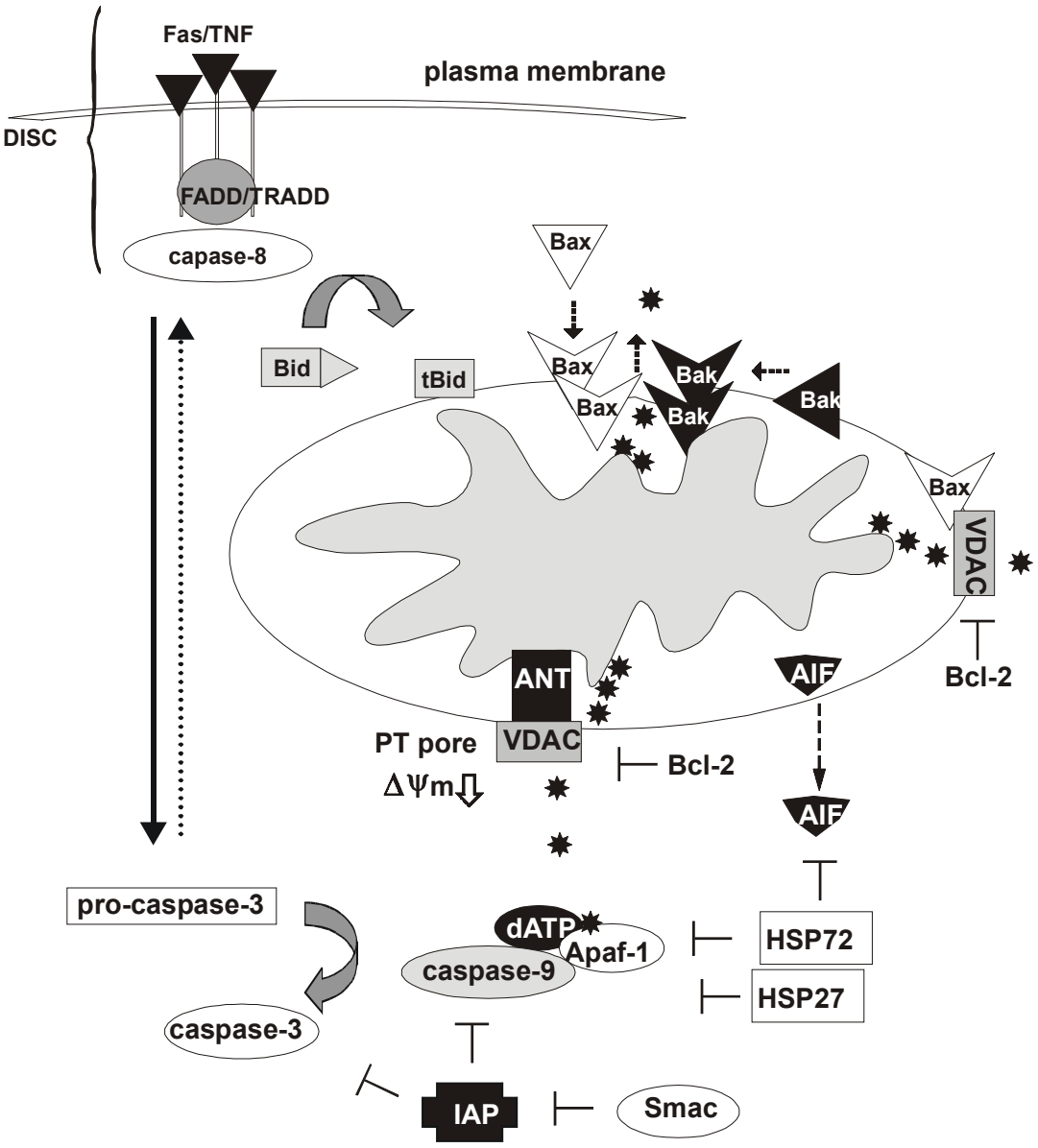


Figure 5

An overview of the major apoptotic signaling pathways. Please see text for details.

✸ = cytochrome *c*

The mitochondria are not only the power plant of the cell but also the major death signal integrators. Mitochondrial membrane permeabilization, and the subsequent release of pro-apoptotic molecules, e.g. cytochrome *c*, initiates the intrinsic pathway. This event is tightly regulated by the Bcl-2 family of proteins. Cytosolic cytochrome *c* induces the assembly of the apoptosome complex, a multimeric holoenzyme consisting of Apaf-1, pro-caspase-9, dATP and (reviewed in Cain et al., 2002). Binding of cytochrome *c* to Apaf-1 is necessary for the induction of conformational change allowing Apaf-1 to, in the presence of dATP, aggregate and form a heptameric platform for pro-caspase-9 assembly (Acehan et al., 2002; Li et al., 1997; Zou et al., 1997). In contrast to other pro-caspases, pro-caspase-9 can be activated by dimerization in the apoptosome complex (Renatus et al., 2001) without the need for processing (Stennicke et al., 1999). The active apoptosome complex in turn activates downstream executioner pro-caspases-3 and -7. The XIAP protein was found to directly interact with the apoptosome complex and can inhibit the activation of pro-caspase-9 within the apoptosome, or prevent the release of active caspase-3 from the complex (Bratton et al., 2001).

Several pro-apoptotic factors (AIF, endonuclease G, HSP60/10, caspases, ARTS, Smac and HtrA2/Omi) reside in the intermembrane space of mitochondria. AIF is a flavoprotein that, upon release from the mitochondrial intermembrane space, translocates to the nucleus and induces peripheral chromatin condensation through an unknown mechanism (Daugas et al., 2000; Susin et al., 1999). The AIF protein was recently proposed to have an anti-apoptotic function as long as it is still in the mitochondria (Lipton & Bossy-Wetzel, 2002). Endonuclease G also translocates to the nucleus and is able to induce nucleosomal DNA fragmentation in CAD-/- mouse embryonic fibroblasts (Li et al., 2001). The complex of HSP60/10 promotes the activation of executioner caspases (Samali et al., 1999a). ARTS (apoptotic response to TGFβ signals) is localized in mitochondria and translocates to the nucleus, especially in the model of TGFβ-induced cell death (Larisch et al., 2000). The exact pro-apoptotic mechanism for ARTS is not clarified, but it was proposed to propagate pro-caspase-3 activation. Smac and HtrA2/Omi are both released from the mitochondria to cytosol where they can remove XIAP from the apoptosome complex or inhibit the effect of IAPs on active caspases (Du et al., 2000; van Loo et al., 2002b).

1.2.2.2 Caspases, the executors of apoptosis

Caspases are a large family (14 mammalian caspases have been discovered so far) of cysteine-proteases that cleaves their substrates C-terminal to aspartic residues (Thornberry & Lazebnik, 1998). In addition to the rare preference for aspartic residues, the three N-terminal neighboring residues add further substrate specificity (Thornberry, 1997). Caspases are synthesized as inactive pro-enzymes with regulatory N-terminal pro-domains. They can be divided into two groups according to the length of the pro-domain. The initiator caspases (pro-caspase-2, -8, -9, -10 and -12) and pro-inflammatory caspases involved in activation/maturation of cytokines (pro-caspase-1, -4, -5, -11 and -13) contain a long pro-domain with protein interaction motifs, while executioner caspases (pro-caspase-3, -6 and -7) have a short pro-

domain. No human homologue has been identified for pro-caspase-11 or -13. Moreover, the gene for human pro-caspase-12 harbors a frame shift mutation causing a premature stop codon, and humans therefore lack pro-caspase-12 protein expression (Fischer et al., 2002).

Caspases are mainly localized in the cytosol, but have also been reported to reside in the mitochondrial intermembrane space (pro-caspase-2, -3 and -9), in the nucleus (pro-caspase-2 and -3) and in the Golgi apparatus (pro-caspase-2) (Chandra & Tang, 2003; O'Reilly et al., 2002; Zhivotovsky et al., 1999). The mitochondrial localization could be cell type specific (van Loo et al., 2002a). During both the initiation and execution of apoptosis, caspases need to relocalize to get in contact with their substrates. For example, nuclear translocation of active caspase-3 is needed for caspase-dependent chromatin digestion (Woo et al., 1998). The exact mechanisms regulating this translocation are not known.

Upon initiation of apoptosis, depending on the activated pathway (extrinsic or intrinsic), initiator pro-caspases are recruited to adaptor proteins FADD, TRADD or Apaf-1 by means of their protein interaction domains, to form the DISC or the apoptosome complex. In these complexes, initiator caspases are activated through dimerization and autocatalytic processing (Boatright et al., 2003). The active initiator caspases process the executioner pro-caspases to form tetramers, consisting of two long and two short subunits (Thornberry & Lazebnik, 1998). These tetramers are the active, mature form of caspases that can execute apoptosis by cleavage of a multitude of cellular substrates. The cleavage and activation of executioner pro-caspases can also be initiated by other enzymes such as granzyme B, cathepsins or calpains via direct and indirect mechanisms (Mathiasen & Jäättelä, 2002).

The caspases are indispensable for normal embryonic development, especially in the nervous and immune systems. In mice with defect or missing alleles for *CASP3*, *CASP9* or *APAF1*, embryonic or perinatal mortality was observed together with severe malformation and gross enlargement of the cerebrum (Kuida et al., 1998; Kuida et al., 1996; Yoshida et al., 1998). Mice lacking *CASP6* develops normally, while *CASP7*, *-8* and *-9* knockout mice die at early gestation (Zheng et al., 1999). Although caspases are important for the execution of apoptosis, there are several examples on *CASP3*-, *CASP9*- or *APAF1*-deficient mammalian cells that can undergo apoptosis-like cell death after a variety of stimuli, although at a reduced rate and with prolonged kinetics. Thus, there is a certain amount of redundancy in the caspase signaling pathways (Borner & Monney, 1999).

The long list of known caspase substrates was recently extensively reviewed (Fischer et al., 2003). The inactivation of DNA repair proteins (e.g. DNA-PKcs, PARP), activation of CAD and disruption of cytoskeletal components (e.g. cytokeratins, actin, fodrin, lamin) are consequences of caspase cleavage that contribute to the apoptotic morphology and cell death. However, proteins involved in cell cycle control, cell adhesion, transcription, RNA synthesis, translation, signal transduction and regulation are also on the long list of known caspase substrates, strongly indicating a wider repertoire of functions for caspases than mere apoptosis execution. Caspases-1, -4 and -5 are involved in the maturation of pro-inflammatory cytokines (Fadeel et al., 2000). The necessity of caspase-3 activity has been implied for normal skeletal muscle differentiation (Fernando et al., 2002). Caspase activity was also detected in the terminal differentiation of keratinocytes (Weil et al., 1999) and the lens fiber cells of the eye (Ishizaki et al., 1998). Moreover, the activity of caspases

seems to be necessary for T-cell maturation and proliferation (Kennedy et al., 1999), erythropoiesis (Zermati et al., 2001), thrombopoiesis and platelet formation (De Botton et al., 2002). Further research is necessary to fully understand the non-apoptotic role of caspases.

1.2.3 Regulation of apoptosis

1.2.3.1 Receptor-mediated apoptosis

In order to be executed, receptor-mediated apoptosis requires several criteria to be fulfilled. First, the receptor needs to be present at the cell surface. Second, an appropriate ligand must be able to bind to the receptor. Third, receptor oligomerization and the recruitment of intracellular adaptor proteins are needed for receptor activation and transduction of the signal. Regulation of receptor-mediated apoptosis has been described that interfere with each of these levels. There are also additional players of importance for regulation of receptor-mediated apoptosis.

FLIP (FLICE-inhibitory protein) was originally discovered as a protein involved in the viral inhibition of receptor-mediated apoptosis (Thome et al., 1997). Cellular FLIP was soon discovered and was shown to be a caspase-8-like protein that lacks both the catalytic active site and the residues that form the substrate binding pocket, and is therefore inactive (Irmler et al., 1997). The FLIP protein contains a death domain (DD) and competes with initiator caspases for binding to the death-receptor/FADD or TRADD complex (Hu et al., 1997), thereby abrogating caspase activation. FLIP expression prevents receptor-mediated apoptosis but not cell death induced by perforin/granzyme, chemotherapeutic drugs or ionizing radiation (Kataoka et al., 1998). Another anti-apoptotic mechanism of FLIP has been described, giving it a more active role than merely inhibiting signal transduction. In T-cells, FLIP was observed to interact with TNF-receptor associated factors (TRAF)-1 and -2 resulting in activation of NF-κB and pro-survival signaling (Kataoka et al., 2000). FLIP could therefore possibly act as a switch between pro- and anti-apoptotic receptor-mediated signaling.

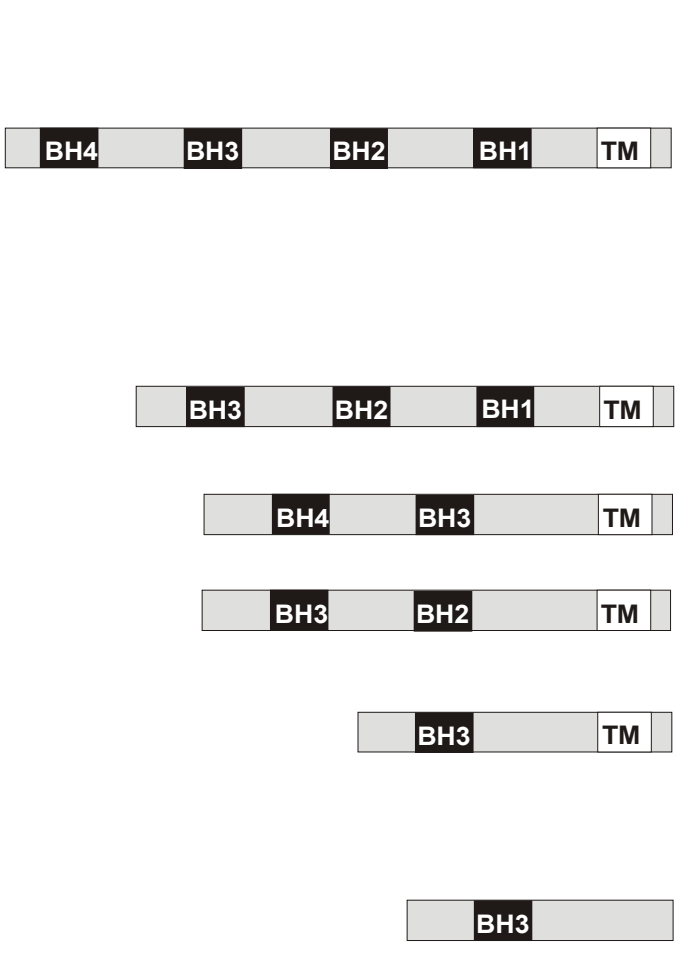
The FAP-1 (Fas-associated protein) is under normal conditions primarily associated with the Golgi complex and peripheral vesicles. FAP-1 protein can bind to the regulatory domain of Fas-R (Sato et al., 1995) and was shown to co-localize with Fas-R in the Golgi complex upon Fas-stimulation of pancreatic tumor cells (Ungefroren et al., 2001). Moreover, the expression of FAP-1 correlated with the sensitivity to Fas-mediated death. The association of FAP-1 to Fas-R was proposed to prevent the translocation of Fas-R from intracellular stores to the cell surface, thereby decreasing the amount of Fas-R on the cell surface.

DAXX is an adaptor protein that can bind to the death domain of the Fas-R (Yang et al., 1997), especially in UV- and TGFβ-induced apoptosis. DAXX is mainly believed to activate the MAPK/SAPK pathway via ASK-1 (Chang et al., 1998). The majority of studies on DAXX-function are based on overexpression, making it difficult to certify its physiologic importance. However, the anti-apoptotic effect of DAXX was recently demonstrated by the use of RNAi to knockdown endogenous DAXX-expression (Michaelson & Leder, 2003). Thus, the recruitment of FADD or DAXX to Fas-R can determine weather the subsequent signaling will be pro-apoptotic or pro-survival, respectively.

In order for the receptor-mediated signals to be transduced, they need to overcome a certain "threshold level". The amount of receptor and ligand molecules present on/at the cell surface determines the achievable amplitude of the signal. The expression of decoy receptors, lacking the intracellular death domain, or even soluble Fas-R has been described (Cheng et al., 1994; Pan et al., 1997; Sheridan et al., 1997). These decoy receptors "dilute" the extra-cellular signal and attenuate the cellular response.

1.2.3.2 The Bcl-2 family

The *BCL-2* oncogene is located at a gene locus on chromosome 18 (band q21) that is involved in a certain type of translocation, t(14:18), found in approximately 60 percent of B-cell lymphomas, hence the name Bcl-2 (Tsujimoto et al., 1984). The Bcl-2 protein family now consists of more than 20 pro- and anti-apoptotic Bcl-2 homologues. An overview of the Bcl-2 family protein members is presented in **Figure 6**.



anti-apoptotic
Bcl-2
Bcl-X_L
Bcl-W
Mcl-1
Blf-1
Boo/Diva
Bcl-2B

pro-apoptotic
Bak
Bax
Bok

Bcl-X_S

Bcl-G_L
Bfk

Bim
Bik
Hrk/DP5
Blk
Bcl-G_S

Bid
Bad
Bmf
Noxa
Puma

Figure 6.

The main protein members of the Bcl-2 protein family. BH, Bcl-2 homology domain; TM, trans-membrane domain.

The regulation of apoptotic signaling by Bcl-2 family members is exerted in several ways (reviewed in Cory & Adams, 2002). Bcl-2 family members are the major regulators of the mitochondrial events that activate the intrinsic pathway. They also govern ER-mediated regulation of the intracellular Ca^{2+} level.

Most Bcl-2 family members possess a C-terminal trans-membrane (TM) domain allowing them to insert into membranes such as the mitochondrial outer membrane, the endoplasmic reticulum and the nuclear envelope. The anti-apoptotic members all have four Bcl-2 homology (BH1-4) domains. The BH4 domain is responsible for specific anti-apoptotic effects and the pro-apoptotic Bcl-2 members all lack this domain except Bcl-X_S that hide its BH4 domain due to the tertiary structure of Bcl-X_S. The BH3 domain is crucial for the interaction between Bcl-2 family members. Caspases (Cheng et al., 1997) and calpains (Gil-Parrado et al., 2002) can cleave Bcl-2 and Bcl-X_L, thereby exposing their BH3 domain and convert them into pro-apoptotic actors. Pro-apoptotic members can be divided into Bax-like proteins (Bak, Bax and Bok) lacking the BH4 domain, Bcl-X_S, and the "BH3-only" proteins (Bim, Bik, Hrk/DP5, Blk, Bid, Bad, Bmf, Noxa, Puma and Bcl-G_s) also lacking the BH1 and -2 domains.

1.2.3.2.1 Bcl-2

Bcl-2 resides as an integral protein in the ER, nuclear envelope and the outer mitochondrial membrane (Krajewski et al., 1993; Lithgow et al., 1994). Its potent antagonistic effect on caspase-dependent as well as caspase-independent and even necrotic cell death is well documented, but do not include "type I" receptor-mediated signaling (Cory & Adams, 2002). Bcl-2 can exert its anti-apoptotic effect by regulation of the calcium homeostasis (Marin et al., 1996), modulation of the cellular redox state (Hockenbery et al., 1993) and stabilization of the mitochondrial membrane. Bcl-2 can also bind active Raf-1, tethering it at the mitochondria where it can phosphorylate pro-apoptotic Bad (Wang et al., 1996). Raf-phosphorylated Bad cannot heterodimerize with anti-apoptotic Bcl-2 or Bcl-X_L, and is dislocated from the mitochondria (Zha et al., 1996). Bad can also be phosphorylated by JNK. This phosphorylation hinders the inactivating phosphorylation of Bad by Raf, thereby resulting in a pro-apoptotic effect. JNK signaling via Bad connects SAPK-signaling to apoptotic regulation (Donovan et al., 2002).

1.2.3.2.2 Bcl-X_L

Bcl-X_L is associated with the outer mitochondrial membrane (Cory & Adams, 2002). The anti-apoptotic activity of Bcl-X_L was proposed to be due to sequestering of BH3-only proteins, thus preventing their interaction with Bax-like proteins (Cheng et al., 2001). DNA damage induces deamidation of Bcl-X_L, disrupting its ability to block the BH3-only proteins (Deverman et al., 2002).

1.2.3.2.3 Bid

Bid is a cytosolic protein that is cleavage-activated to form truncated Bid, tBid. The translocation of tBid to mitochondria induces conformational changes and/or aggregation of Bak and Bax (Gross et al., 1999; Desagher et al., 1999; Wei et al.,

2000). Upon activation, tBid is modified by N-myristoylation, probably important for the targeting of tBid to membranes (Zha et al., 2000). The specific targeting to mitochondrial contact sites is due to a proposed preference for tBid to interact with cardiolipin (Lutter et al., 2000). Casein kinase-1 and -2 can phosphorylate Bid, rendering it immune against cleavage by caspase-8 (Desagher et al., 2001). In addition to caspase-8, Bid can be cleaved by calpain (Chen et al., 2001), cathepsin L (Stoka et al., 2001) or granzyme B (Barry et al., 2000), and may possibly serve as a mediator for caspase-independent induction of apoptosis.

Several other BH3-only members were reported to translocate to mitochondria (e.g. Bim, Bmf, Noxa and Puma) where they may stimulate activation of Bak/Bax or form heterodimers with the anti-apoptotic Bcl-2 members and neutralize their effect.

1.2.3.2.4 Multi-domain proteins Bak and Bax

Bak is normally associated to the mitochondria (Griffiths et al., 1999). Upon apoptosis induction, Bak undergoes a conformational change in its N-terminal, probably allowing it to fully insert into the outer mitochondrial membrane where it can oligomerize (Griffiths et al., 1999; Korsmeyer et al., 2000; Wei et al., 2000).

In contrast to Bak, Bax is mainly cytosolic (Wolter et al., 1997). Upon apoptosis induction, Bax translocates to the nuclear envelope, the Golgi apparatus, ER and the mitochondria (Gajkowska et al., 2001; Godlewski et al., 2001). This event is associated with conformational changes of the C- and N-terminal domains (Nechushtan et al., 1999; Godlewski et al., 2001). Bax oligomerization is crucial for its capacity to release cytochrome *c* (Wieckowski et al., 2001). Oligomeric Bax can form pores in artificial lipid bilayers (Antonsson et al., 2000). Cleavage of Bax by calpain has been shown to generate a fragment with even more potent cytochrome *c* releasing properties that was not inhibited by either Bcl-2 or Bcl-X_L (Gao & Dou, 2000; Wood & Newcomb, 2000). The importance for Bak and Bax in apoptosis signaling was fully revealed in Bak -/- Bax -/- mouse embryo fibroblasts that were resistant to apoptosis induced by a number of stimuli including tBid, staurosporine, ultraviolet radiation, growth factor deprivation, etoposide, and the ER stressors thapsigargin and tunicamycin (Wei et al., 2001). However, the functions of Bak and Bax seem to be partially overlapping, as Bak -/- Bax +/+ and Bak +/+ Bax -/- cells in general were more sensitive to the same agents than were the double knockout cells.

1.2.3.3 Regulation of the mitochondrial membrane permeabilization

Membrane permeabilization is considered the pivotal mitochondrial event in apoptosis. This can occur over the inner and/or outer mitochondrial membrane and is typically followed by loss of $\Delta\psi m$ (Zamzami & Kroemer, 2001). Disruption of $\Delta\psi m$ is not necessarily a direct consequence of OMP but probably reflects secondary respiratory and metabolic disturbances (Bossy-Wetzel et al., 1998). The mechanisms of mitochondrial membrane permeabilization remain under debate, but several hypothetical models have presently emerged.

In the "pore formation" model, cleavage-activated BH3-only proteins translocate to the mitochondria. This induces the oligomerization and full insertion of Bak/Bax proteins into the outer mitochondrial membrane to form cytochrome *c* permeable pores. Cytochrome *c* normally resides on the outer surface of the inner mitochondrial membrane where it is bound to anionic phospholipids, mainly cardiolipin. The release

of cytochrome *c* from mitochondria requires its detachment from cardiolipin as well as OMP (Ott et al., 2002). The mitochondrial contact sites, where the outer and inner membranes are in close contact with each other, are proposed to be rich in cardiolipin and tBid preferentially translocates to these contact sites, probably targeted by the interaction between a three-helix domain in the tBid protein and cardiolipin (Lutter et al., 2000; Lutter et al., 2001). Homo-oligomerization of tBid may induce mitochondrial dysfunction on its own without the need for Bax or Bak (Grinberg et al., 2002). Moreover, also oligomeric Bax seems to require mitochondrial contact site-like structures to be able to release cytochrome *c* (Wieckowski et al., 2001).

Instead of forming new pores in the mitochondrial membrane, Bcl-2 family members may interact with the existing channels. The VDAC (voltage-dependent anion channel) is a channel-forming protein residing in the outer membrane where it is normally responsible for the transport of metabolites. The anti-apoptotic proteins Bcl-2 and Bcl-X_L can interact with VDAC, possibly keeping the channel in a normal conformation, and the BH4 domain seems to be crucial for this interaction (Shimizu et al., 2000b). Forced opening of VDAC was proposed as a mechanism of OMP and cytochrome *c* release (Shimizu et al., 1999). This could be caused by antagonists of Bcl-2/Bcl-X_L, e.g. tBid and/or Bak/Bax. Moreover, the formation of a "novel" large pore by VDAC and Bax was proposed since this was observed to occur under *in vitro* conditions (Shimizu et al., 2000a).

The ANT (adenine nucleotide translocator) is an antiporter protein responsible for ATP/ADP exchange over the inner mitochondrial membrane. It was recently shown that ANT interacts with either Bax or Bcl-2. Bcl-2 was proposed to support the translocase activity of ANT at high levels, whereas Bax would inhibit the translocase activity (Belzacq et al., 2003). Moreover, ANT is able to form a non-specific pore, at least in isolated mitochondria. ANT pore formation can be induced by a variety of different agents (e.g. Ca^{2+}, atractyloside, thiol oxidation) and is enhanced by Bax and inhibited by Bcl-2 as well as by ADP (Vieira et al., 2000). Dysfunction of ANT is proposed to cause dissipation of $\Delta\psi m$, matrix swelling and secondary OMP.

The PT pore is a high-conductance channel localized at the contact sites between the outer and inner mitochondrial membranes. It is believed to consist of a complex of inner and outer mitochondrial membrane proteins, including the channel proteins VDAC and ANT, the matrix protein cyclophilin D, the peripheral benzodiazepine receptor (PBR) and Bcl-2 family members. Opening of the PT pore causes MPT (mitochondrial permeability transition) that includes permeabilization over the inner and outer membrane, release of cytochrome *c* and loss of $\Delta\psi m$ (Scarlett & Murphy, 1997).

