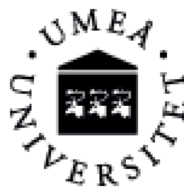
Regulation of apoptosis during treatment and resistance development in tumour cells

Jeanette Blomberg



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ABSTRACT

Induction of apoptosis is the most studied cell death process and it is a tightly regulated physiological event that enables elimination of damaged and unwanted cells. Apoptosis can be induced via activation of either the intrinsic or the extrinsic signalling pathway. The intrinsic pathway involves activation of the mitochondria by stress stimuli, whereas the extrinsic pathway is triggered by ligand induced activation of death receptors such as Fas. Apoptosis induction via Fas activation plays an important role in the function of cytotoxic T lymphocytes and in the control of immune cell homeostasis.

Several studies have shown that anticancer therapies require functional cell death signalling pathways. Irradiation based therapy has been successful in treatment of several malignancies but the usage of high doses has been associated with side effects. Therefore, low dose therapies, that either is optimized for specific delivery or administrated in combination with other treatments, are promising modalities. However, in order to achieve high-quality effects of such treatments, the death effector mechanisms involved in tumour eradication needs to be further explored. Importantly, tumour cells frequently acquire resistance to apoptosis, which consequently allows tumour cells to escape from elimination by the immune system and/or treatment.

Interferons constitute a large family of pleotrophic cytokines that are important for the immune response against viruses and other microorganisms. The interferon signalling pathway mediates transcriptional regulation of hundreds of genes, which result in mRNA degradation, decreased protein synthesis, cell cycle inhibition and induction of apoptosis. Interferon has successfully been used in therapy against some tumours. However, several drawbacks have been reported, such as reduced sensitivity to interferon during treatment.

The aim of this thesis was to elucidate mechanisms that mediate resistance to death receptor or interferon induced apoptosis in human tumour cell models, as well as investigate what molecular events that underlie cell death following radiation therapy of tumour cells.

In order to elucidate mechanisms involved in acquired resistance to Fas- or interferon-induced apoptosis, a Fas- and interferon-sensitive human cell line, U937, was subjected to conditions where resistance to either Fas- or interferon induced apoptosis was acquired. Characterization of the Fas resistant cells showed that multiple resistant mechanisms had been acquired. Reduced Fas expression and increased cFLIP expression, which is an inhibitor of death receptor signalling, were two important changes found. To further examine the importance of these two alterations, clones from the Fas resistant population were established. The reduced Fas expression was determined to account for the resistant phenotype in approximately 70% of the clones. In the Fas resistant clones with normal Fas expression, the importance of an increased amount of the cFLIP protein was confirmed with shRNA interference. A cross-resistance to death receptor induced apoptosis was detected in the interferon resistant variant, which illustrates that a connection between death receptor and interferon induced apoptosis exists. Notably, interferon resistant cells also contained increased cFLIP expression, which were determined to mediate resistance to both interferon and death receptor mediated apoptosis. Finally, when cell death induced by irradiation treatment was investigated in HeLa Hep2 cells we could demonstrate that cell death was mediated by centrosome hyperamplification and mitotic aberrations, which forced the cells into mitotic catastrophes and delayed apoptosis.

In conclusion, we have described model systems where selection for resistance to Fas or interferon induced apoptosis generated a heterogeneous population, where several signalling molecules were altered. Furthermore, we have shown that a complex cell death network was activated by irradiation based therapy.

ABBREVIATIONS

AIF	Apoptosis-inducing factor
AP-1	Transcription complex of Jun/Fos/ATF homo- and heterodimers
ATG	Autophagy-related gene
CaMKII	Calcium/calmodulin-dependent protein kinase II
CARD	Caspase activation and recruitment domain
DC	Dendritic cell
CHX	Cycloheximide
CMA	Chaperone-mediated autophagy
cFLIP	Cellular FLICE-like inhibitory protein
DD	Death domain
DED	Death effector domain
DISC	Death inducing signalling complex
FADD	Fas associated death domain containing protein
FasL	Fas ligand
IAP	Inhibitor of apoptosis protein
IFN	Interferon
IKK	Inhibitor of NF-κB kinase
ISGs	IFN-stimulated genes
ISRE	IFN-stimulated response element
IRF	Interferon regulatory factor
JAK	Janus-kinase
JNK	C-Jun N-terminal kinase
MAP-Kinase	Mitogen-activated protein kinase
MOMP	Mitochondrial outer membrane permeabilization
MQ	Macrophage
NF-κB	Nuclear factor-kappaB
PI3-Kinase	Phosphoinositol-3 kinase
PKR	RNA-dependent protein kinase
SAPK	Stress activated protein kinase
STAT	Signal transducers and activators of transcription
SODD	Suppressor of death domain
TLR	Toll like receptor
TNF	Tumour necrosis factor
TRADD	TNF-R1-associated death domain containing protein
TRAF	TNF-receptor associated factor
RIP	Receptor-interacting serine/threonine-protein kinase
qRT-PCR	Quantitative real-time polymerase chain reaction

PAPERS IN THIS THESIS

1. Blomberg J, Ruuth K, Santos D and Lundgren E. Acquired Resistance to Fas/CD95 Ligation in U937 Cells Is Associated with Multiple Molecular Mechanisms (2008) Anticancer Res 28, 593-600.

2. Blomberg J, Ruuth K, Jacobsson M, Höglund A, Nilsson J and Lundgren E. Reduced Fas transcription in clones of U937 cells that have acquired resistance to Fas induced apoptosis. Submitted manuscript under revision.

3. Blomberg J, Höglund A, Eriksson D, Ruuth K, Jacobsson M, Nilsson J and Lundgren E. Inhibition of cellular FLICE-like inhibitory protein abolishes insensitivity to interferon-α in a resistant variant of the human U937 cell line. Manuscript.

4. Eriksson D, Blomberg J, Lindgren T, Löfroth P.O., Johansson L, Riklund K and Stigbrand T. Iodine-131 induces mitotic catastrophes and activates apoptotic pathways in HeLa Hep2 cells. Accepted for publication in Cancer Biotherapy and radiopharmaceuticals, 2008.

INTRODUCTION

CELL DEATH

Cell death is a fundamental physiological process that is essential for organ formation during development and for maintaining tissue homeostasis by elimination of unwanted cells (1). Induction of cell death is also important for the removal of cells that expose the organism to danger, like virus infected cells or cells with damaged DNA (2).

Cell death was originally classified according to the morphological changes that occur during the lethal progress, and depending on the cell appearance it is often referred to as apoptosis, necrosis, autophagy or mitotic catastrophes (3). Pathological conditions, such as cancer and autoimmunity, are very often associated with defects in the cell death machinery.

Apoptosis

The term apoptosis, which means falling leaves in Greek, was first coined in 1972 by Kerr and colleagues, where it was defined on morphological grounds (4). The characteristic features of apoptosis are cell shrinkage, chromatin condensation, DNA fragmentation, membrane blebbing and formation of apoptotic bodies (3). The apoptotic bodies are engulfed by phagocytes, which prevents the generation of an inflammatory response. Phosphatidylserine, which is normally expressed on the inside of the plasma membrane, is relocated to the outer leaflet of the membrane during apoptosis and this is one of several "eat-me" signals which is recognized by phagocytes (5).

In the early 1990s', a genetically regulated form of apoptosis was described in the nematode, C. elegans. In this experimental model several important molecules involved in apoptosis signalling were discovered (6). Subsequent genetic investigations in mammalian cells identified a more complex system, where the described molecules in C. elegance represented large protein families (7-9). These observations illustrated not only that apoptosis is conserved throughout evolution, but also that specialization, redundancy and regulation at several levels might be crucial in the regulation of mammalian induced apoptosis.

Caspases

In mammalian cells apoptosis is executed by activation of multiple caspases which function in protease cascades. To date 14 mammalian caspases have been identified and based on their function they can be classified in three groups: inflammatory caspases, apoptotic initiator caspases and apoptotic effector caspases (10).

Caspases are resting zymogens that becomes activated by dimerization and/or autocatalytic cleavage. The initiator caspases functions as sensors and transducers of various apoptotic stimuli. They contain either a death effector domain (DED) or a caspase activation and recruitment domain (CARD), which are important for interactions with upstream adaptor molecules. Oligomerization of the initiator caspases through binding of adaptor molecules result in their activation. The activated initiator caspases subsequently cleave and activate the effector caspases, which in turn will cleave multiple cellular substrates leading to the execution of apoptosis (Fig. 1) (11, 12).

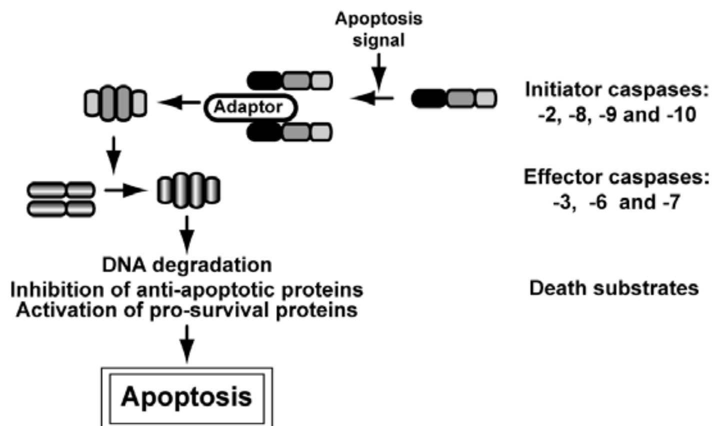

Fig. 1. The activating cascade of apoptotic caspases. Apoptosis inducing signals activate adaptor molecules that recruit initiator caspases. When these procaspases come in close proximity through dimer formation, they assume an active conformation which generally results in autocatalytic cleavage. One large and one small subunit will be released from each precursor molecule and assemble into an active heterotetrameric enzyme. The active initiator caspases subsequently cleave the effector caspases, which resides as inactive pre-associated dimers in the cytoplasm. Catalytic cleavage of the effector caspases leads to the formation of active homo/hetero dimers, which subsequently execute the cell death.

Apoptosis signalling pathways

Apoptosis can be induced via two main signalling pathways, through activation of the extrinsic or the intrinsic pathway.

Extrinsic apoptosis

Death receptors situated on the plasma membrane are responsible for the induction of the extrinsic apoptotic signalling pathway. Activation of the receptors by ligand binding induces a signalling cascade that culminates in degradation of the cell.

Death receptors

Death receptors belong to the tumour necrosis factor (TNF-) receptor superfamily. They are characterized by cysteine-rich repeats in their extra cellular domain and a ~80 amino acid long motif, known as the death domain (DD), within their cytoplasmic region (13). The DD is crucial for the transduction of the apoptotic signal, which is induced by ligand-specific oligomerization of the death receptors (13, 14).

There are eight death receptors characterized to date: TNF-R1 (DR1/CD120a/p55/p60), Fas (Apo-1/CD95), DR3 (APO-3/LARD/TRAMP), TRAIL-R1 (DR4/APO-2), TRAIL-R2 (DR5/KILLER/TRICK2), DR6, ectodysplasin A receptor (EDAR) and nerve growth factor (NGFR). The most studied and well characterized member is Fas (15, 16).

Fas signalling

Activation of Fas via binding of Fas-Ligand (FasL) plays a central role in the function of cytotoxic T lymphocytes and in the control of immune cell homeostasis. Fas is ubiquitously expressed in almost all tissues, whereas constitutive FasL expression is restricted to a few cell types, i.e. activated lymphocytes and cells present in immune-privileged sites like lung, brain, testis and eye (17).

Engagement of the Fas receptor results in the recruitment of several intracellular molecules, which forms a death inducing signalling complex (DISC) together with the intracellular part of the receptor (18). In this complex, the adaptor molecule Fas-associated DD-containing protein (FADD) interacts via its DD with the DD of Fas. Besides the DD, FADD also contains a DED that is homologous to the domain found in caspase-8. Thus, binding of FADD

to the Fas receptor will recruit and induce dimerization of caspase-8, leading to its activation (11, 19, 20). The proteolytically active caspase-8 will in turn activate down stream effector caspases (caspase-3, -6 and -7), which subsequently execute the apoptotic program (12)(Fig. 2A).

Procaspase-10 can also bind to the DISC and become activated as caspase-8 upon Fas ligation. However, the importance of caspase-10 for apoptosis induction in the absence of caspases-8 is still controversial and need to be elucidated (21).

