p38 MAPK Signalling in Endothelial Apoptosis

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p38 MAPK Signalling in Endothelial Apoptosis

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Abstract

Endothelial apoptosis plays an important role in atherosclerosis, and a direct proapoptotic effect of chemotherapeutics on the tumour vasculature emphasises the great potency of antiangiogenic therapy in the treatment of cancer. We investigated signalling pathways in endothelial apoptosis induced by the inflammatory cytokine tumour necrosis factor alpha (TNF), the main target of which is the endothelium, and by the anti-cancer drug doxorubicin. Doxorubicin (also called Adriamycin) is a widely used anthracycline against a broad range of tumours and has been shown to exert its effect via directly targeting the tumour vasculature. However, endothelial apoptosis is also implicated in doxorubicin-mediated cardiotoxicity, an undesirable side effect of the cancer therapy.

The main objective was to elucidate the role of p38 mitogen activated kinase (p38) in endothelial apoptosis, using the endothelial cell line EA.hy926. We found that p38 plays an important proapoptotic role in both TNF- and doxorubicin-induced apoptosis. In TNF-induced cell death, p38 mediates phosphorylation of Bcl-xL, which is followed by Bcl-xL degradation in the proteasomes. Furthermore, we observed that p38 signalling inhibits the MEK/ERK survival pathway and the phosphorylation of its downstream target Bad, which occurs through an increased activity of the protein phosphatase 2A (PP2A). In addition to pharmacological inhibition, we used lentiviral vector transfection of EA.hy926 cells to express a dominant negative mutant Flag-p38 MAPK harbouring T180A and Y182 F amino acid substitutions. Similarly to the TNF- induced cell death, we found a p38-mediated downregulation of Bcl-xL in cells undergoing doxorubicin-induced apoptosis. In contrast, MEK/ERK signalling appeared to be proapoptotic in this system. Interestingly, p38 signalling inhibited the PI3-K/Akt survival pathway and the phosphorylation of Bad. Results from a phosphatase assay showed that doxorubicin-induced p38 activity in endothelial cells could maintain PP2A activity at a normal level and thereby prevent phosphorylation of Akt and Bad. In summary, p38 exerts a strong proapoptotic action in endothelial cells exposed to TNF or doxorubicin by simultaneously decreasing the level of antiapoptotic Bcl-xL and increasing the level of proapoptotic Bad.

Key words: Apoptosis, endothelial cells, p38 MAPK, TNF, doxorubicin, Bcl-xL, Bad, PP2A

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Theorie
Theorie ist Wissen,
Das nicht funktioniert.
Praxis ist, wenn alles funktioniert
Und man weiß nicht warum.
Hermann Hesse, deutscher Dichter,
2.7.1877-9.8.1962

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List of publications

This thesis is based on the following papers, which are referred to in the text by their Roman numerals:

I. p38 MAPK mediates TNF-induced apoptosis in endothelial cells via phosphorylation and downregulation of Bcl-xL.
S. Grethe, M.P.S. Ares, T. Andersson and M.I. Pörn-Ares.
Experimental Cell Research 298:632-642, 2004

II. p38 MAPK regulates phosphorylation of Bad via PP2A-dependent suppression of the MEK1/2-ERK1/2 survival pathway in TNF-α-induced apoptosis.
S. Grethe and M.I. Pörn-Ares.
Cellular Signalling, 2005, in press

III. p38 MAPK downregulates Akt-mediated Bad phosphorylation in doxorubicin-induced endothelial apoptosis.
S. Grethe, N. Coltella, M.F. Di Renzo and M.I. Pörn-Ares.
Manuscript submitted

