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ELUCIDATION OF PRO-APOPTOTIC SIGNALING INDUCED BY CISPLATIN

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TO MY FAMILY

Shoot for the moon. Even if you miss, you will land among the stars.

Les Brown

ABSTRACT

A major clinical problem regarding treatment of malignant tumors with anticancer drugs is inherent or acquired resistance to therapy. One factor contributing to drug resistance of cancer cells is failure to undergo apoptosis. The aim of this thesis was to investigate pro-apoptotic signaling induced by the anti-cancer drug cisplatin in order to contribute to future improvement of cisplatin-based cancer therapy.

The results demonstrate the ability of cisplatin to induce at least two separate proapoptotic pathways, and to affect several cellular organelles. The ERK and the JNK pathways are the two main mitogen-activated protein kinase (MAPK) modules of serial kinase activities stimulated by proliferative signaling as well as by cellular stress. The role of ERK in cisplatin-induced apoptosis was studied in four melanoma cell lines. ERK activation was seen 4 h after cisplatin treatment. Inhibition of the pro-survival ERK pathway resulted in different responses, from sensitizing to slightly protective. The cellular response to a certain type of treatment can thus not always be predicted.

Cisplatin treatment was found to elicit increased intracellular calcium levels and activation of calpain within 1-3 hours. Involvement of calpain in the apoptotic response was demonstrated using calpain inhibitors which blocked caspase activation and nuclear fragmentation. Moreover, the calpain inhibitor calpeptin was able to block cisplatin-induced cleavage of the pro-apoptotic Bid. Recombinant Bid was cleaved in vitro by recombinant calpain as well as lysates of cisplatin-treated cells. Apoptosis via the intrinsic (mitochondrial) pathway is known to require activation of the pro-apoptotic Bcl-2 proteins Bak and Bax. We demonstrate the ability of cisplatin to induce activation of Bak, and suggest a kinase fragment of MEKK1 (?MEKK1) as mediator of Bak activation, since activation was inhibited in cells expressing a kinase-inactive mutant of MEKK1 (dominant negative MEKK1, dnMEKK1). Involvement of ?MEKK1 in Bak activation was supported by the ability of a constitutively active kinase mutant of MEKK1 (dominant positive MEKK1, dpMEKK1) to activate Bak in three cell lines out of four, and to induce apoptosis in two of them. In contrast to dnMEKK1, calpeptin did not affect Bak activation but did block Bid cleavage. On the other hand Bid cleavage was not affected by dnMEKK1. Calpeptin and dnMEKK1 were both able to block nuclear fragmentation and caspase activation by approximately half. When used together, they had an additive inhibitory effect on apoptosis.

Using enucleated cells (cytoplasts) we demonstrated the ability of cisplatin to induce apoptosis in the absence of nuclear DNA. Cisplatin treatment of cytoplasts resulted in calpain-mediated Bid cleavage as well as calcium- and calpain-dependent activation of caspase-3. Moreover, calpeptin-sensitive activation of pro-caspase-12 was found in cisplatin-treated cells and cytoplasts. Caspase-12 is localized at the endoplasmic reticulum (ER) and is activated by ER stress. Cisplatin-induced ER stress was supported by increased expression of grp78, an ER chaperone protein.

In summary, we have characterized two novel pro-apoptotic pathways induced by cisplatin and investigated the time course of their activation. One pathway is activated early and involves calcium-dependent activation of calpain and calpain-mediated cleavage of Bid. The other pathway involves ? MEKK1-mediated activation of Bak shortly before onset of caspase-3. We have also identified ER as a new, non-nuclear target of cisplatin, and suggest ER stress as a potent mediator of cisplatin-induced proapoptotic signaling.

LIST OF PUBLICATIONS

The thesis is based on the following papers:

I. **Mandic A**., Viktorsson K., Heiden T., Hansson J. and Shoshan C.M.
The MEK1 inhibitor PD98059 sensitizes C8161 melanoma cells to cisplatin-induced apoptosis. *Melanoma Research,* 11:11-19

II. **Mandic A***., Viktorsson K*., Molin M., Akusjärvi G., Eguchi H., Hayashi S-I., Toi M., Hansson J., Linder S. and Shoshan C.M.
Cisplatin Induces the Proapoptotic Conformation of Bak in a ?MEKK1-Dependent Manner. *Mol.Cell.Biol.* 21:3684-3691

III. **Mandic A**., Viktorsson K., Strandberg L., Heiden T., Hansson J., Linder S. and Shoshan C.M.
Calpain-Mediated Bid Cleavage and Calpain-Independent Bak Modulation: Two Separate Pathways in Cisplatin-Induced Apoptosis. *Mol.Cell.Biol.* 22:3003-3013

IV. **Mandic A**., Hansson J., Linder S. and Shoshan C.M.
Cisplatin Induces ER Stress and Nucleus-Independent Apoptotic Signaling
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* Authors contributed equally to this work

CONTENTS

ABBREVIATIONS

AIF	Apoptosis inducing factor
ANT	Adenine nucleotide translocator
Apaf-1	Apoptosis protease-activator factor 1
AP-1	Activator protein-1
ASK	Apoptosis Stimulating Kinase
ATF-2	Activating transcription factor-2
ATP	Adenosine three phosphate
BAPTA-AM	Cell permeable calcium chelator
Bcl-2	B cell lymphoma-2
BH	Bcl-2 homology
CAD	Caspase-activated DNase
Caspase	Cysteinyl aspartate proteinases
CREB	cAMP response element binding protein
DED	Death effector domain
DISC	Death-inducing signaling complex
DNA-PK	DNA-dependent protein kinase
dnMEKK1	dominant negative MEKK1
dpMEKK1	dominant positive MEKK1
EGF	Epidermal growth factor
eIF2-α.	Translation initiation factor
ER	Endoplasmic reticulum
ERK	Extracellular signal regulated kinase
FADD	Fas-associated death domain
Fas-L	Fas-ligand
GRP	Glucose-regulated proteins
HMG	high-mobility group
IAP	Inhibitor of apoptosis protein
ICAD	Initiator of CAD
ICE	interleukin -1β converting enzyme
InsP3	Inositol-1,4,5-triphosphate
JNK/SAPK	c-Jun NH2-terminal protein kinase/stress activated protein kinase
MAPK	Mitogen-activated protein kinase
MAPKK	MAPK kinase
MAPKKK	MAPK kinase kinase
MEF	Mouse embryo fibroblasts
MEK	MAPK/ERK kinase
MEKK	MEK kinase
MMP	Mitochondrial membrane permeabilization
MMR	Mismatch repair
NER	Nucleotide excision repair
NFκB	Nuclear factor-kappa B
PARP	Poly (ADP-Ribose) polymerase
PBR	Peripheral benzodiazepine receptor

PCD	Programmed cell death
PKC	Protein kinase C
PTP	Permeability transition pore
PTPC	PTP complex
SH3	Src-homology 3
SEK1	SAPK/ERK kinase
tBid	Truncated Bid
TNF	Tumor necrosis factor
TRADD	TNFR-associated death domain
TRAF	TNF-related apoptosis factor
TRAIL	TNF-related apoptosis inducing ligand
UPR	Unfolded protein response
VDAC	Voltage dependent anion channel
PERK	PKR-like ER kinase
PKA	Protein kinase A
PKR	RNA-activated protein kinase
RyR	Ryanodine receptor
SERCA	Sarcoplasmic/endoplasmic Ca^{2+}-ATP transporter
XIAP	X-linked inhibitor of apoptosis protein
? m	Mitochondrial membrane potential

1 INTRODUCTION

1.1 HALLMARKS OF CANCER

Tumorigenesis is a multistep process consisting of genetic alterations that drive the progressive transformation of normal cells into highly malignant cells. Tumor development proceeds via a succession of genetic changes, each conferring one or another type of growth advantage, leads to progressive conversion of normal cells into cancer cells. Six essential alterations in cell physiology that contribute to malignant growth have been suggested (Hanahan & Weinberg, 2000). These include: self-sufficiency in growth signals, insensitivity to growth-inhibitory signals, limitless replicative potential, sustained angiogenesis, tissue invasion and metastasis and evasion of programmed cell death.

1.2 CISPLATIN

cis-Diamminedichloroplatinum(II), (cisplatin or *cis*-DDP) was the first platinum anticancer drug introduced in the clinic and is one of the most potent chemotherapeutic drugs. It is highly effective in the treatment of many malignancies, including testicular, ovarian, bladder, cervical, head and neck and lung cancers (Eastman, 1991). The cellular uptake of cisplatin is not fully understood. It has been suggested that drug enters the cells partly by passive diffusion through transmembrane channels and partly by facilitated diffusion through an as yet unidentified membrane transport system (Gately & Howell, 1993). Upon diffusion into cells, two chloride ligands are replaced by water molecules, generating a very reactive, positively charged molecule which can interact with nucleophilic sites of cellular proteins, membrane phospholipids, RNA and DNA (Andrews & Howell, 1990).

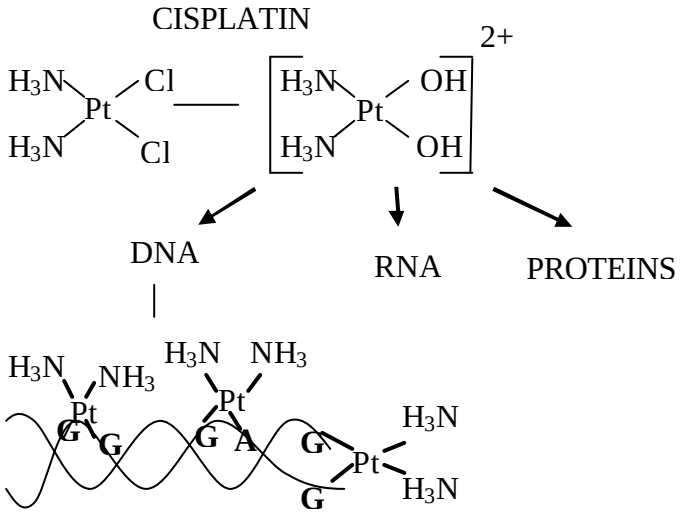

Fig.1. Cisplatin and its intracellular targets

Approximately 1% of the intracellular cisplatin reacts with nuclear DNA to yield intra- and inter-strand DNA crosslinks and DNA-protein crosslinks. The most common adducts are intra-strand crosslinks between adjacent guanines and between neighboring guanine and adenine, representing 65% and 25%, respectively, of the total number of adducts formed (Eastman, 1991).
Although cisplatin is recognized as a DNA-damaging agent, and DNA platination is generally accepted as the essential step in the cytotoxic activity of the drug, the mechanism(s) whereby the DNA damage kills cells is not fully understood. Inhibition of DNA synthesis was initially considered to be the main cause of cisplatin´s cytotoxicity. However, it has been shown that cisplatin-induced cell death does not always correlate with inhibition of DNA synthesis, since cisplatin induces apoptosis at concentrations that do not inhibit synthesis of DNA(Sorenson & Eastman, 1988a; Sorenson & Eastman, 1988b) . Besides inhibition of DNA replication and arrest in G1, cisplatin treatment has been reported to result in inhibition of RNA transcription, arrest in the G2 phase of cell cycle and programmed cell death (apoptosis).

The specific mechanism(s) that trigger apoptosis in response to cisplatin-induced DNA damage have not yet been defined. Such mechanisms must include ways to detect the damage and determine whether it is lethal or not. Proteins that recognize cisplatin-induced DNA damage include: nucleotide excision repair (NER) proteins, mismatch repair (MMR) proteins, DNA-dependent protein kinase (DNA-PK) and high-mobility group (HMG) proteins(Gonzalez et al., 2001) .
Cisplatin adducts are removed from DNA mainly by nucleotide excision repair (NER). The MMR system recognizes but does not remove the cisplatin adducts since it always replaces the incorrect sequence in the daughter strand, leaving the cisplatin adduct unrepaired. This initiates futile repair cycles which may generate DNA breaks and activate pro-apoptotic signals (Perez, 1998).
Binding of DNA-PK to damaged DNA results in phosphorylation and activation of two proteins involved in pro-apoptotic signaling, c-Abl and p53. c-Abl has been reported to be involved in DNA damage-induced activation of the stress-activated protein kinase pathway. Phosphorylation of p53 results in inhibition of its ubiquitination, leading to increased stabilization of the protein. p53 can initiate apoptosis by transcriptionally activating pro-apoptotic Bcl-2 family members such as Bax, Bak, Puma and Noxa, and repressing anti-apoptotic Bcl-2 proteins (Bcl-2, Bcl-xL) and IAPs (survivin). In addition, p53 can transactivate other genes that may contribute to apoptosis, including Apaf-1, PTEN, CD95 and TRAIL receptor 2 (Schuler & Green, 2001). The HMG proteins are a multifunctional family of small non-histone chromatin-associated proteins involved in gene regulation and maintenance of chromatin structure. Binding of HMG proteins to cisplatin adducts may protect the adducts from recognition by DNA repair enzymes, resulting in higher cytotoxicity. Moreover, since these proteins have high affinity for cisplatin-modified DNA, binding to DNA adducts may hijack these proteins away from their normal binding sites, thereby disrupting a variety of cellular processes and potentially leading to cell death (Gonzalez et al., 2001).
The major limitations associated with the use of cisplatin in the clinic are side effects such as nephrotoxicity and neurotoxicity, and resistance of tumor cells to the treatment. Some tumors have intrinsic resistance while others develop resistance during treatment. A defective apoptotic program is one of the major contributors to cisplatin resistance, together with increased drug efflux, decreased drug influx, increased cellular

glutathione and metallothionein levels, increased DNA repair and oncogene expression (Perez, 1998). In order to better understand the cytotoxicity of cisplatin and improve the therapeutic response, it is necessary to elucidate the molecular mechanisms of cisplatin-induced cell death.