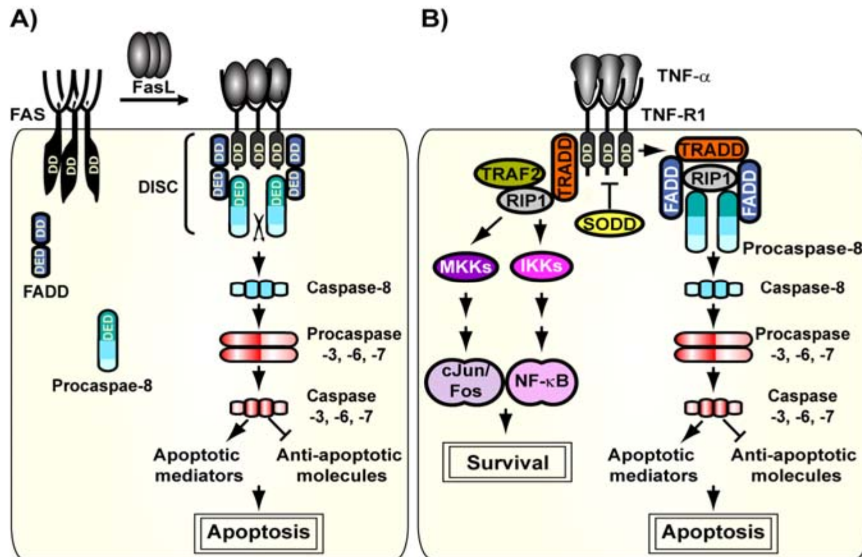

Fig. 2. The extrinsic signalling pathway of Fas and TNF-R1. The death receptors are generally present as pre-associated trimers at the plasma membrane. A) Oligomerization of the Fas receptor by ligand binding results in intracellular clustering of the DD domain, which induces its association with the DD of FADD. The sequential recruitment of FADD and procaspase-8 leads to the formation of DISC. In this complex, the interaction between the DED of FADD and caspase-8 mediates close proximity and dimerization of two caspase-8 molecules, which leads to activation and autoproteolytic cleavage of caspase-8. The released active heterotetrameric molecule cleaves and activates the effector caspases, which subsequently target a range of cellular substrates leading to apoptosis. B) The ability of the TNF-R1 complex to signal is inhibited by the binding to SODD. Engagement of the TNF-R1 by TNF-α homotrimers results in the release of SODD. This allows for the binding of DD containing proteins like TRADD. TRADD serves as an assembly platform by recruiting TRAF2, RIP1 and FADD, which subsequently induce activation of several intracellular signalling pathways. In a first step, the TNF-R1, TRAF2, RIP1 complex induces an anti-apoptotic response by activation of the MAP-Kinase and the NF-κB-Kinase pathways. In a second step, an apoptotic response can be induced by formation of a cytoplasmic complex containing TRADD, RIP1 and FADD which activates caspase-8 in a similar manner as described for Fas.

TRAIL- and TNF-receptor signalling

TRAIL induced signalling via TRAIL-R1 and TRAIL-R2 is almost identical to Fas signalling (22), whereas the TNF-receptor signalling is more complex.

TNF-α is a multifunctional cytokine that is mainly produced by macrophages, NK-cells and T-lymphocytes. There are two TNF-α receptors described that mediate the biological response of TNF-α, namely TNF-R1 and TNF-R2. Whereas TNF-R1 is expressed on almost all cells, TNF-R2 is preferentially expressed on haematopoietic cells (23). Moreover, TNF-R2 does not belong to the death receptor family as it lacks a DD in its cytoplasmic tail. Thus, the TNF-R1 is the receptor that is most important for the pro-apoptotic and inflammatory responses of TNF-α (24).

TNF-R1 is generally pre-associated with an inhibitory protein, SODD, which masks the intracellular recruitment domain of the receptor. TNF-α binding leads to the release of SODD, allowing for the binding of DD-containing adaptor proteins like TRADD. In turn, TRADD recruits TRAF2 and RIP1, which are important activators of NF-κB and/or c-Jun N-terminal kinase (JNK). The NF-κB activation mediates a strong pro-survival signal in the cell and thus, unlike FasL and TRAIL, TNF- α is generally a poor inducer of cell death (25). However, it has been shown that TNF-R1 can induce apoptosis via a two step activation cascade. First, the membrane bound TNF-R1, TRADD, TRAF2 and RIP1 activates NF-κB and/or JNK. Then, in a second step, TRADD and RIP1 associates with FADD in a cytoplasmic complex which subsequently leads to recruitment of caspase-8 (Fig. 2B). Activated NF-κB mediates transcription of a caspase-8 inhibitory protein, cFLIP, which interferes with the apoptosis signalling pathway from TNF-R1 and assure survival of the cell. Lack of NF-κB activity will however result in cell death via caspase-8 activation, as cFLIP is not induced. Thus, TNF-R1 signalling harbours a checkpoint were decision between survival and death can be made (26).

Fas and the TRAIL-receptors are also capable of activating pro-survival signalling such as the NF-κB and JNK pathways. However, these receptors generally show prominent apoptosis induction *in vivo* and the importance of pro-survival responses are still under investigation (25).

Intrinsic apoptosis.

The intrinsic apoptotic pathway is initiated from the inside of the cell and involves activation of the mitochondria (Fig 3). This pathway is triggered by various stress signals, such as damages mediated by irradiation or chemotherapeutic agents.

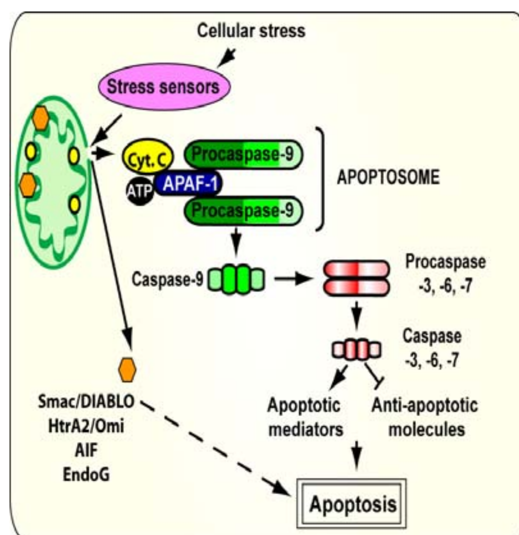

Fig. 3. The intrinsic signalling pathway. Various stress stimuli initiates apoptosis through activation of the intrinsic pathway. In this pathway, disruption of the mitochondrial membrane leads to the release of pro-apoptotic proteins from the mitochondrial intermembrane space into the cytoplasm. The cytosolic presence of cytochrome c generates the formation of an apoptosome complex, which contain the adaptor molecule APAF-1 and caspase-9. Binding of cytochrome c to APAF-1 allow for ATP binding, which induces a conformational change of APAF-1, exposing its CARD domain. Aggregation of caspase-9 through a CARD-CARD interaction with APAF-1 leads to its activation and autoproteolytic cleavages. The released heterotetramers subsequently activate the effector caspases, which then cleave target substrates to induce apoptosis. Other important proteins released from the mitochondria during apoptosis are IAP antagonists, like Smac/DIABLO and HtrA2/Omi, and DNA degrading proteins, e.g. AIF and EndoG.

Stress signals converge on the mitochondria to induce mitochondrial outer membrane permeabilization (MOMP). MOMP in turn causes cell death either via the release of apoptogenic molecules from the mitochondria into the

cytoplasm or indirectly through loss of mitochondrial functions that are important for survival (27). Several pro-apoptotic proteins, such as AIF, EndoG, Smac/Diablo, HtrA2/Omi and cytochrome c, are released from the mitochondria. Cytochrome c, which normally has an important function in the respiratory chain (28), assembles together with the adaptor molecule, APAF-1, ATP and caspase-9 into a complex called the apoptosome. This leads to aggregation and activation of caspase-9 in a similar manner as caspase-8 is activated in the DISC. Active caspase-9 subsequently cleaves and activates downstream effector caspases (casapse-3, -6 and -7), which then execute cell death. Thus, downstream signalling from initiator caspases are the same for the intrinsic and extrinsic pathways.

Cross-talk between the extrinsic and intrinsic pathways

Two models of Fas signalling have been described by which cells can be classified as type I or type II, depending on the engagement of the intrinsic pathway (29). Type I cells have high levels of Fas induced DISC formation and consequently high levels of activated caspase-8, which is sufficient for sustained caspase-3 activation. On the contrary, Type II cells are characterized by low amount of DISC formation and low levels of caspase-8 activation. Therefore, amplification of the signal via activation of the intrinsic pathway is needed. Low amount of caspase-8 is sufficient to cleave Bid into a truncated form (tBid). Bid is a pro-apoptotic member of the Bcl-2 family that upon cleavage causes disruption of the mitochondrial membrane, which subsequently leads to caspase-9 activation (30) (Fig 4).

Caspase-8 activation can be induced during intrinsic apoptosis signalling (30) and it has been shown to accelerate the intrinsic death response (31). Furthermore, cytochrome c mediated activation of caspase-6 has been reported to be an activator of caspase-8 (32). In summary, it is evident that cross-talks exist between the extrinsic and intrinsic apoptosis signalling pathways and they function as amplification loops for each cascade (Fig. 4.).

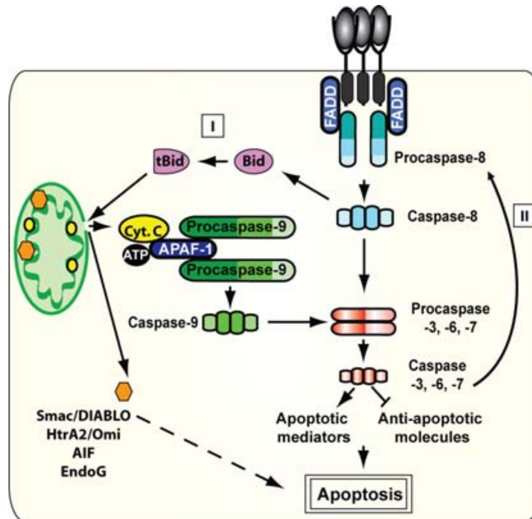

Fig. 4. Cross talk between the extrinsic and the intrinsic signalling pathways. The extrinsic and intrinsic pathways can connect at different levels. I) Active caspase-8 can cleave Bid, which in its truncated form (tBid) activates the intrinsic pathway by disruption of the mitochondrial membrane. Activation of the intrinsic pathway by generation of tBid can serve as an amplification loop for extrinsic signalling in cells with low levels of active caspase-8. II) Activation of the intrinsic pathway can result in the activation of casapse-8, which enhances the intrinsic apoptotic response.

Regulation of apoptosis signalling

When considering the physiological importance of apoptotic responses, it is clear that tight regulations of the signalling cascades are needed. Several regulators of death receptor signalling have been identified and they are briefly described below because of their relevance for apoptosis resistance.

cFLIP

One important family of endogenous inhibitors of the death receptor signalling is the cellular FLICE-like inhibitory proteins (cFLIPs). The viral protein, vFLIP, was first identified in 1997 when investigators were screening for DED-containing proteins that could interact with caspases (33). As the mammalian homologue was cloned by several different groups, it has many names: cFLIP (cellular FLIP), CASH, FLAME, Casper, CLARP; FLAME, I-

FLICE, MRIT and usurpin (34). However, here it will only be referred to as cFLIP.

There are several splice variants of cFLIP reported but only three have been identified to be expressed as proteins. cFLIP-Long (cFLIP-L) is similar to caspase-8, as it contains two N-terminal DEDs and a caspase-like domain. Several important amino acids are however different in the caspase-like domain of cFLIP-L, which abolishes the enzymatic activity. The two other cFLIP splice variants are truncated versions that only contain the two DEDs and they are called cFLIP-Short (cFLIP-S) and cFLIP-Raji (cFLIP-R) (35-37). All three cFLIP isoforms have been demonstrated to inhibit Fas induced apoptosis and the inhibitory effect is a consequence of competition between cFLIPs and caspase-8 for FADD binding (35-37). However, whereas cFLIP-S completely competes out binding and activation of caspase-8, cFLIP-L can under certain conditions have caspase-8-activating properties. cFLIP-L is capable of forming heterodimers with caspase-8 at the DISC, which induces week activation and initial processing of caspase-8 that result in the release of the p10 fragment of caspase-8. The activated enzyme that contains the catalytic part is however believed to be retained within the DISC, which restricts its accessibility of target substrates (38, 39). Association of cFLIP-L with caspase-8 also mediates processing of cFLIP-Long, which generates a 43kDa product from the 55kDa full-length protein (38). The p43cFLIP fragment inhibits Fas induced apoptosis and recent studies have illustrated that the p43cFLIP fragment is a more potent activator of NF-κB then full-length cFLIP-L (40). However, the role of cFLIP-L in NF-κB activation is complex and contradicting studies exist where cFLIP-L has also been shown to inhibit NF-κB activation (41, 42). Nevertheless, the function of cFLIP at the DISC is not only to abolish caspase-8 induced apoptosis but also to regulate other signalling cascades such as survival pathways from the death receptors.