Abbreviations

ASK-1	apoptosis signalling kinase-1
ATF	activating transcription factor
Bcl-2	human B-cell lymphomas
bFGF	basic fibroblast growth factor
BH	Bcl-2 homology domain
CARD	caspase activation and recruitment domain
Caspase	cysteine aspartyl-specific proteases
CHX	cycloheximide
DD	death domain
DED	death effector domain
DISC	death inducing signaling complex
DNR	daunorubicin
DUSP	dual specific phosphatase
ECM	extracellular matrix
ERK	extracellular signal-regulated kinases
FADD	Fas associated DD
FasL	Fas ligand
$FLIP_{L/s}$	FLICE inhibitory protein long/short
FKHR-L	forkhead related transcription factors
GSK-3β	glycogen synthase kinase 3 β
He-PTP	haematopoietic PTP
HCD	hydrophobic C-terminal domain
HGF	hepatocyte growth factor
HMEC	human microvascular endothelial cells
HUVEC	human umbilical vein endothelial cells
Hsp27	heat-shock protein 27
IAP	inhibitor of apoptosis protein
I-CAM	intercellular adhesion molecule-1
IFN-γ	interferon-γ
IL	interleukin
ILK	Integrin-Linked Kinase
JNK	c-Jun NH2-terminal kinase
LPS	lipopolysaccharide
MAPK	mitogen-activated protein kinase
MKK	MAPK kinase
MAPKKK	MAPKK kinase
MEF	mouse embryonic fibroblast
MEF2C	myocyte enhancer factor 2C
MEK	MAPK/ERK kinase
MKP	MAPK phosphatase
MK-2	MAPK-activated protein kinase-2
NFκB	nuclear factor-κB
NO	nitric oxide
oxLDL	oxidised LDL
PCD	programmed cell death
p38	p38 MAPK
PH	pleckstrin homology
PI3K	phosphatidylinositol 3-kinase

PKA,B, C	protein kinase A,B, C
PP2A	protein phosphatase type 2A
PPM	protein phosphatase magnesium dependent
PPP	phosphor-protein phosphatase
PTP	protein tyrosine phosphatase
RIP	Receptor interacting protein kinase
ROS	reactive oxygen species
RSK1	p90 ribosomal S6 kinase 1
SLT-1	Shiga-like toxin-1
SOS	son of sevenless
t-Bid	truncated Bid
TNF	tumour necrosis factor-α
memTNF	transmembrane TNF
sTNF	soluble TNF
TRADD	TNF receptor associated DD
TRAF	TNF-R associated factor
VEGF	vascular endothelial growth factor
XIAP	X-chromosome linked IAP

Introduction

It has become increasingly important to understand signalling pathways involved in endothelial apoptosis, because research has shown that endothelial cell death may limit unwanted neovascularisation of tumors and plays an important role in atherosclerosis. The vascular endothelium is a main target of tumour necrosis factor-α (TNF), a pleiotropic cytokine that is produced by many cells and was originally identified by its cytotoxic effects. Doxorubicin (also called Adriamycin) is an anthracycline with a broad spectrum of antitumor activities which have been shown to be mediated *in vivo* by selective triggering of apoptosis in proliferating endothelial cells. We therefore studied signalling pathways in endothelial apoptosis induced by these agents with focus on the role of the serine/threonine kinase p38 MAPK (p38). p38 has been shown to be both pro- or antiapoptotic depending on cell type and stimuli. We found that p38 plays an important proapoptotic role in both systems, and we investigated p38-mediated regulation of Bcl-2 proteins and survival pathways such as MEK/ERK and PI3-K/Akt, as well as the implication of the protein phosphatase type 2A (PP2A).

Background

1. The endothelium and its functions

A single layer of endothelial cells lines the entire vascular system. In adults, approximately 1 to 6 x 10^{13} cells form an almost 1 kg large secretory and regulatory organ (Stefanec, 2000). Endothelial cells form confluent monolayers with a cobblestone shape and are normally quiescent *in vivo*. It is thought that, in the adult, the average endothelial cell divides approximately twice in a lifetime (Bicknell et al., 1996) in contrast to a doubling time of 1-2 weeks in solid tumours (Bachetti and Morbidelli, 2000). The endothelium was once thought to be a rather inert cell type which merely served as a nonthrombogenic surface over which blood could flow, but actually it is an extremely active tissue that plays an important role in regulating thrombosis, thrombolysis, platelet adherence and blood pressure (Galley and Webster, 2004) and in angiogenesis, the formation of new blood vessels (Chavakis and Dimmeler, 2002). Furthermore, endothelial cells have an important regulatory function in adhesion and transmigration of inflammatory cells out of the vessel into the targeted tissue. The endothelium is one of the major sites of action of TNF and elicits nuclear factor-κB (NF-κB)-dependent transcription of adhesion molecules such as E-selectin, intercellular adhesion molecule-1 (ICAM-1) and vascular cell adhesion molecule (VCAM-1) and cytokines such as