The primary trigger for opening of the PT pore is a rise in intracellular Ca^{2+}. Several factors are known to enhance the sensitivity of the PT pore to Ca^{2+} including oxidative stress, adenine nucleotide depletion and increased inorganic phosphate concentrations (Halestrap et al., 2002). MPT is suggested to involve a conformational change of ANT. The importance of ANT disruption for PT pore opening is illustrated by the effect of MPT inhibitors/inducers. Cyclophilin D binding causes ANT to undergo conformational changes, resulting in ANT pore formation. Well-known MPT inhibitors, cyclosporin A and bongkrekic acid, inhibit the interaction of cyclophilin D with ANT while atractyloside, a plant derived pro-apoptotic ANT

ligand, can induce PT pore opening (Crompton et al., 1988; Vieira et al., 2000; Woodfield et al., 1998).

The involvement of Bax has been implicated in both ANT and PT pore regulation. Immunodepletion of Bax from PT pore complexes made them insensitive to atractyloside. Moreover, PT pore complexes from Bax-deficient mice were also insensitive to atractyloside. Recombinant Bax and purified ANT together, but neither of them alone, efficiently formed atractyloside-responsive channels in artificial membranes (Marzo et al., 1998).

Taken together, there are many possible ways to release cytochrome *c* and/or to disrupt the mitochondrial integrity, as discussed above. The participation of the separate mechanisms may be dependent on both the nature of the stimuli and the particular cell type.

1.2.3.4 The Inhibitor of Apoptosis Protein family

The IAPs are an evolutionary conserved family of proteins. They all contain one to three domains of the zinc-binding baculovirus inhibitor of apoptosis repeats (BIR), crucial for the direct binding of IAPs to active caspases (Deveraux & Reed, 1999). The binding of IAPs creates a steric block for caspases to interact with their substrates (Shiozaki et al., 2003; Sun et al., 1999). Eight members of the mammalian IAP family have been identified so far, cIAP-1, cIAP-2, XIAP (X-linked IAP), Survivin, Livin/ML-IAP, ILP2 and the neuron-specific members NAIP and Apollon (for review see Verhagen et al., 2001). The most potent caspase inhibitors of the IAP family members are XIAP, cIAP-1 and cIAP-2 (Roy et al., 1997).

XIAP directly binds caspase-3, -7 and -9, and inhibits their activity *in vitro* and *in vivo*. This interaction occurs via the binding of the BIR3-domain of XIAP to the small (p10 or p12) subunit of processed caspase-9. Active caspase-9 is thus kept in an inactive, monomeric state (Deveraux et al., 1999; Shiozaki et al., 2003). XIAP can inhibit active caspase-3 or -7 via binding of the BIR1-BIR2 linker region to the catalytic site of the caspase (Deveraux et al., 1999; Suzuki et al., 2001b). XIAP can also interact with oligomerized Apaf-1 and regulate the activity of the apoptosome complex, either by binding to active caspase-9, or by sequestration of activated caspase-3 within the apoptosome complex (Bratton et al., 2002).

cIAP-1 and -2 were originally identified as TRAF-binding proteins (Rothe et al., 1995) and participate in the ubiquitination of TRAF-2 (Li et al., 2002). Both cIAPs can inhibit the enzymatic activity of caspase-3, -7 and -9 *in vitro*, but they are less potent than XIAP (Deveraux et al., 1998; Roy et al., 1997).

cIAP-1, cIAP-2 and XIAP possess ubiquitin ligase activity that promotes their own degradation in the proteasome, along with bound caspases (Deveraux et al., 1997; Huang et al., 2000; Suzuki et al., 2001c). Moreover, cleavage/degradation and inactivation of XIAP by proteases has been reported (Deveraux et al., 1999).

Survivin is the smallest IAP containing only one BIR-domain (Ambrosini et al., 1997). The expression pattern of Survivin is cell cycle dependent with maximal expression in mitosis (Kobayashi et al., 1999) when it co-localizes with components of the mitotic apparatus (Li et al., 1998a). The necessity for Survivin in development was revealed by mitotic defects and embryonic lethality in knockout mice (Uren et al., 2000). Another, more direct, anti-apoptotic role was demonstrated by Survivin downregulation that resulted in caspase-dependent apoptosis (Ambrosini et al., 1998;

Grossman et al., 2001; Li et al., 1998a). However, the exact anti-apoptotic mechanism of Survivin remains elusive.

Overexpression of IAPs has been observed in several types of cancer, but the relation to prognosis and treatment response is not clear (LaCasse et al., 1998; Tamm et al., 2000).

1.2.3.5 Inhibitors of IAPs

The function of IAPs is negatively regulated by the mitochondrial proteins Smac and HtrA2/Omi. Both Smac and HtrA2/Omi each contain a conserved IAP-binding motif, which is exposed after processing of the N-terminal mitochondrial targeting sequence upon import into the mitochondria (Hegde et al., 2002). The mechanism for mitochondrial release of Smac and HtrA2/Omi is unclear. It can be due to OMP (Hegde et al., 2002) but at least Smac release was shown to be separate from the release of cytochrome *c* (Springs et al., 2002).

Smac competes with active caspase-9, -3 and -7 for the BIR3 or BIR2 linker domain of XIAP (Liu et al., 2000; Wu et al., 2000). Smac can also bind to Survivin, cIAP-1 and -2 (Du et al., 2000; Song et al., 2003). However, recent findings suggest that IAP binding is dispensable for the pro-apoptotic function of Smac (Roberts et al., 2001).

The mitochondrial serine protease HtrA2/Omi can inhibit the function of XIAP by binding in a Smac-like manner (Suzuki et al., 2001a; van Loo et al., 2002b). The pro-apoptotic activity of HtrA2/Omi *in vivo* also involves its serine protease activity. Mutation of either the N-terminus of mature HtrA2/Omi, essential for interaction with IAPs, or its catalytic serine residue reduces its ability to promote cell death. A complete loss of pro-apoptotic activity was observed when both sites were mutated (Verhagen et al., 2002).

1.2.3.6 The Heat Shock Protein family

The family of heat shock proteins consists of five sub-families, HSP100, HSP90, HSP70, HSP60 and the small HSP (Garrido et al., 2001). HSPs are cellular chaperones responsible for the proper folding of native and denatured proteins, facilitation of inter-organellar transport, promotion of degradation of denatured proteins, and dissolving of protein aggregates. HSPs can interfere with apoptosis signaling both up- and downstream of the mitochondria and have been shown to even rescue cells with activated caspase-3 (Jäättelä et al., 1998). Moreover, HSPs have been implicated in the inhibition of caspase-independent cell death.

The HSP110/105 proteins have been suggested to participate in both induction and inhibition of apoptosis (Oh et al., 1997; Yamagishi et al., 2002). However, the mechanisms by which these proteins regulate apoptosis are not fully understood.

HSP10 and HSP60 normally reside as a complex in the mitochondria from where they are released upon initiation of apoptosis. These HSPs have been shown to promote the activation of executioner caspases (Samali et al., 1999a). Addition of recombinant HSP60 and HSP10 accelerated the activation of pro-caspase-3 by cytochrome *c* and dATP or different upstream caspases *in vitro* (Samali et al., 1999a; Xanthoudakis et al., 1999). HSP60 and HSP10 could therefore be considered as pro-apoptotic regulators of apoptosis signaling.

The expression of some HSP family members is inducible upon cellular stress such as hyperthermia, oxidative stress and exposure to anti-cancer drugs. HSP90, HSP72 and HSP27 are the main stress-inducible HSPs, and their anti-apoptotic properties are well documented.

HSP90 and HSP72 are ATP-dependent chaperones, but HSP72 can also assert its anti-apoptotic effects in an ATP-independent manner depending on the stimuli (Ravagnan et al., 2001). HSP90 is normally abundantly expressed, but can be further upregulated by cellular stress. The role of HSP90 in apoptosis is stimuli-dependent (Garrido et al., 2001). HSP90 and HSP72 can interfere with formation of the apoptosome complex by binding to Apaf-1 (Pandey et al., 2000b; Saleh et al., 2000). However, HSP72 is also able to prevent apoptosis in Apaf-1-deficient cells, indicating additional anti-apoptotic properties (Ravagnan et al., 2001). The findings that HSP72 can bind and sequester AIF in the cytosol (Ravagnan et al., 2001) and interfere with cathepsin B-mediated cell death (Mathiasen & Jäättelä, 2002) provide an explanation to how HSP72 can abrogate caspase-independent apoptosis.

HSP27 is an ATP-independent chaperone whose main function is to protect against protein aggregation. It is present in the cytoplasm as oligomeric structures, up to 800 kDa in size, made up by tetrameric subunits (Lavoie et al., 1995; Zantema et al., 1992). Phosphorylation of HSP27 by p38MAPK results in re-organization of the oligomeric structures, however, the significance of this event is not fully understood (Stokoe et al., 1992). HSP27 can mediate protection against stress-induced apoptotic signaling by several mechanisms. For example, it can increase the cellular glutathione pool, resulting in an increased ability to deal with elevated intracellular levels of ROS (Mehlen et al., 1996), or aid the refolding of denatured proteins in co-operation with ATP-dependent chaperones (Ehrnsperger et al., 1997). Phosphorylated HSP27 can interfere with receptor-mediated signaling by interaction with DAXX and thereby inhibit Fas-R-induced activation of JNK (Charette et al., 2000). Furthermore, HSP27 has been proposed to interfere with caspase-dependent apoptosis signaling either by binding to cytochrome *c,* which inhibits the formation of the apoptosome complex, or by direct binding to pro-caspase-3, thereby abrogating its activation (Garrido et al., 1999; Pandey et al., 2000a).

Increased expression of HSPs, especially the stress-inducible members, has been observed in many types of tumors, including lung cancer. However, the significance of this elevated expression for tumorigenesis or treatment-resistance is not clarified. Increased expression of HSPs could allow cells to adapt to changes in their environment and to survive under otherwise lethal conditions.

1.2.4 Apoptosis and cancer

Many of the anti-cancer treatments used in the clinic works via induction of apoptosis. However, inherent or acquired resistance to anti-cancer treatment is a common clinical problem. This could result from impaired induction or execution of apoptosis. Moreover, dysregulation of apoptosis is considered a necessary event for tumorigenesis. Once understood, this dysregulation can be exploited as a therapeutic target to restore or even induce apoptotic signaling.

A number of compounds tailored to interfere with apoptotic signaling in tumor cells are already in clinical trials, including Bcl-2 antisense, TRAIL and oncolytic viruses that selectively replicates in p53-deficient cells (reviewed by Reed, 2002).

Other possible targets include mitochondria (reviewed by Debatin et al., 2002), HSPs, IAPs and their antagonists, and several interesting compounds have been developed for interaction with these targets.

HSP90 is presently attracting clinical interest since chemical inhibitors have the potential to provide a simultaneous, combinatorial attack on oncogenic signaling pathways that depend on the chaperoning function of HSP90 (Neckers, 2002).

IAP expression is related to resistance to treatment, at least in some types of tumors (Cheng et al., 2002; Holcik et al., 2001), and could therefore be of interest as a therapeutic target. Survivin is specifically expressed during embryogenesis, in some stem cells and in tumor cells, making it a highly interesting target for anti-cancer treatment (Altieri, 2001). Promising results for Survivin targeting were shown in a xenotransplant melanoma model (Grossman et al., 2001). Survivin could also serve as an antigen for cancer vaccines (Andersen & Thor, 2002). The IAP inhibitors, mainly Smac, are of clinical interest because of the possibility to enhance the apoptotic signaling with mimetic compounds. *In vitro* studies have showed increased sensitivity to anti-cancer treatment for tumor cells when treated with a Smac-like agonistic peptide (Arnt et al., 2002; Fulda et al., 2002; Yang et al., 2003).

Increased understanding of the signals governing induction and regulation of apoptosis could reveal new ways to overcome resistance to anti-cancer treatment.

1.3 BIOCHEMICAL AND BIOLOGICAL EFFECTS OF IONIZING RADIATION

Radiotherapy is one of the oldest weapons in the anti-cancer arsenal and the first report on its use for successful tumor treatment is actually from Stockholm when Dr. Stenbeck in 1899 cured a woman with a basal cell carcinoma by X-ray therapy (Berven, 1962). Today, 50% of patients with cancer are given radiotherapy (source: The Swedish Council on Technology Assessment in Health Care, "Radiotherapy in cancer", 2003). Approximately half of these patients are treated with a curative intent. Treatment can be by radiotherapy alone, for certain types of tumors or when surgery is not applicable, or as combination treatment together with surgery and/or chemotherapy. Radiotherapy is also useful for palliative treatment, especially for pain relief from skeletal metastases.

1.3.1 The physics and chemistry of radiation absorption

Ionizing radiation (IR) used for therapeutic purposes is mainly of the X- or γ-type consisting of electromagnetic energy (photons). The quantity of radiation is expressed in Grays [Gy] and this unit denotes the energy absorption of 1 J /kg. Energy absorption of radiation occurs by interaction of the photon with atoms in the material. This interaction causes the photon to give up some of its energy to produce a fast-moving charged particle. The most common absorption event for high energy X- and γ-rays is the dislocation of an electron from the outer shell of an atom, also known as the Compton process. This interaction causes deflection and energy loss (0-80%) for the photon, which may then participate in further reactions. The knocked-out, fast-moving electrons in turn interact with other atoms, causing ionizations or breaking chemical bonds (Hall, 2000d). The ionization pattern caused by X- and γ-radiation is of low density compared to that of accelerated particles (e.g. nitrogen or carbon ions) that cause dense ionizations along their particle-tracks.

Because of their abundance, water molecules are the main cellular targets for low-density IR. Water molecules are ionized to form ion radicals:

$$H_2O \rightarrow H_2O^+ + e^-$$

The ion radical H_2O^+ reacts with another water molecule to form a highly reactive hydroxyl radical (OH•):

$$H_2O^+ + H_2O \rightarrow H_3O^+ + OH\bullet$$

The hydroxyl radical can then interact with cellular targets, such as DNA, causing damage to the DNA sugar-phosphate backbone, damage the bases of DNA and induce protein-DNA and DNA-DNA cross-links. For the X- and γ-ray type used for radiotherapy, it is estimated that two thirds of the induced DNA damage in a cell is caused by the indirect action of hydroxyl radicals, while one third is induced by direct ionization of DNA.

The initial DNA damage can be restored by a rapid "chemical repair", in which scavenger molecules can donate hydrogen to the ionized molecule to restore its structure. However, molecular oxygen can react with and "fixate" the initial damage, thus preventing the chemical repair (Hall, 2000c). Therefore, the oxygenation status

of the tissue is of importance for the biological effect of IR. Clonogenic survival can differ two- to three-fold due to oxygenation status. From this follows that cellular scavengers (e.g. glutathione, cysteine) play an important role in radiosensitivity due to their ability to take care of free radicals and contribute to chemical repair. Hypoxia is a problem in the clinical reality since most tumors are hypoxic due to fast proliferation and capillary dysfunction.

1.3.2 Ionizing radiation-induced cell death

IR-induced cell death is generally divided into cell cycle-independent (pre-mitotic/interphase) death, and mitotic (reproductive/clonogenic) cell death induced by residual genomic damage. In both cases, morphological features of cell death can resemble apoptosis (Hall, 2000a). The main intrinsic factors governing radiosensitivity include the ability to detect and repair DNA damage and the activation of cellular responses such as cell cycle arrest and induction of apoptosis. The response to IR is also cell type dependent. For example, thymocytes, splenocytes, hematopoietic progenitor cells and intestinal crypt cells undergo apoptosis in response to IR, while fibroblasts undergo irreversible growth arrest. IR-induced apoptosis is not so common in other cell types than the ones mentioned above (Allan, 1992).

IR can also induce cell death by mitotic catastrophe (MC), as observed in many solid tumors (Hendry & West, 1997). In MC, cells fail to undergo successful division due to persistent DNA damage, resulting in giant, multi-nucleated cells. MC may in turn resolve in either necrosis or cell death with apoptotic features (Ianzini & Mackey, 1998).

1.3.2.1 Radiosensitivity, definition and quantification

Radioresistance/Radiosensitivity are relative terms. Radioresistance denotes the preservation of functional integrity at the tissue level, and the ability to maintain viability or clonogenicity at the cellular level. The classic assay for radiosensitivity is the clonogenic assay, in which the clonogenic potential of a cell type is described as a function of the given dose (Hall, 2000a). The surviving fraction for a certain dose, SF(D), is used as a measurement for radiosensitivity. SF(D) is a quotient between the surviving fractions of cells irradiated with the dose (D) and untreated cells. Thus, "SF2 0.25" should be interpreted as "25% of the cells treated with 2 Gy retained clonogenic capacity, as compared to untreated cells". Clonogenic assay is not possible to perform for most primary tumor material due to the poor growth under *in vitro* conditions. However, the intrinsic radiosensitivity, assessed by clonogenic assay, in established tumor cell lines generally reflect the responsiveness of the corresponding tumor (Fertil & Malaise, 1985).

According to radiobiological principles, all cell types and tumors can be eradicated by irradiation, it is just a matter of dose. However, in the clinical situation the dose delivered to the normal tissue surrounding the tumor determines the maximal tolerable dose. SF2 generally correlates with the curability of a tumor by radiotherapy (Fertil & Malaise, 1981). If the SF2 is used for calculation of the total dose needed for cure, tumors with SF2 0.4 or less, which corresponds to 90% of all SCLC tumors, would need a total dose of 60 Gy. This is clinically feasible, and the main problem with SCLC is rather its fast dissemination. The median SF2 for a number of NSCLC cell lines was 0.56, implicating a total dose of at least 85 Gy to

accomplish local control. With modern dose planning techniques, these doses are possible to achieve for small tumors (i.e. stage I) and was shown to be beneficial for local control. However, the majority of patients (~75%) with NSCLC are classified as stage III or more advanced at diagnosis (source: Swedish National Therapy Guidelines for lung cancer, 2001). Toxicity in adjacent tissue/organs limits the achievable dose when the target volume increases. A better understanding of the mechanisms governing intrinsic radiosensitivity could provide ways to specifically enhance tumor radiosensitivity. This would be of great clinical impact since it would allow efficient treatment also of a larger target volume.

1.3.3 Detection and repair of DNA damage

For sparsely ionizing qualities of radiation, such as X- and γ-rays, the majority of DNA breaks are single strand breaks (SSBs) (Hall, 2000b). SSBs are thought to occur frequently in the cell due to replication, transcription and metabolic production of reactive oxygen species. Cells can quickly and efficiently deal with SSBs by utilizing components of the base-excision repair system (Hoeijmakers, 2001). The DNA double-strand break (DSB) is considered the most deleterious DNA damage and the frequency of induced DSBs directly correlates with the manifestation of chromosomal aberrations and loss of clonogenicity (Iliakis, 1991; Radford, 1986). Detection and correct repair of DSBs is of outmost importance for the survival of the cell. In addition, the cellular response to genotoxic stress is coupled to cell cycle arrest at certain checkpoints to allow more time for DNA repair before entry into mitosis. The major IR-induced signaling pathways are presented in **Figure 7**.

1.3.3.1 Detection of DNA double-strand breaks

The main sensors of IR-induced DSBs, or associated changes in chromatin topography, are ATM, ATR and DNA-PKcs, all belonging to the highly conserved PI3-kinase-like family. They are important for the detection of DNA damage and coordination of the cellular response.

ATM is the gene mutated in the ataxia telangiectasia syndrome, a rare recessive disorder characterized by cerebellar degeneration causing ataxia (dysfunctional coordination), telangiectasias (blood vessel dilations) in the eye, immunodeficiency and genomic instability resulting in cancer predisposition and radiation hypersensitivity (Savitsky et al., 1995). The ATM protein was recently discovered to reside in the cell as an inactive dimer or higher-order multimer (Bakkenist & Kastan, 2003). Upon detection of a DSB, ATM is crosswise phosphorylated and released from the dimeric state. This finding explains the extremely rapid activation of ATM. The signal for ATM activation may be alterations the chromatin topography, but ATM may also bind directly to the DSB and possibly function as a scaffold for other enzymes (Bakkenist & Kastan, 2003; Andegeko et al., 2001). p53 and Chk2 are targets of ATM, and activation of these proteins mediates the cellular response to DNA damage including cell cycle-checkpoint arrest, DNA repair and stress response. Moreover, ATM was shown to phosphorylate histone H2AX (Burma et al., 2001), and event associated with induction of "nuclear foci" to which repair and checkpoint protein complexes are recruited (Paull et al., 2000). The exact role of H2AX phosphorylation is not well understood, but it was found necessary for IR-induced cell cycle arrest, as recently demonstrated (Fernandez-Capetillo et al., 2002).

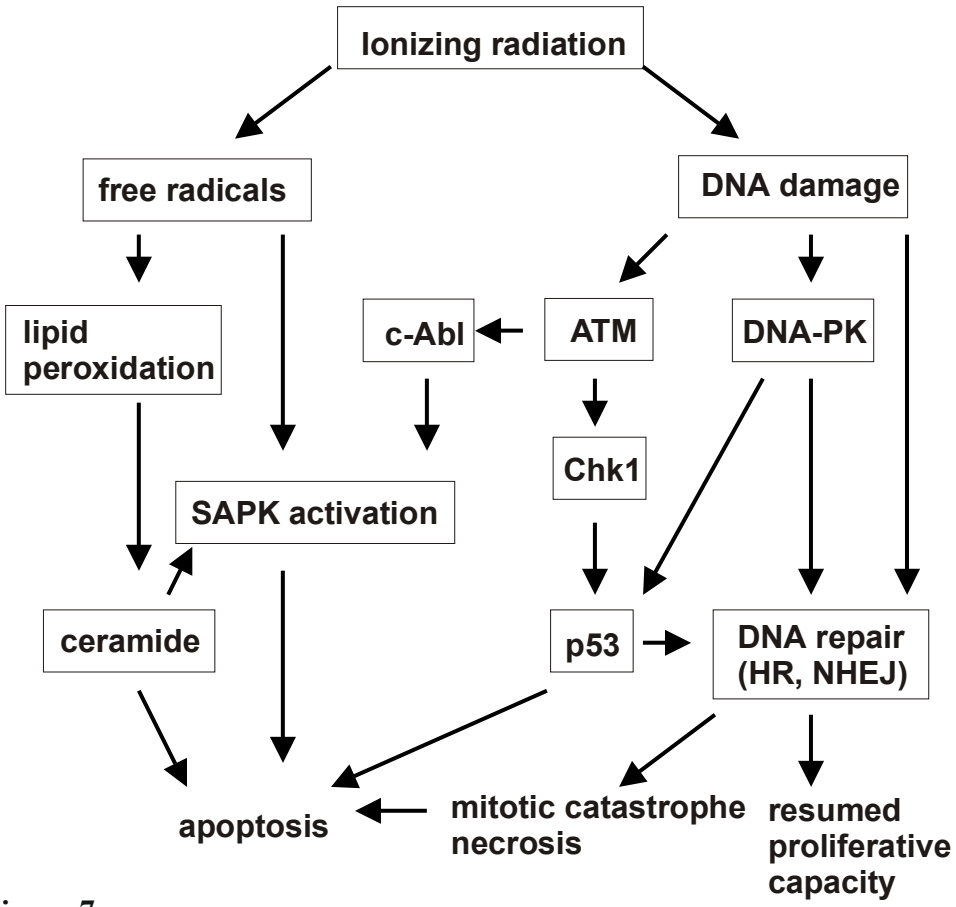


Figure 7.

The mammalian cellular response to ionizing radiation. Ionizing radiation leads to formation of free radicals that can cause DNA damage and trigger the cellular stress response. The cellular survival depends on the ability to arrest the cell cycle, repair DNA, neutralize radicals, deal with cellular stress and inhibit apoptosis signaling. Please see text for further details.

1.3.3.2 Repair of DNA double-strand breaks

Two main pathways have been identified for DNA DSB repair, namely, homologous recombination (HR) and non-homologous end joining (NHEJ).

If the genome is already replicated, during late S and G2-phase, the HR process can utilize the sister chromatide to repair DSBs with high fidelity. After end trimming of the DSB by the Rad50/Mre11/NBS1 complex and binding of Rad52 to the single stranded ends, the Rad51-BRCA2 complex initiates the formation of Rad51 nucleoprotein filaments necessary for strand inversion. This is followed by DNA synthesis, strand displacement and religation (Hoeijmakers, 2001).

However, in mammalian cells, the fast but error-prone process known as NHEJ is the predominant mode of DSB repair (Takata et al., 1998). NHEJ is thought to predominantly function during the G0 to early S-phase of the cell cycle (Lee et al., 1997) where most of the mature cells normally reside. NHEJ serves to quickly seal DSB breaks at the expense of sometimes creating local microdeletions. DNA-PKcs is a serine-threonine kinase crucial for DSB ligation by the NHEJ process. The exposed ends of double stranded DNA are recognized in a sequence-independent manner by

the DNA-binding Ku86/70-dimer (Mimori & Hardin, 1986; Walker et al., 2001) that in turn recruits DNA-PKcs. The DNA-PK complex is believed to keep the DNA ends of a DSB in a juxtaposed position so that the XRCC4/DNA ligase IV complex can join them. The murine SCID phenotype is caused by decreased activity of DNA-PKcs. SCID mice are immunodeficient due to defect V(D)J recombination that is normally mediated by DNA-PK, but also hypersensitive to IR, illustrating the importance of DNA-PK for the repair of IR-induced DNA damage.

Downstream targets of the DNA-PK complex include p53, XRCC4, the Werner protein and interferon regulatory factor 3 (Karmakar et al., 2002; Karpova et al., 2002; Leber et al., 1998). However, the *in vivo* importance of these phosphorylations is not clarified. One of the most interesting targets for DNA-PKcs is Artemis, a protein that possesses exonuclease activity (Moshous et al., 2001). DNA-PKcs regulates Artemis by both phosphorylation and complex formation, which results in the hairpin-opening step during V(D)J recombination and the 5' and 3' overhang processing during NHEJ (Ma et al., 2002). Moreover, a recent report identified Ku70 as a new Bax suppressor. Interaction between cytosolic Ku70 and Bax was reported to block the mitochondrial translocation of Bax, suggesting that Ku70 also have important non-nuclear functions (Sawada et al., 2003).

The efficacy of DNA repair varies during the cell cycle (Sinclair, 1968), probably because of the different topography of the DNA in different cell cycle phases. Cells are normally the most radioresistant in S-phase and the most radiosensitive at mitosis. In S-phase, high-fidelity homologous repair or transcription-coupled repair is possible and the intra S and G2/M checkpoints should ensure the detection of erroneous repair. In the G2/M phase, the chromatin is put under mechanical stress as it is compacted into chromosomes, possibly causing more complex DSBs. Moreover, the DNA repair machinery has little time to act before the chromosome segregation.