The Bcl-2 family

The proteins in the Bcl-2 family are key regulators of the intrinsic apoptotic pathway. The Bcl-2 gene was the first anti-apoptotic gene to be discovered and it was found at the chromosomal breakpoint in a chromosomal translocation present in a B cell lymphoma (43). Today the family consists of 20 members, which can be divided into three subfamilies depending on the presence of conserved Bcl-2 homology (BH) domains (44). One subfamily is constituted of anti-apoptotic proteins, such as Bcl-2, Bcl-X_L, Bcl-w, Mcl-1, A1 and Bcl-B, whereas the other two subfamilies include the pro-apoptotic proteins. Bak, Bax and Bok are three important members of the Bax-like apoptotic subfamily.

These proteins contain three BH domains and they are responsible for mitochondrial membrane permeblization and cytochrome c release. The third subfamily is the more diverse pro-apoptotic BH-3 only family and it includes Bik, Bad, Bim, Bid, Bmf, Hrk, Noxa and Puma (see fig. 5A for overview) (45).

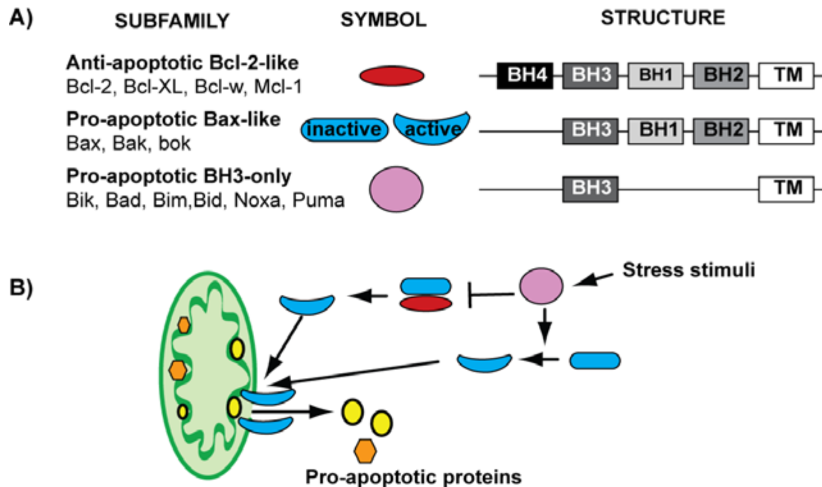

Fig. 5. The Bcl-2 family members and their function at the mitochondrial membrane. A) The Bcl-2 family proteins can be divided into three subgroups depending on how many BH-domains they contain. All four BH-domains are found in the anti-apoptotic Bcl-2 like subfamily, whereas the Bax-like subfamily of the pro-apoptotic proteins only contains three BH-domains. The third subfamily constitute of the BH-3 only proteins, which along with the Bax-like subfamily function as apoptosis inducers. Several proteins in all the subgroups also contain a transmembrane domain (TM), which affects the protein function by anchoring them to intracellular membranes like the mitochondria, nuclear envelope and endoplasmatic reticulum. B) Various stress stimuli result in the activation of the BH-3 only proteins, which function as apoptotic sensors in the cytoplasm. Subsequently, the BH-3 only proteins activate the Bax-like protein directly by changing their conformation or indirectly by liberating their inhibitory binding to anti-apoptotic Bcl-2 protein. Activation of the Bax-like proteins leads to their oligomerization at the mitochondrial membrane, which results in the release of pro-apoptotic proteins like cytochrome c, Smac/DIABLO and HtrA2/Omi, AIF and EndoG into the cytoplasm.

Both pro-apoptotic groups of the Bcl-2 proteins are required for stress-induced apoptosis. The BH3-only proteins function as damage sensors upstream of Bak and Bax, as Bim, Bad and NOXA could not induce apoptosis in Bax and Bak double deficient cells (46, 47). Some BH3-only proteins are activated by transcriptional induction (PUMA, NOXA) or posttranslational modifications, like dephosphorylation (Bad) and cleavage (tBid), whereas others are normally sequestered into specific cell compartments and released upon cell death stimulation (Bim and Bmf) (Jin and El-Deiry 2005; Karst and Li 2007). Active BH3-only proteins mediate conformational changes in Bax and Bak, which induce protein oligomerization at the mitochondrial membrane, thus leading to MOMP and cytochrome c release. There are two models proposed for the activation of Bak and Bax by the BH3-only proteins (Fig. 5B) (44). In the first model a direct association between Bak/Bax and certain BH3-only proteins, i.e. Bim tBid and Puma, has been described. In this model, the rest of the BH3-only proteins are suggested to bind anti-apoptotic proteins and abolish their inhibitory interaction with Bim and tBid. In the indirect activation model, the BH3-only proteins are believed to induce apoptosis merely by interaction with anti-apoptotic proteins, thus preventing them from inhibiting Bax and Bak activation. The functional relevance of these two activation models is currently debated and it is likely that they are of different importance in different cellular systems (48).

IAPs

Both the extrinsic and the intrinsic signalling pathways are under the control of the inhibitors of apoptosis, IAPs. These proteins were originally found in baculoviruses (49) and they are classified on the criteria that they contain one to three baculoviral IAP repeats (BIRs). There are at least 8 different human IAPs identified (Survivin, ILP2, ML-IAP, XIAP, c-IAP1, c-IAP2, NAIP and BRUCE). Recent studies have shown that the IAPs can bind directly to different caspases, mainly caspase-9, -3 and -7, and inhibit their activity. The inhibitory effect is mediated by binding of the BIR domains to the caspases, which sequesters the caspases away from their targets and/or induce their degradation (50).

NF-κB

The NF-κB family of transcription factors regulates many different genes involved in apoptosis, cell growth, inflammation and modulation of the immune system (51). The family consists of five Rel-domain containing proteins (Rel A/p65, Rel B, c-Rel, p50/NF-κB1 and p52/NF-κB2). In general, the proteins are present as dimers that are sequestered into the cytoplasm by inhibitory IκBs. In the classical signalling pathway, activated Inhibitor of NF-κB kinases (IKKs) induce degradation of IκBs by phosphorylation, which result in nuclear translocation of free NF-κB dimers. In the nucleus, NF-κB binds to specific targets sequences and induces gene transcription in cooperation with various activators and repressors. The activation of IKKs is mediated by stimuli-induced activation of surface receptors that induce TRAF/RIP complex formation, which subsequently leads to phosphorylation of IKKs. The classical pathway is usually activated by various inflammatory signals and it has been proposed to regulate apoptosis signalling in a complex way (52). Importantly, TNF-α, TRAIL and FasL induced apoptosis has been shown to be suppressed by NF-κB over-expression (53, 54). Although, NF-κB is generally supposed to promote survival, evidence exists which suggests that NF-κB also can enhance cell death under certain conditions (55).

p53

The p53 transcription factor is often called "the guardian of the genome" and it has a pivotal role in prevention of tumour formation. Mutations, deletions or dysregulation of p53 is some of the most common alterations detected in tumours. The expression of p53 is generally low due to its binding to mdm2, which targets p53 for proteasomal degradation (56). DNA damage and cellular stress induces stabilisation of p53. This together with specific modifications of p53, such as phosphorylations, acetylations and glycosylations, leads to its activation (57). The most important function of p53 is to transcriptionally control a large number of genes that influence cell cycle arrest, DNA repair, apoptosis, senescence and autophagy. p53 has been established as one of the most important DNA damage cell cycle checkpoint proteins and it has for instance a major regulatory role in the response to radiation (58). The apoptotic function of p53 is mainly executed through activation of pro-apoptotic proteins, such as Bax, Noxa, Puma, Bid, Fas, APAF-1 and TRAIL-R2 (59). In addition, p53 mediates transcriptional repression of anti-apoptotic genes like Bcl-2 and survivin (60, 61). Notably, p53 has also been reported to obstruct the

anti-apoptotic function of Bcl-2 and Bcl-X_L as well as to activate the pore-forming function of Bax and Bak by a direct binding (62-64).

Survival signalling pathways

The cell fate can be influenced by several survival signalling pathways following apoptosis stimulation, e.g. the phosphoinositol-3 kinase (PI3-Kinase) and the mitogen-activated protein kinase (MAP-Kinase) pathways.

The PI3-Kinase pathway

The PI3-Kinase pathway has an important role in many cellular processes, such as cell survival, proliferation, migration and vesicular trafficking. Activation of receptor protein tyrosine kinases by various external stimuli leads to the generation of a second messenger called phosphatidylinositol 3,4,5 triphosphate (PIP3), which is generated from phosphatidylinositol 4,5-bisphosphate (PIP2) by activated PI3-Kinase. The PIP3 recruits AKT/PKB to the plasma membrane, where it is activated through phosphorylations (65, 66). When activated, AKT either positively or negatively regulates the function of many different proteins in the cells (Fig. 6) (67). For example, AKT has been shown to phosphorylate Bad and caspase-9 which will lead to inhibition of their involvement in transducing an apoptotic signal (68-70). In addition, AKT activates NF-κB through phosphorylation of IκB, which in turn mediate survival via transcriptional up-regulation of anti-apoptotic proteins such as Bcl-2 and Bcl-X_L (71, 72). AKT has also been shown to enhance cFLIP expression in tumour cell lines (73, 74). Thus, one important function of AKT is to inhibit cell death at multiple steps in the apoptosis signalling pathways.

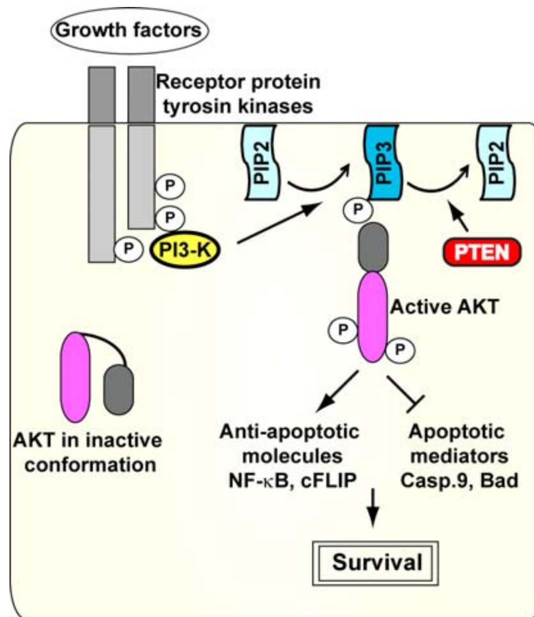

Fig. 6. The PI3-Kinase signalling cascade. Activation of receptor protein tyrosine kinases by various external stimuli, such as growth factors, results in the association of the PI3-kinase to the receptors. This will induce allosteric activation of the kinase that subsequently catalyse the formation of phosphatidylinositol 3,4,5 triphosphate (PIP3) from phosphatidylinositol 4,5-bisphosphate (PIP2) lipids in the membrane. The generated PIP3 then recruits pleckstrin homologue domain containing proteins such as the AKT/PKB kinase. Binding to the PIP3s by AKT induces a conformational change, which results in the exposure of activating phosphorylation sites. When AKT becomes phosphoryalted by other kinases, like the protein serine/treonine kinase 3′-phosphoinositide-dependent klinases (PDKs), it either positively or negatively regulates the function of many target proteins in the cells. PTEN is an important tumour suppressor gene that can dephosphorylate the PIP3s and thereby negatively regulate the PI3-kinase signalling.

The MAP-Kinase pathway

The MAP-kinase pathway is also an important mediator of signal transduction. It is initiated by various stimuli and regulates a multitude of cellular responses. There are three main signalling cascades that respond to somewhat different stimuli, namely the ERK1/ERK2, the c-Jun N-terminal kinase/ stress activated protein kinase (JNK/SAPK) and the p38 pathways. All three pathways include activation of a cascade of at least three different protein kinases: MAP3K,

MAP2K and MAPK (Fig. 7.)(75). Activation of kinases from all three pathways has been implicated to regulate apoptosis signalling.