1.3 APOPTOSIS

The fact that cells die during normal development was recognized during amphibian metamorphosis (Vogt, 1842) and was later found to occur in many developing tissues of both invertebrates and vertebrates. The term programmed cell death (PCD) was suggested in 1965 (Lockshin & Williams, 1965a; Lockshin & Williams, 1965b) and has been used to describe a cell death in which cells follow a sequence of genetically controlled steps towards their own destruction. PCD was found to occur in predictable places and at predictable times during development. It was also discovered that PCD serves as a major mechanism for removal of unwanted and potentially dangerous cells, such as virus-infected cells, self-reactive lymphocytes or tumor cells (Lockshin & Zakeri, 2001).
The term "apoptosis" was proposed by Currie and colleagues (Kerr et al., 1972) and was originally used to describe the morphological characteristics of a certain type of cell death which differed from necrosis.

1.3.1 Morphological and biochemical hallmarks: apoptosis versus necrosis

During necrosis, the cell swells, mitochondria dilate, organelles dissolve and the plasma membrane ruptures, releasing cytoplasmic material into extracellular medium which results in an inflammatory response. By contrast, no rupture of plasma membrane and no release of cellular compounds occur during apoptosis. Two groups have presented evidence that high ATP levels are required during the apoptotic process, and that the level of intracellular ATP determines whether a cell will die by apoptosis or necrosis (Eguchi et al., 1997; Leist et al., 1997). This may explain the importance of maintaining mitochondrial function during early phases of apoptosis.
Morphological changes typical for apoptosis include cell shrinkage, chromatin condensation, blebbing of the cell membrane and exposure of phosphatidylserine. In healthy cells phosphatidylserine is located on the inner surface of the plasma membrane but is exposed on the outer surface in apoptotic cells, and thereby provides a signal for engulfment by adjacent phagocytic cells. In vitro, apoptotic cells fragment into membrane-enclosed vesicles (apoptotic bodies), whereas in vivo they are recognized and removed by phagocytes.
Biochemical hallmarks of apoptosis include the activation of endonucleases, cleavage of DNA into oligonucleosomal fragments and activation of the caspase (Cysteinyl aspartate proteinases) family of proteases(Desagher & Martinou, 2000) .

1.3.2 The biochemistry of apoptosis

The apoptotic process can be divided into several phases: (1) *Initiation phase*. Many different signals, either from within or outside the cell, have been shown to induce cell death. These include receptor-mediated signals from the plasma membrane, genotoxic and physical stress, oncogene expression, etc. In this phase, the signals are detected and multiple signaling pathways are induced in response to the stimuli; (2) *Effector phase*, in which the signals are transduced and amplified within a cell in order to execute the cell's decision to die; (3) *Execution phase.* The activated apoptotic machinery acts on different cellular targets to cleave DNA and specific cellular proteins.

There are two initiator pathways which each may activate the execution of cell death: the death receptor pathway and the mitochondrial pathway. Activation of either of these pathways results in activation of caspases, the main executors of cell death.

1.3.2.1 Caspases: the central executors

Caspases are a family of cysteine proteinases which specifically cleave their substrates after aspartic acid residues. The distinct substrate specificity is determined by the four residues amino-terminal to the cleavage site. Caspases reside in the cell as inactive proenzymes and require proteolytic cleavage for activation. They are divided into two subfamilies; proximal or initiator caspases, and terminal or effector caspases.

Initiator caspases include caspase -1, -2, -4, -5, -8, -9, -10 and -12. They have a long prodomain containing protein interaction motifs which allow interaction of caspases with adaptor proteins. Binding of adaptor proteins promotes activation of initiator caspases. It seems that most of the long prodomain-containing caspases are activated via oligomerization-induced autoproteolysis. Upon activation, these initiator caspases process and activate the effector caspases.
Effector caspases are thus mainly activated by another proteinase, in most cases a caspase, although cleavage of procaspase-12 and -7 by calpain or procaspase-3 by granzyme B has also been reported. Effector caspases possess short prodomains and include caspase-3, -6, -7 , -11 and -13. Upon activation, caspases, mainly caspase-3 and -7, cleave their specific substrates contributing to the morphological and functional changes associated with apoptosis, e.g., nuclear shrinking is caused by cleavage of nuclear lamin, and loss of cellular shape by cleavage of cytoskeletal proteins. DNA fragmentation requires cleavage and inactivation of ICAD, the initiator of CAD (caspase-activated DNase) (Herr & Debatin, 2001) whereas DNA repair is impaired by the caspase-3 mediated cleavage of poly (ADP-Ribose) polymerase (PARP).
Thus, apoptotic signaling induced by various stimuli converges on the activation of caspases followed by prevention of DNA repair, DNA fragmentation and destruction of cellular structure.

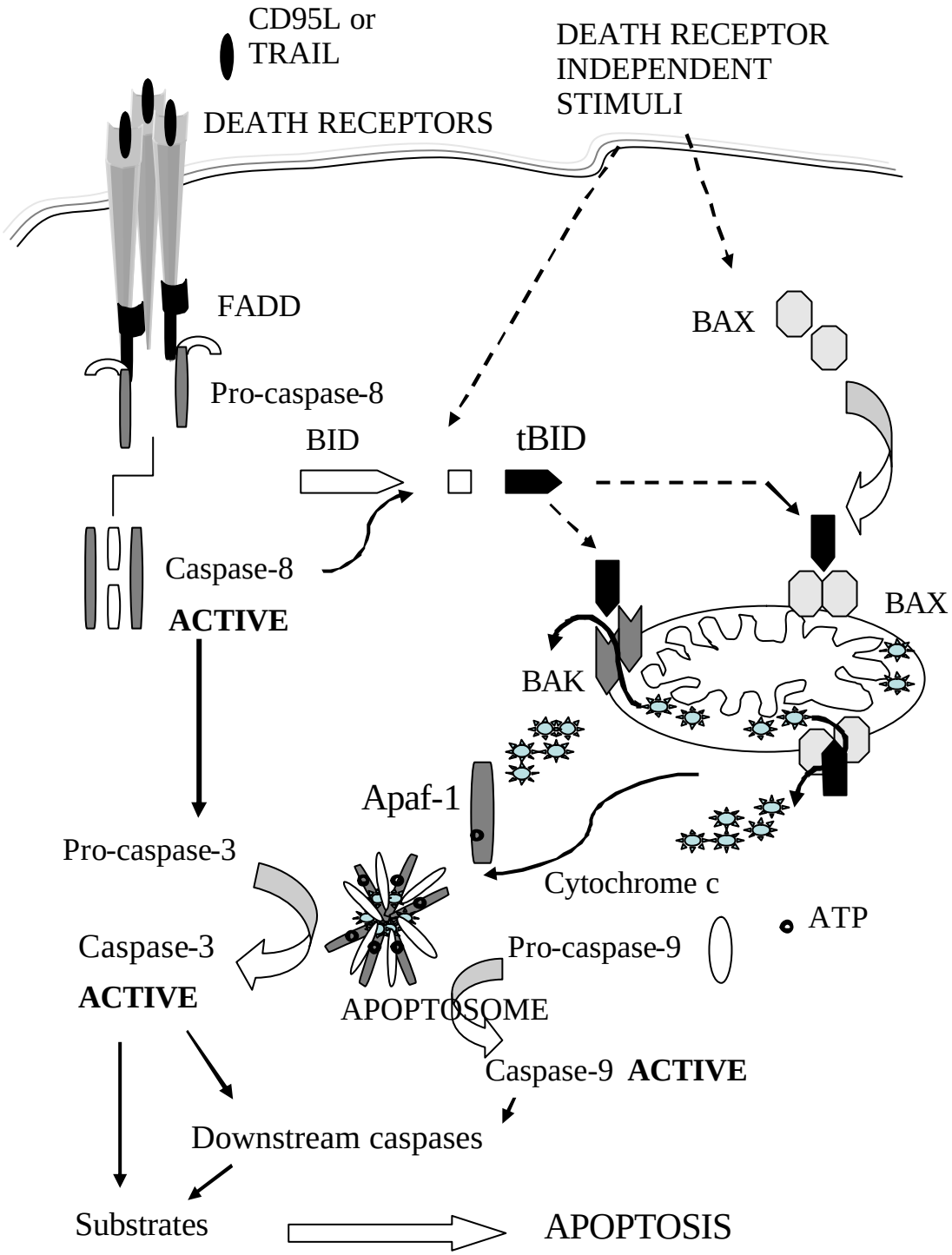

Fig.2. The roads to death: two major apoptotic pathways in mammalian cells

1.3.2.2 Death receptors as initiators of caspase activation

The best-studied members of the death receptor family are Fas (also known as CD95 or Apo-1) and TNF receptor-1 (TNFR1). Death receptors contain an intracellular death domain. Binding of specific ligands to death receptors [CD95L (Fas ligand) or TRAIL (TNF-related apoptosis inducing ligand)] is followed by receptor trimerization and recruitment of adaptor proteins to the cytoplasmic death domain. Specific adaptor proteins (FADD, Fas-associated death domain and TRADD, TNFR-associated death domain) bind to the death domain of the receptor via their own death domains, forming a complex called DISC (death-inducing signaling complex).
FADD carries also a death effector domain (DED) which is responsible for recruitment of DED-containing procaspase-8 to the DISC. Procaspase-8 is proteolytically activated upon binding to DISC and may then cleave various proteins, including procaspase-3 (Krammer, 2000). Cleavage and activation of procaspase-3 is necessary for activation and execution of the cell death program.
This direct activation of a caspase cascade does not necessarily involve mitochondrial events. However, caspase-8 has been shown to mediate cleavage of the cytosolic protein Bid, a proapoptotic member of the Bcl-2 family. Upon cleavage, Bid translocates to mitochondrial membrane where it is involved in cytochrome c release and subsequent caspase activation (Gross et al., 1999b; Luo et al., 1998), demonstrating the integration of the two initiator pathways.

1.3.2.3 The role of mitochondria in the apoptotic response

Various signals induced by stress stimuli such as cytotoxic drugs, DNA-damaging agents, hypoxia, heat shock, growth factor withdrawal, irradiation and death-receptor signaling converge on mitochondria. The mitochondrial events observed in response to cellular stress include permeabilization of the mitochondrial membrane(s) and release of death-promoting proteins located in the intermembrane space.

The mechanism of mitochondrial membrane permeabilization is not completely understood but based on present knowledge, three different models are proposed.
Model I Opening of the permeability transition pore (PTP)
The permeability transition pore is a polyprotein complex formed at the contact sites between the outer and the inner mitochondrial membrane (Marzo et al., 1998). Two core proteins of the PTP complex (PTPC) are VDAC, located in the outer membrane and adenine nucleotide translocator (ANT), in the inner mitochondrial membrane. Other proteins involved in the pore complex are: cyclophilin D, a soluble protein of the mitochondrial matrix, peripheral benzodiazepine receptor (PBR), hexokinase II and creatine kinase. Opening of the PTP, e.g., permeabilization of the outer and the inner mitochondrial membranes, induces a cascade of events such as depletion of ATP, dissipation of mitochondrial membrane potential (?? m) and Ca^{2+} release from the mitochondrial matrix (Zoratti & Szabo, 1995).
Opening of the PTP also allows influx of water into the mitochondrial matrix, causing it to swell and rupture the outer membrane, leading to release of intermembrane proteins. However, disruption of mitochondrial structure is a hallmark of necrosis rather than apoptosis. It has therefore been suggested that opening of the PTP in apoptotic cells is transient, allowing release of proteins from the intermembrane space

but preserving the ATP production and intact mitochondrial structure (Martinou & Green, 2001) .

Model II According to this model, Bcl-2 proteins may interact with proteins in the outer mitochondrial membrane, such as the voltage dependent anion channel (VDAC), and thereby regulate this channel's activity (Shimizu et al., 1999). Since the pore size of VDAC is too small for passage of cytochrome c, it has been suggested that pro apoptotic Bcl-2 members induce a conformational change in VDAC, which leads to an increase in channel size. Anti-apoptotic Bcl-2 members would, according to this model, promote closure of the channel and thereby inhibit cytochrome c release (Shimizu et al., 2000).

Model III suggests insertion of pro-apoptotic Bcl-2 family members into the outer mitochondrial membrane, followed by formation of channels for passage of proteins localized in the mitochondrial intermembrane space. It has been shown that Bcl-2 family proteins can insert into synthetic lipid bilayers, oligomerize and form channels (Reed, 1997) but it remains unclear whether these channels exist in cells and whether they would be large enough for passage of intermembrane proteins.