1.3.4 The cellular stress response to IR

1.3.4.1 The p53 response

A key molecule in the cellular response to DNA damage is the tumor suppressor p53. Activation of p53 can cause cell cycle arrest or trigger apoptosis. Upon DNA damage, p53 can be phosphorylated via the ATM-Chk2 pathway, or by DNA-PKcs (Shieh et al., 1997; Tominaga et al., 1999). Phosphorylation of p53 inhibits its Mdm-2-dependent degradation, resulting in p53 accumulation (Chen et al., 1995). Mdm-2 is a transcriptional target of p53 and this negative feedback loop regulates the level of p53 (Wu et al., 1993). Mdm-2 can also be phosphorylated by ATM or DNA-PK and this inhibits its binding to p53 (Mayo et al., 1997; Shieh et al., 2000). Furthermore, ARF targets Mdm-2 for destruction in the proteasome (Zhang et al., 1998).

p53 has diverse functions (Vousden & Lu, 2002 and references therein). It is a stimulatory transcription factor for the expression of genes encoding pro-apoptotic proteins, e.g. Fas, TNF, TRAIL, Bax, Puma and Noxa, while at the same time being a repressor for expression of anti-apoptotic proteins such as Bcl-2 and IAPs. Moreover, p53 induces cell cycle arrest via upregulation of the cyclin-dependent kinase inhibitor $p21^{WAF1}$. Some data also point to the existence of p53-mediated transcription-independent apoptosis. This could possibly involve activation of pro-caspase-8 (Ding et al., 2000) or initiation of apoptosis via the mitochondria-mediated pathway by direct interaction between p53 and $Bcl\text{-}X_L$ (Mihara et al., 2003).

It is not exactly understood how the p53 response is differentially directed towards cell cycle arrest, repair and survival, or induction of apoptosis. Two models have been proposed for the direction of p53-induced transcription (Vousden, 2000). In the first model ("p53 dumb"), the initial response to p53 induction is the same under all conditions. The outcome of p53-induced transcription is instead regulated by p53-independent factors. The availability of regulatory factors would depend on the cell type, stage of differentiation and phase in the cell cycle. In the second model ("p53 smart"), p53 is itself responsible for the differential expression of genes. This could be determined by the p53 level in the cell or by modifications of p53 that affect its ability to bind DNA or interact with transcriptional co-activators.

In the majority of tumors, the p53 response is inactivated either by direct mutation of p53 or by interference with its regulation of signal transduction. This may be clinically exploited, for example by preferential transcription of oncolytic viruses like the ONYX-015 (Heise et al., 1997). The restoration of p53-function is a wish that have come true, at least for some forms of p53 mutations, with the discovery of small p53 reactivating compounds like PRIMA-1 (Bykov et al., 2002). p53 reactivation by PRIMA-1 was shown to kill tumors cells on its own. However, it is very plausible that p53 reactivation would also sensitize cells to DNA-damaging agents.

1.3.4.2 Stress-activated protein kinase signaling

MAPK-mediated signaling is a key regulator of cell survival and cell death. This pathway transduces signals from the cell surface to the nucleus via the phosphorylation of a triad of cytoplasmic kinases. Typically, stimulation of receptor-mediated signaling activates the MAPKKKs, a group of serine-threonine kinases, to phosphorylate their targets, the MAPKKs. The dual-specificity MAPKKs phosphorylate tyrosine- and threonine-residues in their targets, the MAPKs. MAPKs are serine-threonine kinases that can phosphorylate cytosolic proteins or translocate to the nucleus and regulate transcription (Hagemann & Blank, 2001). Signaling via the MAPK pathways can promote survival or death depending on the stimulus. The most well characterized MAPK signaling pathway is the Raf-MEK-ERK pro-survival pathway that transduces mitogenic extracellular growth signals to the cell nucleus.

In response to cytotoxic drugs, UV irradiation, γ-irradiation, oxidative stress and pro-inflammatory cytokines, a MAPK pathway also known as the stress-induced protein kinase (SAPK) pathway, is activated. SAPK signaling is transduced via JNK/SAPK and p38MAPK (Davis, 2000). Frequent reports can be found documenting IR-induced SAPK signaling in many cell types (Verheij et al., 1998). The major outline of the MAPK/SAPK-cascade is presented in **Figure 8**.

MEKK1 is the major activator of SAPK-signaling and preferentially activates JNK over ERK (Ellinger-Ziegelbauer et al., 1997; Xia et al., 1998). However, MEKK1 also has a direct pro-apoptotic effect. MEKK1 is normally associated with cellular membranes (Xu et al., 1996), but can be cleaved by caspase-3-like proteases to release a pro-apoptotic 91 kDa fragment containing the C-terminal catalytic domain (ΔMEKK1) (Cardone et al., 1997; Widmann et al., 1998). The exact mechanism by which the ΔMEKK1 fragment promotes apoptosis is not known, but expression of a constitutively active MEKK1 fragment induced Bak activation (Mandic et al., 2001) and PT pore opening (Gibson et al., 2002). Mutation of the caspase-cleavage site in

MEKK1 or inhibition of caspase-9 or caspase-8 activity abrogated MEKK1-mediated apoptosis and induction of MPT (Widmann et al., 1998; Schlesinger et al., 2002).

The cytoplasmic tyrosine-kinase c-Abl can activate MEKK1-JNK signaling *in vitro* and *in vivo* in response to IR-induced DNA damage (Kharbanda et al., 2000) and c-Abl-deficient cells fail to activate SAPKs and are resistant to IR (Yuan et al., 1997), implicating an important role for SAPK-signaling in response to DNA damage and induction of apoptosis.

The p38MAPKs exist in four isoforms, α, β, δ and γ. The upstream activators include MKK3 and -6, which in turn are activated by MEKK2 and -3, ASK1 or PAK. The activation of p38MAPK by IR has been reported to vary from no detectible activation to a strong activation (Lee et al., 2002; Taher et al., 2000). The role of p38MAPK signaling depends on the cell type and stimulus. Both growth- as well as cell death-promoting effects have been observed (Dent et al., 2003). Activation of p38 may stimulate the translocation of Bax to mitochondria (Ghatan et al., 2000) or activate transcription of the *CHOP/GADD153* gene, resulting in increased levels of pro-apoptotic Gadd-family proteins (Wang & Ron, 1996).

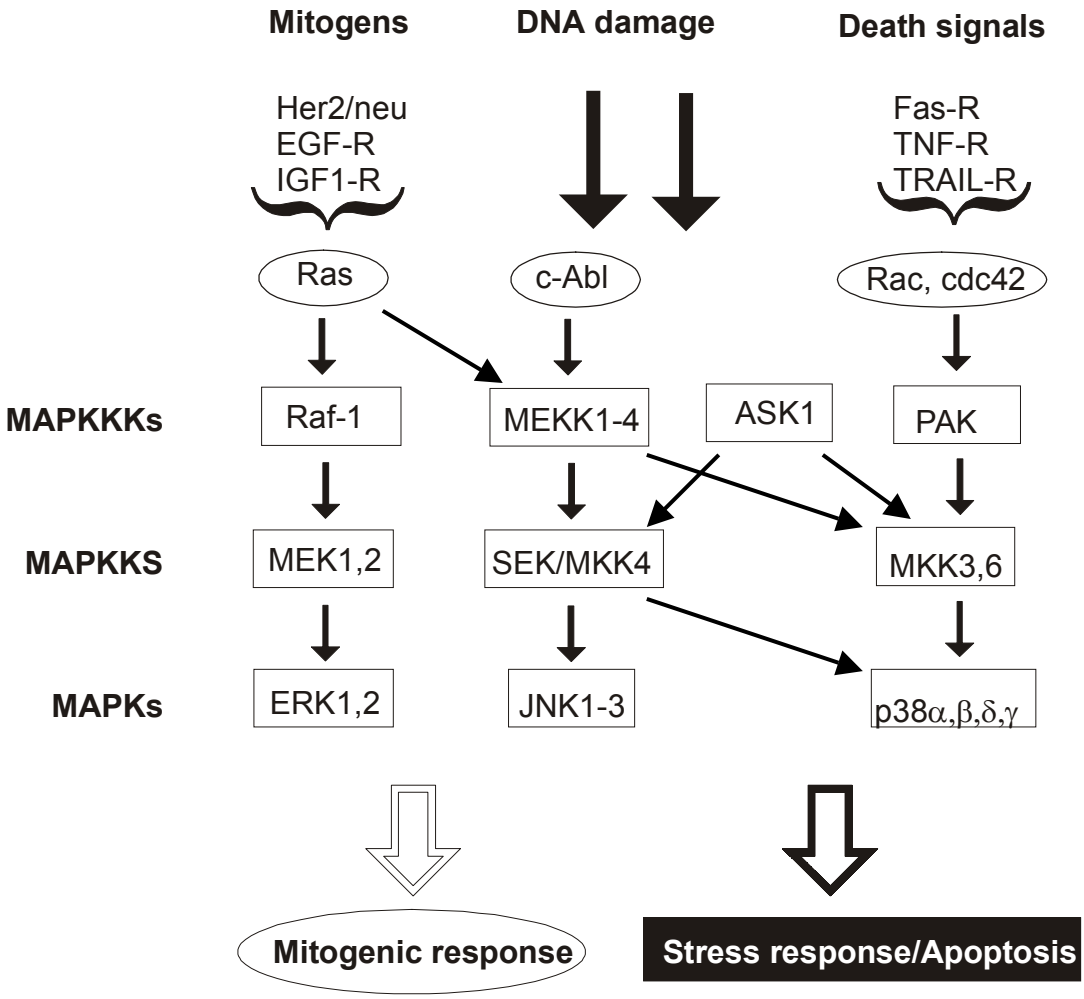


Figure 8.

The MAPK signaling pathways. For explanation of acronyms and abbreviations, please see abbreviation list.

c-Jun N-terminal kinase (JNK) signaling has been implicated in various cellular responses including proliferation, differentiation and stress-induced apoptosis. JNK is predominantly activated by stress signals but the necessity of JNK-signaling for apoptosis induction seems to be cell type- and stimuli-dependent (Verheij et al., 1998). In addition, the duration of JNK signaling can differentiate the outcome. Sustained JNK-signaling was found to correlate to the toxicity of platinum compounds (Sanchez-Perez et al., 1998). Activation of JNK is best characterized for the MEKK1 and ASK1 pathways (Davis, 2000).

The mechanisms by which JNK induces apoptosis are not fully elucidated but several possibilities are revealed in the downstream targets of JNK-phosphorylation.

Nuclear translocation of JNK is observed after UV irradiation, but this does not seem to be necessary for all genotoxic agents to induce apoptosis (Sanchez-Perez et al., 1998). The nuclear JNK targets are the transcription factors c-Jun, ATF-2 and Elk-2 of the AP-1 family (Davis, 2000). Fas-R and TNFα-R are transcriptional targets of c-Jun, and there is extensive evidence for the involvement of c-Jun in apoptosis (Bossy-Wetzel et al., 1997; Ham et al., 1995).

Non-nuclear targets of JNK include Bcl-2 and Bcl-X_L, Bad and Bax. JNK-mediated phosphorylation of Bcl-2 and Bcl-X_L inhibits their anti-apoptotic effect (Fan et al., 2000). Phosphorylation of Bad by JNK antagonizes Akt-mediated inhibition of Bad thus protecting its pro-apoptotic effect (Donovan et al., 2002). Recently, the requirement for Bax-like proteins in JNK-mediated apoptosis signaling was implicated. Activated JNK was sufficient to induce rapid cytochrome *c* release and apoptosis, but failed to do so in cells deficient for members of the Bax subfamily. Furthermore, exposure to stress failed to activate Bax, cause cytochrome *c* release or induce apoptosis in JNK-deficient cells (Lei et al., 2002). A novel link between JNK and mitochondrial dysfunction was recently reported. The BH3-only protein Bim is normally associated with the dynein filaments. Upon phosphorylation by JNK, Bim translocates to mitochondria and can induce the activation of Bax/Bak and subsequent mitochondrial permeabilization. Taken together, these findings suggest that that the involvement of mitochondria is important for pro-apoptotic signal transduction through the JNK pathway.

THE PRESENT STUDY

1.4 AIMS

The general aim of this thesis was to investigate the mechanisms of apoptotic signaling induced by ionizing radiation in lung cancer cells. This aim is based on the hypothesis that tumor cell radioresistance could be caused by failure to initiate or execute apoptosis.

There were several reasons for choosing lung carcinoma as an experimental model. Lung cancer is a common and lethal disease and radiotherapy is a cornerstone in both curative and palliative treatment. Due to few symptoms and early dissemination of lung cancer, the majority of patients have loco-regional lymph node metastases upon diagnosis. To obtain local control, a prerequisite for cure, high dose radiotherapy is required. However, these doses are not always possible to achieve in a target volume that covers both the main tumor and the loco-regional lymph nodes. We believe that an increased understanding of the mechanisms of resistance to IR-induced apoptosis could open new possibilities for pharmacological modulation of this process, serving to improve the efficacy of radiotherapy. Moreover, the clinical difference in responsiveness to radiotherapy between NSCLC and SCLC is mirrored in lung cancer cell lines cultured *in vitro*. Previous studies, in our and other laboratories, have resulted in characterization of molecular changes and radiosensitivity of many of the lung carcinoma cell lines used in the present work. These data enables the search for parameters in apoptotic signaling or regulation that correlate with radiosensitivity.

The specific aims were as follows:

To investigate the expression of proteins participating in activation and regulation of the apoptotic process in a panel of SCLC and NSCLC cell lines displaying various degrees of radiosensitivity.

To assess the functionality of the major apoptotic signaling pathways in a subset of lung cancer cell lines by using agents with different mechanisms of action.

To investigate the expression of anti-apoptotic proteins in the panel of lung cancer cell lines and evaluate their importance for IR-induced apoptosis.

To identify the level(s) at which IR-induced apoptotic signaling in resistant lung cancer cells is abrogated.

1.5 MATERIALS AND METHODS

The techniques used in this thesis are described in detail in **Papers I-V**. Here, materials and methods will be listed and briefly commented upon.

1.5.1 Cell lines

In this study, a panel of SCLC and NSCLC cell lines was utilized. All cell lines were cultured in humidified air at 37°C, 5% CO_2, in RPMI1640 medium supplemented with 10% heat-inactivated fetal bovine serum, 100 IU/ml penicillin and 100 ng/ml streptomycin. Cells were seeded in fresh medium 24 hrs before treatment.

Since tumor cell lines are genetically instable, the DNA content was regularly monitored by flow cytometry according to the method developed by Castro et al., (1993) and compared to a lymphocyte standard to exclude a split population. Viability was routinely monitored by exclusion of trypan blue. All cell lines were tested negative for mycoplasma.

The characteristics of the individual cell lines are summarized in **Table 1**. SCLC cell lines are usually derived from metastatic lesions thus representing the most advanced form of the disease. They are further subclassified as "variant" or "classic" according to *in vitro* growth characteristics and neuroendocrine profiles. The "variant" cell lines have shorter doubling time and are usually less radiosensitive, however, this division is not absolute.

Mutated p53 is found in almost 100% of SCLC and 50% of NSCLC cells. The p53 status in U1285, U1906 and U1810 cell lines was previously characterized by sequencing of all exons in the *TP53* gene (Sirzén et al., 1998b). In U1285 cells, a 26 base pair deletion starting from codon 126 was found. U1906 cells had a CGG → CTG point mutation at codon 248 and U1810 cells harbored a single base pair deletion at codon 172.

Although an *in vitro* system in many ways differs from the conditions *in vivo*, the intrinsic radiosensitivity still correlates in general between cell lines and the corresponding tissue (Fertil & Malaise, 1985). The *in vivo* influence of blood supply, hypoxia, etc. can of course not be mimicked, however, an *in vitro* system may better suite the detailed study and manipulation of intrinsic radiosensitivity.

1.5.2 Treatments

1.5.2.1 Irradiation procedure

Irradiation was from a Co^{60} source and cells were irradiated at ambient room temperature. Adherent cells were irradiated while attached. The dose rate for the given geometry was calculated by a staff physicist, and the treatment time for administration of a certain dose was monthly adjusted to the second decimal for decay. A 4 mm thick sheet of Plexiglas was placed on top of the cell culturing vessels for maximum dose-build-up. The dose-rate was ~1 Gy/min at the end of this study. The dose 8 Gy was chosen since it overcomes the "shoulder-region" in the cell survival curve for U1810 cells and was expected to induce approximately a one-log killing.

Table 1. Histological classification and SF2 values for the panel of lung cancer cell lines

cell line	histology	/subtype	growth pattern	isolated from	prior treatment	SF2	refs.
H69	SCLC	C	clusters	primary tumor	CT	0.23-0.25	Bergh et al. 1982, Carmichael et al. 1989, Carney et al. 1983
H82	SCLC	V	clusters	pleural fluid	CT	0.58-0.73	Carney et al. 1983, Carmichael et al. 1989
N/H592	SCLC	C	clusters	bone marrow	ND	ND	Binaschi M et al. 1990
U1285/dox	SCLC	C	clusters	pleural fluid	none	0.25-0.30	Bergh et al.1982, Brodin et al. 1991, Sirzén et al 1998
U1568	SCLC/L	V	clusters	primary tumor	none	ND	Bergh et al. 1982
U1690	SCLC	V	clusters and monolayer	pleural fluid	RT+CTx3	0.57	Bergh et al 1985, Persson et al. 2002
U1906	SCLC	V	clusters and adherent	brain metastasis	CTx1	0.45-0.59	Brodin et al. 1991, Sirzén et al 1998
U2020	SCLC	V	clusters and adherent	pleural fluid	RT+CTx6	ND	Bergh et al. 1985
U2050	SCLC	C	clusters in suspension	pleural fluid	RT+CTx3	ND	Bergh et al. 1985
A549	NSCLC	AC	monolayer	primary tumor	ND	0.62-0.82	Berg et al 1981, Morstyn et al. 1984, Carmichael et al. 1989
U1752	NSCLC	SCC	monolayer	primary tumor	ND	0.90	Brodin et al. 1991
U1810	NSCLC	LC/AC	monolayer	pleural fluid	none	0.80-0.88	Sirzén et al 1998, Brodin et al. 1991
H23	NSCLC	AC	monolayer	primary tumor	none	0.17-0.20	Carmichael et al. 1989, Morstyn et al. 1984
H125	NSCLC	ASQ	monolayer	soft tissue	ND	0.37	Morstyn et al. 1984
H157	NSCLC	LC	monolayer	pleural fluid	none	0.38-0.8	Carmichael et al. 1989, Morstyn et al. 1984
H661	NSCLC	LC	monolayer	lymph node	none	0.93	Carmichael et al. 1989

Abbreviations: AC, adenocarcinoma; SCC, squamous cell carcinoma; ASQ, adenosquamous mixed type carcinoma, LC, large cell (undifferentiated) carcinoma; SCLC/, large cell variant of SCLC; SF2, surviving fraction after 2 Gy; UD, undetected; ND, not determined; C, classic; V, variant.

1.5.2.2 DNA damaging drugs

In addition to IR, cisplatin (CP, Platinol®, CDDP) and VP16 (etoposide, Vepeside®) were used. Both drugs are cornerstones in combined chemotherapy of both NSCLC and SCLC.

The chemical structure of cisplatin is a platinum molecule surrounded by two chloride atoms and two amino groups in cis-conformation. Upon entering the cell, the decrease in chloride concentration causes hydration of cisplatin to its monoaqua form, which is positively charged and highly reactive with nucleophilic sites e.g. SH2-containing proteins, RNA and DNA. The monoaqua form is then hydrated to the diaqua form which is responsible for adduct formation in DNA or between DNA and proteins. The vast majority (90%) of the DNA crosslinks are intra-strand (Perez, 1998 and references therein). The toxic effect of cisplatin is not limited to the induction of DNA damage, but can also cause ER stress followed by the release of calcium (Mandic et al., 2003).

The nuclear enzyme topoisomerase II generates a transient DNA DSB, enabling one DNA helix to pass through another and thus prevents intertwining of DNA during replication, transcription and mitosis. VP16 causes DNA damage by inhibiting the ATP-dependent topoisomerase II re-ligation of DNA, resulting in a multitude of DNA DSB/topoisomerase II complexes that eventually trigger apoptosis or mitotic catastrophe (Sorensen et al., 1999; Wang et al., 2001). VP16 have also been shown to have a direct toxic effect on mitochondria at high doses (50 μM) (Robertson et al., 2000).

1.5.3 Western blot analysis

Western blotting is a standard method for immunodetection of proteins. It was used in **Papers I-V** to detect proteins involved in apoptosis signaling and the specific cleavage products of caspase target proteins. Proteins are size-separated by SDS-polyacrylamide gel electrophoresis and transferred to a nitrocellulose membrane. Detection of a specific protein is made by the use of a specific antibody. This antibody is in turn bound by a horseradish peroxidase-conjugated secondary antibody. The horseradish peroxidase serves as substrate for the enhanced chemiluminescence (ECL), and the light emission from the chemical reaction is captured on film. The sensitivity and specificity of this method is mainly determined by the quality of the antibody. Densitometric analysis of the bands on the film was used for quantification of protein content.

1.5.4 Detection of gene expression

RT-PCR (reversed-transcription PCR) is a technique for the enzymatic amplification of RNA by the polymerase chain reaction (PCR) method. An oligonucleotide primer, specific for the mRNA of interest, is used to guide the synthesis of the corresponding cDNA using reverse transcriptase (RT). Enzymatic amplification of the cDNA is then performed by PCR and the product is subjected to gel electrophoresis followed by staining of the gel and densitometric quantification of the band. The sensitivity and specificity of this method is determined by the probe specificity and the conditions for transcription. The RT-PCR method was used in **Paper III** for the detection of a subset of IAP transcripts.

Another method for the detection and quantification of specific mRNA is the RNase protection assay (RPA), used in **Papers I** and **III**. This assay allows the simultaneous detection of several transcripts by radiolabeled probes that hybridize to and protect the specific mRNAs from degradation by RNase (added after the hybridization step). The protected probe/mRNA complexes are then size-separated by gel electrophoresis and visualized by autoradiography of the radiolabeled probe.

In these experiments, total RNA was isolated from cells by RNAzol B. The RT-PCR and RPA methods indirectly detect the level of transcription of a gene, however, post-transcriptional regulation of a protein occurs and differences between RNA and protein expression is quite common (Chen et al., 2002). Therefore, these results were compared with data obtained from western blot analysis.

1.5.5 Apoptosis assays

The original definition of apoptosis was based on morphological features of the cell. With the discovery of stereotypic biochemical events taking place during apoptosis execution, caspase activation and the subsequent cleavage of target proteins together with the appearance of characteristic DNA fragmentation (laddering) were considered hallmarks of apoptosis. In the present work, several methods for detection of events associated with apoptosis were used.

1.5.5.1 Morphological analysis

Morphological assessment of apoptotic cells was used in **Papers II**-**V**. For evaluation of nuclear morphology, cells were fixed in phosphate buffered 4% formaldehyde (PFA) and stained with 4',6-diamidino-2-phenylindole dihydrochloride (DAPI) or propidium iodide (PI) and observed by fluorescence microscopy. For visualization of the whole cell, May-Grünwald-Giemsa staining was performed and evaluated by light microscopy.

1.5.5.2 TUNEL-assay

The terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) technique was used for detection of DNA fragmentation in **Paper II**. Cells were fixed in 1% PFA and permeabilized with Triton-X 100. The terminal deoxynucleotidyl transferase is used to introduce fluorescein-labeled dUTP in nicked or broken DNA. Cells were then co-stained with DAPI or PI and observed by fluorescence microscopy. Fluorescent cells with apoptotic nuclear morphology were scored as apoptotic.

1.5.5.3 Cell cycle analysis

Cells were fixed by 4% PFA for more than 6 hrs, washed in 70% ethanol and incubated with subtilisin Carlsberg (0.1% Sigma protease XXIV, 0.1 M Tris, 7.0 mM NaCl, pH 7.2) at 30°C for 30 minutes to release nuclei. Nuclei were stained with DAPI and analyzed using a Partec Flow Cytometer PASII (Castro et al., 1993). The Phoenix Flow Systems multicycle software was used for calculation of the cell cycle composition (**Papers II, IV** and **V**, data not shown). DNA fragmentation was assessed as the sub-G1 content of the cell cycle (**Paper II**).

1.5.5.4 Caspase activity assays

Caspase activity can be monitored by several methods (reviewed in Köhler et al., 2002). One of the most commonly used assays is measurement of the cleavage-release of indicators (e.g. fluorochromes) coupled to a caspase substrate (e.g. a tetrapeptide). The sensitivity allows detection of 5-10% apoptosis in the lysate of one million cells. The specificity is determined by the substrate. Caspases can be divided into groups according to their preferred substrates. Caspases-3 and-7 preferentially cleave after the DEXD motif, while caspases-6, -8 and -9 prefer (I/L/V)EXD. Only caspase-2 preferentially cleaves after the pentapeptide sequence VDVAD. In the substrate-assay method used in the present work, the maximum linear rate of the cleavage-release of the fluorochrome 7-amino-4-methylcoumarin (AMC) was calculated from multiple measurements in a microtiter plate reader (**Paper II** and **IV**). This protocol describes the rate of activity, in contrast to protocols where only one time point is used that assesses the total conversion of substrate.

The active form of caspase-3 can be detected by a specific antibody in fixed, permeabilized intact cells. The intracellular localization of active caspase-3 was investigated by immunocytochemistry in **Paper II**. Cells were grown on coverslips or prepared by cytospin preparation, fixed in acetone, permeabilized with 0.1% Triton X-100 and incubated with a polyclonal biotinylated anti-active caspase-3 antibody. The localization of this antibody was detected by the addition of FITC-conjugated streptavidin and observation by confocal fluorescence microscopy. In **Paper V**, the use of a FITC-conjugated monoclonal anti-active caspase-3 antibody allowed quantification by FACS analysis. Prior to staining, cells were fixed and permeabilized in a 4% PFA/saponin solution (Cytofix/Cytoperm solution) for 20 minutes.

1.5.5.5 Mitochondrial integrity

The release of cytochrome *c* and other proteins from the mitochondria into cytosol can be detected by western blotting of cytosolic extracts (S100), or by immunocytochemistry (both used in **Paper II)**. For immunocytochemistry, cells were grown on coverslips or prepared by cytospin, fixed in 4% PFA and 0,19% piric acid, and permeabilized by 0.1% SDS. A mouse monoclonal antibody against cytochrome *c* was used and visualized by using a FITC-conjugated secondary antibody. The intracellular localization of cytochrome *c* was observed by confocal fluorescence microscopy.

The mitochondrial membrane potential ($\Delta\psi m$) can be indirectly assessed by cell permeable cationic fluorochrome dyes, e.g. tetramethylrhodamine ethyl ester (TMRE) used in **Paper V**. TMRE accumulates in mitochondria with intact $\Delta\psi m$ and this cause a red shift in the fluorescence emission spectra of TMRE that can be quantified by FACS analysis. Upon depolarization of mitochondria, TMRE is released, resulting in a decrease in the red fluorescence. An increased TMRE staining was observed in U1810 cells after IR (**Paper V**). Treatment–induced arrest at the G2/M checkpoint or failure to undergo cell division can cause an increased number of mitochondria per cell. This may increase the fluorescence signal from cells with intact mitochondria.

1.5.6 Assessment of Bak and Bax activation

These pro-apoptotic Bcl-2 family members undergo at least one conformational change during their activation. The conformational changes of both Bak and Bax expose a neo-epitope in the N-terminal that can be recognized by a monoclonal antibody and quantified by FACS analysis (**Paper V**). Cells were fixed in 0.25% PFA for 5 min and permeabilized with digitonin (100 μg/ml) prior to staining.