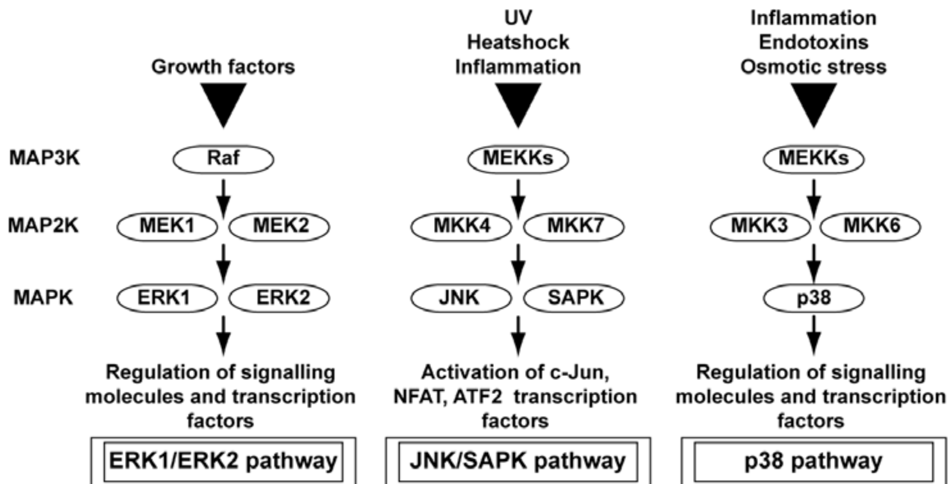

Fig. 7. The MAP-Kinase signalling cascades. Three major types of MAP-kinase signalling cascades have been demonstrated in mammalian cells: the ERK1/ERK2, the JNK/SAPK and the p38 pathways. These signalling cascades involve sequential activation of MAP3Ks, MAP2Ks and MAPKs and they are induced by different upstream signals. It is well established that the ERK pathway is stimulated by growth signals, whereas the JNK/SAPK and p38 pathways are induced by various stress stimuli exemplified in the picture. Activation of the different pathways leads to diverse cellular responses mediated by the MAPKs (see main text for details).

In the ERK1/ERK2 pathway, Ras is the major upstream activator of Raf, which subsequently activates MEK1/MEK2. Activated MEK1/MEK2 phosphorylates and activates ERK1/ERK2. The major targets of activated ERK1/ERK2 are kinases, transcription factors and apoptotic executers. For example, ERK1/ERK2 can repress caspase-9 mediated apoptosis and regulate the activity of Bcl-2 family proteins, such as Bad, Bim and Mcl-1 (68, 76-79). In addition, over-expression of MEK1 has been shown to inhibit FADD mediated apoptosis as a consequence of increased expression of cFLIP (80). As Ras is one of the most frequently mutated oncogenes found in human cancers, increased activity of this pathway is often detected in different malignancies where it mediates increased proliferation, survival and drug resistance (81).

The JNK/SAPK pathway is activated by environmental stress, growth factors and inflammatory cytokines. One important target molecule of this pathway is the c-Jun protein, which function as a transcription factor when activated by phosphorylation. The role of this pathway in apoptosis is highly

controversial as it has been reported to have pro-apoptotic, anti-apoptotic and no apoptotic regulatory potential. The functional outcome is suggested to depend on cell type, the nature and duration of the death signal, as well as the influence of other signalling pathways (82). As mentioned earlier, NF-κB and JNK are activated during TNF-α signalling. The activity of JNK is however transient and it is rapidly down regulated by NF-κB. In the absence of NF-κB activity, sustained JNK activity can enhance TNF-α induced apoptosis but prolonged JNK activity itself is not sufficient for apoptosis induction on its own (83). This illustrates that the JNK signalling pathway can be a modulator rather than a component of the apoptotic machinery in the cell.

The p38 pathway has also been illustrated to have diverse effects on apoptosis signalling, depending on cell type and stimuli (84). For example, Fas signalling has been shown to activate the p38 pathway downstream of caspase activation (85). Furthermore, cell lines established from p38 knockout mice are less sensitive to apoptosis induction by UV, serum withdrawal and Ca^{2+} depletion (86).

Non-classical apoptotic cell death

Necrosis

Necrotic cell death has long been regarded as the opposite to apoptosis. It is considered to be an energy-independent and un-regulated form of cell death, characterized by swelling of organelles and cytoplasm that eventually leads to disruption of the plasma membrane. As the content of the cells leaks out to surrounding tissue, an inflammatory response is normally generated. Necrosis is often induced by acute and severe injuries, like in pathological conditions and ischemia (87). Recently, evidence has emerged showing that necrosis under certain conditions can be programmed (88). Induction of necrosis can be an important alternative if normal apoptosis signalling is impaired. The signalling mechanisms leading to necrosis are not fully understood but some important findings have been made. For example, the RIP-1 kinase has been demonstrated to be an effector molecule in Fas induced necrosis (89) and calcium as well as reactive oxygen species have been shown to be important players in the execution of necrosis (90).

Autophagy

Autophagy is an evolutionary conserved intracellular process that eliminates damaged or dysfunctional cell components. The maintenance of cellular homeostasis by autophagy is important for survival, development and differentiation. Normally, autophagy operates at low speed but it can serve as an adaptive response against several pathologies like cancer, infection and neurodegeneration. Several different forms of autophagy have been described, namely macro-, micro- and chaperone-mediated autophagy (CMA). Whereas macro- and micro autophagy are capable of degrading large structures, CMA only takes care of soluble proteins. During macro-autophagy, double-membrane-containing vesicles are formed that are called phagosomes. These phagosomes enclose organelles and proteins which are subsequently degraded when the vesicles fuse with lysosomes. In contrast, the micro-autophagy and CMA involves direct incorporation of target substrates into lysosmes (91). Autophagy is under the control of a large family of autophagy-related genes (ATG). During stress conditions, like nutrient and growth factor deprivation, autophagy can sustain cell survival by degrading disposable cell components (92). In contrast, under uncontrolled up-regulation of autophagy the outcome can be cell death. For instance, over expression of ATGs, e.g. becline-1, in mammalian cells leads to cell death (93). A potential danger of autophagy has been suggested as it might keep damaged cells alive and thus supporting pathologies like tumour formation (92).

Mitotic catastrophe

Mitotic catastrophe refers to cell death that is occurring during mitosis or caused by mitotic failure (Fig. 8). Several insults may lead to abnormal mitosis through the generation of lagging chromosomal material, anaphase bridging or multiple spindle poles. Due to an impaired mitosis, this form of cell death is often associated with the formation of giant cells that contain nuclei with altered morphology, i.e. multiple nuclei or micronuclei (87). These cells may continue to divide and become polypoid and/or aneuploid, but eventually they die by delayed apoptosis or necrosis (94). Mitotic catastrophe generally results from defects mediated by impaired cell cycle checkpoints (95). Therefore, mitotic catastrophe is particularly prevalent in cells with compromised p53 function, as p53 is a major regulator of both G1 and G2 checkpoints (58). Mitotic catastrophe can for instance be induced as a consequence of premature entry into mitosis with unrepaired DNA damage, due to a compromised G2 checkpoint (58). In addition, it can be caused by anomalous duplication of

centrosomes, as these are crucial for the number of spindle poles formed during mitosis and for accurate chromosome segregation into daughter cells (96).

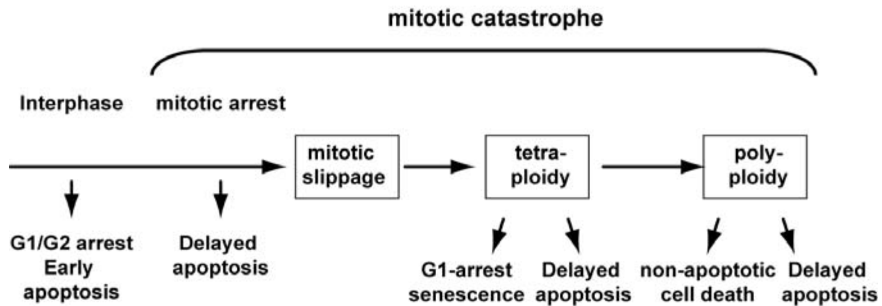

Fig. 8. Mitotic catastrophe. Cell death induced in mitosis or as a consequence of mitotic abnormalitites is often referred to as mitotic catastrophe and it is preferentially induced in cells where the G1/G2 arrest is compromised. It can be detected at multiple stages during the accumulation of DNA alterations depending on the genetic backround of the cell and the intensity of the DNA damageing stimuli applied. This type of cell death has been demonstrated to involve both a delayed type of apoptosis and non-apoptotic events, such as necrosis. (Modified from Eriksson et.al. 2008).

CARCINOGENESIS

Tumour development is a multi step process in humans. It is associated with genetic alterations, like mutations in tumour promoting (oncogenes), tumour suppressing (suppressor) genes and stability genes, which progressively drive transformation of a normal cell into a malignant form. Thus, many cancers are age-dependent and occur later in life. Different insults, like chemical, physiological or viral agents, can induce changes in DNA that results in increased survival and proliferation. At least six important alterations have been reported to be important for tumour development including self-sufficiency in growth signals, evasion of apoptosis, insensitivity to anti-growth signals, limitless replication capacity, sustained angiogenesis and an ability to invade other tissues (97).

Treatment modalities

At present, the traditional ways to treat cancers are through surgery, chemotherapy or radiotherapy. Combinations of these techniques are often applied to achieve optimal results. Several novel therapeutics like gene therapy, immunotherapy, angiogenetic compounds and small molecular inhibitors, are in basic and clinical development. These modalities are aiming to improve target specificity and minimise the toxic side effect on normal tissue.

Chemotherapy

In cancer research, chemotherapy includes all chemical drugs that are used in cancer treatment. Today, there are several different anticancer substances approved for treatment, e.g. DNA-damaging agents, antimetabolites, mitotic inhibitors, nucleotide analogues and inhibitors of topoisomerases. In general, these therapeutic agents induce a cellular stress response that leads to growth inhibition and cell death. Chemotherapeutic drugs generally target fast growing cells, as they are more sensitive to DNA damage and cellular stress. Since tumour cells generally have a higher growth rate than normal cells, they are more severely affected (98). Most drugs induce activation of the intrinsic apoptotic pathway and altered functions of several Bcl-2 family proteins are important for anticancer drug resistance. In addition, several chemical agents have also been shown to activate the death receptors pathway (99), but the

extrinsic pathway is generally not regarded as the major regulator of drug induced apoptosis in tumour cells (100).

Radiotherapy

Radiotherapy is the medical use of ionizing radiation (X and γ-rays), which induce DNA damage and cellular stress. Ionizing radiation is not specifically targeting tumour cells, but neoplastic cells have an increased growth rate and are therefore more effectively eradicated. Radiotherapy can not treat non-identified metastases because it is applied locally to minimize the side effect on healthy tissues. Radiotherapy is continuously improved and combination therapies with other modalities, e.g. chemotherapy and surgery are under development (101).

Radioimmunotherapy

To improve delivery and specificity of radiotherapy, radioimmunotherapy is an attractive alternative. The identification of tumour antigens, i.e. proteins with increased expression in tumour cells, made it possible to deliver radio nuclides or other toxic substances in conjugation with specific tumour-recognising antibodies. This has aided to restrict the delivery to local tumour sites but refinements are still needed, which includes optimisation of the molecular vehicle and the radio nuclides used (102).

Radiation treatment generates pro-apoptotic signalling within target cells through induction of DNA breaks and activation of proteins in the plasma membrane (103). Radiation induced cell death has been shown to be extremely complex since it involves activation of mitotic catastrophe, apoptosis, autophagy and necrosis (104, 105).

Apoptosis resistance in malignant disease

Acquired resistance towards apoptosis is present in most types of cancers and it supports tumour development in a profound way. There are numerous molecular alterations described that promotes both tumour development and progression.

Resistance mechanisms

A significant number of proteins involved in the induction and regulation of cell death have been reported to be altered in various types of cancer. The well-known molecular alterations that are important for apoptosis resistance are listed in table I and they include reduced function of pro-apoptotic molecules, increased expression of anti-apoptotic proteins and increased activity of pro-survival signalling molecules (106).