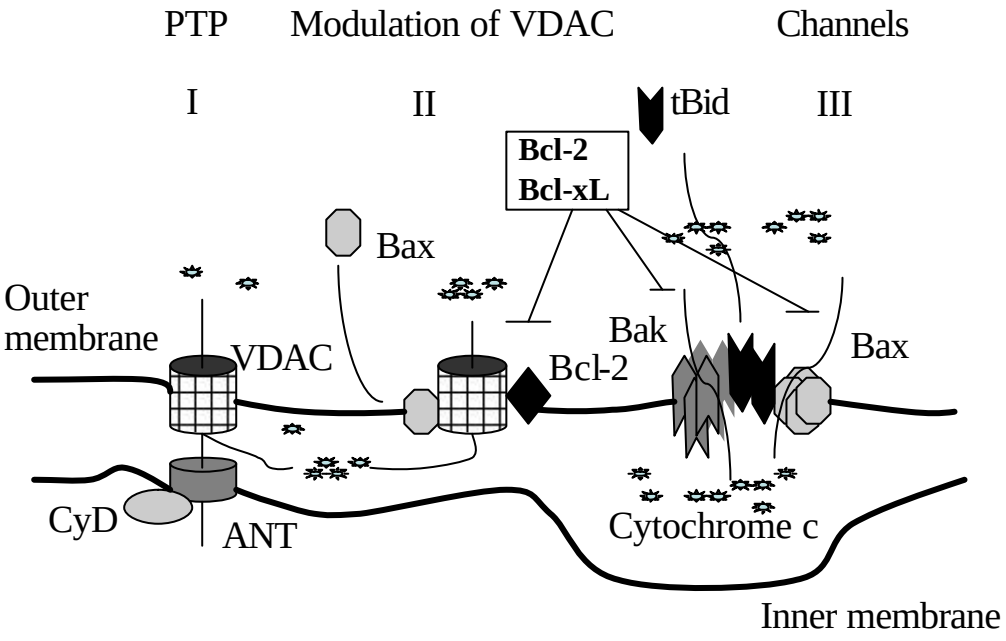

Fig.3. Mechanisms of mitochondrial membrane permeabilization

1.3.2.4 Mitochondrial proteins released during apoptosis

Cytochrome c was the first apoptogenic intermembrane protein shown to be released from mitochondria during apoptosis. Upon release from mitochondria, cytochrome c, together with the apoptosis protease-activator factor 1 (Apaf-1), dATP and caspase-9 forms a caspase-activating complex termed the apoptosome (Li et al., 1997; Zou et al., 1997). The apoptosome is required for processing and activation of caspase-9 which then activates caspase-3 and caspase-7 and thereby initiates the caspase cascade (Slee et al., 1999; Van de Craen et al., 1999).
Smac/DIABLO is a mitochondrial intermembrane protein released during apoptosis, together with cytochrome c. Smac/DIABLO interacts with XIAP (X-linked inhibitor of apoptosis protein) and prevents the inactivation of caspase-3 and caspase-9 (Srinivasula et al., 2001). Besides its interaction with XIAP, Smac/DIABLO binds other IAPs (inhibitor of apoptosis protein) including c-IAP1, c-IAP2 and survivin (Du et al., 2000; Verhagen et al., 2000).
Omi/HtrA2 is a mitochondrial serine protease which shares functional properties with Smac/DIABLO (Faccio et al., 2000; Gray et al., 2000). It is also released from the intermembrane space after an apoptotic stimulus and upon release interacts with cytosolic IAP proteins (Suzuki et al., 2001).
Induction of apoptosis may result also in the release of ***endonuclease G*** from mitochondria and its translocation to the nucleus where it is involved in nuclear breakdown (Li et al., 2001; van Loo et al., 2001). Endonuclease G-induced DNA fragmentation is independent of caspase activation (Li et al., 2001; van Loo et al., 2001) in contrast to fragmentation mediated by CAD/DFF40, the best characterized DNase.
The mitochondrial intermembrane space contains also ***AIF*** (apoptosis inducing factor) which translocates to the nucleus in response to apoptotic stimuli where it mediates fragmentation of DNA into 50 kb fragments (Susin et al., 2000; Susin et al., 1999).AIF-mediated effects are not inhibitable by the caspase-inhibitor zVAD, suggesting a caspase-independent role in apoptosis.

1.3.3 The Bcl-2 family

The family of Bcl-2 related proteins is named after the first identified member, Bcl-2, which was discovered at the chromosomal breakpoint of t(14:18)-bearing human B-cell lymphomas. The primary site of action of Bcl-2 family proteins is mitochondria and modulation of mitochondrial membrane permeability seems to be one of the major mechanisms by which Bcl-2 family proteins regulate apoptosis (Tsujimoto & Shimizu, 2000). In humans, more than twenty members of this family have been discovered to date. They can be divided into two functional groups: anti- and pro-apoptotic proteins. Members of the first group such as Bcl-2, Bcl-xL, Bcl-W, Mcl-1, Bfl-1, are characterized by four short Bcl-2 homology (BH) domains (BH1-BH4). They also possess a C-terminal hydrophobic tail allowing these proteins to be anchored to the membranes of mitochondria, ER and nucleus.
The proapoptotic members can be further subdivided into the Bax subfamily, including Bax, Bak, Bok which contain BH1, BH2 and BH3 domains, and the group named "BH3 only" (Bid, Bad, Bim, Bik, Blk, Hrk) which possess only the BH3 domain, also called the "minimal death domain". Pro-apoptotic family members are normally found

in the cytosol or are loosely associated with membranes. After a death signal, these proteins translocate to the intracellular membranes, mostly the outer mitochondrial membrane, where they either insert into the membrane or interact with other proteins. Most Bcl-2 family proteins can interact with each other, forming homodimers, heterodimers and oligomers, and may in this way act as agonists or antagonists of their binding partners. Dimerization occurs through interaction between the amphiphatic BH3 α-helix of the proapoptotic proteins and the hydrophobic groove of the anti-apoptotic members, created by the α-helices in the BH1, BH2 and BH3 regions (Gross et al., 1999a).

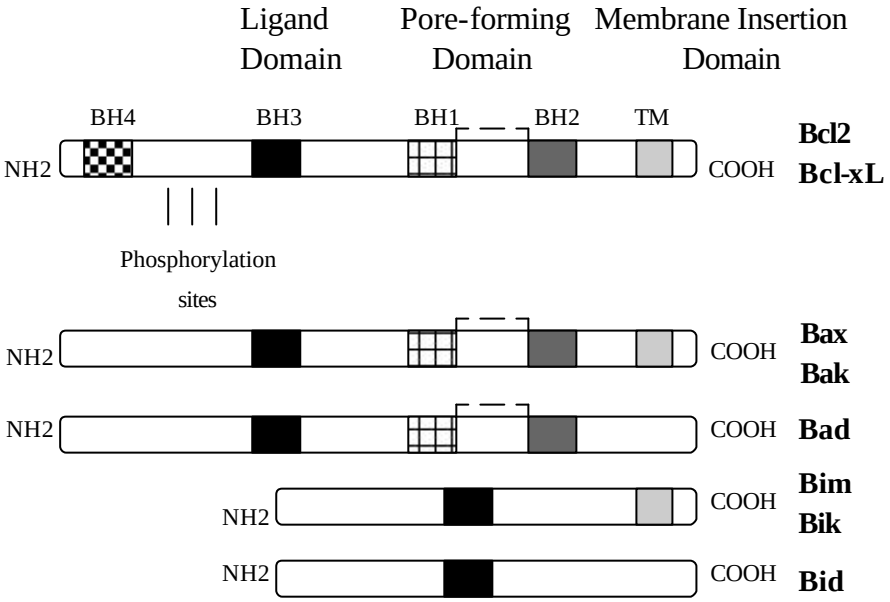

Fig.4. Bcl-2 family members

1.3.3.1 Anti-apoptotic proteins, Bcl-2 and Bcl-xL

Bcl-2 is a powerful inhibitor of cell death with the ability to abrogate both caspase-dependent and -independent modes of cell death. Involvement of Bcl-2 has been demonstrated in various processes, including regulation of calcium homeostasis (Lam et al., 1994; Marin et al., 1996), modulation of antioxidant pathways (Hockenbery et al., 1993), promotion of gluthathione sequestration to the nucleus (Voehringer et al., 1998) and abrogation of cytochrome c release from mitochondria (Kluck et al., 1997; Yang et al., 1997b). Bcl-2 resides in the outer mitochondrial membrane, ER and nuclear membranes (Krajewski et al., 1993; Lithgow et al., 1994). Bcl-2 can bind also non-homologous proteins such as Raf-1 and calcineurin. Bcl-2 mediates translocation of Raf-1 to the vicinity of the mitochondrial membrane. Once there, Raf-1 phosphorylates Bad, a pro-apoptotic BH3 only protein, the role of which is to heterodimerize with Bcl-2 and/or Bcl-xL and abrogate their anti-apoptotic function. Phosphorylated Bad dissociates from Bcl-2 and Bcl-XL and forms a cytosolic complex

with 14-3-3, a scaffold protein that inhibits interference of Bad with anti-apoptotic family members (Wang et al., 1996a; Zha et al., 1996). Moreover, by binding to calcineurin, a calcium-activated serine-threonine phosphatase, Bcl-2 may inhibit dephosphorylation of Bad (Wang et al., 1999).

Bcl-xL is located at the outer mitochondrial membrane. Bcl-xL, together with Bcl-2, has been suggested to prevent mitochondrial membrane permeability either by direct interaction with PTPC proteins, such as VDAC and /or ANT (Shimizu et al., 1999) or by inhibition of pro-apoptotic proteins such as Bax and Bak (Oltvai et al., 1993). Interaction of Bcl-2 and Bcl-xL with Bax and Bak inhibits the oligomerization of the pro-apoptotic proteins and/or their insertion into the mitochondrial membrane. A model wherein anti-apoptotic proteins inhibit activation of Bak and Bax by sequestering BH3-only proteins has also been suggested (Cheng et al., 2001; Zong et al., 2001). Furthermore, Bcl-xL can prevent caspase activation by sequestering Apaf-1 (Hu et al., 1998; Pan et al., 1998).
Posttranslational modifications such as phosphorylation and cleavage regulate the activity of Bcl-2 and Bcl-xL. Chemotherapeutic agents that cause microtubule disruption have been reported to induce phosphorylation of Bcl-2 and Bcl-xL, abrogating their anti-apoptotic function (Haldar et al., 1995; Poruchynsky et al., 1998). It has been suggested that phosphorylation within the loop region of the Bcl-2 protein may determine the susceptibility to the cleavage by altering the conformational change and making the cleavage site more accessible to proteases (Fadeel et al., 1999). Caspase-dependent cleavage of Bcl-2 and Bcl-xL may occur in response to e.g., Fas ligation, etoposide and growth factor withdrawal. Cleavage results in the exposure of the BH3 domains, converting these anti-apoptotic proteins into promoters of cell death (Fadeel et al., 1999). Cleavage of Bcl-2 and Bcl-xL can also be mediated by calpain, a calcium activated protease (Gil-Parrado et al., 2002).

1.3.3.2 Pro-apoptoti proteins, Bax, Bak and Bid

Bax and Bak

In healthy cells, Bax is located in the cytosol or is loosely attached to the outer mitochondrial membrane. In response to apoptotic stimuli, cytosolic Bax translocates to mitochondria, likely as a consequence of a conformational change, which exposes the hydrophobic C- terminal of Bax. This allows the C-terminal domain to insert into the mitochondrial membrane, promoting subsequent cytochrome c release (Nechushtan et al., 1999). Bax has been shown to interact with VDAC and ANT, suggesting a role in opening of the PTP (Shimizu et al., 1999). Binding of Bax to VDAC has been suggested to mediate a conformational change in VDAC, resulting in enlargement of the pore and allowing the passage of cytochrome c into the cytosol (Shimizu et al., 1999). Cleavage of Bax by calpain has been shown to generate an 18 kDa fragment which is even more potent in inducing cytochrome c release than the full-length protein (Gao & Dou, 2000; Wood & Newcomb, 2000). Bcl-2 was not able to interact with cleaved Bax (Gao & Dou, 2000).

In healthy cells, Bak is located in the outer mitochondrial membrane. During apoptosis Bak undergoes conformational changes, leading to exposure of its N-terminal epitope and to dissociation of Bcl-xL from Bak (Griffiths et al., 2001). These conformational changes of Bak were observed upon treatment with staurosporine, etoposide and dexamethasone, (Griffiths et al., 1999). The changes were not triggered in Fas-induced apoptosis, suggesting that signaling cascades may differ depending on the death stimulus. Involvement of Bak in apoptosis was demonstrated by the failure of different apoptotic agents, including cisplatin, etoposide, staurosporine and UV irradiation, to induce cytochrome c release in Bak deficient cells (Wang et al., 2001). However, in another study Bak deficient MEFs showed the same sensitivity to etoposide, staurosporine and UV irradiation as wild-type MEFs (Wei et al., 2001) indicating that Bak is not absolutely required for apoptosis. Both Bak deficient ($Bak^{-/-}$ $Bax^{+/+)}$ and Bax deficient ($Bak^{+/+}$ $Bax^{-/-)}$ cells were susceptible to pro-apoptotic stimuli including etoposide, staurosporine, UV irradiation and also ER stress inducing agents, whereas $Bak^{-/-}$ $Bax^{-/-}$ cells were completely protected (Wei et al., 2001). These results indicate that presence of either Bak or Bax is required for the apoptosis and that each of them efficiently promote the apoptotic response.

Bid

Bid is a BH3-only pro-apoptotic protein first noted for its capacity to bind either Bcl-2 or Bax and promote cell death. A model was then suggested in which Bid may serve as a death ligand which moves to the mitochondrial membrane to inactivate Bcl-2 and/or activate Bax (Wang et al., 1996b). Approximately 50% of endogenous Bid is soluble in the cytosol and 50% is associated with intracellular membranes, especially the ER membrane (Esposti, 2002). Cleavage of Bid by caspase-8 and translocation of the truncated protein (tBid) to the mitochondria represents the main molecular link between death receptor- and mitochondria-mediated proapoptotic signaling. Overexpression of Bcl-2 and Bcl-xL failed to prevent cleavage of Bid and its translocation to mitochondria in response to TNFα/Fas but it did abrogate the release of cytochrome c (Gross et al., 1999b).
The mechanism by which tBid targets mitochondria is not clear. It has been demonstrated that caspase-8-mediated cleavage of Bid is followed by N-myristoylation, which has been suggested to promote targeting of tBid to mitochondria (Zha et al., 2000). Cardiolipin is possibly also involved in tBid translocation since it is found in high concentrations at the contact sites between the outer and inner mitochondrial membranes, where tBid is preferentially targeted (Lutter et al., 2000).
Caspase-8 is not the only protease shown to cleave Bid. Granzyme B, a T-cell specific serine protease (Barry et al., 2000; Heibein et al., 2000; Sutton et al., 2000) as well as calpain, a calcium-activated cysteine protease(Chen et al., 2001; Mandic et al., 2002) have both been shown to cleave Bid and thereby activate the mitochondrial pathway. tBid has been reported to induce a conformational change in Bax, its oligomerization and insertion into the mitochondrial membrane (Desagher et al., 1999). Similarly, the conformational modulation and oligomerization of Bak were also shown to be mediated by tBid (Wei et al., 2000).
Genetic knockout studies confirm that Bax and Bak are downstream substrates of tBid since Bax-Bak double knock-out MEF cells are resistant to overexpression of tBid as well as to other pro-apoptotic stimuli, including staurosporine, etoposide, UV radiation

and ER stress (Wei et al., 2001). However, tBid is not the only activator of Bak and Bax, since Bid deficient cells were shown to be equally susceptible as wild type cells to the same pro-apoptotic stimuli (Wei et al., 2001).
The ability of Bid to bind phospholipids and lysolipids and to mediate transport of these molecules between membranes was recently demonstrated, suggesting a role of Bid in remodeling of the intracellular membranes (Esposti, 2002).