interleukin (IL)-6 and -8. E-selectin mediates the initial interaction between leukocytes and endothelial cells, once adhered they are activated by IL-8 and bind ICAM-1 and other ligands on the endothelial surface. Several proinflammatory cytokines have been shown to induce endothelial apoptosis mediated by death receptor pathways, such as lipopolysaccharide (LPS) (Bannermann et al, 2003), TNF (Grethe et al., 2004; Grethe and Pörn-Ares, 2005) and IL-18 solely or in combination with TNF (Marino and Cardier, 2003; Chandrasekar et al., 2004).

1.1. Endothelial survival factors

Endothelial cell survival is maintained by the presence of haemodynamic shear stress, and its loss results in endothelial apoptosis. It has been reported that blood flow and shear stress mediate endothelial cell survival via activation of Akt (Dimmeler et al., 1998) and subsequent phosphorylation of the endothelial nitric oxide (NO) synthase (Dimmeler et al., 1999). NO, released in response to shear stress, inhibits caspase-3 activation and prevents endothelial apoptosis (Dimmeler et al., 1997).

Another important survival signal for endothelial cells is contact with the extracellular matrix (ECM), mediated by integrin-dependent cell adhesion, and detachment from the ECM leads to endothelial apoptosis (Meredith et al., 1993, Aoudjit and Vuori, 2001). Furthermore, adhesion between endothelial cells, mediated by cadherins, promotes endothelial survival (Carmeliet et al., 1999).

In addition to shear stress and cell adhesion, several growth and survival factors protect endothelial cells from apoptosis. Vascular endothelial growth factor (VEGF) is an important endothelial mitogen and survival factor and signals via its receptors VEGF-R1 and VEGF-R2. Mice deficient in VEGFR-2 or VEGF fail to develop a vascular system and die during embryogenic development (reviewed in Chavakis and Dimmeler, 2002). Interestingly, tumour cells frequently secrete endothelial survival factors such as VEGF-A, and the inhibition of tumour-derived VEGF-A causes endothelial apoptosis and vascular regression in xenografted gliomas and prostate tumours (Benjamin et al., 1997, 1999). Similarly, inhibition of platelet derived growth factor (PDGF)-mediated signalling in endothelial cells could sensitise bone-derived endothelial cells to taxol (Langley et al., 2004). Furthermore, the endothelial-specific survival factor angiopoietin-1 (Ang-1) has been shown to act through activation of PI3K/Akt. The antiapoptotic effects of VEGF and angiopoietin is mediated through interaction with the ECM since the antiapoptotic effects of these growth factors is lost in suspended cells (Fujikawa et al., 1999). In addition, hepatocyte growth factor (HGF) and basic fibroblast growth factor (bFGF) have been shown to inhibit endothelial apoptosis induced by serum

starvation or by radiation treatment in a phosphatidylinositol 3-kinase (PI3K)/Akt and ERK-dependent manner (Ma et al., 2002; Gu Q et al., 2004a,b).

1.2. Endothelial apoptosis in health and disease

In vivo, endothelial cells survive for prolonged periods and apoptosis is difficult to detect because of rapid loss of apoptotic endothelial cells into the blood stream or by phagocytosis. When apoptosis occurs, it may serve to delete cells which have been damaged, infected, senescent or transformed. In dynamic tissues such as ovary, mammary gland, endometrium, growing or regressing tumours, healing wounds and developing embryos, vessels are continuously remodelling, which involves endothelial apoptosis (Duval et al., 2003).

Endothelial apoptosis has been implicated in the pathogenesis of several disease states. Activation of apoptotic pathways in endothelial cells have been suggested to be an important factor in atherosclerosis (Choy et al., 2001) and risk factors for atherosclerosis have been shown to cause endothelial apoptosis. Thus, areas of low shear stress are known to be preferential sites for the development of atherosclerotic plaques and oxidised low density protein (oxLDL) is a well-described endothelial proapoptotic agent (reviewed in Stefanec, 2000). Furthermore, endothelial apoptosis is involved in bacterial sepsis, the main mediator of this is LPS (Bannerman et al., 2003), and in hypertension and congestive heart failure (Stefanec, 2000). Increasing number of reports have shown a direct proapoptotic effect of chemotherapeutics on the tumour vasculature (Bocci et al., 2002; Schiffelers et al 2003), emphasising the great potency of antiangiogenic therapy in the treatment of cancer.