Altered molecules		Example of tumour type	Ref
Anti-apoptotic proteins (increased)			
Bcl-2 members	Bcl-2	Melanoma, B cell lymphoma	(43, 107)
	Mcl-1	Multiple myeloma, *CML	(108)
cFLIPs		Burkitt lymphoma, Pancreatic cancer	(109, 110)
IAPs	c-IAP2	Melanoma, B-cell lymphoma	(111, 112)
	Survivin	Lung-, Colon-, Breast-, Pancreatic-cancer	(113)
Decoy receptors	DcR3	Glioblastoma, melanoma	(114, 115)
Pro-apoptotic proteins (decreased)			
Bcl-2 members	Bax	Colon cancer, Burkitt lymphoma	(116, 117)
Death receptors	Fas	Colon and hepatocellular cancer	(118, 119)
	TRAIL-R1/2	Head- neck and lung cancer	(120)
Caspases	Caspase-8	Neuroblastoma, lung cancer	(121, 122)
APAF-1		Melanoma	(123)
Survival signalling proteins (increased signalling)			
PI3-Kinase pathway	PI3-Kinase	Ovarian cancer	(124)
	PTEN	Brain-, Brest and Prostate cancer	(125)
	AKT	Ovarian and pancreatic cancer	(126)
MAP-Kinase pathway	Ras	Myeloid leukaemia, lung- & colon cancer	(127)
	Raf	Melanoma, colon cancer	(128)
NF-κB		Many solid and haematological tumours	(129)
p53 pathway	p53	50% of all tumours	(130, 131)
	INK4/ARF	Many solid and haematological tumours	(132)

Table I. Examples of important proteins that cause resistance to apoptosis in various tumours. There is overwhelming evidence that many apoptotic signalling molecules and regulators are altered in a wide range of cancers. These alterations contribute to tumour development, progression and survival following treatment. *CML: Chronic myelogenous leukemia.

The molecular alterations that support tumour formation are primarily caused by genomic changes. It is well established that mutations of important tumour suppressor genes and oncogenes is a major driving force of transformation. These mutations can occur through exchange of single nucleotides or rearrangements of larger DNA stretches, such as deletions, inversions and translocations (133). Recently, the impact of epigenetic changes, like DNA methylation and histone modifications, was also appreciated to have a great influence on tumour development (134).

Apoptosis resistance in tumour development and survival

Apoptosis resistance is needed in cooperation with increased proliferation signalling to mediate tumour formation and progression. Furthermore, insensitivity to apoptosis allows tumour cells to escape from elimination by cytotoxic immune cells and cancer therapeutics.

Defects in apoptosis signalling supports enhanced proliferation

The apoptotic program normally counteracts inappropriate cell proliferation or growth by apoptosis induction. During normal development the proliferation rate varies greatly and cells have to distinguish between a normal increase in proliferation and abnormal excessive growth. The proliferation rate is normally regulated by growth factors and nutrient availability. It is proposed that activation of signalling pathways that mediates growth also can lead to cell death. This implies that proliferation is only permitted in the presence of survival factors which simultaneously repress cell death (135). Several oncogenes have been demonstrated to induce proliferation as well as cell death. For example, it was early discovered that induction of c-MYC and E2F1 not only increased proliferation but also promoted cell death (135, 136). Thus, environmental growth factors are important for oncogenic stimulation of cell proliferation and a concomitant blockage of cell death are required for malignant transformation.

Escape of immune surveillance through apoptosis resistance

It there is overwhelming evidence that the immune system has an important cancer-immune surveillance function in mammals (137). As tumour growth and surivial are suppressed by the immune system, neoplastic cells are suggested to acquire a reduced immunogenic phenotype in order to escape elimination. This illustrates that the immune system possess both tumour inhibitory and tumour sculpturing effects, which is referred to as immunoediting (138). It has for example been shown that tumours from immunodeficient mice are less prone to grow in an immunocompetent recipient than tumours from mice with an intact immune system (139, 140).

In general, the cytotoxic effect by T lymphocytes and NK cells are mediated via granule exocytosis and Fas Ligand induced apoptosis of target cells. These two systems have been suggested to be important for immune suppression of tumour formation (141). The importance of Fas in the immune system was established in the so-called lymphoproliferation phenotype mice (*lpr*), when it was discovered that these mice harboured a point mutation in *FAS* (142). Later, several mutations of *FAS* have been detected in humans (12, 143). Besides the generation of an autoimmune lymphoproliferative syndrome, some *FAS* mutations have been shown to enhance tumour formation (144, 145). Thus, resistance to Fas signalling might allow malignant cells to escape from surveillance by the immune system

Apoptosis resistance confers insensitivity to cancer therapy

As chemotherapy and irradiation eliminates tumour cells by induction of apoptotosis, development of resistance towards apoptosis can make tumour cells insensitive to therapy. In addition to the apoptosis resistance acquired during tumour formation and progression, tumour cells also acquire resistance during treatment. The development of drug resistance is troublesome, as it might contribute to cross-resistance against additional chemotherapeutics (146). Recently, novel strategies have been taken to find specific targets in the apoptosis signalling pathways that upon inhibition or activation promote apoptosis in tumour cells. Some promising therapeutic candidates are listed in table II and they include activating substances of molecules in the death signalling pathways as well as drugs that interfere with the anti-apoptotic properties of survival proteins (147, 148).

Target	Agents	ref
Extrinsic specific proteins		
TRAIL-R1, TRAIL-R2	Monoclonal antibodies	(149, 150)
	Recombinant protein, human TRAIL,	(149, 150)
TNF-R1	Recombinant protein, human TNFα	(149)
Intrinsic specific proteins		
Bcl-2, Bcl-XL	Antisense oligonucleotides	(151)
Bcl-2, Bcl-XL, Bcl-W	BH3 peptides	(48)
Mitochondrial function	Arsenite, Lonidamine,	(149)
General pro- and anti-apoptotic proteins		
Caspases	Inducible synthetic caspases	(149)
Survivin	Antisense oligonucleotide	(149)
IAPs	Small molecular inhibitors	(149)
Under investigation	Apoptin	(152)
PML-RARα, TRAIL	All-trans retinoic acid (ATRA)	(153)
Ras	Farnesyl transferase inhibitors	(154)
PI3-K/AKT	PI3-kinases inhibitors, NPV-BEZ235	(155)
mTOR	Rapamycin analogues	(65)
p53	Gene therapy, ONYX-015, INGN 201	(156)
	Small molecular stabilizers, PRIMA-1	(156)
IKK, IκB	Small molecule inhibitors, PS1145	(150)
Proteasome	Bortezomib	(48)

Table II. Novel anti-cancer therapeutics are under development and they target an array of different apopsosis inducing and regulating proteins. The constantly increasing knowledge about tumour progression and resistance stimulates the development of new drugs. Some promising therapeutics that are in pre-clinical or clinical studies are listed in the table.

INTERFERON

Interferons (IFNs) were first described in 1957 by Isaacs and Linderman as antiviral substances (157). Today it is established that IFNs possess a wide range of biological regulatory functions, i.e. antiviral, cell growth inhibitory, anti-angiogenic and immunomodulatory effects. The family comprises of three subfamilies, the type I, II and type III IFNs. The IFN type I family includes multiple IFN-α subtypes, IFN-β, IFN-κ, IFN-ε and IFN-ω, whereas the IFN type II family only consist of IFN-γ. The type I IFNs have a broad biological importance as they are induced by most cell types upon viral infection. On the contrary, IFN-γ is mainly expressed by Th-1 and NK-cells and have important functions in the adaptive immune response (158). The type III IFNs consists of the IFN-λs and they also have antiviral properties. The IFN-λs activate the same main signalling pathway as the type I IFNs but they work through independent receptors, namely IL10R1 and IFNLR1 (159). The type I IFNs will be the main focus in the following chapters, as IFN-α induced apoptosis was the topic of our study.

Production of type I IFNs

Type I IFNs are generally produced through activation of Toll like receptors (TLRs) or cytoplasmic retinoic acid-inducible gene I-like helicases (RLHs), i.e. RIG-1 and Mda5, by viral and bacterial derivates. These pathways activate several transcription factors, including cJun/ATF-2, NF-κB, IRF-3 and IRF-7, which translocate to the nucleus and induce transcription of the type I IFNs. The secreted IFNs then work in paracrine and autocrine ways to induce an antiviral state in the host (Fig. 9A) (160).

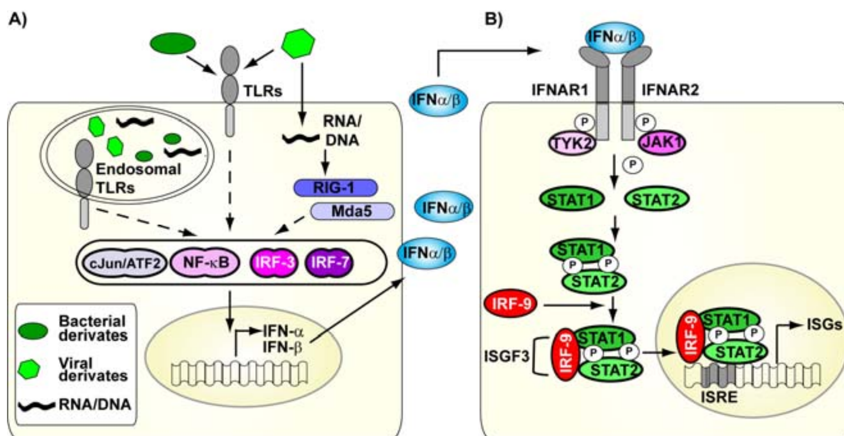

Fig. 9. Production of type I IFNs and their signalling cascade. A) Microbial products like LPS, CpG DNA, and viral RNA-DNA derivates can be recognized by specific TLRs situated on the plasma membrane or in endosomes. Different RNA-DNA derivates can also activate RNA helicases, i.e. RIG-1 and Mda5, in the cytoplasm. Stimulation of both theses systems result in a cascade of signalling events, leading to activation of several transcription factors like c-Jun/ATF-2, NF- NF-κB, IRF-3 and IRF-7. These transcription factors are then responsible for the induction of the type I IFN production. B) The type I IFN signalling is mediated by IFN induced dimerization of the IFNAR1 and IFNAR2 receptors. The activated receptors interact with the TYK2 and JAK1 kinases respectively, which subsequently recruit and activate the STAT1 and STAT2 transcription factors by phosphorylation. The phosphorylated STAT1/STAT2 heterodimer associates with the IRF-9 transcription factor and forms an ISGF3 complex. This complex translocates to the nucleus and induces expression of various ISG-genes by binding to specific IFN-stimulated response elements (ISREs) in their promoters.

The IFN-signalling cascade

Activation of the IFN signalling pathway occurs when soluble IFN proteins bind to and stimulate the IFN receptors, which upon heterodimerization activates the Janus-kinase/Signal transducers and activators of transcription (JAK/STAT) pathway. When activated by phosphorylation, the STATs form homo/hetero dimers that translocate to the nucleus and transcriptionally regulate several hundreds of IFN-stimulated genes (ISGs). IFNs from the

different subfamilies engage specific receptors that activate different STATs and JAKs, thus generating diverse biological responses in various cells (158).

As the IFN-α and IFN-β were among the fist IFNs to be distinguished, their signalling cascade have been well studied (161). In the classical signalling pathway, activation of the IFNAR1 and IFNAR2 receptors by IFN-α/-β binding leads to activation of STAT1 and STAT2 heterodimers, which associate with the IRF-9 protein and translocate to the nucleus (Fig. 9B). The STAT1/STAT2 complex have been established to be the most important molecular mediator of IFN-α signalling (162-165), but several other STATs (STAT-3, -4, -5, and -6) are also activated upon IFN-α/-β signalling (166, 167). In addition, other signalling pathways, like the MAP-kinase and PI3-kinase pathways, are activated by IFN in both STAT-dependent and STAT-independent ways and they affect the IFN signalling cascade at several steps (167). Thus, the signalling from the IFN receptors are much more complex than first anticipated and the biological response can vary depending on which pathways that are engaged.

In the nucleus, the STAT dimmers bind to specific target sequences and transcriptionally regulate more than 300 ISGs (168). These genes are generally quiescent or expressed at low levels during normal conditions and IFN stimulation results in a transient increase in their expression. The general responses mediated by the various ISGs are cell growth inhibition, cytoskeletal remodelling, mRNA degradation, inhibition of protein translation and apoptosis (169). The diversity of the transcriptional induction has made it difficult to identify which gene products that are important for the various responses induced. Some important ISGs and their main function are listed in table III (158, 170, 171).

ISGs (increased by IFN if not otherwise stated)	Function
Antiviral	
PKR OASs/RNASEL MXA ISG-15 RIG-1, MDA5, STAT1 IRF1-9 IRF7	Blocks protein synthesis Degrades RNA Interferes with intracellular trafficking Protein modification by ISGylation Enhanced IFN signalling Amplification of transcription, IFN production IFNα production
Regulation of cell growth	
p21 p202 Down-regulation of cyclins and cycline-dependent kinases	Cell cycle inhibitor Cell cycle inhibitor Mediators of cell cycle progression
Apoptotic	
TRAIL, Fas XAF1 PML Caspases DAP-kinase	Death receptor signalling Inhibition of the anti-apoptotic XIAP Cell death induced by formation of nuclear bodies Apoptosis signalling Caspase-independent cell death
Immunomodulatory	
MHC I and II Perforin, TRAIL ICAM1 TLRs Other cytokines (IL-12, IL-15)	Antigen presentation CTL, NK cytotoxicity Leukocyte trafficking Enhanced IFN production Various functions of immunecells

Table III. IFN induced ISGs. Signalling by the type I IFNs result in the activation of hundreds on ISG-genes, which subsequently mediate the biological response of IFN. Some important ISG and their main function are listed in the table (158, 170, 171).