1.4 THE CELLULAR STRESS RESPONSE

Cells can sense and respond to changes in their environment, including alterations in the amount of nutrients, growth factors, changes induced by mechanical stress, radiation, heat, altered pH, osmolarity, etc. These physical and chemical alterations may affect different cellular functions such as proliferation, migration, differentiation and cell death. The cellular response to stress depends on the type, strength and duration of the stimuli and involves a complex network of signal-transduction pathways.
Among the best characterized pathways regulating cell survival and cell death are those mediated by the mitogen-activated protein kinase (MAPK) family. A MAPK cascade consists of a module of three cytoplasmic kinases: a mitogen-activated protein (MAP) kinase kinase kinase (MAPKKK), a MAP kinase kinase (MAPKK) and a MAP kinase (MAPK) itself. MAPK cascades, whose components are evolutionarily highly conserved in structure and organization, can sense and transduce growth-related as well as stress signals into changes in protein interactions and/or gene expression. MAPKKKs are serine-threonine kinases that receive activating signals and then activate its substrate, a MAPKK, by phosphorylation. MAPKKs are dual-specificity kinases with ability to phosphorylate serine and threonine residues in their substrates, MAPKs. MAPKs represent a family of serine-threonine kinases with ability to phosphorylate both cytoplasmic and nuclear substrates. When translocated to the nucleus, they are involved in control of gene expression by direct activation of transcription factors.
In mammals, there are three well-characterized subfamilies of MAPKs: extracellular signal regulated kinase (ERK), p38 and c-Jun NH2-terminal protein kinase/stress activated protein kinase (JNK/SAPK) groups of MAPKs (Davis, 2000).
These different MAPKs are regulated by distinct extracellular stimuli. JNK and p38 are predominantly activated by chemical and environmental stress and by inflammatory cytokines, and are recognized mainly as part of death-promoting pathways. In contrast, the ERK signaling pathway is activated by mitogens and growth factors, and transduces survival, proliferation and differentiation signals. All MAPKs are involved in the regulation of specific components of transcription factor AP-1(activator protein-1), e.g., c-Jun is regulated by JNK- or ERK-mediated phosphorylation, c-Fos is a substrate for ERK while ATF-2 can be phosphorylated by JNK and p38. The complex regulation of AP-1 may be involved in the broad range of responses on different cellular stimuli.

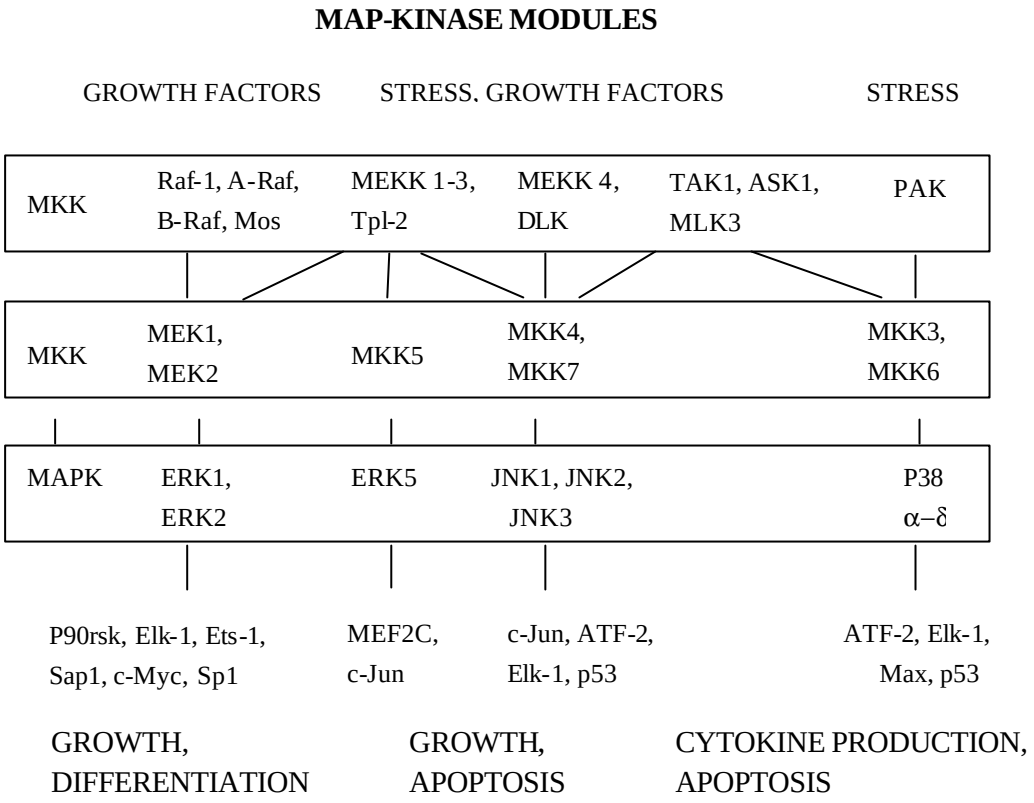

Fig.5. MAPK signaling cascades

1.4.1 Raf and Extracellular regulated kinase, ERK

The best understood MAPK signal transduction pathway in mammalian cells is the Raf-MEK1 (MAPK/ERK kinase)-ERK module. Proliferative signals such as growth factors cause activation and autophosphorylation of receptor tyrosine kinases, which via adaptor proteins and the Ras protein leads to activation of Raf. Activated Raf signals via its substrate MEK to ERK1 and/or ERK2 (Hagemann & Blank, 2001). The role of ERK in proliferation and cell cycle progression is at least in part mediated by induction of cyclin D1, a positive cell cycle regulator (Balmanno & Cook, 1999). However, although the ERK pathway is generally regarded as survival-promoting, ERK activation may also be part of the response to various stress-inducing stimuli including genotoxins, microtubule inhibitors and hydrogen peroxide. ERK activity was also reported to be required for anoikis (apoptosis induced by loss of cell adherance) in mouse embryo fibroblasts (MEF) (Rul et al., 2002). Inhibition of ERK activity correlates with activation of JNK and p38 signaling pathways as well as with induction of apoptosis (Xia et al., 1995). Inhibition of basal ERK activity appears to be sufficient to trigger p38 activity in caspase-dependent manner (Xia et al., 1995). The balance between the activities of survival-promoting pathways such as ERK and pro-apoptotic pathways such as JNK and p38 has been suggested to determine cell fate, indicating that the decision of a cell to live or die depends on the integration of multiple signals (Xia et al., 1995).

1.4.2 MEKKs and c-Jun NH_2-terminal kinase, JNK

The JNK pathway, also known as stress activated protein kinase (SAPK) pathway is activated primarily by cytokines and exposure to various stress stimuli including UV light, ? radiation, DNA-damaging drugs, TNF-α, protein synthesis inhibitors and microtubule-inhibiting drugs (Chen et al., 1996b).

1.4.2.1 The MEKK family

The MEKK proteins (MEKK1-4) are serine-threonine kinases consisting of a C-terminal catalytic domain and N-terminal regulatory domain. The size and sequence of the N-terminal domains differ between the family members. Compared to MEKK1 (196 kDa) and –4 (180 kDa), MEKK2 and 3 are only 80 kDa due to shorter N-terminal domains. The N-terminal domains of MEKK1 and -4 contain proline-rich regions and pleckstrin-homology (PH) domains. Proline-rich sequences are involved in binding of proteins containing Src-homology 3 (SH3) domains, while PH domains associate with polyphosphoinositides and mediate localization of proteins to specific regions of the plasma membrane. The N-terminal domains of MEKK2 and -3 lack specific sequence domains which in turn may explain their cytoplasmic localization.

MEKK1 is the only member of the MEKK family with ability to promote apoptosis (Widmann et al., 1998). The protein received its name after the discovery that it can phosphorylate and activate the MAPK MEK1, which up to then was thought to be activated only by Raf. The phosphorylation sites of MEKK1 and Raf are identical, and both kinases phosphorylate MEK1 on serines 218 and 222. However, whereas Raf-induced MEK1 activation effectively stimulates ERK, MEKK1 seems to induce only modest activation of ERK.
Instead, MEKKs appear to be preferentially involved in regulation of the two other MAPK signaling pathways, the SAPK/JNK and p38-pathways which are not activated by Raf. MEKK1 can directly bind to, phosphorylate and activate MKK4 (SEK1) which seems to be its major substrate. Activated MKK4 binds to JNK and activates it. In addition to MKK4, MEKK1 activates also MKK7, which activates JNK and p38 (Hagemann & Blank, 2001).
Activation of MEKK1 has been reported to be mediated by Ras (Lange-Carter & Johnson, 1994) and by two other small G proteins, members of the Rho-family, Cdc42 and Rac (Fanger et al., 1997), although the mechanism of MEKK1 activation by these proteins remains to be established. c-Abl is a proteine kinase shown to associate with and activate MEKK1 in response to DNA-damage, leading to MEKK1-mediated activation of JNK (Kharbanda et al., 2000). Several isoforms of PKC have also been suggested to participate in MEKK1-dependent activation of JNK but their involvement seems to be cell type- and stimulus-dependent and requires further investigation (Hagemann & Blank, 2001).

1.4.2.2 The role of MEKK1 in apoptosis

A role of MEKK1 in apoptosis was first reported as part of the response to survival factor withdrawal (Xia et al., 1995). Involvement of MEKK1 has since then been demonstrated in apoptosis induced by various agents, including cisplatin, taxol, etoposide, UV irradiation, TNFα and anti-Fas ligation (Widmann et al., 1997; Deak et al., 1995). MEKK1 has been suggested to be involved in both survival- and apoptotic signaling. According to one model, proposed by Widmann and colleagues, full-length MEKK1 mediates transduction of survival signals, whereas its caspase-generated 91 kDa cleavage fragment ?MEKK1 is involved in amplification of the pro-apoptotic signaling (Widmann et al., 1998). The protective role of full-length MEKK1 might thus be mediated by rapid, transient JNK activation and by activation of NF-?B (Hirano et al., 1996; Lee et al., 1997). The pro-apoptotic role of ?MEKK1 is indicated by its ability to induce apoptosis in several cell types and to sensitize cells to genotoxic damage (Johnson et al., 1996; Widmann et al., 1998; Widmann et al., 1997).

The kinase-inactive full-length MEKK1 mutant was not cleaved in response to etoposide treatment or UV irradiation, indicating that the kinase activity of MEKK1 is required for its cleavage (Widmann et al., 1998). Kinase activity is also required for its apoptotic activity since kinase-inactive ?MEKK1 was unable to promote apoptosis (Johnson et al., 1996).
The cleavage of MEKK1 has been suggested to be caspase-mediated since a caspase-3 cleavage-site (DTVD) has been found in the sequence of mouse MEKK1 and the cleavage was blocked in the presence of the viral caspase inhibitors p35 and CrmA (Widmann et al., 1998).
Cleaved MEKK1 has been suggested to potentiate further cleavage of caspase-3 and thereby amplify the caspase-cascade (Widmann et al., 1998) although the mechanism of this process is still unclear.
Cleavage of MEKK1 is not required for activation of JNK since JNK activation has been reported to correlate with phosphorylation/activation of full-length MEKK1 and to precede the appearance of cleavage product. Furthermore, the cleavage resistant MEKK1 mutant, despite its failure to induce apoptosis, activates the JNK pathway as efficiently as the wt protein (Widmann et al., 1998). Moreover, it has been demonstrated that cell death induced by ?MEKK1 is independent of JNK regulation or c-Jun transactivation (Johnson et al., 1996; Schlesinger et al., 2002). These results indicate differential involvement of full-length MEKK1 and its cleavage fragment in JNK activation and apoptosis.
One possible explanation for different substrate specificities may be different intracellular distribution, since full-length MEKK1 is found to be predominantly membrane-associated whereas ?MEKK1 is present in the cytosolic fraction (Widmann et al., 1998). Furthermore, it has been shown that 14-3-3 proteins can bind to full-length MEKK1 but not to the 91 kDa fragment and thus may be responsible for differential locations of MEKK1 and its fragments (Fanger et al., 1998).

1.4.2.3 c-Jun NH_2-terminal kinase, JNK

A major target of the JNK signaling pathway is activation of a transcription factor, AP-1, which is mediated by phosphorylation of c-Jun. JNK binds to the NH_2-terminal activation domain of c-Jun and phosphorylates it on Ser-63 and Ser-73, resulting in increased transcriptional activity of AP-1 (Pulverer et al., 1991; Smeal et al., 1991). The other major substrates are transcription factors: activating transcription factor (ATF)-2 and Elk-1 (Gupta et al., 1996). JNK appears to be essential for AP-1 activation caused by stress and some cytokines, but is not required for AP-1 activation in response to other stimuli (Yang et al., 1997a). Thus, the precise role of AP-1 in the response to JNK activation is not clear and is likely to be modified by the activity of other transcription factors that interact with AP-1 on the promoters of target genes (Davis, 2000).

The JNK protein kinases are encoded by three genes. The *Jnk1* and *Jnk2* genes are expressed ubiquitously whereas *Jnk3* expression is limited to brain, heart and testis. These genes are alternatively spliced resulting in at least ten JNK isoforms. Mice deficient in JNK1 or JNK2 are morphologically normal, but are immunodeficient due to severe defects in T cell function. In contrast, disruption of both *Jnk1* and *Jnk2* genes causes early embryonic death (Davis, 2000). Embryonic fibroblasts isolated from *Jnk1*$^{-/-}$ *Jnk2*$^{-/-}$ mice exhibit defects in AP-1 transcription activity, decreased proliferation and resistance to stress-induced apoptosis (Tournier et al., 2000).

JNK is activated by two protein kinases, MKK4 (SEK1) and MKK7. Although MKK4 and MKK7 are dual specificity kinases and can phosphorylate JNK on both Tyr and Thr, MKK4 and MKK7 appear to preferentially phosphorylate JNK on Tyr and Thr, respectively. The difference in specificity suggest that MKK4 and MKK7 may cooperate to activate JNK (Davis, 2000).