1.3. The EA.hy926 cell line

Detailed study of endothelial functions became feasible with the development in the 1970s of techniques to culture endothelial cells *in vitro* (Jaffe et al., 1973; Lewis et al., 1973). However, it should be kept in mind that cell culture changes endothelial cells from their quiescent *in vivo* state to an activated phenotype with loss of specialised functions associated with diverse vessels and organ functions.

The EA.hy926 cell line used in this study is one of the most frequently used and best characterised permanent human vascular endothelial cell lines (Bouis et al., 2001) derived from human umbilical vein endothelial cells (HUVEC) by fusion with the relatively undifferentiated cell line A549/8 which is originally derived from a human lung carcinoma. Importantly, this cell line shows a cobblestone shape and expresses the endothelial cell

marker protein von Willebrand factor with the same morphological distribution as primary cells (Edgell et al., 1983). Furthermore, this cell line has been shown to be useful for studying many differentiated endothelial functions since a significant fraction of mRNA represents genes that are differentially expressed in endothelium. This extensive differentiation of EA.hy926 cells is noteworthy since tissue-specific gene expression is generally extinguished in hybrids of two different cell types (Rieber et al., 1993). In addition, TNF stimulation of EA.hy926 cells results in upregulation of ICAM-1, VCAM-1 and E-selectin (Thornhill et al., 1993), and leukocyte-adhesion assays also support endothelial cell property retention in these cells (Brown et al., 1993).

2. Apoptosis

The term apoptosis (coming from the Greek and meaning the "falling off" of petals from flowers, or leaves from trees) was coined in 1972 by Kerr, Wyllie and Currie to describe a cell death occurring in a programmed and orderly fashion (Kerr et al., 1972), as opposed to the more non-specific, caspase-independent necrotic cell death caused by overwhelming stress (Nicotera et al., 1999). However, already in the middle of the 19th century Carl Vogt had discovered that cells die in a predictable, "programmed" fashion, by studying dying cells in the neuronal system of developing toad embryos (reviewed by Clarke and Clarke, 1996). In addition, developmental cell death in insect systems was described as "programmed cell death" (PCD) in 1965 by Lockshin and Williams. A genetic understanding of cell death has primarily come from studies of *C. elegans*, in which 131 of the 1090 somatic cells formed in this nematode die during development (Ellis and Horvitz, 1986). Today the terms apoptosis and PCD are often used interchangeably. However, PCD is defined as cell death occurring at a specific point in development (and is therefore programmed) and most examples of PCD occur via apoptosis. The term apoptosis is more descriptive and is characterised by a number of unique distinguishing features. In apoptotic cells, DNA is condensed and then fragmented, cells shrink and are further separated into apoptotic bodies. Finally, the apoptotic bodies are engulfed by phagocytotic cells, thereby avoiding leakage of cellular contents and elicitation of inflammation (Wyllie et al., 1980). In contrast during necrosis, membrane integrity is lost and massive ion influx results in swelling of mitochondria and membranes as well as DNA disruption, and finally, the cells burst and an inflammatory response starts in the area (Majno et al., 1995).

2.1. Caspases

Caspases (cystein aspartate-specific proteases) are a family of cysteine proteases that (as can be deduced from the name) specifically cleave their substrates after an aspartate residue. Caspases specifically recognise and cleave substrates after a sequence of four amino acids with the preferred structure of X-Glu-X-Asp (Thornberry et al., 1997). Currently, eleven human caspases have been identified: caspase 1-10 and -14. Caspase-11- and –12 are murine enzymes that are most likely the homologs of caspase-4 and –5, and the protein initially named caspase-13 was later found to represent a bovine homolog of caspase-4 (reviewed in Degterev et al., 2003). All caspases are synthesised as inactive pro-enzymes (zymogens) containing a prodomain followed by a large and a small subunit. The activation of executioner caspases is mediated by a series of cleavage events, first separating the large and small subunits, followed by the removal of the prodomain (Fig.1). Subsequently, the small and large subunit are assembled into a heterodimer, that is then further connected in a homodimeric fashion to another such heterodimer, forming the active tetrameric caspase (Liang et al., 1997).