IFNs as apoptosis inducers

With the identification of several hundreds of ISGs induced by IFN stimulation, it became apparent that IFN stimulation induced apoptosis in multiple ways (168, 172). For example, some proteins which are important players in the antiviral response of IFN, like PKR, RNAseL and IRFs, also have the capacity to induce apoptosis (170). In addition, IFN stimulation directly increases the expression of pro-apoptotic genes, such as death receptors, death receptor ligands and caspases (173-175). It has been suggested

that IFN induced apoptosis is dependent on functional TRAIL- but not Fas-receptor signalling (176-179). In addition, the PI3-kinase pathway, which normally is involved in survival signalling, has been shown to be needed for IFN induced apoptosis in U266 cells (180). Importantly, the apoptosis response induced by IFN has been shown to depend on caspase activation, as inhibition of caspase activity reduces the apoptotic response (179). Furthermore, FADD has been reported to be required, as expression of a dominant negative FADD abolished apoptosis induced by PKR and IRF-1 over expression (178, 181). Exactly how the caspase cascade is activated and which IFN-inducible genes that are crucial is however not determined and remains to be elucidated.

IFN in clinical treatment

Due to the important biological functions of IFNs in anti-viral and tumour suppressive responses, they have attracted interest as treatment modalities of viral infections, cancer and multiple sclerosis. Today, IFN treatment has shown to be effective against several viral infections, like chronic hepatitis C, herpes zoster and cytomegalovirus (182-184). However, many viruses have developed diverse strategies to circumvent the IFN response and current investigations are focusing on the interplay between virus and IFN signalling in order to improve the usages of IFNs in clinical treatment (185).

In tumour treatment, IFN-α has successfully been used against some cancers, like lymphomas and certain solid tumours (186, 187). Still, several drawbacks have been reported, which includes side effects at high doses (188, 189). As the tumour suppressive function of IFNs is poorly understood, the development of effective treatment has been delayed. Nevertheless, it has become apparent that beside the direct effect on infected and transformed cells, IFN has a strong regulatory effect on both the innate and adaptive immune responses. The multiple biological activities of IFN have been proposed to cooperate to induce a long lasting anti tumour state. Thus, recent obtained knowledge about IFN activation and signalling has stimulated investigators to find new drug targets within the IFN system. For example, activation of endogenous IFN production via activation of the TLRs resembles the biological response that occurs when the host is infected with pathogens and thus generates an immune response which can contribute to tumour elimination (190, 191). In addition, modified soluble IFN proteins, like pegylated and synthetic variants, are currently tested for their improved signalling capacities. Finally, direct activation of molecules in the IFN signalling casacade, like JAKs and ISGs, are also potential strategies proposed for a more direct IFN mediated response (158).

AIMS OF THIS THESIS

The general objective of this thesis is to elucidate the molecular apoptosis signalling mechanisms that occur during resistance development and tumour cell treatment.

The specific objectives are:

- To elucidate what molecular abnormalities that are important for resistance development against death receptor and IFN induced apoptosis.

- To investigate mechanisms of low dose irradiation induced cell death in solid tumours.

RESULTS AND DISCUSSION

APOPTOSIS RESISTANCE IN TUMOURS

Cancer is a genetic disease that develops through continuous accumulation of mutations. Many important tumour promoting genes have been identified during the last two decades and their functions have been well characterized (192). However, recent genome-wide screenings of different tumour samples have pointed out that also more gene mutations are to be discovered and their unique characteristics will be important to elucidate (133).

Evasion of apoptosis by tumour cells has gained much attention since it allow tumour cells to escape from elimination by endogenous default mechanisms, immune surveillance and cancer therapeutics. Several molecular alterations that contribute to apoptosis resistance have been demonstrated but identification of novel apoptotic regulators is still important to improve tumour treatment (193).

Impaired death receptor signalling in tumour progression

The death receptor pathway is important for elimination of tumour cells by the immune system. Consequently, resistance to death receptor signalling has been shown to facilitate tumour formation (fulda debatin 2004, muschen Beckman 2000). In order to increase the knowledge about the molecular abnormalities that contribute to resistance against death receptor induced apoptosis, a resistant human tumour cell model was generated. Fas resistant cells were generated by growth of sensitive U937 cells in progressively increasing concentrations of agonistic Fas antibody for 8 weeks. When the resistant population was characterized, multiple molecular alterations of important apoptosis signalling molecules were detected (Paper I). These aberrations included a decreased Fas expression, an increased cFLIP expression and altered functions of protein tyrosine kinase and phosphatase signalling.

Normally, the oncogenic process will result in the generation of heterogeneous cancer populations, as it occurs over time and involves sequential acquisitions of mutations. In this respect, it was reasonable to suspect that the different molecular alterations detected in our resistant population represented clonal variations. Thus, to further elucidate the importance and contributions of the different molecular alterations detected in the Fas resistant

population, individual clones were established. A schematic illustration of the generation of the resistant cells is visualized in fig .10.

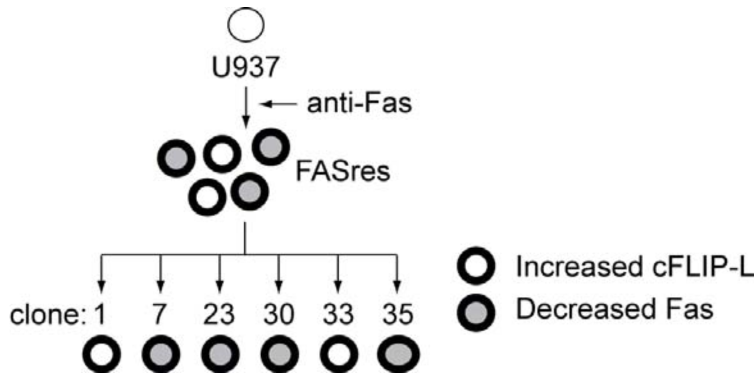

Fig. 10. Generation of Fas resistant cells. Fas resistant cells were generated by growth of U937 cells in increasing concentrations of stimulating Fas antibody for 8 weeks. The established Fas resistant cells (FASres) were a heterogeneous population of cells that contained multiple resistant phenotypes. The resistant population was subcloned and 39 individual clones were established. Six of the 39 resistant clones, 1, 7, 23, 30, 33 and 35, were picked for further studies. Two major resistant phenotypes were detected in these clones, namely a reduced Fas expression and an increased cFLIP-L expression.

In addition to the data in paper I, a proteomic study was made to reveal differences in the global expression profile between parental and Fas resistant cells. 2-dimensional-gel elecrophoresis was made on fluorescent labelled proteins from parental and Fas resistant cells. The DIGE system was used which allows two samples to be run at the same gel to minimize discrepancies. In addition, a third dye was included as an internal control on duplicate gels to minimize gel-to-gel variability within one experiment. The fluorescent stained gels were scanned in a Typhoon 9400 scanner (GE-Healthcare) and the fluorescent intensity was analysed with the DeCyder DIA and BVA software (GE-Healthcare). Proteins identified to have an altered expression (>±1.5 fold) compared to parental cells were isolated and sent for Trypsin digestion followed by mass spectrometric analyses, which were carried out by the WCN Expression Proteomics Facility (IMBIM, Uppsala University, Sweden). An example of a Coomassie stained gel that illustrates the protein profile of parental cells is shown in Fig. 11A.

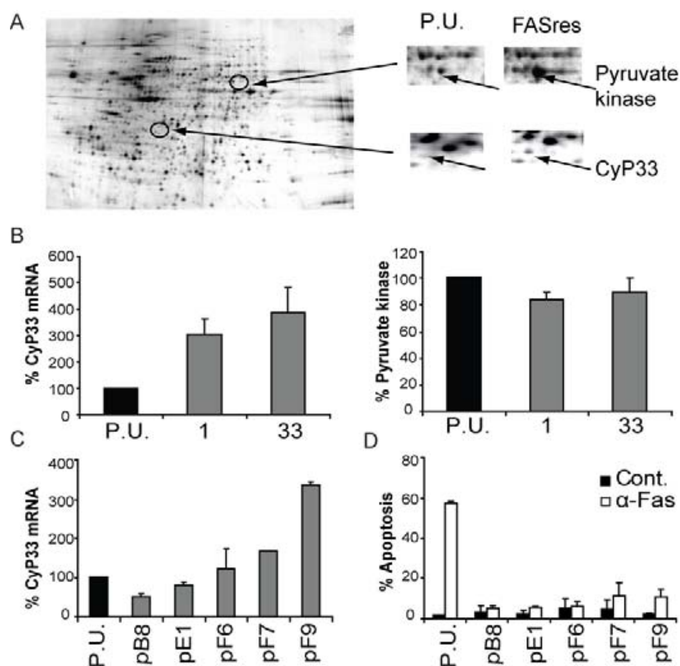

Fig. 11. Increased CyP33 expression in Fas resistant cells does not mediate Fas resistance. Global protein expression pattern was analysed with 2D-PAGE gel electrophoresis in parental U937 cells and the maternal Fas resistant population. Total lysate was run on Immobiline-DryStrip gels in the first dimension, before separation in the second dimension was made on a 12% SDS-PAGE gels. A) Illustrative picture on the protein expression profile of parental cells is shown by Coomassie staining and the two proteins with the highest increased expression identified in the Fas resistant cells (FASres) are indicated with arrows. B) qRT-PCRs were made to confirm increased expression of CyP33 and Pyruvate Kinase in the Fas resistant clones. Cells from clone 1 was infected with lentivirus that carried different shRNA against CyP33 (pB8, pE1, pF6, pF7 and pF9). Stable transfectants were selected by puromycine treatment and subsequently analysed. C) Relative expression of CyP33 mRNA was measured with qRT-PCR in cells of clone 1 that had been infected with the different shRNA constructs against CyP33. D) Fas induced apoptosis was measured after 15h of treatment with 15ng/ml anti-Fas in CyP33 shRNA containing cells and compared to parental cells. Apoptosis was scored by PI-staining and flow cytometry.

Two proteins that were up-regulated more than 1.75 fold in the resistant cells were identified and confirmed in three independent experiments. The protein whose expression differed the most was Pyruvate Kinase, which is an important enzyme that catalyse the generation of ATP in the second step of the glycolytic pathway (1.78 fold, $p<0.001$). The next highest protein expressed in resistant cells was Cyclophilin 33/E (Cyp33) (1.76 fold, $p<0.01$). CyP33 belongs to the

peptidyl-propyl cis-trans-isomerase (PPIase) class of proteins, and is thus proposed to be involved in accelerating protein folding. In addition, CyP33 is the only protein of this family that have been reported to have a dsDNA and mRNA binding capacity (194). Notably, the biological function of Cyp33 is still not established and thus, we found this protein interesting to study further. Interestingly, an increased mRNA expression could be confirmed for CyP33 but not Pyruvate kinase in the resistant clones (1 and 33) (Fig. 11B). However, a reduced Cyp33 expression did not correlate to a restored Fas sensitivity when CyP33 knockdown was made with 5 different shRNA constructs that target 5 different sites in the CyP33 transcript. QRT-PCR analysis showed that the different shRNA constructs (pB8, pE1, pF6, pF7 and pF9) were differently efficient to reduce the Cyp33 expression. The degree of knockdown ranged from more than a 6 fold decrease (pB8) to no decrease at all (pF9) (Fig. 11C). Importantly, reduction of CyP33 expression did not mediate a restored Fas sensitivity in the resistant cells of clone 1 (Fig. 11D). This demonstrates that the increased Cyp33 expression represents a secondary irrelevant event not linked to the acquired Fas resistance.

The other proteins with altered expression patterns indentified in the 2-dimensional-gel screen were not further investigated because we suspected that many of them might as Cyp33 represent secondary alterations, reflecting the heterogenicity of the population. Instead, investigations were made to determine whether the decreased Fas expression and increased cFLIP-L expression was important mediators of Fas resistance and if they were coupled to each other.