In addition to MEKKs, several other MAPKKKs have been reported to activate the JNK signaling pathway, e.g., the Apoptosis Stimulating Kinase (ASK) group (ASK1 and ASK2), TAK1, TPL2 and the mixed-lineage protein kinase group (MLK1-3, DLK and LZK) (Davis, 2000). Involvement of MEKK1 in JNK signaling has been demonstrated in fibroblasts derived from Mekk1$^{-/-}$ mice (Yujiri et al., 1998) and in ES cells with homozygous insertion of LacZ in the kinase domain of MEKK1 (Xia et al., 2000). Defects in JNK activation were observed in these cells upon treatment with a number of stimuli including microtubule destabilizing drugs, serum, reovirus infection and cold shock.

Involvement of ASK1 in JNK activation has been studied in *Ask*$^{-/-}$ embryonic fibroblasts. The results indicate that ASK1 is not required for acute JNK activation, but seems to be involved in sustained JNK activation and apoptosis caused by long-term treatment with TNFα (Tobiume et al., 2001). ASK1 activation has been observed in cisplatin-treated ovarian carcinoma cells (Chen et al., 1999). Tpl-2 and TAK1, however, do not seem to be involved in physiological regulation of JNK signal transduction pathway (Weston & Davis, 2002).

1.4.2.4 The role of JNK in apoptosis

The role of JNK in pro-apoptotic signaling has been investigated by identification of target genes induced by stress. The JNK/AP-1 pathway has been proposed to promote apoptosis by increasing the expression of pro-apoptotic genes such as Bak and TNFα and decreasing the expression of p53 and its target p21, which would prevent cell cycle arrest and promote apoptosis (Fan & Chambers, 2001). JNK has also been suggested to regulate the stability of p53 protein. JNK-mediated phosphorylation of p53 has been reported to inhibit ubiquitin-mediated degradation, and thereby stabilize the protein (Fuchs et al., 1998). However, more recent studies demonstrate that JNK is not required for UV radiation-induced accumulation of p53 (Tournier et al., 2000) and that p53 is not required for JNK-induced apoptosis (Chen & Tan, 2000). The potential role of p53 as a target of JNK signaling is therefore unclear.

JNK has also been observed to increase expression of Fas-L (Fas-ligand) (Faris et al., 1998; Kasibhatla et al., 1998). However, murine embryo fibroblasts prepared from *Jnk1*$^{-/-}$ and *Jnk2* $^{-/-}$ embryos (*Jnk* null MEFs) exhibit no defects in Fas-induced apoptosis, indicating that JNK is not required for Fas-mediated apoptosis but it can contribute by increasing the expression of Fas-ligand. In contrast, *Jnk* null MEFs did exhibit a defective apoptotic response to stress-induced stimuli, including UV radiation, the DNA alkylating agent methyl methansulfonate and the translational inhibitor anisomycin. The defect in apoptosis correlated with failure to induce mitochondrial depolarization, cytochrome c release and subsequent caspase activation (Tournier et al., 2000). Translocation of JNK to mitochondria has been reported in response to DNA damage (Kharbanda et al., 2000) supporting the involvement of mitochondria in JNK-mediated apoptosis. The Bcl-2 proteins are potential targets of JNK involved in regulation of cytochrome c release (Davis, 2000). Phosphorylation of Bcl-2 and Bcl-xL by JNK has been shown in vitro and is suggested to abrogate their anti-apoptotic functions (Maundrell et al., 1997; Yamamoto et al., 1999). JNK is also reported to phosphorylate the pro-apoptotic protein Bad resulting in abrogation of its pro-apoptotic function (Donovan et al., 2002).

Although involvement of JNK in pro-apoptotic signaling is generally accepted, apoptosis does not represent the only possible outcome of JNK activation, since most forms of stress do not cause apoptosis under conditions that are sufficient for JNK activation (Davis, 2000). This may be due to parallel activation of survival-mediating pathways such as ERK, Akt/PKB, NFκB that can block pro-apoptotic signaling (Xia et al., 1995). Increasing evidence in the literature suggests that the duration of JNK activation is important for the outcome, i.e., sustained JNK activation is associated with apoptosis, whereas transient activation primarily mediates pro-survival signaling (Chen et al., 1996a; Chen & Tan, 2000; Sanchez-Perez et al., 1998).

1.4.3 Cellular responses to ER stress

The endoplasmic reticulum (ER) is involved in regulation of protein synthesis, folding and trafficking of proteins, but also in regulation of intracellular calcium levels and cellular response to stress. Agents that block protein folding or export, inhibitors of protein glycosylation, and agents that affect calcium uptake and release from the ER can all lead to ER stress and ultimately cell death.
There are at least two functionally different mechanisms for protection of cells against ER stress. One response, known as the unfolded protein response (UPR), involves up-regulation of genes encoding ER chaperone proteins in order to increase protein folding activity and prevent protein aggregation. Another mechanism involves attenuation of translation to prevent further accumulation of malfolded proteins. However, prolonged ER stress and severe impairment of ER functions finally result in apoptosis.

1.4.3.1 Unfolded protein response (UPR)

The unfolded protein response results in increased expression of molecular chaperones such as Grp94 and Grp78/Bip that promote proper protein folding. These chaperones belong to a family of proteins whose expression was discovered under conditions of glucose deprivation and which were therefore named glucose-regulated proteins or GRPs. The GRPs are expressed constitutively in all cells. Increased transcription is induced in response to disruption of ER function, e.g., calcium depletion from the ER lumen, inhibition of asparagine (N)-linked glycosylation, protein misfolding and accumulation of unfolded proteins in the ER (Kaufman, 1999).
Ire1α and Ire1β are transmembrane serine/threonine kinases which are recognized as the ER stress sensor proteins and play important roles in transducing the stress signals from the ER to the cytoplasm and nucleus. Binding of unfolded proteins, accumulated in the lumen of ER to Grp78 competitively disrupts interaction between Grp78 and Ire1α, which upon dissociation from Grp78 oligomerizes and undergoes autophosphorylation via its cytosolic kinase domain. Upon activation, Ire1α recruits the cytosolic adapter protein TRAF2, which in turn activates the JNK pathway although the link between TRAF2 and proximal components of the JNK pathway is still unknown (Urano et al., 2000).
Ire1β possesses ribonuclease activity and cleaves 28S rRNA, thereby inhibiting protein translation (Iwawaki et al., 2001).

1.4.3.2 Attenuation of translation

ER stress activates an ER transmembrane protein, PERK (for PKR-like ER kinase, where PKR is RNA-activated protein kinase). During UPR, PERK undergoes oligomerization and autophosphorylation followed by phosphorylation of the general translation initiation factor eIF2-α.This results in inhibition of eIF2-α and downregulation of overall protein synthesis which protects from further accumulation of unfolded proteins.

1.4.3.3 Apoptosis

Apoptosis induced by ER stress involves transcriptional activation of genes possessing an ER stress element in the promoter region and activation of ER-associated caspase-

12. ER stress results in the cleavage of ER transmembrane protein ATF-6, followed by translocation of its cytosolic domain to the nucleus, where it functions as a basic leucine zipper transcription factor of the ATF/CREB family. ATF-6 leads to the activation of genes possessing an ER stress element in the promoter region. It has been shown that ATF-6 regulates transcription of proteins that increase protein folding in the ER lumen (including chaperones such as BiP/Grp78 and calreticulin) and CHOP/GADD153, a transcription factor that decreases expression of Bcl-2 and can therefore induce apoptosis.
Grp78 has been shown to bind to and form a complex with pro-caspase-12 and pro-caspase-7, thereby preventing their activation and release. Excessive ER stress, together with (d)ATP binding to the complex, may disrupt the complex, leading to translocation of active caspase-12 to the cytosol (Rao et al., 2002b).

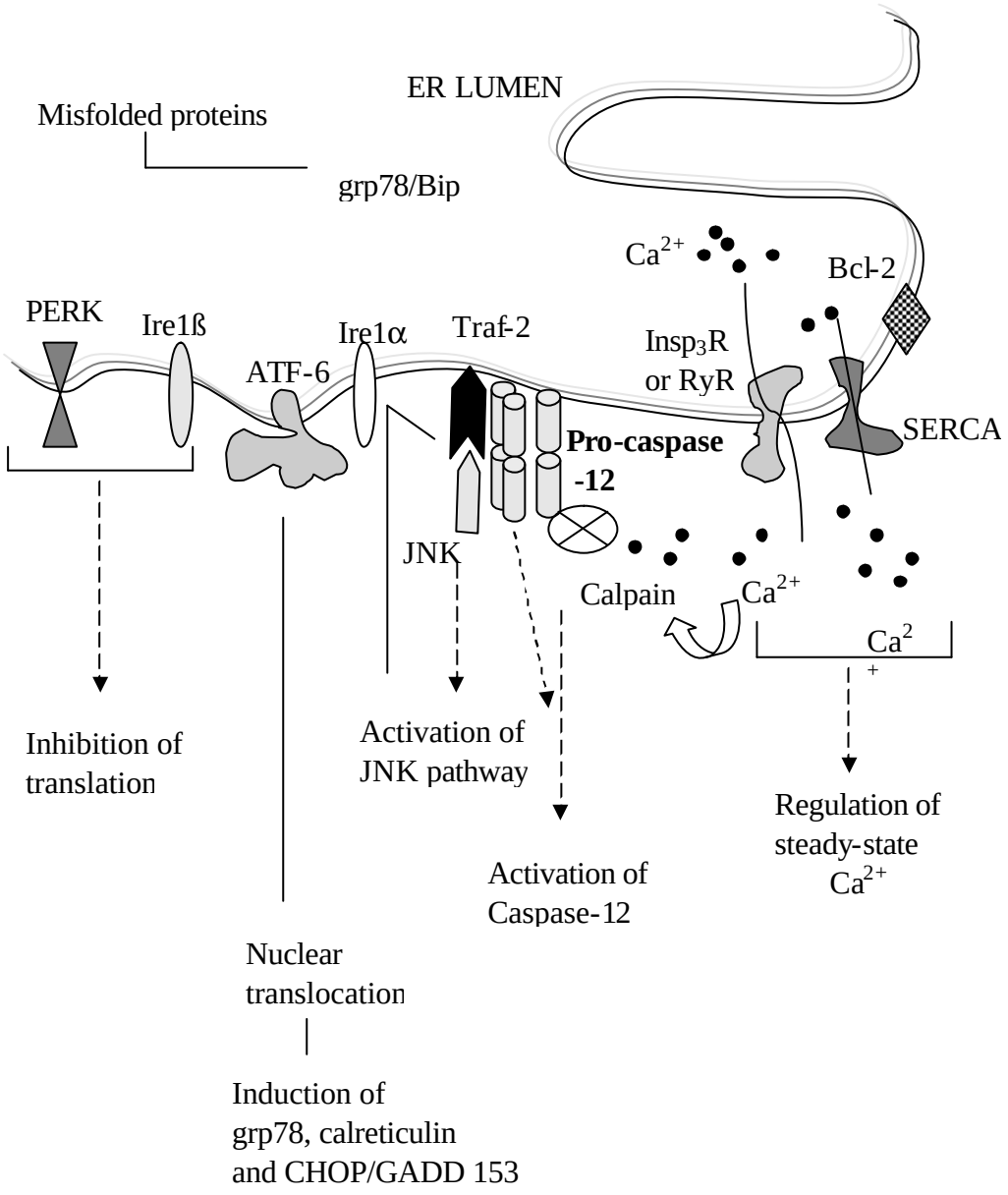

Fig.6. ER stress-mediated pro-apoptotic signaling

1.4.3.3.1 Caspase-12

Caspase-12 is a recently identified caspase predominantly localized at the ER and specifically activated by ER stress (Nakagawa et al., 2000). Apoptosis triggered through pathways that do not involve ER, such as serum deprivation, tumor necrosis factor (TNF) and Fas activation does not result in activation of caspase-12 (Nakagawa & Yuan, 2000; Nakagawa et al., 2000). In contrast, treatment with agents known to induce ER stress such as brefeldin A (inhibits ER-Golgi transport), tunicamycin (blocks N-linked protein glycosylation), thapsigargin (depletes intralumenal calcium stores by inhibition of SERCA, a Ca^{2+}-ATP transporter) and the calcium ionophore A23187, resulted in cleavage/activation of pro-caspase-12 (Bitko & Barik, 2001; Nakagawa et al., 2000). Caspase-12 deficient cells were resistant to tunicamycin-induced cell death but not to staurosporine or trophic factor deprivation (Nakagawa et al., 2000) supporting specific involvement of caspase-12 in ER stress-induced apoptosis.

Activation of caspase-12 requires its cleavage from the ER membrane surface by proteases that may be present on the ER surface or it may involve a translocation of cytoplasmic proteases to the ER. Recent studies have indicated that elevation of intracellular calcium levels causes activation and movement of calpain to the ER surface where it activates pro-caspase-12 (Nakagawa & Yuan, 2000). Similarly, ER stress was shown to induce translocation of caspase-7 to the ER where it participated in the cleavage of pro-caspase-12 (Rao et al., 2001).
Activation of caspase-12 may also occur without calpain or caspase-7-mediated proteolytic cleavage and in that case it involves Ire-1α and TRAF-2-mediated dimerization or oligomerization of pro-caspase-12, followed by its autoactivation (Yoneda et al., 2001).
Activated caspase-12 has been suggested to translocate to the cytoplasm where it may interact with caspase-9 and promote activation of cytosolic caspase cascade(Rao et al., 2001) . Recent studies have demonstrated the ability of caspase-12 to cleave and activate caspase-9 in vitro and in vivo (Morishima et al., 2002; Rao et al., 2002a). A proteolytic signal is in this way transmitted from caspase-12 to caspase-3, via caspase-9 without involvement of mitochondria.

1.4.3.4 ER calcium

Intracellular calcium concentrations are regulated by different mechanisms involving calcium channels, calcium binding proteins and sequestration to the ER and other intracellular spaces. The ER is the major organelle involved in calcium storage. Ca^{2+} in the ER lumen is either free or bound to luminal proteins such as calreticulin and calnexin. Ca^{2+} is taken up from the cytosol by the sarcoplasmic/endoplasmic Ca^{2+}-ATPase (SERCA), the ATP-dependent Ca^{2+} pump that transport calcium against its concentration gradient into the lumen of the ER. Inositol-1,4,5-triphosphate (InsP3) receptor/ Ca^{2+} channels or ryanodine receptor (RyR)/ Ca^{2+}channels are responsible for calcium release from the ER (Berridge et al., 2000).