1.5.7 SAPK-activity assays

The activation of p38MAPK was assessed by western blotting using a phospho-specific antibody (**Paper V**).

An immunokinase assay was used to assess JNK activity. JNK-immunocomplexes were precipitated from cell lysates and incubated with recombinant GST-Jun together with P^{32}-labeled ATP. The phosphorylation of GST-Jun was quantified by gel electrophoresis of GST-Jun and densitometry of autoradiographs (**Paper V**).

1.5.8 Transfection methods

1.5.8.1 Adenoviral transfer and expression of MEKK1 fragments

The pro-apoptotic conformational change in Bak can be induced via the expression of a constitutively active MEKK1 fragment (Mandic et al., 2001). Adenoviral vectors were used for the inducible expression of a kinase-dead (dnMEKK1) or a constitutively active (dpMEKK1) fragment of MEKK1 (**Paper V**). A co-infected vector encodes the transactivating protein, Adeno-rtTa, that in the presence of doxycycline undergo a conformational change allowing it to bind to and activate the promotor region for the protein of interest. The adenoviral transfer system is a very efficient way of gene transfer. Some non-specific effects of adeno-rtTA alone were noticed, like a slight change in cell morphology and very low-grade toxicity, but there were no remarkable effects on cell proliferation or cell cycle composition.

1.5.8.2 Transfection of cDNA

For the downregulation of HSP72, U1810 cells were transfected with a cDNA plasmid encoding HSP72 antisense (**Paper IV**). Co-transfection (1/10) with a plasmid coding for green-fluorescent protein was made to allow the detection of transfected cells by fluorescence microscopy or FACS analysis. The transfection reagents used in this work (Lipofectamine PLUS and Lipofectamine 2000) complex the plasmid DNA into lipsomes that will be taken up by the cells. The transfection efficiency varies between cell lines. For U1810 cells, transfection efficiency was approximately 30%, with efficient down regulation of the target protein. However, ~20% unspecific cell death, also in GFP-negative cells, was induced at 24 hrs. Therefore, this method was not used for evaluating the effect of HSP72 down regulation on treatment-induced apoptosis.

1.5.8.3 Transfection of siRNA

RNAi (RNA-interference) is a recently developed method for mammalian cells to specifically and efficiently knockdown gene expression. This is done by the

introduction of a siRNA (short interfering RNA), a 19-21-mer double-stranded RNA, which will bind to the specific transcript and induce its degradation by nucleases. RNAi was used for specific and efficient knockdown of HSP72 protein expression in U1810 cells (**Paper IV**). Transfection with siRNA induced non-specific effects, such as growth retardation and even cell death at late time point (more than 72 hrs), despite careful optimization.

1.5.9 Cellular fractionation

Isolation of nuclei, mitochondria and cytosol was used in **Paper III**. To isolate cell nuclei, cells were incubated in hypotonic buffer containing cytochalasine B (to inhibit actin polymerization), 0.01% Triton X-100 and PMSF (to inhibit serine and cysteine proteases) for 30 min and then homogenized on ice to break the plasma membrane. Nuclei were separated from intact cells and debris by centrifugation using a 50% sucrose cushion.

To isolate the heavy membrane fraction containing mitochondria, cells were homogenized on ice in a buffer containing 0.01% digitonin (to permeabilize the plasma membrane but not mitochondria) and protease inhibitors. Intact cells and nuclei were pelleted by centrifugation, and the supernatant was centrifuged at 15.000 x g to pellet the heavy membrane fraction containing mitochondria. This supernatant was cleared by further centrifugation at 100.000 x g to obtain the cytosolic fraction.

The purity of fractions was analyzed using western blot and immunodetection of compartment-specific proteins.

1.6 SUMMARY OF THE PAPERS

Paper I

The absence of spontaneous and IR-induced apoptosis in NSCLC cells was previously described by our group (Sirzén et al., 1998a; Sirzén et al., 1998b). It was hypothesized that the radioresistance of NSCLC cells might relate to the deficiency of one or more proteins important for the activation and/or execution of the apoptotic process. Therefore, the expression of pro-caspases, μ-calpain and the Bcl-2 family proteins Bcl-2, Bcl-X_L and Bax, was analyzed in a panel of 10 SCLC and 6 NSCLC cell lines by RPA and western blotting.

Bcl-X_L and Bax protein were present in all cell lines at equal levels. Bcl-2 was only strongly expressed in 1/5 NSCLC (H157), but in 5/5 SCLC cell lines investigated. SCLC cells were thus characterized by a high ratio Bcl-2/Bax.

While protein expression of μ-calpain and pro-caspases-2, -3, -7 and -9 was observed in all NSCLC and SCLC cell lines investigated, pro-caspase-8 was not detected in 8/10 SCLC cell lines. The only SCLC cell lines in which pro-caspase-8 was detected was U1285 and its doxorubicin resistant daughter line, U1285DOX900. All NSCLC cell lines expressed pro-caspase-8.

Lack of pro-caspase-8 mRNA was found in the same SCLC cell lines that were missing pro-caspase-8 protein expression, indicating silencing of the gene as a probable cause. In addition, pro-caspases-10, -4 and -1 mRNA was not detected in the majority of the five SCLC cell lines, and in half of the NSCLC cell lines.

These findings were unexpected since SCLC cells in general are more apoptosis prone than NSCLC cells, but here were shown to lack the expression of several pro-caspases and have a high level of Bcl-2 protein. There results suggest that the resistance of NSCLC cells to undergo IR-induced apoptosis cannot simply be explained by the absence of executioner caspases or by the ratio of Bcl-2 family members.

Paper II

In this study, the functionality of the major apoptosis signaling pathways in lung cancer cells was studied in more detail. Three different apoptosis-inducing agents were used to investigate if dysfunctional signaling could explain the resistance of NSCLC to anti-cancer treatment. VP16 (1.5 μg/ml) and IR are both DNA-damaging agents, but generate different types of lesions, while agonistic Fas-antibodies (αFas) activate the receptor-mediated apoptotic pathway. A subset of cell lines from the panel, representing differences in radiosensitivity and expression of pro-caspase-8, was chosen for this study. U1285 was the only SCLC cell line with intact pro-caspase-8 expression. It is also one of the most radiosensitive cell lines in the panel. H82 is an intermediate radiosensitive SCLC cell line that lack expression of pro-caspase-8. U1810 is the most radioresistant of the NSCLC cell lines. Several apoptotic parameters were analyzed including pro-caspase processing, caspase activity, cytochrome *c* release, *DNA* fragmentation and nuclear morphology.

U1810 cells were found to be resistant, i.e. did not undergo apoptotic nuclear morphological changes, to either of the three treatments, whereas H82 cells were only

resistant to αFas-induced apoptosis. U1285 cells underwent apoptosis in response to all three treatments.

Apoptotic processing of pro-caspase-8, as well as increased caspase-8 activity, occurred in both U1285 and U1810 cells after either of the three treatments. However, this was not enough to drive U1810 cells into apoptosis.

Cytosolic cytochrome *c* was detected by western blotting of cytosolic extracts from all cell lines after IR and VP16 treatment, although to a lesser degree in U1810 compared with U1285 cells. Immunocytochemistry of cytochrome *c* in U1285 and U1810 cells revealed a change from the punctate pattern, indicating mitochondrial localization, to a more diffuse one after IR-treatment. This coincided with apoptotic nuclear morphology in U1285, but not in U1810 cells.

Increased caspase-9 activity was detected in both SCLC and NSCLC cells after treatment with IR, but not to the same extent after VP16 or αFas treatment. Disappearance of the band corresponding to pro-caspase-9 was observed in western blots of irradiated U1285 and U1810 cells.

A substantial increase in caspase-3-like activity was detected in both SCLC and NSCLC cells after all treatments, with the exception of H82 cells treated with αFas (since they lack pro-caspase-8 protein). This was accompanied by the detection of a fragment corresponding to processed caspase-3 in both U1285 and U1810 cells by western blot analysis. The cleavage of nuclear targets for caspase-3 (PARP and ICAD/DFF45) was detected only in SCLC cells. Moreover, relocalization of active caspase-3 from the cytosol into the nucleus was observed in the SCLC cell line, but not in three NSCLC cell lines, after IR-treatment.

Taken together, these data demonstrate activation of apoptotic signaling after IR in both NSCLC and SCLC cells. However, nuclear translocation of active caspase-3, DNA fragmentation and nuclear apoptotic morphology was only induced in SCLC cells. Thus, inhibition of apoptosis signaling in NSCLC cells might occur downstream of mitochondrial changes and caspase activation, but upstream of nuclear apoptotic events.

Paper III

Members of the inhibitor of apoptosis family of proteins are able to bind and inhibit active caspases, thus keeping caspase activation below the critical threshold. The absence of nuclear apoptotic morphology in NSCLC cells in spite of IR-induced apoptotic signaling, including caspase activity, was observed in **Paper II**. This phenomenon could possibly be explained by the intervention of IAPs. Therefore, the expression of IAPs was determined in the panel of lung cancer cells. The levels of cIAP-1, cIAP-2 and XIAP, the most potent caspase inhibitors, were further investigated in three cell lines after VP16- or IR-treatment.

An RNase Protection Assay (RPA) kit, including probes for Survivin, cIAP-1, cIAP-2, XIAP and NAIP, was used for detection of IAP mRNAs. The expression of cIAP-2 mRNA was higher in the NSCLC cell lines compared with the SCLC cell lines. cIAP-1 and XIAP mRNAs were expressed by almost all lung cancer cell lines, however, no general difference was observed between the NSCLC and the SCLC group. Survivin mRNA was detected at high levels in both SCLC and NSCLC cells. No NAIP mRNA was detected in any of the cell lines, as expected, since NAIP is a neuronal-specific IAP. The RPA findings were confirmed by RT-PCR using primers for cIAP-1, cIAP-2 and XIAP.

The expression of cIAP-1, cIAP-2 and XIAP proteins was determined in the panel of lung cancer cell lines by western blot analysis. cIAP-1 was present in all cell lines at different level but with no significant difference between the NSCLC and SCLC groups. cIAP-2 was detected at a significantly higher level in the NSCLC compared to the SCLC group of cell lines. XIAP was expressed more than four-fold in the SCLC group compared to the NSCLC group.

To investigate if treatment would change the expression of IAPs, mRNA and protein levels of cIAP-1, cIAP-2 and XIAP were analyzed after treatment with VP16 or IR in U1810, H82 and U1285 cells together with Jurkat cells. XIAP protein decreased after treatment in Jurkat and U1285 cells, especially in response to VP16, but no change in XIAP mRNA level was detected indicating degradation of the protein. An increase in XIAP transcripts after VP16 treatment was observed in U1810 cells, but there was no change in the protein level.

Moreover, the intracellular localization of IAPs was investigated using subcellular fractionation of untreated, VP16- or IR-treated cells (U1810 and U1285) followed by western blot analysis. cIAP-1 and XIAP were present in the cytosolic fraction, but XIAP was also found in the heavy membrane and nuclear fractions. Surprisingly, cIAP-2 was mainly detected in the heavy membrane fraction containing mitochondria. In response to treatment, the IAPs did not change their sub-cellular localization.

In summary, when comparing IAP expression in the two types of lung cancer cells, NSCLC cell lines had a stronger cIAP-2 expression, while the SCLC cell lines were characterized by higher level of XIAP protein. Although differences in IAP expression were observed between the groups of NSCLC and SCLC cells, no correlation could be made between the level of IAP expression and the radiosensitivity of individual cell lines. Neither the expression level nor the intracellular localization of IAPs pre- and post-treatment changed in a way to explain the radioresistance of U1810 cells. These results indicate that IAPs alone are not the main factor responsible for the resistance of NSCLC cells to treatment.

Paper IV

The heat shock protein family members (HSPs), some of which can be upregulated by stress, are cellular chaperones. HSP27 and HSP72 are the most well known stress-induced proteins with anti-apoptotic properties and their expression and influence on IR-induced apoptosis was investigated.

HSP72 protein was detected at high levels in all cell lines. HSP27 was also abundantly expressed in all NSCLC and a majority (6/9) of the SCLC cell lines. While mild heat shock further increased the HSP expression, treatment with IR or VP16 did not cause HSP upregulation. Pre-treatment with heat shock did not protect radiosensitive cells from IR-induced apoptosis nor did it attenuate IR-induced caspase activity.

It was suggested that HSP72 is required for the viability of tumor cells, and down-regulation, by antisense HSP72, was shown to induce apoptosis and tumor regression *in vivo* (Nylandsted et al., 2002). Therefore, the effect of HSP72 downregulation in U1810 cells, the most IR-resistant NSCLC cell line, was investigated in the present work. HSP72 protein expression was knocked down by both cDNA antisense and RNAi. cDNA transfection of U1810 cells induced non-specific cell death at 24 hrs. Instead, the RNAi method was used for specific and

efficient knockdown of HSP72 protein expression. Interestingly, downregulation of HSP72 by siRNA did not sensitize U1810 cells to either CP-, VP16- or IR-induced apoptosis. Non-specific side effects, such as morphologic changes, slight growth retardation and (after four days) increasing cell death with apoptotic morphology, were observed also with this method. It was therefore not possible to make any conclusions concerning the requirement of retained HSP72 expression for U1810 cell survival. In summary, these results do not support an important contribution of HSP72 to the IR-resistance of U8110 cells.

Paper V

Loss of mitochondrial integrity is a key event in apoptosis. Bax and Bak are pro-apoptotic Bcl-2 protein family members that can induce MPT, and their role in IR-induced apoptosis was investigated in U1810 and U1285 cells. Furthermore, the IR-induced activation of SAPK signaling was studied. In these experiments, IR was compared with cisplatin (CP) that induces caspase-dependent apoptosis. CP has also been shown to induce activation of Bak and SAPKs in tumor cells (Mandic et al. 2001).

In U1285 cells, both CP- and IR-treatment induced pro-apoptotic conformational changes in Bak and, to a lesser extent Bax. This was followed by a drop in $\Delta\psi m$, detection of active caspase-3 and apoptotic nuclear morphological changes, in a time dependent manner. These changes were observed in U1810 cells after treatment with CP, but not IR.

The upstream signaling leading to Bak activation is not well elucidated, but MEKK1 has been shown to regulate Bak activation. Expression of dnMEKK1 in U1285 cells partially blocked IR-induced apoptosis, pointing to the involvement of other upstream signaling pathways. In U1810 cells, dpMEKK1 expression or CP-treatment induced Bak activation, mitochondrial depolarization, caspase-3 activation and nuclear apoptotic morphology, demonstrating a functional Bak signaling pathway.

An early and sustained activation of SAPKs p38MAPK and JNK was detected in U1285, but not in U1810 cells, in response to IR. However, CP treatment substantially induced SAPK activation also in U1810 cells. The blocking of SAPK activation in U1285 cells by chemical inhibitors totally abrogated IR-induced apoptosis.

These results point to a failure of IR to initiate apoptotic mitochondrial events in U1810 cells. The upstream events leading to activation of the mitochondrial apoptotic pathway could be associated with SAPK signaling.

1.7 DISCUSSION

In this thesis, the IR-induced apoptotic signaling in lung cancer cells was studied. The goal was not only to achieve a better understanding of the process, but also to identify possible targets for pharmacological modulation. Several differences in the apoptotic signaling of NSCLC and SCLC cells were identified, and their possible importance for radioresistance will be the subject of this discussion.

1.7.1 Prologue. IR-induced cell death, not only apoptosis?

IR-induced cell death is a complicated process that can involve several types of cell death, such as mitotic catastrophe, apoptosis and necrosis, evolving with different kinetics. Thus, the fundamental question of the actual role of apoptosis in IR-induced cell death immediately arises. To assess the contribution of apoptosis to the total cell death induced by IR in the cell lines used in the present study, a subset of lung cancer cell lines with known SF2 were irradiated with 8 Gy. Loss of plasma membrane integrity, indicating necrosis or late-stage apoptosis, was assessed by trypan blue exclusion. Apoptosis was assessed by evaluation of nuclear morphology (**Figure 9**). In the SCLC cell lines, a fraction of spontaneous apoptotic and trypan blue positive cells were present in the untreated cells, as previously described (Sirzén et al., 1998a). A time-dependent increase in the fraction of trypan blue positive cells was observed in the radiosensitive U1285 cells (two-fold) after IR, but only to a very small extent in the intermediately radiosensitive H82 and the radioresistant U1810 cells. Apoptosis was induced by IR in both SCLC cell lines in a time dependent manner and was most pronounced in U1285 cells. Only a few percent cells with apoptotic nuclear morphology was observed in U1810 cells after IR.

Thus, the amount of IR-induced apoptosis correlated with radiosensitivity for these three lung cancer cell lines. Moreover, the increase in trypan blue positive cells reflects the kinetics and magnitude of apoptosis induction. This indicates that IR-induced apoptosis is an important contributor to cell death in the radiosensitive lung cancer cells, and that early necrosis is not a predominant form of cell death in these cells.

1.7.2 Act I. The works of apoptotic signaling

1.7.2.1 Setting the scene

Earlier studies in our group showed that IR does not induce apoptosis in NSCLC cells (Sirzén et al., 1998b). We asked whether this could be due to a lack of proteins important for the induction and/or execution of the apoptotic process. The expression of several pro-caspases, μ-calpain, Bcl-2, Bcl-X_L and Bax was analyzed in **Paper I** to investigate the possible connection between radioresistance and the presence of these proteins.

The most striking observation was the lack of pro-caspase-8 protein and mRNA expression in 8/10 SCLC cell lines. This was the first published observation of *CASP8* silencing and was later confirmed for SCLC cell lines and tumor biopsies (Shivapurkar et al., 2002). Lack of pro-caspase-8 expression was also found in neuroblastoma cells (Teitz et al., 2000) as well as in many types of pediatric tumors and their corresponding cell lines (Harada et al., 2002). The silencing was

demonstrated to be due to promoter methylation, gene deletion or mutation, and is now considered a characteristic feature of neuroendocrine tumors, like SCLC and neuroblastoma, and a possible oncogene.

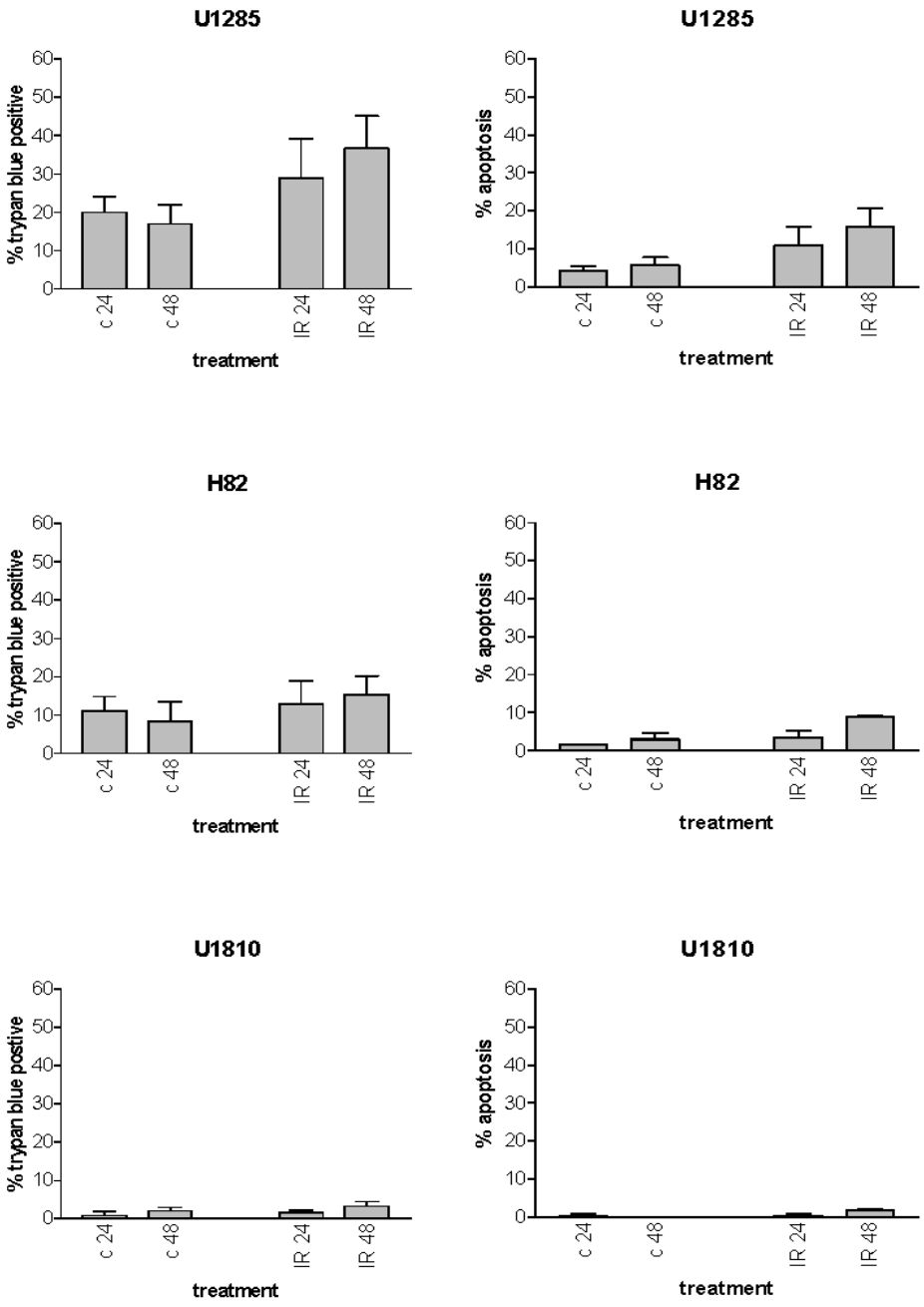


Figure 9.

IR-induced cell death in lung cancer cells. Cells were irradiated with 8 Gy. After the indicated times (hrs), the percentage of cells with disrupted plasma membrane integrity (left-hand panels) and nuclear apoptotic morphology (right-hand panel) was assessed. Error bars denoted standard deviation from three separate experiments.
This is a modified version of Figure 4 in Paper III.

It would be interesting to see if restoration of pro-caspase-8 expression would help to amplify IR-induced apoptotic signaling in SCLC cells, as was shown for the response to drug- and receptor-induced apoptosis in other tumor types (Fulda et al., 2001). Demethylation of promoter regions can be chemically induced. However, the recent findings that general hypomethylation contributes to genomic instability and tumor development in mice (Gaudet et al., 2003) point to a need for specific tumor targeting of the demethylating effect before this mechanism can be utilized in a clinical setting. More interesting is the finding that interferon-γ, that is already utilized in the clinic, was shown to increase the level of pro-caspase-8 protein in cells with low or no expression, even when the *CASP8* promotor was methylated (Fulda & Debatin, 2002).

A strong Bcl-2 protein expression was observed in SCLC compared to NSCLC cells (**Paper I**). Since Bax and Bcl-X_L were equally expressed in both types of lung cancers, SCLC cells were characterized by a higher Bcl-2/Bax ratio. This reflects the clinical situation where 75-80% of primary SCLC tumor specimens have a strong Bcl-2 immunostaining (Ben-Ezra et al., 1994; Kaiser et al., 1996). The high level of Bcl-2 in SCLC is paradoxical since they are usually more sensitive to treatment. However, a recent study on SCLC cell lines found a correlation between acquired drug resistance and strong upregulation of Bcl-2 (Sartorius & Krammer, 2002). So far, no strong prognostic value has been demonstrated for Bcl-2 expression in lung cancer (Sekido et al., 1998). Bcl-2 expression is associated with neuroendocrine features of lung cancer, suggesting a role for Bcl-2 during malignant transformation (Jiang et al., 1996). This is further supported by the findings of strong Bcl-2 expression in dys- or metaplasia *in situ* (Kikuchi et al., 1997; Tesfaigzi et al., 1998). Although of unclear prognostic value, a high level of Bcl-2 in SCLC can still be of clinical use. Down regulation of Bcl-2 inhibits tumor growth *in vitro* and in xenotransplanted tumors (Cotter et al., 1994; Jansen et al., 1998). In addition, the simultaneous downregulation of Bcl-2 and Bcl-X_L was successful in inducing apoptosis in both NSCLC and SCLC cells *in vitro* (Zangemeister-Wittke et al., 2000).

Several clinical trials on Bcl-2 antisense therapy, alone or in combination with chemotherapy, are ongoing for a variety of cancers including NSCLC and SCLC (Ciardiello & Tortora, 2002). The treatment is well tolerated and clinically feasible. A recent pilot study on patients with chemo-refractory SCLC treated with Bcl-2 antisense in combination with paclitaxel reports on disease stabilization in two of twelve patients, one of which lasted for 30 weeks (Rudin et al., 2002). This may not sound impressive, but considering the bad prognosis for this category of patients, these results are promising.

1.7.2.2 The role of mitochondria in IR-induced apoptosis

Mitochondrial disturbance seems to be pivotal for IR-induced damage to initiate apoptosis. To study the importance of mitochondrial apoptotic events for IR-induced apoptosis in lung cancer cells, cytochrome *c* release, activation of pro-apoptotic Bcl-2 proteins Bak and Bax, and loss of $\Delta\psi m$ were investigated.

Cytosolic cytochrome *c* was observed already at 8 hrs post-IR in U1810 cells as well as in U1285 and H82 cells (**Paper II**). Loss of $\Delta\psi m$ was assessed by TMRE staining in **Paper V**. At 16 hours post-treatment, a small increase in U1285 cells with depolarized mitochondria was observed that could explain the cytosolic appearance

of cytochrome *c*. Δψm was stable in U1810 cells after IR at least up to 48 hrs (**Paper V** and (Joseph et al., 2002) in agreement with the absence of apoptotic cells. However, an early and transient drop in Δψm, allowing the release of cytochrome *c*, cannot be excluded.

Persistent loss of Δψm was always observed together with the appearance of nuclear apoptotic morphology, indicating this to be the crucial event for the induction of apoptosis in U1810 cells (**Paper V**). The pro-apoptotic Bcl-2 family members Bak and Bax are able to induce MPT and release of cytochrome *c*. No substantial Bax activation was observed in any of the cell lines up to 20 and 30 hrs post-IR in U1285 and U1810 cells, respectively. In a recent study, Ku70 was found to suppress the apoptotic translocation of Bax to mitochondria. However, Ku70 expression was earlier demonstrated to be at similar levels in U1285 and U1810 cells (Sirzén et al., 1999).

IR induced Bak activation in U1285, but not in U1810 cells, prior to the drop in Δψm. Moreover, treatment with CP or dpMEKK1 caused Bak activation, loss of Δψm, caspase activation and apoptosis in U1810 cells, further underlining the importance of mitochondrial dysfunction for apoptosis to take place in these cells.