Reduced Fas expression in apoptosis resistance

(Paper II)

Characterization of six Fas resistant clones determined that two major phenotypic populations could be distinguished, i.e. one that contained reduced amount of Fas and one that had increased expression of the casaspe-8 regulatory protein cFLIP-L. Decreased Fas expression was the major resistant phenotype, which was found in approximately 70% of the clones (Fig. 10). Further investigations demonstrated that the reduced Fas expression was caused by impaired transcription of *FAS*. Reduced expression of death receptors have been detected in many different tumours and it has been described to occur both at protein and mRNA levels. Several alterations, such as mutations, epigenetic silencing or altered regulation by transcription factors, have been demonstrated to decrease the Fas expression (195-197). The frequency and importance of these molecular mechanisms are not well defined and needs to be further investigated.

Mutations of *FAS* have been detected in a wide range of solid and lymphoid tumours. Apart from a few mutations that cause a translational stop codon in *FAS* and as a consequence abolish Fas expression (198, 199), the majority of the mutations have been found to result in the expression of a dysfunctional Fas protein (29). Loss-of function mutations are most frequently found in exons 8 and 9, which constitutes the intracellular part of Fas (29). Sequencing of *FAS* in our resistant cells did not reveal any mutations in either the coding sequence or the promoter of *FAS*. In summary, this suggests that repression of *FAS* expression more frequently depend on other factors than gene mutations.

Changes in epigenetic regulation, by altered promoter methylations or global chromatin modifications, are often detected in tumours. Methylation of DNA occurs at cytosine at the 5-carbon position in CpG dinecleotides and regions with high CpG content is referred to as CpG islands (134). Interestingly, hypermethylations of *FAS* have been shown to regulate Fas expression in colon carcinoma (197). Furthermore, it was recently demonstrated that oncogenic Ras mediates epigenetic silencing of the *FAS* promoter (196). However, genomic sequencing of bisulfite-modified DNA in our resistant cells showed that there were no changes in the methylation pattern of a 650bp CpG island in the *FAS* promoter. Moreover, treatment of the resistant cells with a demethylating agent did not restore Fas surface expression or Fas sensitivity. Thus, although other regions than the 650pb island studied could have been methylated, this indicates that the reduced Fas expression is not mediated by epigenetic silencing through methylations.

FAS transcription can be regulated by several transcription factors that are under the control of important signalling molecules regulating growth and survival. For example, p53, AP-1, YB-1, STAT3, YY1 and NF-κB can regulate *FAS* transcription (200-205). The regulation and interplay between different transcription factors is complex and several factors can have multiple binding sites within one promoter. For example, c-Jun in cooperation with STAT3 has been shown to suppress *FAS* transcription by binding to the core region of the *FAS* promoter (203). In contrast, c-Jun was found to induce *FAS* expression by binding to the enhancer region in another study (204). Thus, the importance of different transcription factors for the regulation of *FAS* expression is not fully established. Moreover, the transcription factors involved in stimuli induced up-regulation of *FAS* are generally more investigated then those that mediate basal transcription of *FAS* (173, 200, 205, 206). In this respect it would be interesting to compare the binding of transcription factors to the *FAS* promoter in our resistant versus parental cells.

Survival pathways such as the PI3-Kinase and the MAP-Kinase pathway can interfere with death receptor signalling in multiple ways, which includes activation of various transcription factors such as c-Jun and NF-κB (65, 71, 72, 207). Notably, we detected increased signalling of the PI3-Kinase and the ERK

pathways in our Fas resistant cells. However, treatment with specific inhibitors of these two pathways, either separately or in combination, did not abolish the Fas resistance. This suggested that increased signalling from the PI3-Kinase and the ERK pathways was not important for the acquired resistant phenotype or regulation of Fas expression.

Increased cFLIP expression in Fas resistance

(Paper III)

There are numerous reports showing that the Fas signalling cascade can be disrupted at multiple sites and increased expression of anti-apoptotic proteins has been reported in several cancers (106). In the resistant clones with normal Fas expression we could detect an impaired activation of caspase-8 (paper II). This made us hypothesise that an early event downstream of Fas receptor activation was impaired in these resistant clones. Recently, it has become apparent that cFLIPs are important modulators of death receptor signalling at the DISC (34). Interestingly, we could detect increased protein expression of cFLIP-L in our resistant clones with normal Fas expression. cFLIPs are as many other anti-apoptotic proteins short lived and can therefore be inhibited with translational inhibitors like cycloheximide (CHX) (208). Inhibition with CHX restored sensitivity to Fas induced apoptosis in our resistant cells and correlated to a reduced expression of cFLIP-L. This indicates that cFLIP-L functions as an inhibitor of Fas induced apoptosis in our resistant cells. To confirm that cFLIP-L was mediating the resistance to Fas induced apoptosis, shRNA interference were used to specifically target cFLIP expression. As inhibition of cFLIP-L abolished the Fas resistance and resulted in re-established activation of caspase-8, we conclude that the resistance to Fas induced apoptosis in the clones with normal Fas expression is caused by an increased cFLIP-L expression. This is in agreement with other studies showing that increased expression of cFLIP is associated with resistance to apoptosis.

In our resistant clones, the cFLIP-L expression was only increased by 2-fold. Importantly, very small amount of cFLIP-L is sufficient for caspase-8 regulation and cFLIP-L is recruited more efficiently than caspase-8 to the DISC upon Fas activation (37, 209). Thus, a two fold increase in cFLIP-L might be enough for inhibition of Fas signalling. Heterodimerization of cFLIP-L with caspase-8 has been demonstrated to accelerate the initial activation of caspase-8 but then inhibit its complete processing into a fully active state. The partial activated caspase-8 is also retained within the DISC, promoting caspase-8 non-apoptotic signalling (38, 210). The restricted activation of caspase-8 leads to processing of the cFLIP-L molecule, generating a p43-cFLIP-L fragment (37,

38). Recently, this fragment was demonstrated to more efficiently recruit the TRAF2 and RIP-1 kinases and subsequently activate NF-kB (40, 211). This could explain why a 2 fold increase of the p43-cFLIP-L fragment but not of the full-length cFLIP-L was detected in the DISC of our resistant cells upon Fas activation.

The expression of cFlip can be regulated by transcription factors, such as NF-κB, NFAT, p53 and c-Myc (34). However, the increased expression of cFLIP-L in our Fas resistant clones is regulated at the protein level as qRT-PCR of cFLIP-L mRNA detected equal amounts of transcript in both resistant and parental cells. Protein expression of cFLIP-L has been reported to be under the control of several signalling pathways, including the ERK- and PI3-Kinase pathways (74, 80, 212). In paper II, enhanced signalling of these pathways were detected in the resistant cells. Importantly, inhibition of PI3-Kinase and ERK did not abolish the resistance to Fas stimulation, which suggests that the increased cFLIP-L expression is caused by other regulatory events. An additional way to regulate cFLIP-L expression is through ubiquitination and proteasomal degradation (34). However, the degradation rate of cFLIP-L was similar in parental and resistant cells after CHX treatment for 0, 6, 12 and 24h, which indicated that there was no decreased stability or increased degradation rate of cFLIP-L in the resistant cells. In summary, we have not yet been able to indentify the reason for the increased cFLIP-L expression in the resistant cells and forthcoming studies will hopefully increase the knowledge about the regulation of cFLIP-L expression.

General aspects of Fas resistance

(Paper II and III)

Tumours develop heterogeneity because of their genomic and epigenetic instability. In this respect, it was not surprising to find alterations of several unrelated proteins in our resistant cells. The U937 cell line is derived from a patient suffering from histiocytic lymphoma, which means that the parental cells are a heterogenic population with increased capacity to grow and survive. Thus, the adaption of Fas resistance in this system is either generated by additional genomic and epigenetic abnormalities or outgrowth of cells that already had gained intrinsic survival advantages in the patient.

Notably, the resistant clones that contained decreased Fas expression also had an increased activity of AKT and ERK signalling. Importantly, re-introduction of Fas restored sensitivity whereas inhibition of AKT and ERK had no effect on the resistance (Paper II). In addition, an increased expression of cFLIP-L was confirmed to mediate the resistance by inhibition through shRNA

knockdown (paper III). However, increased expression of Cyp33 (discussed above) as well as enhanced AKT and ERK signalling (paper II) were not involved in mediating the Fas resistance. In summary this illustrates the complexity of resistance development in tumour cells. Even though several abnormalities can be detected, which of them that is functionally important for tumour survival is the important question. The large-scale mutational analyses that are under development will certainly facilitate the identification of genomic abnormalities but the importance and contributions of each alteration will be challenging to elucidate.

When the susceptibility to apoptosis induced by other death receptors was investigated in the resistant cells, we could detect a complete cross resistance in the clones with increased expression of cFLIP-L and a partial resistance to TNF-α in the clones with reduced Fas expression. The fact that increased expression of cFLIP-L mediates resistance to TRAIL and TNF-α is not surprising since these two death receptors induce apoptosis in a similar way as the Fas receptor, i.e. through caspase-8 activation (16). However, the reduced sensitivity to TNF-α in the resistant clones with decreased amount of Fas was unexpected. QRT-PCR demonstrated that the resistant cells expressed normal levels of the TNF-receptors, which excluded that the resistance is caused by a general defect in the regulation of death receptor transcription. Interplay between different death receptors have been suggested as they share some of the intracellular signalling molecules (213-216). In addition, as TNF-α is a cytokine, it induces activation of transcription factors like NF-κB and c-jun, which regulates expression of death receptors and their ligands (17, 217, 218). Notably, we could exclude that Fas expression was needed for TNF-α induced apoptosis because restoration of Fas expression abolished only Fas and not TNF-α resistance. Therefore, we speculate that the resistance to TNF-α either represents an independent molecular alteration or that it is caused by impaired functions of factors regulating Fas transcription as well as TNF-α apoptotic signalling.

Impaired IFN-α signalling in tumour progression

(Paper III)

IFNs are induced by pathogens and they have a critical function in the development and function of the immune system. Moreover, IFNs can directly induce growth inhibition and apoptosis in target cells (158). IFNs have therefore been shown to be useful in the treatment of viral infections and cancers. However, the biological properties of IFNs are extremely complex and they need to be thoroughly investigated in order to improve their therapeutic

potential. Only a few human tumour cell lines respond to IFN-α with apoptosis. To investigate what molecular events that mediates reduced sensitivity to IFN-α induced apoptosis, the IFN-α sensitive U937 cell line was used. Resistant cells were selected by growth in increasing concentrations of IFN-α. The resistant population (IFNres) was subcloned and four clones were isolated (Fig. 12).

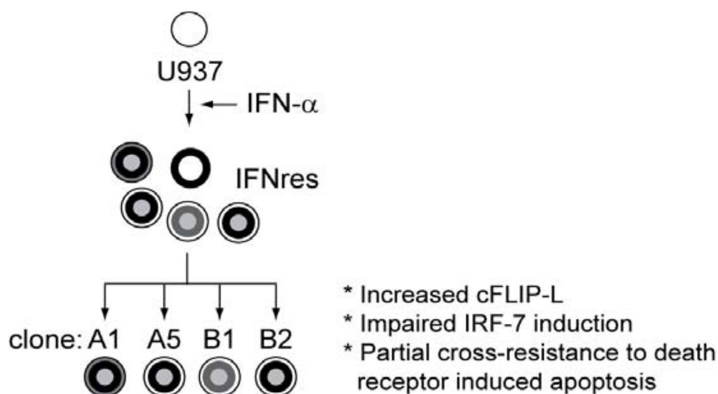

Fig. 12. Generation of IFN resistant cells. To generate IFN-α resistant cells, sensitive U937 cells were grown in the presence of gradually increasing concentrations of IFN-α for 9 weeks. Four individual clones were isolated from the resistant population, A1, A5, B1 and B2. Characterization of these clones showed that they diverged slightly in their protein expression profiles but some common alterations could be detected in all four clones. These included an increased cFLIP-L expression, an impaired induction of IRF-7 following IFN-α treatment and a partial cross resistance to death receptor induced apoptosis.

A large-scale genomic screen by micro array analysis would probably have been the best way to indentify genes with altered expression in the IFN resistant cells but the fact that IFN-α regulates the expression of several hundreds of genes restrained us from taking this approach. Instead, key signalling molecules in the IFN signalling cascade were studied. We found that the resistant cells contained a decreased expression of important IFN signalling molecules that normally are activated by the IFN receptors (STAT2, TYK2 and JAK1). However, downstream signalling events, such as increased expression of STAT1 and induced expression of PKR, were not impaired. This suggests that other molecular alterations were causing the resistance, which is in contrast to several studies that have shown that altered expression of STATs are involved in tumour resistance against IFN-α (219-222). However, resistance to IFN-α in a melanoma cell line has been suggested to depend on effects downstream or independent of the JAK/STAT pathway, which are in agreement with our study

(223). This illustrates that there is heterogeneity in the mechanisms regarding resistance to IFN-α in different cellular systems.