Overexpression or alterations in membrane proteins may disrupt the ER membrane and permit calcium to leak out. Alternatively, membrane alterations may block the activity of the Ca^{2+}-ATPase and thereby block calcium uptake by the ER resulting in increased cytosolic calcium concentration (Kaufman, 1999). Depletion of the ER calcium store has been reported in apoptosis induced by various agents such as thapsigargin, calcium ionophores, cadmium (Kaufman, 1999).
Several answers have been suggested to the question: how is the ER calcium pool depletion coupled to the apoptotic machinery?
Increased cytosolic calcium concentration is a well- known activator of calpain, a Ca^{2+} -dependent cystein protease. The multiple role of calpain in apoptosis is described separately in this thesis and involves activation of caspase-12 (Nakagawa & Yuan, 2000), cleavage of Bid (Chen et al., 2001; Mandic et al., 2002), Bax (Gao & Dou, 2000; Wood et al., 1998), Bcl-xL and Bcl-2 (Gil-Parrado et al., 2002). An elevation in cytosolic Ca^{2+} concentration can also lead to activation of calcineurin, a phosphatase responsible for dephosphorylation and activation of Bad, a pro-apoptotic Bcl 2- family protein ((Wang et al., 1999).
Finally, calcium release from the ER results in the formation of high local concentrations of a Ca^{2+} sites of close contact between ER and mitochondria (Rizzuto et al., 1993). This may lead to mitochondrial calcium overload which is a potent stimulus for opening of PTP (Bernardi et al., 1998) resulting in cytochrome c release and activation of apoptotic cascade.

1.4.3.5 The role of mitochondria in ER stress-mediated cell death

There are conflicting reports in the literature regarding the involvement of mitochondria in ER stress-mediated apoptosis.
Mitochondrial events such as mitochondrial membrane permeabilization (MMP) and cytochrome c release has been reported in ER stress-induced apoptosis (Boya et al., 2002; Hacki et al., 2000). Mitochondrial calcium uptake during physiological conditions is of importance for regulation of cytosolic calcium concentrations, since it prevents activation of Ca^{2+} sensitive IP3 receptors (InsP3R) and further release of calcium from the ER (Duchen, 2000).
Mitochondrial Ca^{2+} uptake sites are concentrated in regions of the membrane apposed to the ER (Szalai et al., 1999) which may result in mitochondrial Ca^{2+} overload during ER stress when calcium release from ER is strongly increased. This in turn may lead to depolarization of mitochondria followed by cytochrome c release.

ER-targeted Bcl-2 protein has been shown to inhibit brefeldine A- and tunicamycin-induced cytochrome c release and caspase-3 activation (Hacki et al., 2000). It was also found to inhibit radiation-induced caspase-9 and caspase-3 activation and cell death to the same extent as mitochondria-targeted Bcl-2 and wt Bcl-2 (Rudner et al., 2001). Moreover, ER-targeted Bcl-2 inhibited loss of mitochondrial membrane potential, suggesting a molecular crosstalk between the ER and mitochondria during radiation-induced apoptosis (Annis et al., 2001).
Mouse embryonic fibroblasts lacking both Bak and Bax become resistant to apoptosis induced by tunicamycin and brefeldin A. It is still unclear if this depends on

involvement of Bak and Bax in the permeabilization of mitochondrial membrane and cytochrome c release, or on a still unidentified function of these proteins at the level of ER (Wei et al., 2001).

However, there are also contradictory reports demonstrating the independence of ER stress-induced apoptosis and mitochondria. It has been reported that tunicamycin and thapsigargin-induced activation of caspase-12 initiates a cascade, involving activation of caspase-9 and caspase-3 which is independent of the cytochrome c release (Morishima et al., 2002). Furthermore, Apaf-1 deficient fibroblasts, which are known to be partially resistant to apoptosis induced by tamoxifen, UV radiation, etoposide, cisplatin and staurosporine were susceptible to thapsigargin and brefeldin A-induced proapoptotic signaling (Rao et al., 2002a). Moreover, activation of pro-caspase 12 in Apaf-1 deficient cells following ER stress did not require cytochrome c indicating the presence of ER stress-mediated, apoptosome-independent activation of pro-caspase-12 and subsequent activation of effector caspases (Rao et al., 2002a).

1.4.4 Calpain

Calpains are a family of cytosolic cysteine proteases whose enzymatic activities depend on Ca^{2+} Members of the calpain family are found in organisms ranging from mammals to *Drosophila melanogaster* and *Caenorhabditis elegans* with homologs in yeast and bacteria. The mammalian calpain family includes at least 12 different members with ubiquitous and tissue-specific expression patterns. The best characterized members are the ubiquitous m- and µ-calpains which have similar substrate specificities but differ in the calcium concentration required for their activation in vitro; m-calpain requires mM levels of Ca^{2+} while µ-calpain requires µM Ca^{2+} concentration (Kawasaki & Kawashima, 1996). Both enzymes are heterodimers composed of a homologous 80 kDa catalytic subunit and an identical 30 kDa regulatory subunit. The large subunit consists of four domains (I-IV), while the small subunit has two domains (V and VI).

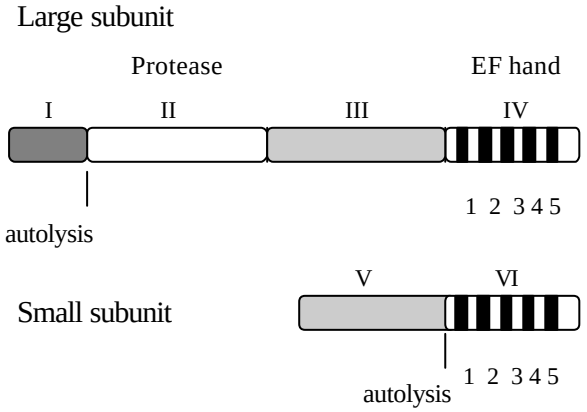

Fig.7. Schematic figure of human calpain

Domain I consists of a single L helix anchored in a cavity of domain VI, thereby stabilizing the circular domain arrangement of the protein. Domain II contains the catalytic site and contains two recently identified Ca^{2+} binding sites. Domain III is

found in various proteins and is known to interact with Ca^{2+} and phospholipids. Domains IV and VI are Ca^{2+} binding domains, each containing five EF-hand motifs. Interaction between EF-hand motifs of these domains is responsible for heterodimerization. Domain V of the small subunit contains a cluster of Gly residues in the N-terminal region.

Calpains are implicated in a wide range of cellular functions including cytoskeletal remodeling, differentiation, cell migration, proliferation and apoptosis (Suzuki & Sorimachi, 1998). The critical importance of the ubiquitous m- and µ calpains for normal development has been demonstrated in transgenic mice that lack the calpain regulatory subunit and thereby calpain activity. These mice die during embryonic development (Arthur et al., 2000).
Unlike caspases and granzyme B, calpain does not recognize a well-defined cleavage motif. It has been observed that the last three residues of the N-terminal fragment are often aliphatic and P1 on the C-teminal fragment is aliphatic or basic. However, substrate recognition seems to be determined by the overall three-dimensional structure rather than by particular sequence motifs (Carafoli & Molinari, 1998).

1.4.4.1 Regulation of calpain activity

The involvement of calpain in a wide range of cellular functions and its ability to cleave many intracellular signaling and structural proteins requires complex temporal and spatial regulation of its activity. Calpain activity is regulated by different mechanisms including autoproteolytic cleavage, phosphorylation, calcium requirement and inhibition by an endogenous inhibitor, calpastatin (Molinari & Carafoli, 1997). Calcium concentrations required for calpain activation in vitro are in the µM to mM range which is unphysiologically high. Thus, some mechanism(s) for reduction of Ca^{2+} requirement and/or sensitization of calpain to physiological Ca^{2+} levels must exist in cells. Several models of calpain sensitization to intracellular calcium levels have been discussed including association with membrane phospholipids (Carafoli & Molinari, 1998) and the existence of activator proteins which might increase Ca2+ affinity of calpain and thereby promote calpain activation (Melloni et al., 2000).

Active calpain is found predominantly at the membranes, suggesting that membrane localization may be an important factor in calpain activation. Translocation of calpain to the plasma membrane may be a way to escape control by calpastatin, the endogenous inhibitor localized in the cytoplasm, and may indicate the presence of membrane-located factors required for calpain activation.
It has been observed that cleavage of substrates *in vitro* may be preceded by autolysis of calpain subunits suggesting that autolysis would free the active site making it accessible to the substrates. The requirement of autolysis for calpain activation is controversial since unproteolyzed calpain can also cleave the substrates (Molinari et al., 1994). In addition, N-terminal mutations of the catalytic subunit prevent autolysis but not the cleavage of substrates (Elce et al., 1997).
Calpain contains many possible phosphorylation sites which may be relevant for its activation and/or inactivation. Activation of calpain by the ERK/MAP kinase signaling pathway has been observed in cells stimulated with epidermal growth factor (EGF)

(Glading et al., 2000). Interferon inducible chemokine IP-10 has been shown to inhibit m-calpain activity via a PKA-dependent phosphorylation (Shiraha et al., 1999). The endogenous calpain inhibitor calpastatin is also a substrate for kinase activity (Kuo et al., 1994). Two forms of cytosolic calpastatin differing in phosphorylation state have been described (Salamino et al., 1994) suggesting an additional way to modulate calpain activity, namely by phosphorylation and dephosphorylation of its endogenous inhibitor.

1.4.4.2 Involvement of calpain in apoptosis

Although the possible involvement of calpains in apoptosis was first suggested in 1993 (Sarin et al., 1993), the role of calpains in common apoptotic models is still not well understood. The discovery of caspases and their efficient role in apoptosis contributed to decreased interest in calpains and their role in cell death. Involvement of calpain has been observed in response to, e.g., radiation (Waterhouse et al., 1998), dexamethasone, cycloheximide (Squier et al., 1999), serum deprivation (Lu et al., 2002), staurosporine, etoposide (Gao & Dou, 2000), and cisplatin (Mandic et al., 2002).
The list of calpain substrates involved in apoptotic signaling is growing. It has been suggested that calpain-mediated cleavage of the Bcl-2 family proteins Bax, Bid, Bcl-2 and Bcl-xL results in conformational changes causing dissociation from their binding partners, translocation to mitochondria and subsequent cytochrome c release followed by caspase activation (Gil-Parrado et al., 2002).
Calpain-mediated cleavage of Bax was observed in early stages of etoposide- and staurosporine-induced apoptosis, before or in association with cytochrome c release. The generated 18 kDa fragment (Bax/p18) was shown to induce cytochrome c release but was unable to interact with Bcl-2. Accordingly, Bax/p18-induced apoptosis was not blocked by overexpression of Bcl-2, confirming that calpain-induced cleavage inhibits interaction between Bcl-2 family proteins (Gao & Dou, 2000). Calpain-mediated cleavage of Bax was also shown in HL-60 cells treated with 9-AC, a topoisomerase I inhibitor. However, in these cells calpain activation and calpain-mediated Bax cleavage were preceded by caspase activation and DNA fragmentation (Wood & Newcomb, 2000; Wood et al., 1998). Calpain activation has been shown to occur before, and to potentiate, caspase activation in radiation-induced apoptosis (Waterhouse et al., 1998). The suggestion that calpain activation is upstream of caspase activation was confirmed by the ability of calpain to directly activate caspase-7, in vitro and in vivo. It has been demonstrated that the initial calpain-mediated processing of caspase-7 is necessary for caspase activation during B cell clonal deletion (Ruiz-Vela et al., 1999). In addition, calpain was shown to regulate caspase activation (caspase-3 and DEVD-ase activation) in etoposide-induced apoptosis in T cells (Varghese et al., 2001).
Calpain cleavage of Bid, another pro-apoptotic Bcl-2 family protein has been shown in vivo and in vitro (Chen et al., 2001; Mandic et al., 2002). The pro-apoptotic function of calpain-cleaved Bid was demonstrated by its ability to induce cytochrome c release from isolated mitochondria (Gil-Parrado et al., 2002; Mandic et al., 2002).
Calpain has also been reported to cleave Bcl-2 and Bcl-xL (Gil-Parrado et al., 2002). Removal of the N-terminal domain of Bcl-2 and Bcl-xL by cleavage renders these proteins pro-apoptotic, as they are then able to induce cytochrome c release (Cheng et

al., 1997; Clem et al., 1998). Calpain-cleaved Bcl-2 was shown to induce cytochrome c release from isolated mitochondria (Gil-Parrado et al., 2002).
Recently, calpain was found to be involved in cleavage and activation of caspase-12 during ER stress-induced apoptosis (Nakagawa & Yuan, 2000). Thapsigargin and the calcium ionophore A23187, both reported to induce ER stress, induce calpain activation and cell death which is inhibitable by calpastatin overexpression (Lu et al., 2002). Furthermore, in support of calpain involvement in ER stress-mediated cell death, calpain has been shown to cleave Grp94, an ER chaperone protein (Reddy et al., 1999). Cleavage of p53 by calpain has been demonstrated in vitro, although the cleavage fragment was not detectable in cells (Kubbutat & Vousden, 1997). The response to DNA damage may involve modifications of p53 which inhibit recognition and /or cleavage by calpain (Kubbutat & Vousden, 1997). Today, the contribution of calpains in apoptosis is thus generally accepted, although their exact role is still not completely elucidated.

2 THE PRESENT STUDY

2.1 AIM(S)

Inherent or acquired resistance to therapy is a major clinical problem in treatment of malignant tumors with anticancer drugs. The aim of this thesis was to investigate the mechanisms of cisplatin-induced cell death and to characterize components involved in the pro-apoptotic signaling in order to understand functions and interactions between different pathways involved in cisplatin-induced apoptosis.
Improved understanding of the mechanisms involved in cisplatin-induced pro-apoptotic signaling may identify novel targets for clinical intervention. This knowledge can be used as a powerful tool to modify cellular response to therapy by stimulation of pro-apoptotic and inactivation of anti-apoptotic signals and thereby contribute to decreased resistance and better clinical response.