1.7.2.3 IR-induced caspase activity

Treatment with αFas or IR caused pro-caspase-8 activation in both U1285 and U1810 cells (**Paper II**). However, αFas-induced caspase-8 activation was not followed by the release of cytochrome *c* or apoptosis in U1810 cells, demonstrating that the activation of pro-caspase-8 is not enough to trigger fulminant apoptosis in U1810 cells.

The appearance of cytosolic cytochrome *c* enables formation of the apoptosome complex in which pro-caspase-9 is activated. Processing of pro-caspase-9 and increased caspase-9 activity was observed after IR in all lung cancer cell lines investigated, including U1810 cells (**Papers II** and **IV**).

Caspase-9 in turn activates executioner caspases-3 and –7. Activation of caspase-3 was observed after IR treatment in all cell lines investigated (**Papers II** and **IV**). However, despite apparent caspase activation in U1810 cells, no apoptosis was detected.

1.7.3 Intermission I

As was demonstrated by the use of cytotoxic drugs, such as VP16 and CP (**Papers IV** and **V**) as well as staurosporine (Joseph et al., 2002), the radioresistant U1810 cells can undergo apoptosis in response to these agents. However, the apoptotic signaling is indeed activated after IR but apparently not sufficient to trigger the actual execution of apoptosis in U1810 cells. One of the major differences observed between SCLC and NSCLC cells is that NSCLC cells were able to maintain Δψm after IR. The loss of Δψm strongly correlates with the ability of an agent to induce apoptosis in U1810 cells.

IR-induced cytochrome *c* release and caspase activation were detected in both NSCLC and SCLC cells, implying that apoptotic inhibition also could occur at or downstream of the level of executioner caspases in NSCLC cells. In the next section, possible mechanisms for this inhibition will be discussed.

1.7.4 Act II. The cellular bag of anti-apoptotic tricks

1.7.4.1 Inhibitor of Apoptosis Proteins, pouring oil on the waves

IAP overexpression can make cells more radioresistant, as shown for H661 NSCLC cells (Holcik et al., 2000). The IAP family of proteins can inhibit active caspases. IAPs interfere with apoptosis signaling by suppressing the caspase activity below a certain "critical threshold". However, the "threshold level" for caspase activity to be deleterious is not known and is in addition probably cell type and stimuli dependent.

The expression of IAPs in the panel of lung cancer cell line was investigated in **Paper III**. Although differences in the level of IAP expression were found when comparing the groups of NSCLC and SCLC cells, a detailed analysis did not reveal a correlation between the level of expression of these proteins and the radiosensitivity of individual cell lines. The SCLC cell lines had a strong protein expression of XIAP. This is in parallel to the "Bcl-2 paradox", and makes XIAP an interesting therapeutic target in lung cancer. XIAP can interact with the apoptosome by binding to the small subunit of caspase-9, inhibiting downstream caspase activation, or sequester active caspase-3 (Bratton et al., 2002). The apoptosome activity can be restored by a Smac-like peptide that disrupts the XIAP/caspase-9 binding. Such a peptide was shown to potentiate chemotherapy in xenotransplanted tumors (Arnt et al., 2002; Fulda et al., 2002). The requirement of IAP-antagonism for the execution of apoptosis was also demonstrated in a study where mutant XIAP that could bind Smac, but not caspase-9 or caspase –3 and thus only sequestered Smac, was able to inhibit UV-induced apoptosis (Silke et al., 2002). The high XIAP expression in SCLC could indicate a dependence of IAP-antagonists for the propagation of apoptosis. It would therefore be very interesting to see what effect Smac-mimetic peptides would have on IR-induced death in SCLC as well as in NSCLC cells.

1.7.4.2 HSP72, Jack of all trades

The strong expression of constitutive and stress-inducible HSPs in tumors and tumor cell line is a well-known phenomenon. Stress-inducible HSPs have been shown to inhibit cell death in caspase-dependent and -independent manners. The therapeutic use for HSPs has so far mainly been as an antigen to raise an immune response against the tumor. However, recent studies point to a direct requirement of HSP72 for tumor cell survival (Nylandsted et al., 2000a). Downregulation of HSP72 expression caused caspase-independent cell death with apoptotic morphology (Nylandsted et al., 2000b). Furthermore, downregulation of HSP72 sensitized prostate cancer cells to IR-induced apoptosis (Gibbons et al., 2000). This makes HSP72 a therapeutic target of great interest, especially for treatment-resistant tumors like NSCLC.

We investigated the role of HSP72 in IR-induced apoptosis in lung cancer cells in **Paper IV**. The constitutive expression of HSP72 was high in all lung cancer cell lines and was not increased by IR-treatment in any of the cell lines investigated. Further upregulation of the stress-inducible HSP27 and HSP72 by heat shock did not protect cells against IR-induced apoptosis. Surprisingly, knockdown of HSP72 expression by siRNA did not sensitize U1810 cells either to IR-, CP- or VP16-induced apoptosis.

This strongly argues against the importance of HSP72 for radioresistance, at least in U1810 cells.

1.7.4.3 The nucleus, the cellular sanctum

The typical nuclear morphological changes in apoptosis are caused by the action of executioner caspases in conjunction with nucleases and possibly other factors. To execute this function, caspases must enter the nucleus. After IR, relocalization of active caspase-3 from cytosol into the nucleus was observed by immunocytochemistry in radiosensitive the U1285 cells, but not in three NSCLC cell lines (**Paper II**). This indicates a maintained nuclear integrity in NSCLC cells. Further supporting this assumption was the disappearance of the band representing the nuclear caspase target ICAD/DFF45 on western blots in U1285 but not in U1810 cells after IR-treatment. Moreover, the cleavage of DFF45 would release the nuclease DFF40, and no DNA fragmentation was observed in NSCLC cells after IR (**Paper II**).

Nuclear transport is conducted via the nuclear pores, i.e. large protein complexes that form conduits through the nuclear envelope, by soluble transporter molecules, e.g. importins and Ran. The small GTPase Ran binds to the importin and, depending on its association with GTP or GDP, determines in which direction the transport will go (reviewed by Adam, 2001). To use this transport system, proteins must display a special nuclear localization signal (NLS) to enter, or a nuclear exit signal (NES) to exit the nucleus. Neither NLS nor NES has been found in caspase-3, arguing for its nuclear transport either to be in complex with another protein possessing an NLS, or by passive diffusion. During apoptosis, the nuclear transport process is disrupted, possibly in a caspase-dependent manner (Faleiro & Lazebnik, 2000; Ferrando-May et al., 2001) and the diffusion limit of the nuclear pores increases. This would allow the passive diffusion of active caspases into the nucleus. Microinjection of wheat germ agglutinin, a nuclear pore "plugger", rescued cells from Fas-R-mediated apoptosis (Yasuhara et al., 1997) and prevented the nuclear condensation of chromatin induced by AIF (Susin et al., 1999) and Acinus (Sahara et al., 1999).

Taken together, the nuclear entry for caspases is probably via the nuclear pores. The exclusion of active caspase-3 from the nucleus in NSCLC cells could be interpreted either as a failure of caspase-3 to actively enter the nucleus, or that the IR-induced apoptosis signaling is insufficient to trigger the nuclear transport dysfunction observed in apoptotic cells. Since irradiated U1810 cells display caspase activation but do not undergo apoptosis, the nuclear pore relaxation is probably not caspase-dependent in these cells.

1.7.5 Intermission II

The signal(s) connecting IR-induced cellular damage to the induction of apoptosis are not well understood. Since IR-induced apoptosis in lung cancer cells seems to be connected to loss of mitochondrial function, possible upstream signals linking the IR-induced damage to mitochondrial apoptotic events will be discussed in the last part.

1.7.6 Act III. In search for the missing link

1.7.6.1 Is it a caspase?

Pro-caspase-2 was pointed out as a possible candidate for the nuclear signaling of apoptosis via mitochondria (Robertson et al., 2002). Pro-caspase-2 is present in several intracellular compartments but is the only pro-caspase constitutively located in the nucleus (Zhivotovsky et al., 1999). Moreover, a cell specific requirement for caspase-2-dependent cytochrome *c* release and apoptosis in response to VP16, CP and UV-irradiation was demonstrated in several cell lines, including NSCLC A549 cells (Lassus et al., 2002). The effect of caspase-2 on mitochondria was not dependent on other cytosolic factors, as was demonstrated in a cell-free system (Robertson et al., 2002). The event responsible for the activation of pro-caspase-2 upon DNA damage is not clear, nor is the mechanism. Nothing is known about the importance of pro-caspase-2 for IR-induced apoptosis and this could be further investigated.

1.7.6.2 Is it a kinase?

The activation of JNK after IR is well documented, but the exact mechanism of JNK-induced apoptosis remains elusive. Translocation of JNK to mitochondria is associated with the release of Smac (Chauhan et al., 2003) and induction of OMP (Aoki et al., 2002; Ito et al., 2001). A novel link between JNK and mitochondrial dysfunction was recently reported. The BH3-only proteins Bim and Bmf are normally sequestered by binding to dynein and myosin V motor complexes. Upon phosphorylation by JNK, these BH3-only proteins can translocate to mitochondria and induce the activation of Bax/Bak and subsequent mitochondrial permeabilization (Lei & Davis, 2003).

Induction of sustained SAPK activation correlated with loss of $\Delta\psi m$ and induction of apoptosis (**Paper V**). Chemical inhibitors of JNK and p38MAPK efficiently abrogated IR-induced apoptosis in U1285. CP, but not IR, induced both SAPK activation and apoptosis in U1810 cells. These findings point to another difference between radioresistant and radiosensitive cells that demands further investigation of the upstream regulation of SAPK activation. The possible upstream kinases implicated in DNA damage are c-Abl, MEKK1 and ASK1.

The non-receptor tyrosine kinase c-Abl is a plausible link between DNA damage and apoptosis. c-Abl is activated by phosphorylation of ATM and/or DNA-PKcs in response to DNA damage (Baskaran et al., 1997; Kharbanda et al., 1997). c-Abl has been shown to activate JNK (Raitano et al., 1995) and MEKK1 (Kharbanda et al., 2000) in response to IR, while c-Abl-deficient cells failed to activate JNK (Kharbanda et al., 1995). Since an inactive fragment of MEKK1 only partially blocked IR-induced apoptosis in U1285 cells, additional pathways are probably involved in JNK activation in these cells. Another pathway to SAPK signaling could be via the apoptosis signaling kinase 1 (ASK1). ASK1 is activated by oxidative stress (Saitoh et al., 1998), and have been shown to induce SAPK signaling (Ichijo et al., 1997) and to initiate apoptosis via the mitochondrial pathway (Hatai et al., 2000).

The importance of kinase signaling in IR-induced apoptosis needs further investigation and future research will be directed to this matter.

1.7.6.3 Is it stress?

The pivotal event for IR-induced apoptosis seems to be the loss of $\Delta\psi m$ (**Papers II** and **V**). PT pore opening can be triggered by a rise in intracellular Ca^{2+}. The intracellular Ca^{2+} level is mainly governed by the ER that is the major Ca^{2+} storing organelle. Cellular stress and chemical compounds can cause disruption of the ATP-dependent Ca^{2+} pump residing in the ER membrane, or opening of Ca^{2+} channels, resulting in an increased intracellular level of Ca^{2+} (Berridge et al., 2000). Bcl-2 is believed to modulate intracellular Ca^{2+} levels and ER-targeted Bcl-2 has been shown to inhibit IR-induced MPT as efficiently as mitochondria-targeted Bcl-2 (Rudner et al., 2001). In addition, oxidation of ANT thiol groups can cause ANT dysfunction and subsequent MPT, but can also enhance the sensitivity of the PT pore to Ca^{2+} (and thus indirectly to ER stress). It is possible that U1810 cells are more efficient in handling the ER stress and elevated intracellular Ca^{2+} than are SCLC cells, however this probably not by the action of Bcl-2 since it was expressed at very low levels in most NSCLC cell lines. The change in intracellular Ca^{2+} after IR and the mitochondrial capacity to buffer Ca^{2+} could be investigated. This would elucidate the involvement of ER stress in IR-induced apoptosis.

Reactive oxygen species (ROS) is directly induced by IR, but could also arise from mitochondrial dysfunction. The role of oxidative stress for induction of apoptosis was demonstrated in studies where addition of ROS induced apoptosis, while various antioxidants inhibited cell death (for review, see Curtin et al., 2002). Moreover, the radioprotective effect of pharmacological antioxidants is well documented. IR-induced ROS can oxidize phospholipids in the cellular membranes. The oxidized lipids are hydrolyzed by lipases, producing lipid "second messengers" that can activate further signaling. One of these second messengers is ceramide that has been implicated in IR-induced apoptosis, especially of the interphase type (Chmura et al., 1997; Michael et al., 1997), although its importance may be cell type specific (Burek et al., 2001). Ceramide, or rather its degradation product sphingosine, can activate the SAPK pathway (Verheij et al., 1996) and induce apoptosis via the mitochondrial pathway (Rodriguez-Lafrasse et al., 2002; Tepper et al., 1999). Ceramide is produced either by *de novo* synthesis or by degradation of the membrane lipid sphingomyelin by the sphingomyelinase lipase. There are two variants of sphingomyelinase with different pH optima (acid and neutral). Lymphocytes from patients with an inherited deficiency in sphingomyelinase activity (Niemann-Pick syndrome) do not respond to IR with SAPK activation and apoptosis (Santana et al., 1996). Restoration of acid sphingomyelinase activity reverses this resistance. Moreover, mice deficient in acid sphingomyelinase are also defective in IR-induced ceramide generation and apoptosis *in vivo*, further arguing that sphingomyelinase activity rather that *de novo* synthesis is of importance in IR-induced ceramide signaling. Further investigation of the role of ceramide in IR-induced apoptotic signaling in lung cancer cells is needed to elucidate its importance for apoptosis induction.

Since ROS generation occurs during the normal metabolism, the cellular defense to oxidative stress is well developed. There are several molecules that can act as hydrogen-donors e.g. thioredoxin (Trx), glutathione (GSH) and superoxide dismutases (SOD). Manganese SOD (MnSOD) is normally present in the mitochondrial matrix at high concentrations. Overexpression of MnSOD, but not CuZnSOD or glutathione peroxidase, was shown to protect CHO cells from IR (Sun

et al., 1998). Overexpression of MnSOD was also shown to delay early translocation of Bax and SAPKs (p38MAPK and JNK1) to the mitochondria and significantly decrease the induction of MPT, cytochrome *c* release and apoptosis in hematopoietic cells (Epperly et al., 2002). The importance of the cellular antioxidants, especially MnSOD, could be a determinant for IR-induced apoptosis and should be further investigated.

1.7.7 Epilogue. General conclusions and future directions

In this thesis, the hypothesis that failure of induction or execution of apoptosis is responsible for the radioresistance observed in NSCLC cells was investigated. The data presented here demonstrate that IR-induced apoptotic signaling in radioresistant cells is abrogated at several levels, including SAPK activation, Bak activation, mitochondrial depolarization and nuclear relocalization of active caspase-3. Interestingly, in response to other agents initiation and execution of apoptosis can be activated also in radioresistant cells. This suggests that the apoptotic machinery is functional in NSCLC cells, but is not activated vigorously enough to trigger apoptosis in response to IR. Mitochondrial dysfunction was always observed in cells undergoing apoptosis, suggesting that this is the pivotal event for apoptosis induction in lung carcinoma cells. The mitochondrial changes might be under the control of SAPK activation, but the upstream signaling conveying the IR-induced damage to the mitochondria is still unclear. The search for this "missing link" is the most important task ahead. Specifically, the roles for intracellular antioxidants, c-Abl, ASK1 and JNK, need to be investigated in IR-induced apoptosis.

The enhancement of IR-induced apoptosis could drastically improve the effect of radiotherapy. To be able to eradicate radioresistant cells, one could either try to amplify the induced pro-apoptotic signaling to overcome the "execution threshold", or to modulate the threshold itself. Compounds acting on mitochondria could lower the "apoptotic threshold", or even induce apoptosis on their own, and should be evaluated in combination with IR in NSCLC cells. However, more knowledge of the signaling linking IR-induced damage to the pivotal mitochondrial events is needed to be able to identify additional therapeutic targets.

ACKNOWLEDGEMENTS

This work was funded by grants from: The Swedish and Stockholm Cancer Societies, The Jubilee Foundation of King Gustav V, The European Community RTD grant no QLK3-CT-2002-01956, The Heart and Lung Foundation and The Swedish Research Council.

The Institute of Environmental Medicine (IMM) and the Department of Oncology-Pathology, Karolinska Institutet, are acknowledged for providing a stimulating research environment during my years as a graduate student.

I wish to express my gratitude to the following persons:

My parents, Ulla and Björn Ekedahl, for endless love and encouragement. Without you, I would have accomplished none of this. I dedicate this thesis to you.

Professor Rolf Lewensohn, my supervisor, or luring me into science and sharing your never-ending flow of ideas and visions. For encouragement, trust and support all the way and for being a generous and appreciative supervisor. For reminding me of the importance of having a life outside the lab (although not always following you own advice…).

Professor Boris Zhivotovsky, my supervisor, for always taking the time, even when you don't have it. For being dedicated, all knowing, and always striving for perfection. Спасибо.

Dr. Bertrand Joseph, for kick-starting me on my thesis work and for nice collaboration. For bringing coffee, tea and a nice atmosphere to the Blue Group!!!

My collaborators: Associate Professor Mimmi Shoshan, Dr. Kristina Viktorsson, Professor Stig Linder and Dr. Annelie Meijer at the department of Oncology and Pathology, Karolinska Institutet. Professor Pierre Formstecher, Ph. D student Harold Fauvel and Associate Professor Philippe Marchetti at the INSERM U459 laboratory in Lille, France.

The Rolf Lewensohn group:
Marianne "Meeri" Langéen, for guiding my first steps in the lab and in the cell culture room. For being my role model on how to conduct lab work. For sharing and caring through the ups and downs in life.
Anders Nilsson, "The eternally curious", for always having something interesting to discuss - and a big laugh to go with it.
Ingrid Welander, for good co-work and for making the lab a nice (and ordered!) place to work.
Florin Sirzén, for paving the way.
Åsa Holgersson, Anya Polischouk and Annica Jernberg, for nice company through the years.
Lillemor Laurén, for help when needed, esprit and "turtle-sitting".
Jeffery Yachnin, Birgitta Mörk, Anica Dricu, Daria Cosaceanu, Signe Friesland, Roger Tell, Catharina Beskow, Josefin Paulsson, Lukas Orre and Annica Sterner, for making the Lewensohn group a nice place to work!

Professor Sten Orrenius and former and present members of the "Blue Group" at the Institute of Environmental Medicine (IMM): Joya Chandra, Bettina Gleiss, Hélène Stridh, Malin Müller, Kari Högstrand, Carina Magnusson, Afshin Samali, Adrienne Gorman, Camilla Köhler, Eva Ahlbom, Mari Enoksson, John Robertsson, Jan Schmidt-Mende, Vladimir Gogvadze, Annie Gahm, Elisabeth Petersson, the eternally esprit Birgitta Blacker, Sandra Ceccatelli, Ping Huang, Elisabetta Daré, Margareta Sandström, Leta Nutt, Suzanne Iversen, Christoffer Tamm, Pavel Grudinkin, Andrej Kropotov, Wanlaya Uthaisang, Bengt Fadeel, Roshan Tofighi, Agnieszka Sobczak-Pluta, Maria "Marsha" Karpova, Therese Hemström and the queen of neatness Babett Bartling, for making good science and sharing the fun!

Former and present companions at the CCK ground floor:
Malin Andersson, for letting me practice my teaching skills on your master thesis project.
Mats Ljungman, my "roomie". For interesting and helpful discussions and very valuable comments on the thesis.
Galina Selivanova for interesting discussions on life and science over late suppers at CCK.
Ingegerd Hedlöf -the queen of cell culturing, for help and advice.
Teresia Svensson, Per Kogner, Frida Ponthan, Lena Klevenvall, Nina Karnehed, Magnus Lindskog and John-Inge. For nice company in the cell culturing room.
Margareta Edgren, Bim Eriksson, Cecilia Glöckner, Jenny Flygare, Eva Andersson, Maude Lindgren, Eva Liljemark, Marianne Frostvik-Stolt, Rong Zhou, Yuying Wang, Tomas Heiden, Naining Wang, Peter Biberfeld, Charles Massambu, Pavan Pakuyrel, Ali Moshfegh, Torsten Hägerström, Natalia Issaeva, Marina Protopopova and Martin Enge, for making the "ground floor" a nice place to work.

Professor Tina Dalianis, Prefect of the Department of Oncology and Pathology, for being a role model, caring for the students and for making the department a stimulating place to work.
Evi Gustavsson-Kadaka, for always being helpful with the administrative things (and they are many!).

Drs Erika Isaksson Friman and Nina Cavalli-Björkman, for excellently guiding my first steps as a "real" doctor at Radiumhemmet. A special thanks to Nina for introducing Ebba to us.
The patients I met at Radiumhemmet, for giving me some extra research motivation.

Everyone who made me develop my skills as a "computer wiz". Trial and error can accomplish quite a lot!

Alexander Freudenthal, my love, my life and husband to be! For cheering me on while covering all bases at home. For being patient, loving me and keeping me sane during this time.
The Freudenthal family (Peter, Solveig, Jakob, Yael) and my friends, for cheering me on, being supportive and patient.

REFERENCES

Acehan, D., Jiang, X., Morgan, D.G., Heuser, J.E., Wang, X. and Akey, C.W. (2002). Three-dimensional structure of the apoptosome: implications for assembly, procaspase-9 binding, and activation. *Mol Cell*, 9 (2); 423-32.

Adam, S.A. (2001). The nuclear pore complex. *Genome Biol*, 2 (9); REVIEWS0007.

Allan, D.J. (1992). Radiation-induced apoptosis: its role in a MADCaT (mitosis-apoptosis-differentiation-calcium toxicity) scheme of cytotoxicity mechanisms. *Int J Radiat Biol*, 62 (2); 145-52.

Altieri, D.C. (2001). The molecular basis and potential role of survivin in cancer diagnosis and therapy. *Trends Mol Med*, 7 (12); 542-7.

Ambrosini, G., Adida, C. and Altieri, D.C. (1997). A novel anti-apoptosis gene, survivin, expressed in cancer and lymphoma. *Nat Med*, 3 (8); 917-21.

Ambrosini, G., Adida, C., Sirugo, G. and Altieri, D.C. (1998). Induction of apoptosis and inhibition of cell proliferation by survivin gene targeting. *J Biol Chem*, 273 (18); 11177-82.

Andersen, M.H. and Thor, S.P. (2002). Survivin--a universal tumor antigen. *Histol Histopathol*, 17 (2); 669-75.

Antonsson, B., Montessuit, S., Lauper, S., Eskes, R. and Martinou, J.C. (2000). Bax oligomerization is required for channel-forming activity in liposomes and to trigger cytochrome c release from mitochondria. *Biochem J*, 345 (Pt 2); 271-8.

Aoki, H., Kang, P.M., Hampe, J., Yoshimura, K., Noma, T., Matsuzaki, M. and Izumo, S. (2002). Direct activation of mitochondrial apoptosis machinery by c-Jun N- terminal kinase in adult cardiac myocytes. *J Biol Chem*, 277 (12); 10244-50.

Arnt, C.R., Chiorean, M.V., Heldebrant, M.P., Gores, G.J. and Kaufmann, S.H. (2002). Synthetic Smac/DIABLO peptides enhance the effects of chemotherapeutic agents by binding XIAP and cIAP1 in situ. *J Biol Chem*, 277 (46); 44236-43.

Bakkenist, C.J. and Kastan, M.B. (2003). DNA damage activates ATM through intermolecular autophosphorylation and dimer dissociation. *Nature*, 421 (6922); 499-506.

Barry, M., Heibein, J.A., Pinkoski, M.J., Lee, S.F., Moyer, R.W., Green, D.R. and Bleackley, R.C. (2000). Granzyme B short-circuits the need for caspase 8 activity during granule-mediated cytotoxic T-lymphocyte killing by directly cleaving Bid. *Mol Cell Biol*, 20 (11); 3781-94.

Baskaran, R., Wood, L.D., Whitaker, L.L., Canman, C.E., Morgan, S.E., Xu, Y., Barlow, C., Baltimore, D., Wynshaw-Boris, A., Kastan, M.B. and Wang, J.Y. (1997). Ataxia telangiectasia mutant protein activates c-Abl tyrosine kinase in response to ionizing radiation. *Nature*, 387 (6632); 516-9.

Belzacq, A.S., Vieira, H.L., Verrier, F., Vandecasteele, G., Cohen, I., Prevost, M.C., Larquet, E., Pariselli, F., Petit, P.X., Kahn, A., Rizzuto, R., Brenner, C. and Kroemer, G. (2003). Bcl-2 and Bax modulate adenine nucleotide translocase activity. *Cancer Res*, 63 (2); 541-6.

Ben-Ezra, J.M., Kornstein, M.J., Grimes, M.M. and Krystal, G. (1994). Small cell carcinomas of the lung express the Bcl-2 protein. *Am J Pathol*, 145 (5); 1036-40.

Berridge, M.J., Lipp, P. and Bootman, M.D. (2000). The versatility and universality of calcium signalling. *Nat Rev Mol Cell Biol*, 1 (1); 11-21.

Berven, E. (1962). The development and organization of therapeutic radiology in Sweden. *Radiology*, 79 829-841.

Boatright, K.M., Renatus, M., Scott, F.L., Sperandio, S., Shin, H., Pedersen, I.M., Ricci, J.E., Edris, W.A., Sutherlin, D.P., Green, D.R. and Salvesen, G.S. (2003). A unified model for apical caspase activation. *Mol Cell*, 11 (2); 529-41.

Bonomi, P. (2002). Matrix metalloproteinases and matrix metalloproteinase inhibitors in lung cancer. *Semin Oncol*, 29 (1 Suppl 4); 78-86.

Borner, C. and Monney, L. (1999). Apoptosis without caspases: an inefficient molecular guillotine? *Cell Death Differ*, 6 (6); 497-507.

Bossy-Wetzel, E., Bakiri, L. and Yaniv, M. (1997). Induction of apoptosis by the transcription factor c-Jun. *Embo J*, 16 (7); 1695-709.

Bossy-Wetzel, E., Newmeyer, D.D. and Green, D.R. (1998). Mitochondrial cytochrome c release in apoptosis occurs upstream of DEVD- specific caspase activation and independently of mitochondrial transmembrane depolarization. *Embo J*, 17 (1); 37-49.

Bratton, S.B., Lewis, J., Butterworth, M., Duckett, C.S. and Cohen, G.M. (2002). XIAP inhibition of caspase-3 preserves its association with the Apaf-1 apoptosome and prevents CD95- and Bax-induced apoptosis. *Cell Death Differ*, 9 (9); 881-92.

Bratton, S.B., Walker, G., Srinivasula, S.M., Sun, X.M., Butterworth, M., Alnemri, E.S. and Cohen, G.M. (2001). Recruitment, activation and retention of caspases-9 and -3 by Apaf-1 apoptosome and associated XIAP complexes. *Embo J*, 20 (5); 998-1009.