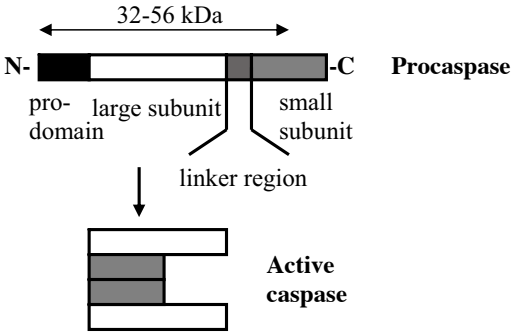

Fig. 1. Schematic presentation of caspase activation.

Most commonly, caspases are divided into initiator and executioner caspases. Initiator caspases possess long prodomains containing one of two characteristic protein-protein interaction motifs: either the death effector domain (DED) (caspase-8 and –10) or the caspase activation and recruitment domain (CARD) (caspase-1, -2, -4, -5, -9, -11 and –12). These provide the basis for interaction with upstream adapter molecules (reviewed in Degterev et al., 2003). Among the initiator caspases, caspase-1, -5 and –11 form a subclass of caspases that can control both apoptosis and certain inflammatory responses. Caspase-2 is unique in that it possesses a long, CARD-containing prodomain, but its substrate preferences are similar to

executioner caspases. Caspase-2 has been reported as an upstream initiator of mitochondrial permeabilisation, but under certain conditions caspase-2 appears to be downstream of caspase-9 and –3, making it difficult to assign caspase-2 to the effector or initiator caspases (Troy and Shelanski, 2003). The executioner caspases (caspase-3, -6, -7) are the main effectors of the death signal, cleaving more than 280 substrates (Fischer et al., 2003), which finally leads to the phenotypic changes characteristic for apoptosis. Executioner caspases are characterised by the presence of a short prodomain and are processed and activated by upstream caspases. In addition, noncaspase proteases have been shown to directly cleave and activate caspases. Among them, granzyme B, a serine protease with substrate specificity for aspartate residues, is important in killing virally-infected cells and directly activates caspase-3 in target cells (Darmon et al., 1995). The calcium-activated protease m-calpain has been reported to process caspase-12 following ER stress (Nakagawa and Yuan, 2000), and cathepsin B was shown to process caspase-1 and –11 *in vivo* (Schotte et al., 1998; Van Compernolle et al., 1998).

Besides their important role as main executioners in apoptotic cell death, caspases have an essential role in the differentiation of macrophages, epidermal cells, erythrocytes, sperm cells and platelets and they are important for B and T cell proliferation (reviewed in Garrido and Kroemer, 2004).

2.2. Caspase-independent cell death and necrosis

Caspases are thought to be the main executioners of apoptotic cell death. However, it has been shown that apoptosis-like cell death occurs without the activation of caspases in some instances. Such caspase-independent cell death pathways might have evolved to fulfil the same purpose as proposed for classical apoptosis, that is to exert a safe and non-inflammatory cell death, since it has been shown that non-apoptotically dying eukaryotic cells can be efficiently phagocytosed in contrast to necrotic cells (reviewed in Leist and Jäättelä, 2001). For example, necrosis seems predominant in the fibroblastic cell line L929 treated with TNF, despite the fact that stimulation of Fas leads to caspase-dependent apoptosis in the same cells (Vercammen et al., 1997).

Chipuk and Green (2005) recently defined caspase-independent death as a cell death induced by proapoptotic conditions, which despite inhibition or disruption of caspase function mediates cell death. Proteases such as cathepsins (lysosomal proteases) and calpains (Ca^{2+}-dependent cysteine proteases) have been shown to participate in this kind of cell death.

Caspase inhibition is known to sensitise mice to a rapid, ROS- dependent death by TNF treatment (Cauwels et al., 2003). Regarding endothelial cells, inhibition of PI3-K sensitised cells to cathepsin B-mediated cell death upon treatment with IL-1 and TNF (Madge et al, 2003). Furthermore, calpains have the ability to mediate oxLDL-induced endothelial cell death in the absence of caspase-3 activation (Pörn-Ares et al., 2003).