2.2 SUMMARY OF PAPERS

Paper I

The MEK1 inhibitor PD98059 sensitizes C8161 melanoma cells to cisplatin-induced apoptosis

Several studies have reported involvement of SAPK/JNK pathway in apoptosis induced by different stimuli, including cisplatin (Sanchez-Perez et al., 1998; Zanke et al., 1996). It has been suggested that sustained JNK activation is of particular importance for the apoptotic response (Chen et al., 1996a; Sanchez-Perez et al., 1998). The MEK1/ERK pathway is activated in response to growth stimulation and is involved in cell proliferation. Inhibition of this pathway has been reported to induce apoptosis on its own or enhance apoptosis induced by other agents. Moreover, the MEK1 inhibitor, PD98059 has been shown to sensitize ovarian cancer cells to cisplatin (Persons et al., 1999).
In this study we wanted to investigate whether cisplatin-induced cell death can be similarly potentiated in human melanoma cells. The effect of the MEK1 inhibitor PD98059 was studied in four human melanoma cell lines, C8161, AA, DFW and M5. Treatment with inhibitor retarded cell growth in all cell lines, although C8161 was most affected. Cisplatin treatment resulted in increased phosphorylation of JNK1 and ERKs in all cell lines except for M5, in which no or very weak activation was observed. Activation of JNK1 was approximately equal in C8161, AA and DFW cell lines in accordance with similar sensitivity to cisplatin while JNK2 activation was noticeably higher in DFW and AA than in C8161. Induction of ERK1 phosphorylation was similar in these three cell lines. On contrary, activation of ERK2 was much more prominent in C8161 than in the other two cell lines. Pretreatment of C8161 cells with PD98059 significantly decreased activation of ERK and sensitized these cells to cisplatin. However, in AA, DFW and M5 cell lines the inhibitor did not have any sensitizing effect; rather in AA cells it even protected slightly against cisplatin-induced

cytotoxicity. Our results demonstrate that although the MEK1/ERK signaling is viability-promoting, blocking of this pathway can not be regarded as a general tool for sensitizing cancer cells to drug-induced apoptosis.

Paper II

Cisplatin induces the proapoptotic conformation of Bak in a ?MEKK1-dependent manner

Proteolytic cleavage of MEKK1, resulting in a constitutively active kinase fragment has been reported in apoptosis induced by various agents, including cisplatin (Widmann et al., 1998; Widmann et al., 1997). The cleavage fragment, ?MEKK1, has been shown to induce apoptosis by its own and to sensitize cells to genotoxic damage (Johnson et al., 1996; Widmann et al., 1998) and is suggested to be involved in the amplification of the apoptotic signaling. However, the mechanism of ?MEKK1-mediated apoptosis is still unclear.

In this study we investigated the role of the proapoptotic ?MEKK1 pathway in cisplatin-induced apoptosis. Using kinase-active and kinase-inactive mutants of ?MEKK1 we examined the involvement of ?MEKK1 in cisplatin-induced activation of Bak and Bax, caspase activation and nuclear fragmentation.

Using antibodies recognizing specific conformational changes characteristic for active Bak and Bax, we showed the ability of cisplatin to activate the pro-apoptotic protein Bak in all cell lines tested. Bak activation was observed prior to the onset of the execution phase of apoptosis. In contrast to Bak, activation of Bax occurred late in the apoptotic process, after caspase activation and nuclear fragmentation.

The role of Bak in cisplatin-induced apoptosis was confirmed by treatment of MCF-7 cells expressing an antisense Bak. Two sublines stably expressing antisense Bak showed decreased apoptosis in response to cisplatin, assessed as caspase-mediated cleavage of cytokeratin 18 and mitochondrial depolarization.

Furthermore, we demonstrated that cisplatin-induced activation of Bak is mediated by ?MEKK1. A kinase-inactive ?MEKK1 mutant, dnMEKK1, was found to block cisplatin-induced activation of Bak, cytochrome c release and DNA fragmentation. It also abrogated activation of DEVD-ases, caspase-3 and -7, as well as activation of caspase-9. Interestingly, nuclear fragmentation and caspase activation were reduced by approximately half whereas Bak activation was almost completely blocked. This suggested the existence of additional signal(s) induced by cisplatin which contribute to Bak-independent, dnMEKK1-insensitive caspase activation and nuclear fragmentation.

We have also investigated the ability of a constitutively active kinase mutant, dpMEKK1 to induce modulation of Bak. Our results demonstrated that expression of dpMEKK1 was on its own sufficient to induce Bak activation in three out of four cell lines tested. However, the apoptosis was induced in only two of these cell lines.

Paper III

Calpain-mediated Bid cleavage and calpain-independent Bak modulation: two separate pathways in cisplatin-induced apoptosis

In this study we report involvement of calpain, a calcium-activated cysteine protease, in cisplatin-induced apoptosis. Our results demonstrate that activation of calpain is an early event in cisplatin-induced apoptotic signaling and is preceded by an increase in intracellular calcium. Calpain activation was blocked by the calcium chelator BAPTA-AM, in accordance with a role of calcium in calpain activation.
Co-treatment of cells with the calpain inhibitor calpeptin blocked cisplatin-induced DEVD-ase activation and nuclear fragmentation by approximately half. Cisplatin-induced nuclear fragmentation was also abrogated by co-treatment with BAPTA-AM. Moreover, we showed that calpain inhibitor, calpeptin was able to block cleavage of pro-apoptotic protein Bid in cisplatin-treated cells. Recombinant Bid was cleaved in vitro by both recombinant calpain and by lysates of cisplatin-treated cells. Calpain-cleaved Bid was able to induce cytochrome c release from isolated mitochondria.
In this study we present evidence that support the hypothesis of two separate pro-apoptotic pathways activated by cisplatin. First, dnMEKK1 completely blocks activation of Bak but does not affect cleavage of Bid. Secondly, inhibition of calpain with calpeptin inhibits cleavage of Bid but does not affect modulation of Bak. Third, both calpeptin and dnMEKK1 abrogate apoptosis by approximately half but when combined these inhibitors have an additive effect on inhibition of apoptosis. These results suggest that cisplatin induces at least two separate proapoptotic pathways, which result in calpain-mediated cleavage of Bid and MEKK1-mediated activation of Bak, respectively. Both pathways are involved in caspase activation and contribute to the execution of apoptosis.

Paper IV

Cisplatin induces ER stress and nucleus-independent apoptotic signaling

It is generally accepted that nuclear DNA is the critical target of cisplatin although only 1% of the intracellular cisplatin has been shown to form DNA adducts. The role of platination of intracellular proteins and RNA in cisplatin-induced cytotoxicity is still unclear. This prompted us to investigate the ability of cisplatin to induce apoptosis in the absence of nuclear DNA. We show here that cisplatin treatment induces activation of caspase-3 in enucleated cells (cytoplasts).This was shown in a human melanoma cell line, 224 and in the human colon carcinoma cell lines, HCT116 (wt and p53 mutant). We have previously observed activation of the pro-apoptotic proteins Bak and Bid upon cisplatin treatment. We have also shown that Bid cleavage is mediated by calpain in cisplatin-treated cells. Here we show that cleavage of Bid also occurs in cisplatin-treated cytoplasts. A role of calpain in apoptotic cytoplasts was supported by the finding that co-treatment with the calpain inhibitor calpeptin blocked cisplatin-induced activation of caspase-3. Furthermore, activation of caspase-3 in cytoplasts was abrogated by the calcium chelator BAPTA-AM.

Both increase in intracellular calcium and calpain activation have been reported during ER stress, suggesting the involvement of ER stress also in cisplatin-induced apoptosis. Apoptosis induced by ER stress has furthermore been shown to involve activation of caspase-12 (Nakagawa et al., 2000) and increased expression of grp78, an ER chaperone protein. Both caspase-12 activation and up-regulation of grp78 were observed in cisplatin-treated cells, indicating the involvement of ER stress in cisplatin-induced apoptosis. Cleavage of pro-caspase-12 has been reported to be mediated by calpain (Nakagawa & Yuan, 2000). Accordingly, both calpeptin and BAPTA-AM blocked cleavage of pro-caspase-12 in cisplatin-treated cells. Cleavage of pro-caspase-12 was also observed in cytoplasts suggesting the ER as a non-nuclear target for cisplatin.

In contrast to cisplatin, the topoisomerase II inhibitor etoposide, required the presence of nuclei for its apoptotic effect. Etoposide failed to induce activation of caspase-3 in cytoplasts. We also demonstrated that etoposide-induced apoptosis did not involve ER stress since treatment with this drug did not induce activation of caspase-12 nor did it lead to increased expression of grp78.

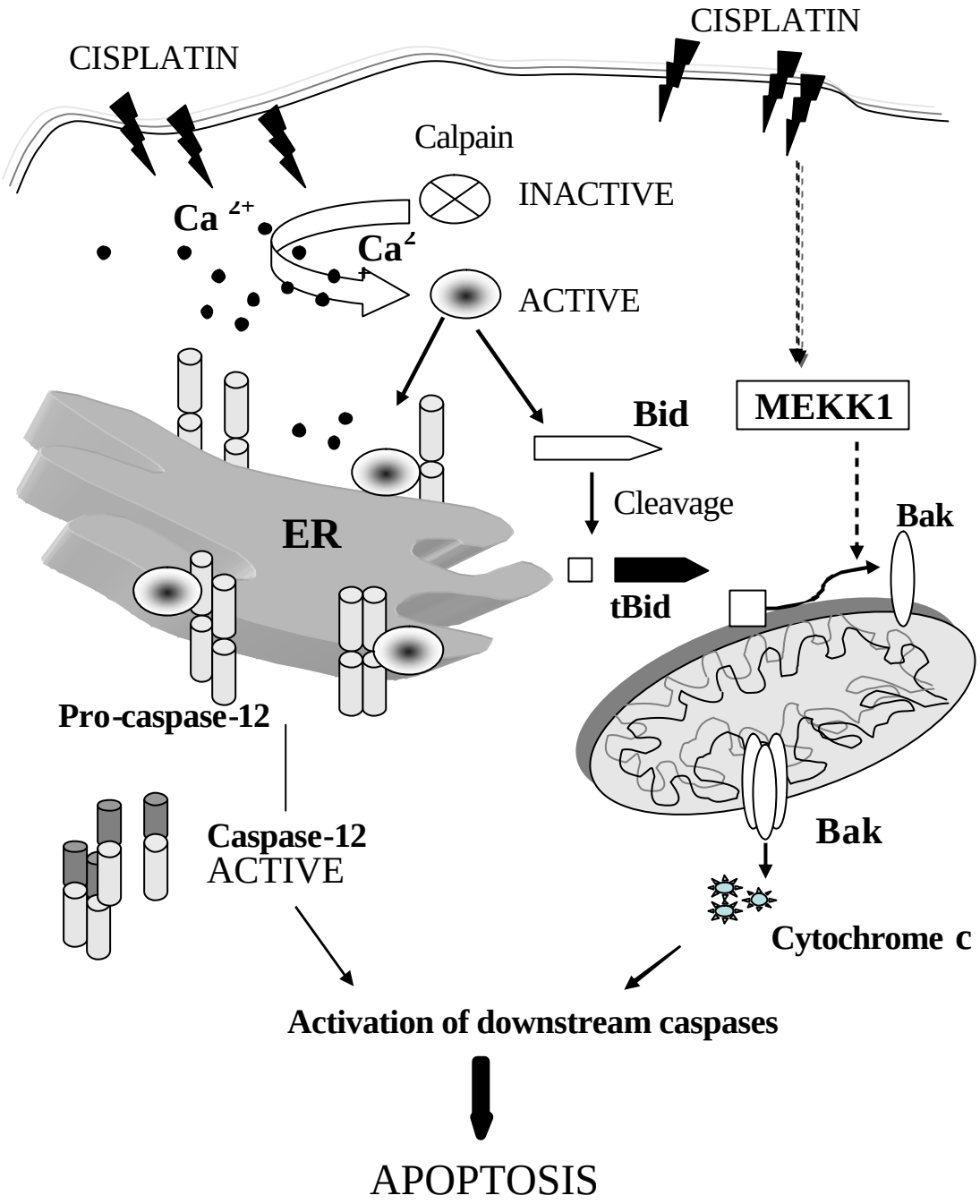

Fig.8. Two separate pro-apoptotic pathways induced by cisplatin

2.3 GENERAL DISCUSSION

Regulation of apoptosis is considered too complex to be explained by a simple "ratio" between pro- and anti-apoptotic proteins. The apoptotic response is rather determined by a complex network of signals involving a variety of regulators and executors of pro-apoptotic signaling.
This concept is exemplified in this thesis by findings that cisplatin-induced apoptosis involves activation of both anti-apoptotic and pro-apoptotic pathways. Furthermore, inhibition of the ERK pathway, regarded as a pro-survival pathway, was found to differentially affect the cisplatin response, indicating that the cellular response to a certain type of treatment cannot always be predicted. A complex cellular strategy for the temporal and spatial regulation of pro-apoptotic proteins is also required and contributes to the complexity of intracellular signaling. One example is the complex regulation of calpain activity, which involves translocation of the protein to cellular membranes, the presence of activator proteins, calcium requirements, the presence of the endogenous inhibitor calpastatin, phosphorylation and cleavage of calpastatin,…etc. Correct regulation is also necessary for successful cross-talk between different pathways, here exemplified by cross-talk between members of two protease families, caspases and calpain, or cross-talk between organelles, e.g. ER and mitochondria.
An additional level of complexity is introduced when a drug induces several pro-apoptotic pathways. The present work demonstrates the ability of a widely used anti-cancer drug, cisplatin, to induce at least two separate proapoptotic pathways, and to affect several cellular organelles.