Burek, C., Roth, J., Koch, H.G., Harzer, K., Los, M. and Schulze-Osthoff, K. (2001). The role of ceramide in receptor- and stress-induced apoptosis studied in acidic ceramidase-deficient Farber disease cells. *Oncogene*, 20 (45); 6493-502.

Burma, S., Chen, B.P., Murphy, M., Kurimasa, A. and Chen, D.J. (2001). ATM phosphorylates histone H2AX in response to DNA double-strand breaks. *J Biol Chem*, 276 (45); 42462-7.

Bykov, V.J., Issaeva, N., Shilov, A., Hultcrantz, M., Pugacheva, E., Chumakov, P., Bergman, J., Wiman, K.G. and Selivanova, G. (2002). Restoration of the tumor suppressor function to mutant p53 by a low- molecular-weight compound. *Nat Med*, 8 (3); 282-8.

Cain, K., Bratton, S.B. and Cohen, G.M. (2002). The Apaf-1 apoptosome: a large caspase-activating complex. *Biochimie*, 84 (2-3); 203-14.

Cardone, M.H., Salvesen, G.S., Widmann, C., Johnson, G. and Frisch, S.M. (1997). The regulation of anoikis: MEKK-1 activation requires cleavage by caspases. *Cell*, 90 (2); 315-23.

Castro, J., Heiden, T., Wang, N. and Tribukait, B. (1993). Preparation of cell nuclei from fresh tissues for high-quality DNA flow cytometry. *Cytometry*, 14 (7); 793-804.

Chandra, D. and Tang, D.G. (2003). Mitochondrially-localized active caspase-9 and caspase-3 result mostly from translocation from the cytosol and partly from caspase-mediated activation in the organelle --- Lack of evidence for Apaf-1-mediated procaspase-9 activation in the mitochondria. *J Biol Chem*, 28 M300750200.

Chang, H.Y., Nishitoh, H., Yang, X., Ichijo, H. and Baltimore, D. (1998). Activation of apoptosis signal-regulating kinase 1 (ASK1) by the adapter protein Daxx. *Science*, 281 (5384); 1860-3.

Charette, S.J., Lavoie, J.N., Lambert, H. and Landry, J. (2000). Inhibition of Daxx-mediated apoptosis by heat shock protein 27. *Mol Cell Biol*, 20 (20); 7602-12.

Chauhan, D., Li, G., Hideshima, T., Podar, K., Mitsiades, C., Mitsiades, N., Munshi, N., Kharbanda, S. and Anderson, K.C. (2003). JNK-dependent release of mitochondrial protein, SMAC during apoptosis in multiple myeloma (MM) cells. *J Biol Chem*, 28 C300076200.

Chen, G., Gharib, T.G., Huang, C.C., Taylor, J.M., Misek, D.E., Kardia, S.L., Giordano, T.J., Iannettoni, M.D., Orringer, M.B., Hanash, S.M. and Beer, D.G. (2002). Discordant protein and mRNA expression in lung adenocarcinomas. *Mol Cell Proteomics*, 1 (4); 304-13.

Chen, J., Lin, J. and Levine, A.J. (1995). Regulation of transcription functions of the p53 tumor suppressor by the mdm-2 oncogene. *Mol Med*, 1 (2); 142-52.

Chen, M., He, H., Zhan, S., Krajewski, S., Reed, J.C. and Gottlieb, R.A. (2001). Bid is cleaved by calpain to an active fragment in vitro and during myocardial ischemia/reperfusion. *J Biol Chem*, 276 (33); 30724-8.

Cheng, E.H., Kirsch, D.G., Clem, R.J., Ravi, R., Kastan, M.B., Bedi, A., Ueno, K. and Hardwick, J.M. (1997). Conversion of Bcl-2 to a Bax-like death effector by caspases. *Science*, 278 (5345); 1966-8.

Cheng, E.H., Wei, M.C., Weiler, S., Flavell, R.A., Mak, T.W., Lindsten, T. and Korsmeyer, S.J. (2001). BCL-2, BCL-X(L) sequester BH3 domain-only molecules preventing BAX- and BAK-mediated mitochondrial apoptosis. *Mol Cell*, 8 (3); 705-11.

Cheng, J., Zhou, T., Liu, C., Shapiro, J.P., Brauer, M.J., Kiefer, M.C., Barr, P.J. and Mountz, J.D. (1994). Protection from Fas-mediated apoptosis by a soluble form of the Fas molecule. *Science*, 263 (5154); 1759-62.

Cheng, J.Q., Jiang, X., Fraser, M., Li, M., Dan, H.C., Sun, M. and Tsang, B.K. (2002). Role of X-linked inhibitor of apoptosis protein in chemoresistance in ovarian cancer: possible involvement of the phosphoinositide-3 kinase/Akt pathway. *Drug Resist Updat*, 5 (3-4); 131-46.

Chmura, S.J., Nodzenski, E., Beckett, M.A., Kufe, D.W., Quintans, J. and Weichselbaum, R.R. (1997). Loss of ceramide production confers resistance to radiation-induced apoptosis. *Cancer Res*, 57 (7); 1270-5.

Ciardiello, F. and Tortora, G. (2002). Inhibition of bcl-2 as cancer therapy. *Ann Oncol*, 13 (4); 501-2.

Clarke, P.G. and Clarke, S. (1996). Nineteenth century research on naturally occurring cell death and related phenomena. *Anat Embryol (Berl)*, 193 (2); 81-99.

Conradt, B. and Horvitz, H.R. (1998). The C. elegans protein EGL-1 is required for programmed cell death and interacts with the Bcl-2-like protein CED-9. *Cell*, 93 (4); 519-29.

Cory, S. and Adams, J.M. (2002). The Bcl2 family: regulators of the cellular life-or-death switch. *Nat Rev Cancer*, 2 (9); 647-56.

Cotter, F.E., Johnson, P., Hall, P., Pocock, C., al Mahdi, N., Cowell, J.K. and Morgan, G. (1994). Antisense oligonucleotides suppress B-cell lymphoma growth in a SCID-hu mouse model. *Oncogene*, 9 (10); 3049-55.

Croce, C.M., Sozzi, G. and Huebner, K. (1999). Role of FHIT in human cancer. *J Clin Oncol*, 17 (5); 1618-24.

Crompton, M., Ellinger, H. and Costi, A. (1988). Inhibition by cyclosporin A of a Ca2+-dependent pore in heart mitochondria activated by inorganic phosphate and oxidative stress. *Biochem J*, 255 (1); 357-60.

Curtin, J.F., Donovan, M. and Cotter, T.G. (2002). Regulation and measurement of oxidative stress in apoptosis. *J Immunol Methods*, 265 (1-2); 49-72.

Daugas, E., Susin, S.A., Zamzami, N., Ferri, K.F., Irinopoulou, T., Larochette, N., Prevost, M.C., Leber, B., Andrews, D., Penninger, J. and Kroemer, G. (2000). Mitochondrio-nuclear translocation of AIF in apoptosis and necrosis. *Faseb J*, 14 (5); 729-39.

Davis, R.J. (2000). Signal transduction by the JNK group of MAP kinases. *Cell*, 103 (2); 239-52.

De Botton, S., Sabri, S., Daugas, E., Zermati, Y., Guidotti, J.E., Hermine, O., Kroemer, G., Vainchenker, W. and Debili, N. (2002). Platelet formation is the consequence of caspase activation within megakaryocytes. *Blood*, 100 (4); 1310-7.

Debatin, K.M., Poncet, D. and Kroemer, G. (2002). Chemotherapy: targeting the mitochondrial cell death pathway. *Oncogene*, 21 (57); 8786-803.

Dent, P., Yacoub, A., Contessa, J., Caron, R., Amorino, G., Valerie, K., Hagan, M.P., Grant, S. and Schmidt-Ullrich, R. (2003). Stress and radiation-induced activation of multiple intracellular signaling pathways. *Radiat Res*, 159 (3); 283-300.

Desagher, S., Osen-Sand, A., Montessuit, S., Magnenat, E., Vilbois, F., Hochmann, A., Journot, L., Antonsson, B. and Martinou, J.C. (2001). Phosphorylation of bid by casein kinases I and II regulates its cleavage by caspase 8. *Mol Cell*, 8 (3); 601-11.

Desagher, S., Osen-Sand, A., Nichols, A., Eskes, R., Montessuit, S., Lauper, S., Maundrell, K., Antonsson, B. and Martinou, J.C. (1999). Bid-induced conformational change of Bax is responsible for mitochondrial cytochrome c release during apoptosis. *J Cell Biol*, 144 (5); 891-901.

Deveraux, Q.L., Leo, E., Stennicke, H.R., Welsh, K., Salvesen, G.S. and Reed, J.C. (1999). Cleavage of human inhibitor of apoptosis protein XIAP results in fragments with distinct specificities for caspases. *Embo J*, 18 (19); 5242-51.

Deveraux, Q.L. and Reed, J.C. (1999). IAP family proteins--suppressors of apoptosis. *Genes Dev*, 13 (3); 239-52.

Deveraux, Q.L., Roy, N., Stennicke, H.R., Van Arsdale, T., Zhou, Q., Srinivasula, S.M., Alnemri, E.S., Salvesen, G.S. and Reed, J.C. (1998). IAPs block apoptotic events induced by caspase-8 and cytochrome c by direct inhibition of distinct caspases. *Embo J*, 17 (8); 2215-23.

Deveraux, Q.L., Takahashi, R., Salvesen, G.S. and Reed, J.C. (1997). X-linked IAP is a direct inhibitor of cell-death proteases. *Nature*, 388 (6639); 300-4.

Deverman, B.E., Cook, B.L., Manson, S.R., Niederhoff, R.A., Langer, E.M., Rosova, I., Kulans, L.A., Fu, X., Weinberg, J.S., Heinecke, J.W., Roth, K.A. and Weintraub, S.J. (2002). Bcl-xL deamidation is a critical switch in the regulation of the response to DNA damage. *Cell*, 111 (1); 51-62.

Ding, H.F., Lin, Y.L., McGill, G., Juo, P., Zhu, H., Blenis, J., Yuan, J. and Fisher, D.E. (2000). Essential role for caspase-8 in transcription-independent apoptosis triggered by p53. *J Biol Chem*, 275 (49); 38905-11.

Donovan, N., Becker, E.B., Konishi, Y. and Bonni, A. (2002). JNK phosphorylation and activation of BAD couples the stress-activated signaling pathway to the cell death machinery. *J Biol Chem*, 277 (43); 40944-9.

Du, C., Fang, M., Li, Y., Li, L. and Wang, X. (2000). Smac, a mitochondrial protein that promotes cytochrome c-dependent caspase activation by eliminating IAP inhibition. *Cell*, 102 (1); 33-42.

Ehrnsperger, M., Gräber, S., Gaestel, M. and Buchner, J. (1997). Binding of non-native protein to Hsp25 during heat shock creates a reservoir of folding intermediates for reactivation. *Embo J*, 16 (2); 221-9.

Ellinger-Ziegelbauer, H., Brown, K., Kelly, K. and Siebenlist, U. (1997). Direct activation of the stress-activated protein kinase (SAPK) and extracellular signal-regulated protein kinase (ERK) pathways by an inducible mitogen-activated protein Kinase/ERK kinase kinase 3 (MEKK) derivative. *J Biol Chem*, 272 (5); 2668-74.

Ellis, H.M. and Horvitz, H.R. (1986). Genetic control of programmed cell death in the nematode C. elegans. *Cell*, 44 (6); 817-29.

Ellis, R.E., Jacobson, D.M. and Horvitz, H.R. (1991). Genes required for the engulfment of cell corpses during programmed cell death in Caenorhabditis elegans. *Genetics*, 129 (1); 79-94.

Epperly, M.W., Sikora, C.A., DeFilippi, S.J., Gretton, J.A., Zhan, Q., Kufe, D.W. and Greenberger, J.S. (2002). Manganese superoxide dismutase (SOD2) inhibits radiation-induced apoptosis by stabilization of the mitochondrial membrane. *Radiat Res*, 157 (5); 568-77.

Erridge, S.C. and Murray, N. (2003). Thoracic radiotherapy for limited-stage small cell lung cancer: issues of timing, volumes, dose, and fractionation. *Semin Oncol*, 30 (1); 26-37.

Fadeel, B., Orrenius, S. and Zhivotovsky, B. (1999). Apoptosis in human disease: a new skin for the old ceremony? *Biochem Biophys Res Commun*, 266 (3); 699-717.

Fadeel, B., Orrenius, S. and Zhivotovsky, B. (2000). The most unkindest cut of all: on the multiple roles of mammalian caspases. *Leukemia*, 14 (8); 1514-25.

Faleiro, L. and Lazebnik, Y. (2000). Caspases disrupt the nuclear-cytoplasmic barrier. *J Cell Biol*, 151 (5); 951-9.

Fan, M., Goodwin, M., Vu, T., Brantley-Finley, C., Gaarde, W.A. and Chambers, T.C. (2000). Vinblastine-induced phosphorylation of Bcl-2 and Bcl-XL is mediated by JNK and occurs in parallel with inactivation of the Raf-1/MEK/ERK cascade. *J Biol Chem*, 275 (39); 29980-5.

Fernandez-Capetillo, O., Chen, H.T., Celeste, A., Ward, I., Romanienko, P.J., Morales, J.C., Naka, K., Xia, Z., Camerini-Otero, R.D., Motoyama, N., Carpenter, P.B., Bonner, W.M., Chen, J. and Nussenzweig, A. (2002). DNA damage-induced G2-M checkpoint activation by histone H2AX and 53BP1. *Nat Cell Biol*, 4 (12); 993-7.

Fernando, P., Kelly, J.F., Balazsi, K., Slack, R.S. and Megeney, L.A. (2002). Caspase 3 activity is required for skeletal muscle differentiation. *Proc Natl Acad Sci U S A*, 99 (17); 11025-30.

Ferrando-May, E., Cordes, V., Biller-Ckovric, I., Mirkovic, J., Gorlich, D. and Nicotera, P. (2001). Caspases mediate nucleoporin cleavage, but not early redistribution of nuclear transport factors and modulation of nuclear permeability in apoptosis. *Cell Death Differ*, 8 (5); 495-505.

Fertil, B. and Malaise, E.P. (1981). Inherent cellular radiosensitivity as a basic concept for human tumor radiotherapy. *Int J Radiat Oncol Biol Phys*, 7 (5); 621-9.

Fertil, B. and Malaise, E.P. (1985). Intrinsic radiosensitivity of human cell lines is correlated with radioresponsiveness of human tumors: analysis of 101 published survival curves. *Int J Radiat Oncol Biol Phys*, 11 (9); 1699-707.

Fischer, H., Koenig, U., Eckhart, L. and Tschachler, E. (2002). Human caspase 12 has acquired deleterious mutations. *Biochem Biophys Res Commun*, 293 (2); 722-6.

Fischer, U., Jänicke, R.U. and Schulze-Osthoff, K. (2003). Many cuts to ruin: a comprehensive update of caspase substrates. *Cell Death Differ*, 10 (1); 76-100.

Fulda, S. and Debatin, K.M. (2002). IFNgamma sensitizes for apoptosis by upregulating caspase-8 expression through the Stat1 pathway. *Oncogene*, 21 (15); 2295-308.

Fulda, S., Kufer, M.U., Meyer, E., van Valen, F., Dockhorn-Dworniczak, B. and Debatin, K.M. (2001). Sensitization for death receptor- or drug-induced apoptosis by re- expression of caspase-8 through demethylation or gene transfer. *Oncogene*, 20 (41); 5865-77.

Fulda, S., Wick, W., Weller, M. and Debatin, K.M. (2002). Smac agonists sensitize for Apo2L/TRAIL- or anticancer drug-induced apoptosis and induce regression of malignant glioma in vivo. *Nat Med*, 8 (8); 808-15.

Gajkowska, B., Motyl, T., Olszewska-Badarczuk, H. and Godlewski, M.M. (2001). Expression of BAX in cell nucleus after experimentally induced apoptosis revealed by immunogold and embedment-free electron microscopy. *Cell Biol Int*, 25 (8); 725-33.

Gao, G. and Dou, Q.P. (2000). N-terminal cleavage of bax by calpain generates a potent proapoptotic 18-kDa fragment that promotes bcl-2-independent cytochrome C release and apoptotic cell death. *J Cell Biochem*, 80 (1); 53-72.

Garrido, C., Gurbuxani, S., Ravagnan, L. and Kroemer, G. (2001). Heat shock proteins: endogenous modulators of apoptotic cell death. *Biochem Biophys Res Commun*, 286 (3); 433-42.

Gaudet, F., Hodgson, J.G., Eden, A., Jackson-Grusby, L., Dausman, J., Gray, J.W., Leonhardt, H. and Jaenisch, R. (2003). Induction of Tumors in Mice by Genomic Hypomethylation. *Science*, 300 (5618); 489-492.

Ghatan, S., Larner, S., Kinoshita, Y., Hetman, M., Patel, L., Xia, Z., Youle, R.J. and Morrison, R.S. (2000). p38 MAP kinase mediates bax translocation in nitric oxide-induced apoptosis in neurons. *J Cell Biol*, 150 (2); 335-47.

Gibbons, N.B., Watson, R.W., Coffey, R.N., Brady, H.P. and Fitzpatrick, J.M. (2000). Heat-shock proteins inhibit induction of prostate cancer cell apoptosis. *Prostate*, 45 (1); 58-65.

Gibson, E.M., Henson, E.S., Villanueva, J. and Gibson, S.B. (2002). MEK kinase 1 induces mitochondrial permeability transition leading to apoptosis independent of cytochrome c release. *J Biol Chem*, 277 (12); 10573-80.

Gil-Parrado, S., Fernandez-Montalvan, A., Assfalg-Machleidt, I., Popp, O., Bestvater, F., Holloschi, A., Knoch, T.A., Auerswald, E.A., Welsh, K., Reed, J.C., Fritz, H., Fuentes-Prior, P., Spiess, E., Salvesen, G.S. and Machleidt, W. (2002). Ionomycin-activated calpain triggers apoptosis. A probable role for Bcl- 2 family members. *J Biol Chem*, 277 (30); 27217-26.

Godlewski, M.M., Motyl, M.A., Gajkowska, B., Wareski, P., Koronkiewicz, M. and Motyl, T. (2001). Subcellular redistribution of BAX during apoptosis induced by anticancer drugs. *Anticancer Drugs*, 12 (7); 607-17.

Greenblatt, M.S., Bennett, W.P., Hollstein, M. and Harris, C.C. (1994). Mutations in the p53 tumor suppressor gene: clues to cancer etiology and molecular pathogenesis. *Cancer Res*, 54 (18); 4855-78.

Griffiths, G.J., Dubrez, L., Morgan, C.P., Jones, N.A., Whitehouse, J., Corfe, B.M., Dive, C. and Hickman, J.A. (1999). Cell damage-induced conformational changes of the pro-apoptotic protein Bak in vivo precede the onset of apoptosis. *J Cell Biol*, 144 (5); 903-14.

Grinberg, M., Sarig, R., Zaltsman, Y., Frumkin, D., Grammatikakis, N., Reuveny, E. and Gross, A. (2002). tBID Homooligomerizes in the mitochondrial membrane to induce apoptosis. *J Biol Chem*, 277 (14); 12237-45.

Grossman, D., Kim, P.J., Schechner, J.S. and Altieri, D.C. (2001). Inhibition of melanoma tumor growth in vivo by survivin targeting. *Proc Natl Acad Sci U S A*, 98 (2); 635-640.

Gudkov, A.V. and Komarova, E.A. (2003). The role of p53 in determining sensitivity to radiotherapy. *Nat Rev Cancer*, 3 (2); 117-29.

Hagemann, C. and Blank, J.L. (2001). The ups and downs of MEK kinase interactions. *Cell Signal*, 13 (12); 863-75.

Halestrap, A.P., McStay, G.P. and Clarke, S.J. (2002). The permeability transition pore complex: another view. *Biochimie*, 84 (2-3); 153-66.

Hall, E.J. (2000a). *Radiobiology for the radiologist*. Lippincott Willliams&Wilkins, pp 32-45.

Hall, E.J. (2000b). *Radiobiology for the radiologist*. Lippincott Willliams&Wilkins, pp 17-21.

Hall, E.J. (2000c). *Radiobiology for the radiologist*. Lippincott Willliams&Wilkins, pp 91-109.

Hall, E.J. (2000d). *Radiobiology for the radiologist*. Lippincott Willliams&Wilkins, pp 5-14.

Ham, J., Babij, C., Whitfield, J., Pfarr, C.M., Lallemand, D., Yaniv, M. and Rubin, L.L. (1995). A c-Jun dominant negative mutant protects sympathetic neurons against programmed cell death. *Neuron*, 14 (5); 927-39.

Hanahan, D. and Weinberg, R.A. (2000). The hallmarks of cancer. *Cell*, 100 (1); 57-70.

Harada, K., Toyooka, S., Shivapurkar, N., Maitra, A., Reddy, J.L., Matta, H., Miyajima, K., Timmons, C.F., Tomlinson, G.E., Mastrangelo, D., Hay, R.J., Chaudhary, P.M. and Gazdar, A.F. (2002).

Deregulation of caspase 8 and 10 expression in pediatric tumors and cell lines. *Cancer Res*, 62 (20); 5897-901.

Hatai, T., Matsuzawa, A., Inoshita, S., Mochida, Y., Kuroda, T., Sakamaki, K., Kuida, K., Yonehara, S., Ichijo, H. and Takeda, K. (2000). Execution of apoptosis signal-regulating kinase 1 (ASK1)-induced apoptosis by the mitochondria-dependent caspase activation. *J Biol Chem*, 275 (34); 26576-81.

Hegde, R., Srinivasula, S.M., Zhang, Z., Wassell, R., Mukattash, R., Cilenti, L., DuBois, G., Lazebnik, Y., Zervos, A.S., Fernandes-Alnemri, T. and Alnemri, E.S. (2002). Identification of Omi/HtrA2 as a mitochondrial apoptotic serine protease that disrupts inhibitor of apoptosis protein-caspase interaction. *J Biol Chem*, 277 (1); 432-8.

Heise, C., Sampson-Johannes, A., Williams, A., McCormick, F., Von Hoff, D.D. and Kirn, D.H. (1997). ONYX-015, an E1B gene-attenuated adenovirus, causes tumor-specific cytolysis and antitumoral efficacy that can be augmented by standard chemotherapeutic agents. *Nat Med*, 3 (6); 639-45.

Hendry, J.H. and West, C.M. (1997). Apoptosis and mitotic cell death: their relative contributions to normal-tissue and tumour radiation response. *Int J Radiat Biol*, 71 (6); 709-19.

Hengartner, M.O., Ellis, R.E. and Horvitz, H.R. (1992). Caenorhabditis elegans gene ced-9 protects cells from programmed cell death. *Nature*, 356 (6369); 494-9.

Henson, P.M., Bratton, D.L. and Fadok, V.A. (2001). Apoptotic cell removal. *Curr Biol*, 11 (19); R795-805.

Hirsch, F.R., Franklin, W.A. and Bunn, P.A., Jr. (2002). Expression of target molecules in lung cancer: challenge for a new treatment paradigm. *Semin Oncol*, 29 (3 Suppl 9); 2-8.

Hirsch, F.R., Franklin, W.A., Gazdar, A.F. and Bunn, P.A., Jr. (2001). Early detection of lung cancer: clinical perspectives of recent advances in biology and radiology. *Clin Cancer Res*, 7 (1); 5-22.

Hockenbery, D.M., Oltvai, Z.N., Yin, X.M., Milliman, C.L. and Korsmeyer, S.J. (1993). Bcl-2 functions in an antioxidant pathway to prevent apoptosis. *Cell*, 75 (2); 241-51.

Hoeijmakers, J.H. (2001). Genome maintenance mechanisms for preventing cancer. *Nature*, 411 (6835); 366-74.

Holcik, M., Gibson, H. and Korneluk, R.G. (2001). XIAP: apoptotic brake and promising therapeutic target. *Apoptosis*, 6 (4); 253-61.

Holcik, M., Yeh, C., Korneluk, R.G. and Chow, T. (2000). Translational upregulation of X-linked inhibitor of apoptosis (XIAP) increases resistance to radiation induced cell death. *Oncogene*, 19 (36); 4174-7.

Hu, S., Vincenz, C., Ni, J., Gentz, R. and Dixit, V.M. (1997). I-FLICE, a novel inhibitor of tumor necrosis factor receptor-1- and CD-95-induced apoptosis. *J Biol Chem*, 272 (28); 17255-7.

Huang, H., Joazeiro, C.A., Bonfoco, E., Kamada, S., Leverson, J.D. and Hunter, T. (2000). The inhibitor of apoptosis, cIAP2, functions as a ubiquitin-protein ligase and promotes in vitro monoubiquitination of caspases 3 and 7. *J Biol Chem*, 275 (35); 26661-4.

Ianzini, F. and Mackey, M.A. (1998). Delayed DNA damage associated with mitotic catastrophe following X- irradiation of HeLa S3 cells. *Mutagenesis*, 13 (4); 337-44.

Ichijo, H., Nishida, E., Irie, K., ten Dijke, P., Saitoh, M., Moriguchi, T., Takagi, M., Matsumoto, K., Miyazono, K. and Gotoh, Y. (1997). Induction of apoptosis by ASK1, a mammalian MAPKKK that activates SAPK/JNK and p38 signaling pathways. *Science*, 275 (5296); 90-4.

Iliakis, G. (1991). The role of DNA double strand breaks in ionizing radiation-induced killing of eukaryotic cells. *Bioessays*, 13 (12); 641-8.

Irmler, M., Thome, M., Hahne, M., Schneider, P., Hofmann, K., Steiner, V., Bodmer, J.L., Schroter, M., Burns, K., Mattmann, C., Rimoldi, D., French, L.E. and Tschopp, J. (1997). Inhibition of death receptor signals by cellular FLIP. *Nature*, 388 (6638); 190-5.

Ishizaki, Y., Jacobson, M.D. and Raff, M.C. (1998). A role for caspases in lens fiber differentiation. *J Cell Biol*, 140 (1); 153-8.

Ito, Y., Mishra, N.C., Yoshida, K., Kharbanda, S., Saxena, S. and Kufe, D. (2001). Mitochondrial targeting of JNK/SAPK in the phorbol ester response of myeloid leukemia cells. *Cell Death Differ*, 8 (8); 794-800.

Jansen, B., Schlagbauer-Wadl, H., Brown, B.D., Bryan, R.N., van Elsas, A., Müller, M., Wolff, K., Eichler, H.G. and Pehamberger, H. (1998). bcl-2 antisense therapy chemosensitizes human melanoma in SCID mice. *Nat Med*, 4 (2); 232-4.

Jiang, S.X., Kameya, T., Sato, Y., Yanase, N., Yoshimura, H. and Kodama, T. (1996). Bcl-2 protein expression in lung cancer and close correlation with neuroendocrine differentiation. *Am J Pathol*, 148 (3); 837-46.