Increased cFLIP expression in IFN resistance

When a partial cross resistance to death receptor signalling was found in the IFN resistant cells, we hypothesized that the resistance was caused by alterations of the core apoptotic machinery. Indeed, an increased expression of cFLIP-L was detected in the IFN-resistant cells and inhibition of cFLIP-L through shRNA interference restored sensitivity to IFN-α and death receptor induced apoptosis. Pervious studies have suggested that cFLIP could negatively regulate IFN induced apoptosis, as increased cFLIP expression inhibits apoptosis induced by PKR and IRF-1 over-expression as well as dsRNA treatment (178, 181, 224). Notably, this is the first time increased cFLIP-L expression have been demonstrated to mediate acquired resistance to IFN-α induced apoptosis.

As tumour cells have an unstable genome they easily adapt to various stimuli and thus, have an increased capacity to induce resistance with time. The observation that selection for resistance to IFN-α induced apoptosis generated cells displaying cross-resistance to death receptor induced apoptosis is problematic because it indicates that molecules in the central part of apoptotic machinery was targeted rather than IFN specific molecules. This might complicate and reduce the treatment efficiency of tumour cells with IFN-α as well as other apoptosis inducing agents.

The pathway leading to IFN-α mediated apoptosis is not completely determined but several important factors have been identified. For example, caspase activation is important for IFN-α induced apoptosis since specific inhibitors abolished the apoptotic response by IFN-α. Some studies have suggested that FADD and caspase-8 are key regulators of IFN-α induced apoptosis, whereas others have claimed that the intrinsic pathway and caspase-9 activation are the most important events (179, 225, 226). As cFLIP-L is an inhibitor of caspase-8, it is evident that caspase-8 activation in necessary for IFN-α induced apoptosis in U937 cells.

Preliminary data have indicated that the induction of one important ISG, IRF-7, is not induced at mRNA and protein level following IFN stimulation in the IFN resistant cells (Fig. 13A and 13B). In contrast, the induction of PKR, which is an important pro-apoptotic protein induced by IFN stimulation, was detected to similar extent in IFN resistant and parental cells (Fig. 13C). IRF-7 is an interferon response factor that has been reported to be one of the key regulators of IFN-α production and it is a central regulator of type I IFN-regulated immune responses (227, 228). Over-expression of IRF-7 affects the

expression of hundreds of genes and some of these are involved in apoptosis signalling (229). The role of IRF-7 in IFN induced apoptosis has however not been thoroughly investigated. Recently, it was shown that FADD-/- murine embryonic fibroblasts displayed an impaired induction of *Irf7* upon viral infection and dsRNA treatment. Moreover, the presence of viral FLIP blocked complex formation of signalling molecules upstream of IRF-7 and abolished *Irf7* induction (230). This raises the question whether cFLIP-L can interfere with FADD and diminish *Irf7* induction upon IFN-α treatment. However, if the impaired induction of IRF-7 expression is involved in the IFN resistance and in that case how, is yet to be determined.

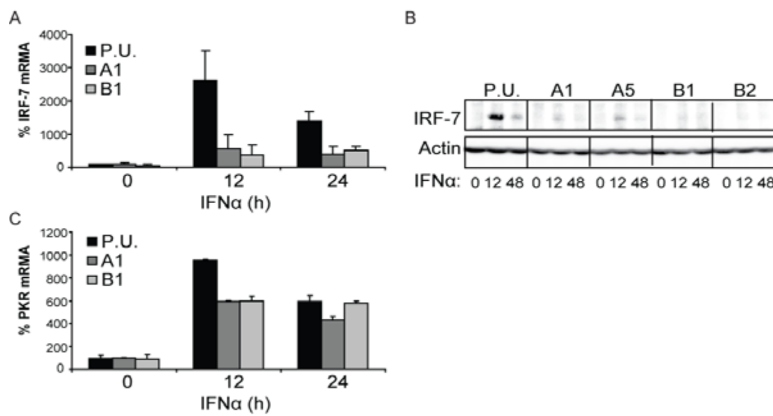

Fig. 13. Reduced induction of IRF-7 following IFN-α treatment of resistant cells. Parental and IFN resistant cells were stimulated with 1000 U/ml IFN-α for indicated time points in the figures. A) Induction of IRF-7 mRNA expression was measured with qRT-PCR and relative quantification was made by normalization to GAPDH. B) The IRF-7 protein expression was investigated by immunoblot analysis. Actin was used as a control for equal loading. C) Relative quantification of PKR expression was made by qRT-PCR where each sample was normalized to GAPDH.

IFN induced cell growth inhibition versus apoptosis

It is generally accepted that apoptosis and cell cycle regulation are intimately linked. For example, several oncogenes and tumour suppressor genes, such as c-Myc, Ras, p53, Bcl-2, NF-κB and pRb have been reported to regulate both apoptosis and proliferation (231). Many cancer therapies, including IFN, induce both inhibition of cell growth as well as activation of apoptosis (158, 232). The pro-apoptotic and anti-growth properties of IFN-α depend mainly on STAT1

and STAT2 activation (163, 165, 233), , which suggests that the two different responses are regulated through induction of various ISGs. The dual resistance to growth inhibition and apoptosis induction by IFN in our resistant cells could not be explained by a dysfunctional activation of STAT1. Thus, the decreased susceptibility to IFN induced growth arrest and apoptosis is mediated by events either downstream of ISG activation or independent of the JAK/STAT pathway. Interestingly, a correlation between resistance to growth inhibition and apoptosis induced by IFN could not be found in three haematopoietic tumour cells lines, which is in contrast to our results. However, the fact that the experimental setup selected for apoptosis resistant cells with normal growth potential makes it impossible to conclude whether the resistance against IFN induced apoptotsis and growth inhibition are linked or if they represent two independent events.

General aspects of acquired resistance in U937 cells

It was interesting to find that separate selection for apoptosis resistance against both IFN-α and Fas stimulation, generated resistant cells with increased expression of the same anti-apoptotic protein, cFLIP-L. cFLIP-L is abundantly expressed in normal tissue, e.g. neurons, endothelial cells, dendritic cells (DC), macrophages (MQ), monocytes and cardiac myocytes (34). Notably, monocytes contain relatively low amount of cFLIP and differentiation to DCs or MQs are associated with a substantial increase in its expression. This correlates to the Fas sensitivity, as monocytes are sensitive to Fas stimulation while DCs and MQs are Fas resistant (234, 235).

The increased expression of cFLIP in our resistant cells could be explained in at least two different ways. First, it is tempting to speculate that U937 cells might be in a primed state for increased expression of cFLIP-L as they are of monocytic origin. Thus, various stress stimuli like IFN-α and Fas stimulation might easily increase the expression of cFLIP-L. Secondly, the heterogenicity of the U937 population may include cells that have acquired an altered phenotype and therefore have increased amount of cFLIP-L. Selection for survivors from such a population would generate cells with increased cFLIP-L independent of the death stimuli as long as it involves activation of caspase-8. Nevertheless, it is important to notice that cFLIP-L has a profound relevance for acquisition of apoptosis resistance in U937 cells. The survival promoting property of cFLIP-L is in accordance to several studies showing that increased cFLIP-L expression correlates to apoptosis resistance in various tumours (236). The function and regulation of cFLIP-L has gained a lot of attention and the development of inhibitors that directly target cFLIP-L is an

important issue in tumour treatment, since it can be used in combination with other therapies for treatment of apoptosis resistant tumours.

CELL DEATH BY LOW DOSE IRRADIATION

(Paper IV)

Although significant progress has been made in the treatment of cancers, more specific therapeutic agents are needed to minimize cancer morbidity and mortality. Thus, increased understanding of the molecular mechanisms that mediates cell death in response to various therapeutic modalities is needed in order to improve the efficiency of tumour eradication. To increase the specificity of drug delivery to tumours, radioimmunotherapy has emerged as an alternative treatment of radiation therapy. β-emitting radionuclides, like ^{131}I and ^{90}Y, conjugated to tumour specific antibody derivates have been used in preclinical and clinical trails (102). In order to extend the knowledge about cell death induced by ^{131}I in solid tumours, the human tumour cell line, HeLa Hep2, was used. Our results show that ^{131}I treatment induces a transient G2/M arrest which is followed by induction of an abnormal mitotic phenotype. After DNA damage, a sustained G2/M arrest is generally induced by p53 and p21, which allow for DNA repair or death if the damages are too severe (58). However, as the activity of p21 and p53 are repressed by human Papilloma virus proteins in HeLa Hep2 cells, only a transient G2-arrest will be induced which might be insufficient for successful DNA repair (237). Thus, the cells will re-enter the cell cycle prematurely with damaged DNA. Therefore, we could detect multiple abnormalities like multinuclei, multiple centrosomes and mulitpolar spindles in ^{131}I treated cells. Notably, hyperamplification of centrosomes has been linked to p53 deficiency, which is in agreement to our study (238).

Cell death executed during or after mitosis as a consequence of mitotic abnormalities is often referred to as mitotic catastrophe. In accordance with our study, it has been suggested that mitotic catastrophe is the main form of cell death mediated by ionizing radiation (94). Mitotic catastrophe that occurs during mitosis may include caspase activation and engagement of the intrinsic apoptosis signalling pathway. For example, this cell death can be suppressed through inhibition of caspases (95). However, mitotic catastrophe is preferentially followed by non-apoptotic cell deaths, as caspase activation and DNA fragmentations is often absent (105). One explanation for this can be that tumour cells that acquire an increased susceptibility to mitotic catastrophe, via the loss of p53, at the same time become more reluctant to apoptosis induction. In our study, we could observe activation of caspase-2, -8 and -9 after the induction of mitotic catastrophes. This indicates that a delayed form of

apoptosis is associated with mitotic catastrophe after treatment with ^{131}I in HeLa Hep-2 cells. Caspase-2 has been postulated to be an important initiator caspase in DNA damaged induced cell death, as caspase-2 depletion inhibits mitotic death upstream of the mitochondria (239). Hence, we argue that the increased activities of caspase-2, -8 and -9 detected in the HeLa Hep2 cells after ^{131}I treatment contribute to the delayed p53 independent cell death of abnormal mitotic cells.

Mitotic catastrophe is generally induced in cells with impaired regulation of cell cycle checkpoints because proper cell cycle arrests normally allow for DNA reparation or cell death if the damage is extensive. Notably, the regulation of cell cycle progression is often dysfunctional in tumours cells and induction of mitotic catastrophe might therefore be an effective way to target tumour cells.

CONCLUDING REMARKS

- Acquisition of Fas resistance in the human monocytic cell line U937 generated cells with multiple alterations. However, only two of the molecular abnormalities detected could be connected to the resistant phenotype, namely decreased Fas expression and increased cFLIP-L expression.
- The reduced Fas expression was demonstrated to be due to impaired transcription in the resistant cells and re-established Fas expression abolished the Fas resistance. Notably, this resistance mechanism accounted for the major resistant phenotype, which indicates that suppression of Fas expression is a likely outcome during sustained Fas stimulation of U937 cells.
- An increased cFLIP-L expression was not only detected in Fas resistant clones but also in U937 cells selected for resistance to IFN-α induced apoptosis. Inhibition of cFLIP-L expression by shRNA interference confirmed that the resistance to Fas and IFN was mediated by cFLIP-L. This demonstrates that U937 cells are prone to increase cFLIP-L expression upon stress stimulation with apoptosis inducing agents and it highlights the pivotal role of cFLIP-L in apoptosis resistance.
- We detected multiple mitotic aberrations when the cell death response induced by the β-emitting radionuclides ^{131}I was investigated in the HeLa-Hep2 tumour cell line. The alterations included centrosome amplification, multipolar mitotic spindles and nuclear abnormalities. These mitotic aberrations induced a mitotic catastrophe which was accompanied by delayed caspase activation.

In conclusion, this thesis reflects on the complexity of apoptosis regulation during development of tumour resistance and treatment of malignant cells. Identification of which altered molecules that is important for the establishment of resistance to cell death is a key issue for effective tumour treatment. Our studies demonstrate that cFLIP-L inhibition as well as restored death receptor expression could be two important approaches. Furthermore, mitotic catastrophe executed by a delayed type of apoptosis is an important cell death modality following low dose irradiation. Increased understanding of this cell death will hopefully aid to improve the efficacy of cancer treatment.

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