The first paper illustrates the complexity of cellular signaling in that the outcome of a given treatment likely depends on balances between the activities of several different pathways. Sustained JNK activation has been reported in apoptosis induced by various agents including cisplatin (Sanchez-Perez et al., 1998). Activation of JNK1 was similar in the four tested human melanoma cell lines and correlated with similar cisplatin sensitivity. In contrast, no correlation was found between cisplatin-induced apoptosis and ERK activation.
Inhibitors of the ERK pathway, such as the MEK1 inhibitor PD98059, have been used to investigate the role of ERK in apoptosis. In two different ovarian carcinoma cell lines PD98059 was found to potentiate the cytotoxic effect of cisplatin, indicating a protective role of ERK in cisplatin-mediated cell death (Persons et al., 1999). This protective effect has in turn been suggested to depend on ERK-mediated phosphorylation and inactivation of the pro-apoptotic protein Bad (Hayakawa et al., 2000). In contrast, in HeLa cells MEK1 inhibitors were found to block cisplatin-induced apoptosis, indicating a death-promoting role of ERK in these cells (Wang et al., 2000). Interestingly, hydrogen peroxide-induced activation of ERK in HeLa cells was found to mediate a pro-survival signal (Wang et al., 2000). One important difference between cisplatin- and hydrogen peroxide-induced ERK activation was the timing and duration of activity. With hydrogen peroxide, ERK activation was more rapid and transient, whereas cisplatin-induced activation appeared later but remained highly elevated. In contrast, another study showed that modest and transient activation of ERK is required for apoptosis while sustained ERK signaling promotes survival (Rul

et al., 2002). Thus, the role of ERK in proliferation and apoptosis is still not well defined and seems to depend on cell type as well as on strength, duration and type of stimuli.

We found that pretreatment with PD98059 had different effects on the cisplatin responses of the four tested human melanoma cell lines, from slightly protective to sensitizing. The protective effect of PD98059 might at least in part be explained by growth retardation which was observed in all four cell lines. However, this effect was overruled in C8161 cells, in which PD98059 was found to potentiate cisplatin-induced cell death. Because pre-incubation with the inhibitor was required for its effect, this sensitization was probably dependent on down-regulation of viability-promoting proteins regulated by MEK/ERK pathway. Thus, we show that inhibition of a single survival pathway cannot be used as a general strategy for sensitizing cancer cells.

Cisplatin has previously been reported to induce apoptosis although the specific mechanisms that trigger the apoptotic response are still unclear.
Most of the work presented in the thesis was done on 224 human metastatic melanoma cell line. In these cells, 20µM cisplatin induces caspase-3 activation starting at 16 hours post treatment and at 20-24 hours, DNA fragmentation is observed in approximately 50% of cells. We present here the molecular characterization of two novel pro-apoptotic pathways in cisplatin-induced apoptosis.
One of these pathways is activated early in the apoptotic process and involves an increase in intracellular calcium levels, seen at 1h post-treatment. This is followed by calpain activation, at 3-5h post treatment and results in calpain-mediated cleavage of Bid at 7h. Two calpain inhibitors, calpeptin and PD150606 could each block cleavage of Bid and reduce nuclear fragmentation as well as DEVD-ase activation by approximately half. Importantly, these inhibitors have different mechanisms of action; calpeptin acts on the active site of calpain while PD150606 blocks its calcium-binding site.

Cleavage of Bid to the active, truncated form, tBid, has been reported in caspase-8-mediated apoptosis induced via death receptors (Gross et al., 1999b; Luo et al., 1998). We show here that cleavage of Bid also occurs in non-death receptor mediated apoptosis. Importantly, human recombinant m-calpain cleaved human recombinant Bid generating a 14.4 kDa fragment which was able to induce cytochrome c release from isolated mitochondria. The calpain cleavage site was mapped between Gly70 and Arg71, or 11 residues C-terminal to the caspase-8 cleavage site. It is thus difficult to distinguish between a calpain cleavage fragment and caspase cleavage fragment on a Western blot. This might contribute to underestimation of calpain involvement in many apoptosis studies.

Although Bax has also been reported as a substrate for calpain (Gao & Dou, 2000; Wood et al., 1998) we have not observed cleavage of Bax in any of the tested cell lines. There are conflicting reports in the literature regarding the timing of calpain-mediated cleavage of Bax and the contribution of calpain-cleaved Bax to the apoptotic signaling. In our system, calpain activation is one of the earliest events and occurs long before caspase activation. Thus, any calpain-mediated cleavage of Bax should be expected before the execution phase of apoptosis. No activation of Bax, assessed by FACS, was

seen prior to cytochrome c release and caspase-activation indicating that Bax is not required for cisplatin-induced apoptosis. Instead, Bax activation was detected much later, after nuclear fragmentation, suggesting that activation of Bax is rather a consequence than a cause of cisplatin-induced cell death.

MEKK1 is a 196 kDa cytosolic kinase, shown to be involved in apoptosis mediated by a number of different agents, including cisplatin. MEKK1 is cleaved in apoptotic cells generating a constitutively active kinase fragment, ?MEKK1 which is suggested to have a role in amplification of pro-apoptotic signaling (Cardone et al., 1997; Johnson et al., 1996; Widmann et al., 1998; Widmann et al., 1997).
Because of the suggested pro-apoptotic role of ?MEKK1, we used a kinase-inactive, dominant negative mutant (dnMEKK1) and a kinase-active, dominant positive mutant (dpMEKK1) of ?MEKK1 to investigate the relationship between MEKK1 and pro-apoptotic events such as activation of caspases, Bak and Bax in cisplatin-induced apoptosis.
We found that cisplatin-induced apoptosis involves induction of the pro-apoptotic conformation/activation of Bak at 14-16h post treatment, i.e. shortly before the onset of execution phase of apoptosis. Activation of Bak was observed in four tested human melanoma cell lines and occurred in cells expressing wild-type as well as mutant p53. Similarly, we have not found any correlation between p53 status and cisplatin sensitivity in a panel of nine melanoma cell lines.
Induced expression of dnMEKK1 was found to inhibit cisplatin-induced activation of Bak and cytochrome c release and to reduce caspase activation and nuclear fragmentation by approximately half.
This prompted us to examine if dpMEKK1 would have effects similar to those of cisplatin. We found that expression of dpMEKK1 was sufficient to induce activation of Bak in three out of four cell lines, in agreement with the reports that it is potentially sufficient to induce apoptosis. Apoptosis was, however, induced in two of these cell lines but not in the third one, suggesting that activation of Bak is not always sufficient to induce apoptosis by itself. Absence of apoptosis in cells with activated Bak may at least in part be explained by the finding that some metastatic melanoma cells have lost expression of Apaf-1 by gene silencing due to methylation (Soengas et al., 2001) which results in failure to activate the caspase cascade.

Cisplatin-induced apoptosis has been reported to require JNK activation. Interestingly, dnMEKK1 did not block activation of JNK suggesting that JNK is not involved in ?MEKK1-mediated signaling.
Differential involvement of full-length MEKK1 and its cleavage fragment in JNK activation and apoptosis has been reported previously (Johnson et al., 1996; Widmann et al., 1998). A cleavage resistant MEKK1 mutant, despite its failure to induce apoptosis, activated JNK as efficiently as did the wt protein. Moreover, it has been demonstrated that cell death induced by catalytic fragment of MEKK1 is independent of JNK regulation or c-Jun transactivation (Johnson et al., 1996; Schlesinger et al., 2002). A possible explanation for different substrate specificities might be the different subcellular localizations of full-length MEKK1 (flMEKK1) and ?MEKK1. It has been shown that full length MEKK1 is predominantly membrane-bound while the cleavage fragment is mostly free in cytoplasm. It has also been found that 14-3-3 proteins can

bind to full-length MEKK1 but not to the cleavage fragment and might thus contribute to the different locations of flMEKK1 and its fragment (Fanger et al., 1998).

Interestingly, calpeptin, which blocked cleavage of Bid, caspase activation and nuclear fragmentation did not affect activation of Bak, indicating either that Bak activation occurs before its interaction with tBid and is then not affected by calpeptin, or that Bak may promote cytochrome c release also independently of tBid. In fact, it has been shown that tBid is not absolutely required for activation of Bak and Bax, since Bid $^{-/-}$ cells were susceptible to various pro-apoptotic stimuli (Wei et al., 2001). Accordingly, we have observed the same degree of nuclear fragmentation after cisplatin treatment in Bid deficient MEFs as in the wt MEFs. Thus, Bak may be activated by at least two distinct signals, at least one of which is independent of tBid.
Conversely, dnMEKK1 blocked activation of Bak and abrogated caspase activation and nuclear fragmentation to the same extent as calpeptin. However, it was unable to block cleavage of Bid.
Thus, we have here presented evidence for induction of at least two separate pro-apoptotic pathways involved in cisplatin-induced apoptosis. One is mediated by calpain and results in cleavage of Bid, and the other is ? MEKK1 dependent and involves activation of Bak. Both pathways contribute to caspase activation and nuclear fragmentation. Accordingly, the combination of both inhibitors, calpeptin and dnMEKK1 had an additive effect on apoptosis-inhibition.

Nuclear DNA is regarded as the critical target of cisplatin. Although the cisplatin molecule is a highly reactive electrophile and can interact with nucleophilic sites on cellular proteins and RNA, the role of protein- and RNA-adducts in cisplatin-induced apoptosis has not been investigated. The ability of cisplatin to bind to mitochondrial DNA (mtDNA) has also been reported, but the contribution of mtDNA-damage to apoptosis has not been studied. To our knowledge, no molecular sensors have been identified which would link mtDNA damage to activation of the apoptotic machinery. Moreover, findings that only 1% of intracellular cisplatin reacts with DNA and that mtDNA constitutes about 1/1,000 of the cell´s DNA suggest that mtDNA is not a major target for cisplatin.
We report here the ability of cisplatin to induce apoptosis in the absence of nuclear DNA, showing that cisplatin-induced apoptosis is not exclusively dependent on DNA damage and the presence of nuclei.
For this study we used enucleated cells (cytoplasts). The cytoplast preparations were found to contain a small amount of intact cells. The contamination did not affect analysis of cytoplasts, since these two populations can be separated by staining with propidium iodide and subsequent electronic gating. The presence of intact cells in the sample was rather an advantage, since these cells were treated in the same way as cytoplasts, including incubation with cytochalasin B and gradient centrifugation, and could be directly compared with cytoplasts.

Cisplatin-induced caspase-3 activation in cytoplasts was found to be higher than in intact cells. This may be due to the inability of enucleated cells to activate “a protective response” such as expression of heat-shock proteins. It has been shown that heat-shock protein 70 (Hsp70) can block apoptosis by interfering with Apaf-1(Saleh et al., 2000)

and AIF (Ravagnan et al., 2001). Also, X-linked inhibitor of apoptosis protein (XIAP) can abrogate apoptosis by inhibiting active caspase-3 and caspase-9 (Bratton et al., 2002). Thus, presence of these proteins can block or at least delay caspase activation. Cisplatin-induced caspase-3 activity in cytoplasts was blocked by calpeptin and by BAPTA-AM, indicating the involvement of calpain-mediated signaling in nucleus-independent apoptosis. This was confirmed by cleavage of Bid.
Bak activation was, in contrast to caspase-3, only slightly increased in cisplatin-treated cytoplasts compared to intact cells. A possible explanation is that ?MEKK1-mediated signaling is the major regulator of Bak activation and requires the presence of DNA damage for activation. It would thus be inactive in cytoplasts. The relatively low Bak activation observed in cytoplasts might instead represent activation mediated by tBid.

Our findings demonstrate involvement of ER stress in cisplatin-induced apoptosis, assessed as cleavage/activation of pro-caspase-12 and as increased expression of an ER chaperone protein, grp78/BiP. In accordance with calpain-mediated activation of pro-caspase-12, both calpeptin and BAPTA-AM were able to block cisplatin-induced cleavage of caspase-12. Although caspase-7 has also been reported to cleave pro-caspase-12, it is probably not involved in our system, since no DEVD-ase activity was detected at 4h post-treatment, when caspase-12 was already cleaved. We also present evidence for activation of pro-caspase-12 in cytoplasts in accordance with the ER as a non-nuclear target for cisplatin.

Caspase-12 is localized to the ER membrane and is activated by agents that induce ER stress but not by membrane- or mitochondrial-targeted apoptotic signals(Nakagawa et al., 2000). Caspase-12 protein is usually not seen in humans since transcription of the caspase-12 gene which contains a stop codon in exon 3 results in expression of a truncated protein containing only a prodomain. However, the same antibody used to identify caspase-12 in murine cells also recognizes a human protein with the same molecular mass and the same localization as murine caspase-12, in human HeLa cells, A549 human lung carcinoma cells and 293T cells (Bitko & Barik, 2001; Nakagawa et al., 2000). This suggests the presence of a human caspase-12 like protein with similar molecular mass and similar cellular distribution as caspase-12 in mice.
Murine caspase-12 shares sequence homology with human caspase-4 (48% identity) and caspase-5 (45% identity) which might be responsible for caspase-12-like functions in human cells. However, the exact identity of the caspase-12-like protein in humans remains unknown.

grp78/BiP is an ER chaperone protein induced in response to accumulation of unfolded proteins during ER stress. Induction of chaperone proteins cannot protect against excessive ER stress which ultimately results in apoptosis.
Interestingly, the basal expression levels of grp78 varied widely in 224 cells. Induction of grp78 expression by cisplatin was not caspase-dependent, since it was not affected by the pan-caspase inhibitor zVAD-fmk. Although cisplatin induced a general increase in the grp78 expression, the population of cells with high basal levels may be more resistant to cisplatin since caspase-3 activation appeared to be delayed in these cells. Thus, with its protective role, grp78 might contribute to cisplatin resistance, and the level of grp78 protein might be used as a predictive marker for the clinical response to

cisplatin treatment. In our system, induction of grp78 expression occurs between 8h and 16h post treatment, after caspase-12 activation and cleavage of Bid. It would be interesting to evaluate the more exact timing of grp78 induction and to investigate the correlation between grp78 expression and cisplatin sensitivity.

Cisplatin is known as a very efficient anti-cancer agent. The work presented in this thesis suggests that this efficacy may depend on the ability of cisplatin to target DNA as well as the ER and to induce at least two distinct pro-apoptotic pathways. Understanding of the individual tumor response to anti-cancer therapy is a great challenge and will undoubtedly be the focus of much future research. We believe that our findings will contribute to an improved understanding of the mechanisms involved in the pro-apoptotic signaling mediated by platinum-based chemotherapy. Identification of components involved in cell death-mediated signaling and elucidation of their interactions will help in identification of new targets for therapy and in prediction of clinical response as well as in designing new and better combination therapies.

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