Jiang, S.X., Sato, Y., Kuwao, S. and Kameya, T. (1995). Expression of bcl-2 oncogene protein is prevalent in small cell lung carcinomas. *J Pathol*, 177 (2); 135-8.

Joseph, B., Marchetti, P., Formstecher, P., Kroemer, G., Lewensohn, R. and Zhivotovsky, B. (2002). Mitochondrial dysfunction is an essential step for killing of non-small cell lung carcinomas resistant to conventional treatment. *Oncogene*, 21 (1); 65-77.

Jäättelä, M., Wissing, D., Kokholm, K., Kallunki, T. and Egeblad, M. (1998). Hsp70 exerts its anti-apoptotic function downstream of caspase-3-like proteases. *Embo J*, 17 (21); 6124-34.

Kaiser, U., Schardt, C., Brandscheidt, D., Wollmer, E. and Havemann, K. (1993). Expression of insulin-like growth factor receptors I and II in normal human lung and in lung cancer. *J Cancer Res Clin Oncol*, 119 (11); 665-8.

Kaiser, U., Schilli, M., Haag, U., Neumann, K., Kreipe, H., Kogan, E. and Havemann, K. (1996). Expression of bcl-2--protein in small cell lung cancer. *Lung Cancer*, 15 (1); 31-40.

Kam, P.C. and Ferch, N.I. (2000). Apoptosis: mechanisms and clinical implications. *Anaesthesia*, 55 (11); 1081-93.

Karmakar, P., Piotrowski, J., Brosh, R.M., Jr., Sommers, J.A., Miller, S.P., Cheng, W.H., Snowden, C.M., Ramsden, D.A. and Bohr, V.A. (2002). Werner protein is a target of DNA-dependent protein kinase in vivo and in vitro, and its catalytic activities are regulated by phosphorylation. *J Biol Chem*, 277 (21); 18291-302.

Karpova, A.Y., Trost, M., Murray, J.M., Cantley, L.C. and Howley, P.M. (2002). Interferon regulatory factor-3 is an in vivo target of DNA-PK. *Proc Natl Acad Sci U S A*, 99 (5); 2818-23.

Kataoka, T., Budd, R.C., Holler, N., Thome, M., Martinon, F., Irmler, M., Burns, K., Hahne, M., Kennedy, N., Kovacsovics, M. and Tschopp, J. (2000). The caspase-8 inhibitor FLIP promotes activation of NF-kappaB and Erk signaling pathways. *Curr Biol*, 10 (11); 640-8.

Kataoka, T., Schröter, M., Hahne, M., Schneider, P., Irmler, M., Thome, M., Froelich, C.J. and Tschopp, J. (1998). FLIP prevents apoptosis induced by death receptors but not by perforin/granzyme B, chemotherapeutic drugs, and gamma irradiation. *J Immunol*, 161 (8); 3936-42.

Kelley, M.J., Nakagawa, K., Steinberg, S.M., Mulshine, J.L., Kamb, A. and Johnson, B.E. (1995). Differential inactivation of CDKN2 and Rb protein in non-small-cell and small-cell lung cancer cell lines. *J Natl Cancer Inst*, 87 (10); 756-61.

Kennedy, N.J., Kataoka, T., Tschopp, J. and Budd, R.C. (1999). Caspase activation is required for T cell proliferation. *J Exp Med*, 190 (12); 1891-6.

Kerr, J.F., Wyllie, A.H. and Currie, A.R. (1972). Apoptosis: a basic biological phenomenon with wide-ranging implications in tissue kinetics. *Br J Cancer*, 26 (4); 239-57.

Kharbanda, S., Pandey, P., Jin, S., Inoue, S., Bharti, A., Yuan, Z.M., Weichselbaum, R., Weaver, D. and Kufe, D. (1997). Functional interaction between DNA-PK and c-Abl in response to DNA damage. *Nature*, 386 (6626); 732-5.

Kharbanda, S., Pandey, P., Yamauchi, T., Kumar, S., Kaneki, M., Kumar, V., Bharti, A., Yuan, Z.M., Ghanem, L., Rana, A., Weichselbaum, R., Johnson, G. and Kufe, D. (2000). Activation of MEK kinase 1 by the c-Abl protein tyrosine kinase in response to DNA damage. *Mol Cell Biol*, 20 (14); 4979-89.

Kharbanda, S., Ren, R., Pandey, P., Shafman, T.D., Feller, S.M., Weichselbaum, R.R. and Kufe, D.W. (1995). Activation of the c-Abl tyrosine kinase in the stress response to DNA- damaging agents. *Nature*, 376 (6543); 785-8.

Kikuchi, Y., Dinjens, W.N. and Bosman, F.T. (1997). Proliferation and apoptosis in proliferative lesions of the colon and rectum. *Virchows Arch*, 431 (2); 111-7.

Kitanaka, C. and Kuchino, Y. (1999). Caspase-independent programmed cell death with necrotic morphology. *Cell Death Differ*, 6 (6); 508-15.

Klasa, R.J., Gillum, A.M., Klem, R.E. and Frankel, S.R. (2002). Oblimersen Bcl-2 antisense: facilitating apoptosis in anticancer treatment. *Antisense Nucleic Acid Drug Dev*, 12 (3); 193-213.

Kobayashi, K., Hatano, M., Otaki, M., Ogasawara, T. and Tokuhisa, T. (1999). Expression of a murine homologue of the inhibitor of apoptosis protein is related to cell proliferation. *Proc Natl Acad Sci U S A*, 96 (4); 1457-62.

Kok, K., Osinga, J., Carritt, B., Davis, M.B., van der Hout, A.H., van der Veen, A.Y., Landsvater, R.M., de Leij, L.F., Berendsen, H.H., Postmus, P.E. and et al. (1987). Deletion of a DNA sequence at the chromosomal region 3p21 in all major types of lung cancer. *Nature*, 330 (6148); 578-81.

Korsmeyer, S.J., Wei, M.C., Saito, M., Weiler, S., Oh, K.J. and Schlesinger, P.H. (2000). Pro-apoptotic cascade activates BID, which oligomerizes BAK or BAX into pores that result in the release of cytochrome c. *Cell Death Differ*, 7 (12); 1166-73.

Krajewski, S., Tanaka, S., Takayama, S., Schibler, M.J., Fenton, W. and Reed, J.C. (1993). Investigation of the subcellular distribution of the bcl-2 oncoprotein: residence in the nuclear envelope, endoplasmic reticulum, and outer mitochondrial membranes. *Cancer Res*, 53 (19); 4701-14.

Kuida, K., Haydar, T.F., Kuan, C.Y., Gu, Y., Taya, C., Karasuyama, H., Su, M.S., Rakic, P. and Flavell, R.A. (1998). Reduced apoptosis and cytochrome c-mediated caspase activation in mice lacking caspase 9. *Cell*, 94 (3); 325-37.

Kuida, K., Zheng, T.S., Na, S., Kuan, C., Yang, D., Karasuyama, H., Rakic, P. and Flavell, R.A. (1996). Decreased apoptosis in the brain and premature lethality in CPP32-deficient mice. *Nature*, 384 (6607); 368-72.

Kuwana, T., Smith, J.J., Muzio, M., Dixit, V., Newmeyer, D.D. and Kornbluth, S. (1998). Apoptosis induction by caspase-8 is amplified through the mitochondrial release of cytochrome c. *J Biol Chem*, 273 (26); 16589-94.

Köhler, C., Orrenius, S. and Zhivotovsky, B. (2002). Evaluation of caspase activity in apoptotic cells. *J Immunol Methods*, 265 (1-2); 97-110.

LaCasse, E.C., Baird, S., Korneluk, R.G. and MacKenzie, A.E. (1998). The inhibitors of apoptosis (IAPs) and their emerging role in cancer. *Oncogene*, 17 (25); 3247-59.

Larisch, S., Yi, Y., Lotan, R., Kerner, H., Eimerl, S., Tony Parks, W., Gottfried, Y., Birkey Reffey, S., de Caestecker, M.P., Danielpour, D., Book-Melamed, N., Timberg, R., Duckett, C.S., Lechleider, R.J., Steller, H., Orly, J., Kim, S.J. and Roberts, A.B. (2000). A novel mitochondrial septin-like

protein, ARTS, mediates apoptosis dependent on its P-loop motif. *Nat Cell Biol*, 2 (12); 915-21.

Lassus, P., Opitz-Araya, X. and Lazebnik, Y. (2002). Requirement for caspase-2 in stress-induced apoptosis before mitochondrial permeabilization. *Science*, 297 (5585); 1352-4.

Lavoie, J.N., Lambert, H., Hickey, E., Weber, L.A. and Landry, J. (1995). Modulation of cellular thermoresistance and actin filament stability accompanies phosphorylation-induced changes in the oligomeric structure of heat shock protein 27. *Mol Cell Biol*, 15 (1); 505-16.

Leber, R., Wise, T.W., Mizuta, R. and Meek, K. (1998). The XRCC4 gene product is a target for and interacts with the DNA- dependent protein kinase. *J Biol Chem*, 273 (3); 1794-801.

Lee, S.E., Mitchell, R.A., Cheng, A. and Hendrickson, E.A. (1997). Evidence for DNA-PK-dependent and -independent DNA double-strand break repair pathways in mammalian cells as a function of the cell cycle. *Mol Cell Biol*, 17 (3); 1425-33.

Lee, Y.J., Soh, J.W., Dean, N.M., Cho, C.K., Kim, T.H., Lee, S.J. and Lee, Y.S. (2002). Protein kinase Cdelta overexpression enhances radiation sensitivity via extracellular regulated protein kinase 1/2 activation, abolishing the radiation-induced G(2)-M arrest. *Cell Growth Differ*, 13 (5); 237-46.

Lei, K. and Davis, R.J. (2003). JNK phosphorylation of Bim-related members of the Bcl2 family induces Bax-dependent apoptosis. *Proc Natl Acad Sci U S A*, 100 (5); 2432-7.

Lei, K., Nimnual, A., Zong, W.X., Kennedy, N.J., Flavell, R.A., Thompson, C.B., Bar-Sagi, D. and Davis, R.J. (2002). The Bax subfamily of Bcl2-related proteins is essential for apoptotic signal transduction by c-Jun NH(2)-terminal kinase. *Mol Cell Biol*, 22 (13); 4929-42.

Leist, M., Single, B., Castoldi, A.F., Kuhnle, S. and Nicotera, P. (1997). Intracellular adenosine triphosphate (ATP) concentration: a switch in the decision between apoptosis and necrosis. *J Exp Med*, 185 (8); 1481-6.

Li, F., Ambrosini, G., Chu, E.Y., Plescia, J., Tognin, S., Marchisio, P.C. and Altieri, D.C. (1998a). Control of apoptosis and mitotic spindle checkpoint by survivin. *Nature*, 396 (6711); 580-4.

Li, H., Zhu, H., Xu, C.J. and Yuan, J. (1998b). Cleavage of BID by caspase 8 mediates the mitochondrial damage in the Fas pathway of apoptosis. *Cell*, 94 (4); 491-501.

Li, L.Y., Luo, X. and Wang, X. (2001). Endonuclease G is an apoptotic DNase when released from mitochondria. *Nature*, 412 (6842); 95-9.

Li, X., Yang, Y. and Ashwell, J.D. (2002). TNF-RII and c-IAP1 mediate ubiquitination and degradation of TRAF2. *Nature*, 416 (6878); 345-7.

Lipton, S.A. and Bossy-Wetzel, E. (2002). Dueling activities of AIF in cell death versus survival: DNA binding and redox activity. *Cell*, 111 (2); 147-50.

Lithgow, T., van Driel, R., Bertram, J.F. and Strasser, A. (1994). The protein product of the oncogene bcl-2 is a component of the nuclear envelope, the endoplasmic reticulum, and the outer mitochondrial membrane. *Cell Growth Differ*, 5 (4); 411-7.

Liu, Z., Sun, C., Olejniczak, E.T., Meadows, R.P., Betz, S.F., Oost, T., Herrmann, J., Wu, J.C. and Fesik, S.W. (2000). Structural basis for binding of Smac/DIABLO to the XIAP BIR3 domain. *Nature*, 408 (6815); 1004-8.

Lutter, M., Fang, M., Luo, X., Nishijima, M., Xie, X. and Wang, X. (2000). Cardiolipin provides specificity for targeting of tBid to mitochondria. *Nat Cell Biol*, 2 (10); 754-61.

Lutter, M., Perkins, G.A. and Wang, X. (2001). The pro-apoptotic Bcl-2 family member tBid localizes to mitochondrial contact sites. *BMC Cell Biol*, 2 (1); 22.

Ma, Y., Pannicke, U., Schwarz, K. and Lieber, M.R. (2002). Hairpin opening and overhang processing by an Artemis/DNA-dependent protein kinase complex in nonhomologous end joining and V(D)J recombination. *Cell*, 108 (6); 781-94.

Macaluso, M., Russo, G., Cinti, C., Bazan, V., Gebbia, N. and Russo, A. (2002). Ras family genes: an interesting link between cell cycle and cancer. *J Cell Physiol*, 192 (2); 125-30.

Mandic, A., Hansson, J., Linder, S. and Shoshan, M.C. (2003). Cisplatin Induces Endoplasmic Reticulum Stress and Nucleus-independent Apoptotic Signaling. *J Biol Chem*, 278 (11); 9100-6.

Mandic, A., Viktorsson, K., Molin, M., Akusjärvi, G., Eguchi, H., Hayashi, S.I., Toi, M., Hansson, J., Linder, S. and Shoshan, M.C. (2001). Cisplatin induces the proapoptotic conformation of Bak in a deltaMEKK1- dependent manner. *Mol Cell Biol*, 21 (11); 3684-91.

Marin, M.C., Fernandez, A., Bick, R.J., Brisbay, S., Buja, L.M., Snuggs, M., McConkey, D.J., von Eschenbach, A.C., Keating, M.J. and McDonnell, T.J. (1996). Apoptosis suppression by bcl-2 is correlated with the regulation of nuclear and cytosolic Ca2+. *Oncogene*, 12 (11); 2259-66.

Marzo, I., Brenner, C., Zamzami, N., Jurgensmeier, J.M., Susin, S.A., Vieira, H.L., Prevost, M.C., Xie, Z., Matsuyama, S., Reed, J.C. and Kroemer, G. (1998). Bax and adenine nucleotide translocator cooperate in the mitochondrial control of apoptosis. *Science*, 281 (5385); 2027-31.

Mathiasen, I.S. and Jäättelä, M. (2002). Triggering caspase-independent cell death to combat cancer. *Trends Mol Med*, 8 (5); 212-20.

Mayo, L.D., Turchi, J.J. and Berberich, S.J. (1997). Mdm-2 phosphorylation by DNA-dependent protein kinase prevents interaction with p53. *Cancer Res*, 57 (22); 5013-6.

Medema, J.P., Scaffidi, C., Kischkel, F.C., Shevchenko, A., Mann, M., Krammer, P.H. and Peter, M.E. (1997). FLICE is activated by association with the CD95 death-inducing signaling complex (DISC). *Embo J*, 16 (10); 2794-804.

Mehlen, P., Kretz-Remy, C., Preville, X. and Arrigo, A.P. (1996). Human hsp27, Drosophila hsp27 and human alphaB-crystallin expression- mediated increase in glutathione is essential for the protective activity of these proteins against TNFalpha-induced cell death. *Embo J*, 15 (11); 2695-706.

Michael, J.M., Lavin, M.F. and Watters, D.J. (1997). Resistance to radiation-induced apoptosis in Burkitt's lymphoma cells is associated with defective ceramide signaling. *Cancer Res*, 57 (16); 3600-5.

Michaelson, J.S. and Leder, P. (2003). RNAi reveals anti-apoptotic and transcriptionally repressive activities of DAXX. *J Cell Sci*, 116 (Pt 2); 345-52.

Mihara, M., Erster, S., Zaika, A., Petrenko, O., Chittenden, T., Pancoska, P. and Moll, U.M. (2003). p53 Has a Direct Apoptogenic Role at the Mitochondria. *Mol Cell*, 11 (3); 577-90.

Mimori, T. and Hardin, J.A. (1986). Mechanism of interaction between Ku protein and DNA. *J Biol Chem*, 261 (22); 10375-9.

Minuto, F., Del Monte, P., Barreca, A., Fortini, P., Cariola, G., Catrambone, G. and Giordano, G. (1986). Evidence for an increased somatomedin-C/insulin-like growth factor I content in primary human lung tumors. *Cancer Res*, 46 (2); 985-8.

Moorehead, R.A., Sanchez, O.H., Baldwin, R.M. and Khokha, R. (2003). Transgenic overexpression of IGF-II induces spontaneous lung tumors: a model for human lung adenocarcinoma. *Oncogene*, 22 (6); 853-7.

Moshous, D., Callebaut, I., de Chasseval, R., Corneo, B., Cavazzana-Calvo, M., Le Deist, F., Tezcan, I., Sanal, O., Bertrand, Y., Philippe, N., Fischer, A. and de Villartay, J.P. (2001). Artemis, a novel DNA double-strand break repair/V(D)J recombination protein, is mutated in human severe combined immune deficiency. *Cell*, 105 (2); 177-86.

Neckers, L. (2002). Heat shock protein 90 inhibition by 17-allylamino-17- demethoxygeldanamycin: a novel therapeutic approach for treating hormone-refractory prostate cancer. *Clin Cancer Res*, 8 (5); 962-6.

Nylandsted, J., Brand, K. and Jäättelä, M. (2000a). Heat shock protein 70 is required for the survival of cancer cells. *Ann N Y Acad Sci*, 926 (1); 122-5.

Nylandsted, J., Rohde, M., Brand, K., Bastholm, L., Elling, F. and Jäättelä, M. (2000b). Selective depletion of heat shock protein 70 (Hsp70) activates a tumor-specific death program that is independent of caspases and bypasses Bcl-2. *Proc Natl Acad Sci U S A*, 97 (14); 7871-6.

Nylandsted, J., Wick, W., Hirt, U.A., Brand, K., Rohde, M., Leist, M., Weller, M. and Jäättelä, M. (2002). Eradication of glioblastoma, and breast and colon carcinoma xenografts by Hsp70 depletion. *Cancer Res*, 62 (24); 7139-42.

Oh, H.J., Chen, X. and Subjeck, J.R. (1997). Hsp110 protects heat-denatured proteins and confers cellular thermoresistance. *J Biol Chem*, 272 (50); 31636-40.

O'Reilly, L.A., Ekert, P., Harvey, N., Marsden, V., Cullen, L., Vaux, D.L., Hacker, G., Magnusson, C., Pakusch, M., Cecconi, F., Kuida, K., Strasser, A., Huang, D.C. and Kumar, S. (2002). Caspase-2 is not required for thymocyte or neuronal apoptosis even though cleavage of caspase-2 is dependent on both Apaf-1 and caspase-9. *Cell Death Differ*, 9 (8); 832-41.

Ott, M., Robertson, J.D., Gogvadze, V., Zhivotovsky, B. and Orrenius, S. (2002). Cytochrome c release from mitochondria proceeds by a two-step process. *Proc Natl Acad Sci U S A*, 99 (3); 1259-63.

Pan, G., Ni, J., Wei, Y.F., Yu, G., Gentz, R. and Dixit, V.M. (1997). An antagonist decoy receptor and a death domain-containing receptor for TRAIL. *Science*, 277 (5327); 815-8.

Pandey, P., Farber, R., Nakazawa, A., Kumar, S., Bharti, A., Nalin, C., Weichselbaum, R., Kufe, D. and Kharbanda, S. (2000a). Hsp27 functions as a negative regulator of cytochrome c-dependent activation of procaspase-3. *Oncogene*, 19 (16); 1975-81.

Pandey, P., Saleh, A., Nakazawa, A., Kumar, S., Srinivasula, S.M., Kumar, V., Weichselbaum, R., Nalin, C., Alnemri, E.S., Kufe, D. and Kharbanda, S. (2000b). Negative regulation of cytochrome c-mediated oligomerization of Apaf-1 and activation of procaspase-9 by heat shock protein 90. *Embo J*, 19 (16); 4310-22.

Parkin, D.M., Bray, F.I. and Devesa, S.S. (2001). Cancer burden in the year 2000. The global picture. *Eur J Cancer*, 37 Suppl 8 S4-66.

Paull, T.T., Rogakou, E.P., Yamazaki, V., Kirchgessner, C.U., Gellert, M. and Bonner, W.M. (2000). A critical role for histone H2AX in recruitment of repair factors to nuclear foci after DNA damage. *Curr Biol*, 10 (15); 886-95.

Perez, R.P. (1998). Cellular and molecular determinants of cisplatin resistance. *Eur J Cancer*, 34 (10); 1535-42.

Porrello, C., Alifano, M., Forti Parri, S.N., Sanna, S., Lacava, N., Corneli, G. and Boaron, M. (2002). Surgical treatment of stage I lung cancer. Results and prognostic factors. *J Cardiovasc Surg (Torino)*, 43 (5); 723-7.

Radford, I.R. (1986). Evidence for a general relationship between the induced level of DNA double-strand breakage and cell-killing after X-irradiation of mammalian cells. *Int J Radiat Biol Relat Stud Phys Chem Med*, 49 (4); 611-20.

Raitano, A.B., Halpern, J.R., Hambuch, T.M. and Sawyers, C.L. (1995). The Bcr-Abl leukemia oncogene activates Jun kinase and requires Jun for transformation. *Proc Natl Acad Sci U S A*, 92 (25); 11746-50.

Ravagnan, L., Gurbuxani, S., Susin, S.A., Maisse, C., Daugas, E., Zamzami, N., Mak, T., Jäättelä, M., Penninger, J.M., Garrido, C. and Kroemer, G. (2001). Heat-shock protein 70 antagonizes apoptosis-inducing factor. *Nat Cell Biol*, 3 (9); 839-43.

Reed, J.C. (2002). Apoptosis-based therapies. *Nat Rev Drug Discov*, 1 (2); 111-21.

Reeve, J.G., Payne, J.A. and Bleehen, N.M. (1990). Production of immunoreactive insulin-like growth factor-I (IGF-I) and IGF-I binding proteins by human lung tumours. *Br J Cancer*, 61 (5); 727-31.

Reissmann, P.T., Koga, H., Figlin, R.A., Holmes, E.C. and Slamon, D.J. (1999). Amplification and overexpression of the cyclin D1 and epidermal growth factor receptor genes in non-small-cell lung cancer. Lung Cancer Study Group. *J Cancer Res Clin Oncol*, 125 (2); 61-70.

Renatus, M., Stennicke, H.R., Scott, F.L., Liddington, R.C. and Salvesen, G.S. (2001). Dimer formation drives the activation of the cell death protease caspase 9. *Proc Natl Acad Sci U S A*, 98 (25); 14250-5.

Richardson, G.E. and Johnson, B.E. (1993). The biology of lung cancer. *Semin Oncol*, 20 (2); 105-27.

Roberts, D.L., Merrison, W., MacFarlane, M. and Cohen, G.M. (2001). The inhibitor of apoptosis protein-binding domain of smac is not essential for its proapoptotic activity. *J Cell Biol*, 153 (1); 221-8.

Robertson, J.D., Enoksson, M., Suomela, M., Zhivotovsky, B. and Orrenius, S. (2002). Caspase-2 acts upstream of mitochondria to promote cytochrome c release during etoposide-induced apoptosis. *J Biol Chem*, 277 (33); 29803-9.

Robertson, J.D., Gogvadze, V., Zhivotovsky, B. and Orrenius, S. (2000). Distinct pathways for stimulation of cytochrome c release by etoposide. *J Biol Chem*, 275 (42); 32438-43.

Rodriguez-Lafrasse, C., Alphonse, G., Aloy, M.T., Ardail, D., Gerard, J.P., Louisot, P. and Rousson, R. (2002). Increasing endogenous ceramide using inhibitors of sphingolipid metabolism maximizes ionizing radiation-induced mitochondrial injury and apoptotic cell killing. *Int J Cancer*, 101 (6); 589-98.

Rothe, M., Pan, M.G., Henzel, W.J., Ayres, T.M. and Goeddel, D.V. (1995). The TNFR2-TRAF signaling complex contains two novel proteins related to baculoviral inhibitor of apoptosis proteins. *Cell*, 83 (7); 1243-52.

Roy, N., Deveraux, Q.L., Takahashi, R., Salvesen, G.S. and Reed, J.C. (1997). The c-IAP-1 and c-IAP-2 proteins are direct inhibitors of specific caspases. *Embo J*, 16 (23); 6914-25.

Rudin, C.M., Otterson, G.A., Mauer, A.M., Villalona-Calero, M.A., Tomek, R., Prange, B., George, C.M., Szeto, L. and Vokes, E.E. (2002). A pilot trial of G3139, a bcl-2 antisense oligonucleotide, and paclitaxel in patients with chemorefractory small-cell lung cancer. *Ann Oncol*, 13 (4); 539-45.

Rudner, J., Lepple-Wienhues, A., Budach, W., Berschauer, J., Friedrich, B., Wesselborg, S., Schulze-Osthoff, K. and Belka, C. (2001). Wild-type, mitochondrial and ER-restricted Bcl-2 inhibit DNA damage- induced apoptosis but do not affect death receptor-induced apoptosis. *J Cell Sci*, 114 (Pt 23); 4161-72.

Sahara, S., Aoto, M., Eguchi, Y., Imamoto, N., Yoneda, Y. and Tsujimoto, Y. (1999). Acinus is a caspase-3-activated protein required for apoptotic chromatin condensation. *Nature*, 401 (6749); 168-73.

Saitoh, M., Nishitoh, H., Fujii, M., Takeda, K., Tobiume, K., Sawada, Y., Kawabata, M., Miyazono, K. and Ichijo, H. (1998). Mammalian thioredoxin is a direct inhibitor of apoptosis signal-regulating kinase (ASK) 1. *Embo J*, 17 (9); 2596-606.

Saleh, A., Srinivasula, S.M., Balkir, L., Robbins, P.D. and Alnemri, E.S. (2000). Negative regulation of the Apaf-1 apoptosome by Hsp70. *Nat Cell Biol*, 2 (8); 476-83.

Samali, A., Cai, J., Zhivotovsky, B., Jones, D.P. and Orrenius, S. (1999a). Presence of a pre-apoptotic complex of pro-caspase-3, Hsp60 and Hsp10 in the mitochondrial fraction of jurkat cells. *Embo J*, 18 (8); 2040-8.

Samali, A., Zhivotovsky, B., Jones, D., Nagata, S. and Orrenius, S. (1999b). Apoptosis: cell death defined by caspase activation. *Cell Death Differ*, 6 (6); 495-6.

Sanchez-Perez, I., Murguia, J.R. and Perona, R. (1998). Cisplatin induces a persistent activation of JNK that is related to cell death. *Oncogene*, 16 (4); 533-40.

Santana, P., Peña, L.A., Haimovitz-Friedman, A., Martin, S., Green, D., McLoughlin, M., Cordon-Cardo, C., Schuchman, E.H., Fuks, Z. and Kolesnick, R. (1996). Acid sphingomyelinase-deficient human lymphoblasts and mice are defective in radiation-induced apoptosis. *Cell*, 86 (2); 189-99.