Yet another type of caspase-independent cell death is autophagy (also called type II cell death), which is characterised by the formation of large lysosome-derived cytosolic vacuoles and can be triggered by classical apoptotic stimuli. Autophagy has been shown to be important during development and in neurodegenerative disorders, and may negatively regulate tumorigenesis (reviewed in Jäättelä and Tschopp, 2003).

2.3. The intrinsic and extrinsic pathways

The apoptotic machinery can be triggered by a plethora of signals, which are believed to proceed along two main pathways: the intrinsic pathway (mediated by the mitochondria) and the extrinsic pathway (mediated by death receptors) (Fig.2). The extrinsic pathway is initiated by ligation of death receptors (which will be discussed in chapter 6). Engagement of these receptors delivers a rapid proapoptotic signal through a death domain (DD)-mediated recruitment of the adapter protein Fas-associated death domain (FADD) and the formation of a so called death-inducing signalling complex (DISC) (Medema et al., 1997). FADD in turn, mediates the recruitment of procaspase-8 via its death effector domain (DED). In addition to procaspase-8, procaspase-2 and –10 may be recruited to and oligomerised in the DISC (Degterev et al., 2003.) Close proximity of zymogens in the DISC leads to their catalytic activity, presumably by allosteric mechanisms, involving dimerization of caspase-8 or-10 molecules (Salvesen and Dixit, 1999; Boatright et al., 2003). The initiator caspase-8 activates further caspases (including the executioner caspases-3, 6 and –7) or alternatively triggers the activation of caspase-3 in an indirect way, through the cleavage of the proapoptotic Bcl-2 family member, Bid. This protein then translocates to the mitochondria where it causes release of cytochrome c through oligomerisation of the proapoptotic Bcl-2 family members Bax and Bak (Cheng et al., 2001). Thus, Bid provides a link between the extrinsic and the intrinsic pathway. Cells which activate an efficient amount of caspase-8 to directly activate the executioner caspases-3 and -7 are also called type I cells in contrast to type II cells, which depend on a mitochondrial amplification step. In addition to its role as an initiator caspase, caspase-8 can also be activated by downstream caspases, such as caspase-6 in a positive feedback loop (Cowling and Downward, 2002).

The intrinsic or mitochondrial pathway is triggered by activation of caspases from inside the cell by stimuli such as cytotoxic drugs, cytokine withdrawal or anoikis. Central to this pathway is the formation of an intracellular caspase-9-activating complex, the apoptosome. The apoptosome is formed after the release of cytochrome c from the mitochondria and was shown to be a heptamer comprised of seven Apaf-1 adapter molecules, each bound to one molecule of cytochrome c and a dimer of the initiator caspase-9. After its activation through an apoptosome-induced conformational change, caspase-9 further activates executioner caspases-3 and –7 (reviewed in Degterev et al., 2003).

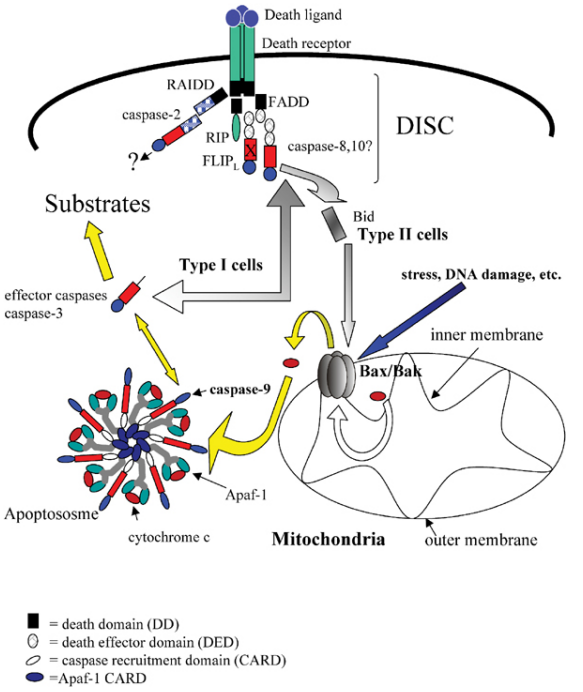

Fig. 2. Schematic presentation of the intrinsic and extrinsic pathways of caspase activation. (Adapted from Degterev et al., 2003).

2.4. Regulation of caspase activity

The apoptotic pathway needs to be highly controlled, since dysregulation of apoptosis is associated with several disorders, such as autoimmune diseases, degenerative disorders (if

excessive) and cancer (if impaired). Therefore some of the mechanisms how caspases can be regulated should be mentioned here.

2.4.1. Phosphorylation

Caspases have been shown to be inhibited upon phosphorylation. Human pro-caspase-9 can be phosphorylated at Ser-196 by Akt leading to its impaired processing (Cardone et al., 1998). Caspase-9 has been further shown to be phosphorylated by ERK, decreasing its activity in transfected cell lines (Allan et al., 2003). In addition, p38 mediates phosphorylation of caspase-3 and –8 resulting in neutrophil survival (Alvarado-Kristensson et al., 2004).

2.4.2. Nitrosylation

The catalytic cysteine of caspases is very active and susceptible to modifications. Caspases can be S-nitrosylated at their active site resulting in inhibition of activity (Mannik et al., 1999). NO has been shown to inhibit endothelial cell apoptosis (Dimmeler and Zeiher, 1999), and TNF treatment was shown to lead to denitrosylation of caspase-3, indicating that S-nitrosylation/denitrosylation could play an important regulatory role in endothelial apoptosis (Hoffmann et al., 2001). Furthermore, NO was suggested to protect hepatocytes from TNF/actinomycin D-induced apoptosis via caspase-8 nitrosylation (Kim et al., 2000). However, NO has also been implicated in apoptosis, thus the outcome of its generation seems to depend on the level of its production and the cellular context (Kim et al., 2002)

2.4.3. Ubiquitination and degradation

IAPs (inhibitor of apoptosis proteins) are the only endogenous proteins that regulate both initiator (caspase-9) and executioner caspases (caspase-3 and -7). Examples of IAP family members include XIAP (X-chromosome linked IAP), c-IAP, c-IAP2 and survivin. An important mechanism of caspase inhibition mediated by XIAP and c-IAP2 is the ubiquitination of caspase-3 and-7 and their degradation in the proteasomes. Furthermore, XIAP, c-IAP1 and c-IAP2 have been shown to ubiquitinate the IAP antagonists Smac/Diablo and Omi/HtrA2. In addition to their ubiquitination and degradation, caspase-3 and-7 can be inhibited by XIAP, by steric hindrance in which substrate entry is blocked. In contrast, binding of XIAP to caspase-9 prevents homodimerization and stabilises the enzyme in an inactive state similar to its monomeric form (Liston et al., 2003). Recently it was reported that XIAP can also interact with and inhibit processed caspase-9 in the apoptosome complex (Zou et al., 2003). Thus, IAPs can prevent or delay apoptosis, but can in addition participate in cell

cycle regulation and in modulation of receptor-modulated signal transduction (Liston et al., 1997).

2.5. The Bcl-2 family

As implied by its name, the bcl-2 gene was first discovered as an oncogene in human B-cell lymphomas and was later shown to inhibit apoptosis (Pegoraro et al., 1984; Hockenberry et al., 1990). To date more than 25 Bcl-2 family members have been defined, and although the sequence homology of the family members is relatively low, they contain a few highly conserved regions, named Bcl-2 homology (BH) domains 1-4 (Adams and Cory, 1998). The antiapoptotic Bcl-2 proteins contain BH1-4, while the proapoptotic can be subdivided into the BH3-only group (containing only the BH3 domain) and a group containing BH 1-3 (Fig.3). Most Bcl-2 members possess a hydrophobic C-terminal domain (HCD) for membrane targeting, except Bad and Bid. In the absence of a death signal, antiapoptotic Bcl-2 members are initially integral membrane proteins found especially in the mitochondria, endoplasmic reticulum and nuclear membranes (Borner, 2003). The large majority of proapoptotic proteins is localised in the cytosol, but following a death signal, a conformational change enables them to integrate into the outer membrane of the mitochondria (Griffiths et al., 1999). The relative amounts or equilibrium between these pro- and antiapoptotic proteins influence the susceptibility of cells to a death signal.