Sartorius, U.A. and Krammer, P.H. (2002). Upregulation of Bcl-2 is involved in the mediation of chemotherapy resistance in human small cell lung cancer cell lines. *Int J Cancer*, 97 (5); 584-92.

Sato, T., Irie, S., Kitada, S. and Reed, J.C. (1995). FAP-1: a protein tyrosine phosphatase that associates with Fas. *Science*, 268 (5209); 411-5.

Sawada, M., Sun, W., Hayes, P., Leskov, K., Boothman, D.A. and Matsuyama, S. (2003). Ku70 suppresses the apoptotic translocation of Bax to mitochondria. *Nat Cell Biol*, 5 (4); 320-329.

Savitsky, K., Bar-Shira, A., Gilad, S., Rotman, G., Ziv, Y., Vanagaite, L., Tagle, D.A., Smith, S., Uziel, T., Sfez, S. and et al. (1995). A single ataxia telangiectasia gene with a product similar to PI-3 kinase. *Science*, 268 (5218); 1749-53.

Scaffidi, C., Fulda, S., Srinivasan, A., Friesen, C., Li, F., Tomaselli, K.J., Debatin, K.M., Krammer, P.H. and Peter, M.E. (1998). Two CD95 (APO-1/Fas) signaling pathways. *Embo J*, 17 (6); 1675-87.

Scarlett, J.L. and Murphy, M.P. (1997). Release of apoptogenic proteins from the mitochondrial intermembrane space during the mitochondrial permeability transition. *FEBS Lett*, 418 (3); 282-6.

Schmitz, I., Kirchhoff, S. and Krammer, P.H. (2000). Regulation of death receptor-mediated apoptosis pathways. *Int J Biochem Cell Biol*, 32 (11-12); 1123-36.

Sekido, Y., Fong, K.M. and Minna, J.D. (1998). Progress in understanding the molecular pathogenesis of human lung cancer. *Biochim Biophys Acta*, 1378 (1); F21-59.

Sheridan, J.P., Marsters, S.A., Pitti, R.M., Gurney, A., Skubatch, M., Baldwin, D., Ramakrishnan, L., Gray, C.L., Baker, K., Wood, W.I., Goddard, A.D., Godowski, P. and Ashkenazi, A. (1997). Control of TRAIL-induced apoptosis by a family of signaling and decoy receptors. *Science*, 277 (5327); 818-21.

Shieh, S.Y., Ahn, J., Tamai, K., Taya, Y. and Prives, C. (2000). The human homologs of checkpoint kinases Chk1 and Cds1 (Chk2) phosphorylate p53 at multiple DNA damage-inducible sites. *Genes Dev*, 14 (3); 289-300.

Shieh, S.Y., Ikeda, M., Taya, Y. and Prives, C. (1997). DNA damage-induced phosphorylation of p53 alleviates inhibition by MDM2. *Cell*, 91 (3); 325-34.

Shields, P.G. (2002). Molecular epidemiology of smoking and lung cancer. *Oncogene*, 21 (45); 6870-6.

Shimizu, S., Ide, T., Yanagida, T. and Tsujimoto, Y. (2000a). Electrophysiological study of a novel large pore formed by Bax and the voltage-dependent anion channel that is permeable to cytochrome c. *J Biol Chem*, 275 (16); 12321-5.

Shimizu, S., Konishi, A., Kodama, T. and Tsujimoto, Y. (2000b). BH4 domain of antiapoptotic Bcl-2 family members closes voltage- dependent anion channel and inhibits apoptotic mitochondrial changes and cell death. *Proc Natl Acad Sci U S A*, 97 (7); 3100-5.

Shimizu, S., Narita, M. and Tsujimoto, Y. (1999). Bcl-2 family proteins regulate the release of apoptogenic cytochrome c by the mitochondrial channel VDAC. *Nature*, 399 (6735); 483-7.

Shiozaki, E.N., Chai, J., Rigotti, D.J., Riedl, S.J., Li, P., Srinivasula, S.M., Alnemri, E.S., Fairman, R. and Shi, Y. (2003). Mechanism of XIAP-Mediated Inhibition of Caspase-9. *Mol Cell*, 11 (2); 519-27.

Shivapurkar, N., Toyooka, S., Eby, M.T., Huang, C.X., Sathyanarayana, U.G., Cunningham, H.T., Reddy, J.L., Brambilla, E., Takahashi, T., Minna, J.D., Chaudhary, P.M. and Gazdar, A.F. (2002). Differential inactivation of caspase-8 in lung cancers. *Cancer Biol Ther*, 1 (1); 65-9.

Silke, J., Hawkins, C.J., Ekert, P.G., Chew, J., Day, C.L., Pakusch, M., Verhagen, A.M. and Vaux, D.L. (2002). The anti-apoptotic activity of XIAP is retained upon mutation of both the caspase 3- and caspase 9-interacting sites. *J Cell Biol*, 157 (1); 115-24.

Sinclair, W.K. (1968). Cyclic x-ray responses in mammalian cells in vitro. *Radiat Res*, 33 (3); 620-43.

Sirzén, F., Nilsson, A., Zhivotovsky, B. and Lewensohn, R. (1999). DNA-dependent protein kinase content and activity in lung carcinoma cell lines: correlation with intrinsic radiosensitivity. *Eur J Cancer*, 35 (1); 111-6.

Sirzén, F., Zhivotovsky, B., Nilsson, A., Bergh, J. and Lewensohn, R. (1998a). Higher spontaneous apoptotic index in small cell compared with non- small cell lung carcinoma cell lines; lack of correlation with Bcl- 2/Bax. *Lung Cancer*, 22 (1); 1-13.

Sirzén, F., Zhivotovsky, B., Nilsson, A., Bergh, J. and Lewensohn, R. (1998b). Spontaneous and radiation-induced apoptosis in lung carcinoma cells with different intrinsic radiosensitivities. *Anticancer Res*, 18 (2A); 695-9.

Song, Z., Yao, X. and Wu, M. (2003). Direct interaction between survivin and Smac is essential for the anti- apoptotic activity of survivin during Taxol-induced apoptosis. *J Biol Chem*, 26 M300957200.

Sorensen, M., Sehested, M. and Jensen, P.B. (1999). Effect of cellular ATP depletion on topoisomerase II poisons. Abrogation Of cleavable-complex formation by etoposide but not by amsacrine. *Mol Pharmacol*, 55 (3); 424-31.

Sozzi, G., Veronese, M.L., Negrini, M., Baffa, R., Cotticelli, M.G., Inoue, H., Tornielli, S., Pilotti, S., De Gregorio, L., Pastorino, U., Pierotti, M.A., Ohta, M., Huebner, K. and Croce, C.M. (1996). The FHIT gene 3p14.2 is abnormal in lung cancer. *Cell*, 85 (1); 17-26.

Springs, S.L., Diavolitsis, V.M., Goodhouse, J. and McLendon, G.L. (2002). The kinetics of translocation of Smac/DIABLO from the mitochondria to the cytosol in HeLa cells. *J Biol Chem*, 277 (48); 45715-8.

Stennicke, H.R., Deveraux, Q.L., Humke, E.W., Reed, J.C., Dixit, V.M. and Salvesen, G.S. (1999). Caspase-9 can be activated without proteolytic processing. *J Biol Chem*, 274 (13); 8359-62.

Stoka, V., Turk, B., Schendel, S.L., Kim, T.H., Cirman, T., Snipas, S.J., Ellerby, L.M., Bredesen, D., Freeze, H., Abrahamson, M., Bromme, D., Krajewski, S., Reed, J.C., Yin, X.M., Turk, V. and Salvesen, G.S. (2001). Lysosomal protease pathways to apoptosis. Cleavage of bid, not pro-caspases, is the most likely route. *J Biol Chem*, 276 (5); 3149-57.

Stokoe, D., Engel, K., Campbell, D.G., Cohen, P. and Gaestel, M. (1992). Identification of MAPKAP kinase 2 as a major enzyme responsible for the phosphorylation of the small mammalian heat shock proteins. *FEBS Lett*, 313 (3); 307-13.

Sun, C., Cai, M., Gunasekera, A.H., Meadows, R.P., Wang, H., Chen, J., Zhang, H., Wu, W., Xu, N., Ng, S.C. and Fesik, S.W. (1999). NMR structure and mutagenesis of the inhibitor-of-apoptosis protein XIAP. *Nature*, 401 (6755); 818-22.

Sun, J., Chen, Y., Li, M. and Ge, Z. (1998). Role of antioxidant enzymes on ionizing radiation resistance. *Free Radic Biol Med*, 24 (4); 586-93.

Susin, S.A., Lorenzo, H.K., Zamzami, N., Marzo, I., Snow, B.E., Brothers, G.M., Mangion, J., Jacotot, E., Costantini, P., Loeffler, M., Larochette, N., Goodlett, D.R., Aebersold, R., Siderovski,

D.P., Penninger, J.M. and Kroemer, G. (1999). Molecular characterization of mitochondrial apoptosis-inducing factor. *Nature*, 397 (6718); 441-6.

Suzuki, Y., Imai, Y., Nakayama, H., Takahashi, K., Takio, K. and Takahashi, R. (2001a). A serine protease, HtrA2, is released from the mitochondria and interacts with XIAP, inducing cell death. *Mol Cell*, 8 (3); 613-21.

Suzuki, Y., Nakabayashi, Y., Nakata, K., Reed, J.C. and Takahashi, R. (2001b). X-linked inhibitor of apoptosis protein (XIAP) inhibits caspase-3 and -7 in distinct modes. *J Biol Chem*, 276 (29); 27058-63.

Suzuki, Y., Nakabayashi, Y. and Takahashi, R. (2001c). Ubiquitin-protein ligase activity of X-linked inhibitor of apoptosis protein promotes proteasomal degradation of caspase-3 and enhances its anti-apoptotic effect in Fas-induced cell death. *Proc Natl Acad Sci U S A*, 98 (15); 8662-7.

Taher, M.M., Hershey, C.M., Oakley, J.D. and Valerie, K. (2000). Role of the p38 and MEK-1/2/p42/44 MAP kinase pathways in the differential activation of human immunodeficiency virus gene expression by ultraviolet and ionizing radiation. *Photochem Photobiol*, 71 (4); 455-9.

Takanami, I., Imamura, T., Hashizume, T., Kikuchi, K., Yamamoto, Y., Yamamoto, T. and Kodaira, S. (1996). Immunohistochemical detection of basic fibroblast growth factor as a prognostic indicator in pulmonary adenocarcinoma. *Jpn J Clin Oncol*, 26 (5); 293-7.

Takata, M., Sasaki, M.S., Sonoda, E., Morrison, C., Hashimoto, M., Utsumi, H., Yamaguchi-Iwai, Y., Shinohara, A. and Takeda, S. (1998). Homologous recombination and non-homologous end-joining pathways of DNA double-strand break repair have overlapping roles in the maintenance of chromosomal integrity in vertebrate cells. *Embo J*, 17 (18); 5497-508.

Tamm, I., Kornblau, S.M., Segall, H., Krajewski, S., Welsh, K., Kitada, S., Scudiero, D.A., Tudor, G., Qui, Y.H., Monks, A., Andreeff, M. and Reed, J.C. (2000). Expression and prognostic significance of IAP-family genes in human cancers and myeloid leukemias. *Clin Cancer Res*, 6 (5); 1796-803.

Teitz, T., Wei, T., Valentine, M.B., Vanin, E.F., Grenet, J., Valentine, V.A., Behm, F.G., Look, A.T., Lahti, J.M. and Kidd, V.J. (2000). Caspase 8 is deleted or silenced preferentially in childhood neuroblastomas with amplification of MYCN. *Nat Med*, 6 (5); 529-35.

Tepper, A.D., de Vries, E., van Blitterswijk, W.J. and Borst, J. (1999). Ordering of ceramide formation, caspase activation, and mitochondrial changes during CD95- and DNA damage-induced apoptosis. *J Clin Invest*, 103 (7); 971-8.

Tesfaigzi, J., Hotchkiss, J.A. and Harkema, J.R. (1998). Expression of the Bcl-2 protein in nasal epithelia of F344/N rats during mucous cell metaplasia and remodeling. *Am J Respir Cell Mol Biol*, 18 (6); 794-9.

Thome, M., Schneider, P., Hofmann, K., Fickenscher, H., Meinl, E., Neipel, F., Mattmann, C., Burns, K., Bodmer, J.L., Schroter, M., Scaffidi, C., Krammer, P.H., Peter, M.E. and Tschopp, J. (1997). Viral FLICE-inhibitory proteins (FLIPs) prevent apoptosis induced by death receptors. *Nature*, 386 (6624); 517-21.

Thompson, C.B. (1995). Apoptosis in the pathogenesis and treatment of disease. *Science*, 267 (5203); 1456-62.

Thornberry, N.A. (1997). The caspase family of cysteine proteases. *Br Med Bull*, 53 (3); 478-90.

Thornberry, N.A. and Lazebnik, Y. (1998). Caspases: enemies within. *Science*, 281 (5381); 1312-6.

Tominaga, K., Morisaki, H., Kaneko, Y., Fujimoto, A., Tanaka, T., Ohtsubo, M., Hirai, M., Okayama, H., Ikeda, K. and Nakanishi, M. (1999). Role of human Cds1 (Chk2) kinase in DNA damage checkpoint and its regulation by p53. *J Biol Chem*, 274 (44); 31463-7.

Tsujimoto, Y., Finger, L.R., Yunis, J., Nowell, P.C. and Croce, C.M. (1984). Cloning of the chromosome breakpoint of neoplastic B cells with the t(14;18) chromosome translocation. *Science*, 226 (4678); 1097-9.

Ungefroren, H., Kruse, M.L., Trauzold, A., Roeschmann, S., Roeder, C., Arlt, A., Henne-Bruns, D. and Kalthoff, H. (2001). FAP-1 in pancreatic cancer cells: functional and mechanistic studies on its inhibitory role in CD95-mediated apoptosis. *J Cell Sci*, 114 (Pt 15); 2735-46.

Uren, A.G., Wong, L., Pakusch, M., Fowler, K.J., Burrows, F.J., Vaux, D.L. and Choo, K.H. (2000). Survivin and the inner centromere protein INCENP show similar cell- cycle localization and gene knockout phenotype. *Curr Biol*, 10 (21); 1319-28.

Walker, J.R., Corpina, R.A. and Goldberg, J. (2001). Structure of the Ku heterodimer bound to DNA and its implications for double-strand break repair. *Nature*, 412 (6847); 607-14.

van Loo, G., Saelens, X., Matthijssens, F., Schotte, P., Beyaert, R., Declercq, W. and Vandenabeele, P. (2002a). Caspases are not localized in mitochondria during life or death. *Cell Death Differ*, 9 (11); 1207-11.

van Loo, G., van Gurp, M., Depuydt, B., Srinivasula, S.M., Rodriguez, I., Alnemri, E.S., Gevaert, K., Vandekerckhove, J., Declercq, W. and Vandenabeele, P. (2002b). The serine protease Omi/HtrA2 is released from mitochondria during apoptosis. Omi interacts with caspase-inhibitor XIAP and induces enhanced caspase activity. *Cell Death Differ*, 9 (1); 20-6.

Wang, H., Mao, Y., Zhou, N., Hu, T., Hsieh, T.S. and Liu, L.F. (2001). Atp-bound topoisomerase ii as a target for antitumor drugs. *J Biol Chem*, 276 (19); 15990-5.

Wang, H.G., Rapp, U.R. and Reed, J.C. (1996). Bcl-2 targets the protein kinase Raf-1 to mitochondria. *Cell*, 87 (4); 629-38.

Wang, X.Z. and Ron, D. (1996). Stress-induced phosphorylation and activation of the transcription factor CHOP (GADD153) by p38 MAP Kinase. *Science*, 272 (5266); 1347-9.

Wei, M.C., Lindsten, T., Mootha, V.K., Weiler, S., Gross, A., Ashiya, M., Thompson, C.B. and Korsmeyer, S.J. (2000). tBID, a membrane-targeted death ligand, oligomerizes BAK to release cytochrome c. *Genes Dev*, 14 (16); 2060-71.

Wei, M.C., Zong, W.X., Cheng, E.H., Lindsten, T., Panoutsakopoulou, V., Ross, A.J., Roth, K.A., MacGregor, G.R., Thompson, C.B. and Korsmeyer, S.J. (2001). Proapoptotic BAX and BAK: a requisite gateway to mitochondrial dysfunction and death. *Science*, 292 (5517); 727-30.

Weil, M., Raff, M.C. and Braga, V.M. (1999). Caspase activation in the terminal differentiation of human epidermal keratinocytes. *Curr Biol*, 9 (7); 361-4.

Verhagen, A.M., Coulson, E.J. and Vaux, D.L. (2001). Inhibitor of apoptosis proteins and their relatives: IAPs and other BIRPs. *Genome Biol*, 2 (7); 3009.1-3009.10.

Verhagen, A.M., Silke, J., Ekert, P.G., Pakusch, M., Kaufmann, H., Connolly, L.M., Day, C.L., Tikoo, A., Burke, R., Wrobel, C., Moritz, R.L., Simpson, R.J. and Vaux, D.L. (2002). HtrA2 promotes cell death through its serine protease activity and its ability to antagonize inhibitor of apoptosis proteins. *J Biol Chem*, 277 (1); 445-54.

Verheij, M., Bose, R., Lin, X.H., Yao, B., Jarvis, W.D., Grant, S., Birrer, M.J., Szabo, E., Zon, L.I., Kyriakis, J.M., Haimovitz-Friedman, A., Fuks, Z. and Kolesnick, R.N. (1996). Requirement for ceramide-initiated SAPK/JNK signalling in stress- induced apoptosis. *Nature*, 380 (6569); 75-9.

Verheij, M., Ruiter, G.A., Zerp, S.F., van Blitterswijk, W.J., Fuks, Z., Haimovitz-Friedman, A. and Bartelink, H. (1998). The role of the stress-activated protein kinase (SAPK/JNK) signaling pathway in radiation-induced apoptosis. *Radiother Oncol*, 47 (3); 225-32.

Viallet, J. and Minna, J.D. (1990). Dominant oncogenes and tumor suppressor genes in the pathogenesis of lung cancer. *Am J Respir Cell Mol Biol*, 2 (3); 225-32.

Widmann, C., Gerwins, P., Johnson, N.L., Jarpe, M.B. and Johnson, G.L. (1998). MEK kinase 1, a substrate for DEVD-directed caspases, is involved in genotoxin-induced apoptosis. *Mol Cell Biol*, 18 (4); 2416-29.

Wieckowski, M.R., Vyssokikh, M., Dymkowska, D., Antonsson, B., Brdiczka, D. and Wojtczak, L. (2001). Oligomeric C-terminal truncated Bax preferentially releases cytochrome c but not adenylate kinase from mitochondria, outer membrane vesicles and proteoliposomes. *FEBS Lett*, 505 (3); 453-9.

Vieira, H.L., Haouzi, D., El Hamel, C., Jacotot, E., Belzacq, A.S., Brenner, C. and Kroemer, G. (2000). Permeabilization of the mitochondrial inner membrane during apoptosis: impact of the adenine nucleotide translocator. *Cell Death Differ*, 7 (12); 1146-54.

Williams, M.D. and Sandler, A.B. (2001). The epidemiology of lung cancer. *Cancer Treat Res*, 105 31-52.

Volm, M., Koomägi, R. and Mattern, J. (1997). Prognostic value of vascular endothelial growth factor and its receptor Flt-1 in squamous cell lung cancer. *Int J Cancer*, 74 (1); 64-8.

Volm, M. and Rittgen, W. (2000). Cellular predictive factors for the drug response of lung cancer. *Anticancer Res*, 20 (5B); 3449-58.

Wolter, K.G., Hsu, Y.T., Smith, C.L., Nechushtan, A., Xi, X.G. and Youle, R.J. (1997). Movement of Bax from the cytosol to mitochondria during apoptosis. *J Cell Biol*, 139 (5); 1281-92.

Woo, M., Hakem, R., Soengas, M.S., Duncan, G.S., Shahinian, A., Kagi, D., Hakem, A., McCurrach, M., Khoo, W., Kaufman, S.A., Senaldi, G., Howard, T., Lowe, S.W. and Mak, T.W. (1998). Essential contribution of caspase 3/CPP32 to apoptosis and its associated nuclear changes. *Genes Dev*, 12 (6); 806-19.

Wood, D.E. and Newcomb, E.W. (2000). Cleavage of Bax enhances its cell death function. *Exp Cell Res*, 256 (2); 375-82.

Woodfield, K., Ruck, A., Brdiczka, D. and Halestrap, A.P. (1998). Direct demonstration of a specific interaction between cyclophilin-D and the adenine nucleotide translocase confirms their role in the mitochondrial permeability transition. *Biochem J*, 336 (Pt 2); 287-90.

Vousden, K.H. (2000). p53: death star. *Cell*, 103 (5); 691-4.

Vousden, K.H. and Lu, X. (2002). Live or let die: the cell's response to p53. *Nat Rev Cancer*, 2 (8); 594-604.

Wu, G., Chai, J., Suber, T.L., Wu, J.W., Du, C., Wang, X. and Shi, Y. (2000). Structural basis of IAP recognition by Smac/DIABLO. *Nature*, 408 (6815); 1008-12.

Wu, X., Bayle, J.H., Olson, D. and Levine, A.J. (1993). The p53-mdm-2 autoregulatory feedback loop. *Genes Dev*, 7 (7A); 1126-32.

Xanthoudakis, S., Roy, S., Rasper, D., Hennessey, T., Aubin, Y., Cassady, R., Tawa, P., Ruel, R., Rosen, A. and Nicholson, D.W. (1999). Hsp60 accelerates the maturation of pro-caspase-3 by upstream activator proteases during apoptosis. *Embo J*, 18 (8); 2049-56.

Xia, Y., Wu, Z., Su, B., Murray, B. and Karin, M. (1998). JNKK1 organizes a MAP kinase module through specific and sequential interactions with upstream and downstream components mediated by its amino-terminal extension. *Genes Dev*, 12 (21); 3369-81.

Xu, S., Robbins, D.J., Christerson, L.B., English, J.M., Vanderbilt, C.A. and Cobb, M.H. (1996). Cloning of rat MEK kinase 1 cDNA reveals an endogenous membrane- associated 195-kDa protein with a large regulatory domain. *Proc Natl Acad Sci U S A*, 93 (11); 5291-5.

Yamagishi, N., Saito, Y., Ishihara, K. and Hatayama, T. (2002). Enhancement of oxidative stress-induced apoptosis by Hsp105alpha in mouse embryonal F9 cells. *Eur J Biochem*, 269 (16); 4143-51.

Yang, L., Mashima, T., Sato, S., Mochizuki, M., Sakamoto, H., Yamori, T., Oh-Hara, T. and Tsuruo, T. (2003). Predominant suppression of apoptosome by inhibitor of apoptosis protein in non-small cell lung cancer H460 cells: therapeutic effect of a novel polyarginine-conjugated Smac peptide. *Cancer Res*, 63 (4); 831-7.

Yang, X., Khosravi-Far, R., Chang, H.Y. and Baltimore, D. (1997). Daxx, a novel Fas-binding protein that activates JNK and apoptosis. *Cell*, 89 (7); 1067-76.

Yasuhara, N., Eguchi, Y., Tachibana, T., Imamoto, N., Yoneda, Y. and Tsujimoto, Y. (1997). Essential role of active nuclear transport in apoptosis. *Genes Cells*, 2 (1); 55-64.

Yoshida, H., Kong, Y.Y., Yoshida, R., Elia, A.J., Hakem, A., Hakem, R., Penninger, J.M. and Mak, T.W. (1998). Apaf1 is required for mitochondrial pathways of apoptosis and brain development. *Cell*, 94 (6); 739-50.

Yuan, J., Shaham, S., Ledoux, S., Ellis, H.M. and Horvitz, H.R. (1993). The C. elegans cell death gene ced-3 encodes a protein similar to mammalian interleukin-1 beta-converting enzyme. *Cell*, 75 (4); 641-52.

Yuan, Z.M., Huang, Y., Ishiko, T., Kharbanda, S., Weichselbaum, R. and Kufe, D. (1997). Regulation of DNA damage-induced apoptosis by the c-Abl tyrosine kinase. *Proc Natl Acad Sci U S A*, 94 (4); 1437-40.

Zamzami, N. and Kroemer, G. (2001). The mitochondrion in apoptosis: how Pandora's box opens. *Nat Rev Mol Cell Biol*, 2 (1); 67-71.

Zangemeister-Wittke, U., Leech, S.H., Olie, R.A., Simões-Wüst, A.P., Gautschi, O., Luedke, G.H., Natt, F., Häner, R., Martin, P., Hall, J., Nalin, C.M. and Stahel, R.A. (2000). A novel bispecific antisense oligonucleotide inhibiting both bcl-2 and bcl-xL expression efficiently induces apoptosis in tumor cells. *Clin Cancer Res*, 6 (6); 2547-55.

Zantema, A., Verlaan-De Vries, M., Maasdam, D., Bol, S. and van der Eb, A. (1992). Heat shock protein 27 and alpha B-crystallin can form a complex, which dissociates by heat shock. *J Biol Chem*, 267 (18); 12936-41.

Zermati, Y., Garrido, C., Amsellem, S., Fishelson, S., Bouscary, D., Valensi, F., Varet, B., Solary, E. and Hermine, O. (2001). Caspase activation is required for terminal erythroid differentiation. *J Exp Med*, 193 (2); 247-54.

Zha, J., Harada, H., Yang, E., Jockel, J. and Korsmeyer, S.J. (1996). Serine phosphorylation of death agonist BAD in response to survival factor results in binding to 14-3-3 not BCL-X(L). *Cell*, 87 (4); 619-28.

Zha, J., Weiler, S., Oh, K.J., Wei, M.C. and Korsmeyer, S.J. (2000). Posttranslational N-myristoylation of BID as a molecular switch for targeting mitochondria and apoptosis. *Science*, 290 (5497); 1761-5.

Zhang, A., Zheng, C., Lindvall, C., Hou, M., Ekedahl, J., Lewensohn, R., Yan, Z., Yang, X., Henriksson, M., Blennow, E., Nordenskjöld, M., Zetterberg, A., Björkholm, M., Gruber, A. and Xu, D. (2000). Frequent amplification of the telomerase reverse transcriptase gene in human tumors. *Cancer Res*, 60 (22); 6230-5.

Zhang, Y., Xiong, Y. and Yarbrough, W.G. (1998). ARF promotes MDM2 degradation and stabilizes p53: ARF-INK4a locus deletion impairs both the Rb and p53 tumor suppression pathways. *Cell*, 92 (6); 725-34.

Zheng, T.S., Hunot, S., Kuida, K. and Flavell, R.A. (1999). Caspase knockouts: matters of life and death. *Cell Death Differ*, 6 (11); 1043-53.

Zhivotovsky, B., Samali, A., Gahm, A. and Orrenius, S. (1999). Caspases: their intracellular localization and translocation during apoptosis. *Cell Death Differ*, 6 (7); 644-51.