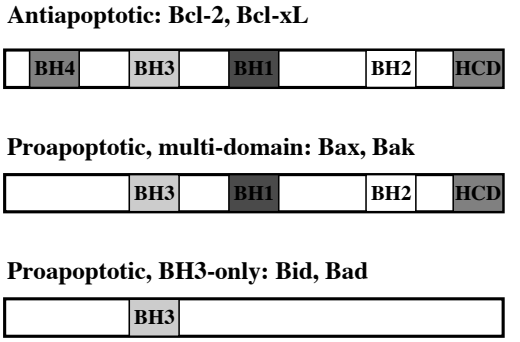

Fig.3. Structure and domain organization of the different groups of Bcl-2 family proteins.

2.5. Bcl-2, Bcl-xL and A1

The antiapoptotic group of proteins include members such as Bcl-2, Bcl-xL, A1 and Mcl-1. Under normal conditions, most antiapoptotic Bcl-2 proteins are associated to organelles due to their membrane anchoring hydrophobic C-terminal domain. Bcl-2 localises to the outer mitochondrial membrane, ER and nuclear membranes, while Bcl-xL is found in the cytosol and in the outer mitochondrial membrane (Vander Heiden et al., 2001; Borner C, 2003). The classical anti-apoptotic Bcl-2 proteins Bcl-2 and Bcl-xL possess four BH domains. BH1-BH3 form a hydrophobic groove which is stabilized by BH4 domain. The binding of BH3-only proteins (except Bid) to Bcl-2 and Bcl-xL occurs via their amphipathic α-helix which optimally fits into this hydrophobic groove. Besides inhibition of the action of the proapoptotic Bcl-2 family members, the antiapoptotic function of Bcl-2 has been explained by several actions: Bcl-2 regulates Ca^{2+} homeostasis by either decreasing ER Ca^{2+} content and flux to the cytosol, or by increasing mitochondrial storage and rate of Ca^{2+} uptake (Rudner et al., 2002). Bcl-2 and Bcl-xL reveal high structural homology with bacterial pore-forming toxins such as colicin and diphteria toxin (Muchmore et al., 1997; Petros et al., 2001), and they exhibited ion channel activities when incorporated into liposomes (Schendel et al., 1998). However, evidence of actual pore formation *in vivo* is missing and even with recombinant proteins in vitro, these channels only form at non-physiological, low pH (pH 4.0) (Minn et al., 1997). Therefore a popular theory on regulating the release of intermembrane space proteins involves the regulation of an existing mitochondrial membrane channel called VDAC (voltage dependent anion channel) (Shimizu et al., 1999). Bcl-2 and Bcl-xL have further been found to regulate the cell cycle by delaying entry into S-phase (Borner et al., 1996), to have anti-inflammatory functions through inhibiting NF-κB, (Badrichani et al., 1999) and to function as antioxidants (Majno et al., 1995).

Phosphorylation of Bcl-2 and Bcl-xL regulates their stability, by either targeting these proteins to proteasomal degradation (Chadebech et al., 1999; Grethe et al., 2004) or preventing degradation and promoting cell survival (Kazi et al., 2002). As in the case of Bid, the N-terminus of Bcl-2 and Bcl-xL has been shown to be cleaved by caspases which converts them into proapoptotic molecules (Cheng et al., 1997; Clem et al., 1998).

A1 differs from Bcl-2 and Bcl-xL in that it is a smaller protein only containing the BH1 and BH2 domain. Since a typical transmembrane domain at the C-terminus of A1 is missing, its localisation has been a bit unclear (Karsan et al., 1996), however it has been shown to be at

least partly localised to the mitochondria (Werner et al., 2002). A1 is expressed in endothelial cells (Ackermann et al., 1999; Grethe et al., 2004), but there are contrasting reports about its role in TNF-induced endothelial apoptosis (Karsan et al., 1996; Ackermann et al, 1999; Duriez et al., 2000). A1 has been shown to delay apoptosis in human microvascular endothelial cells (HMEC, Karsan et al., 1996; Duriez et al., 2000), but not in HUVEC (Ackermann et al., 1999). Since A1 inhibits activation of the mitochondrial pathway, the described discrepancy could possibly be explained by the different involvement of a mitochondrial amplification loop for caspase activation.

2.5.2. Bad and Bid

There are multiple mammalian BH3-only proteins, all which have in common that they are produced constitutively and are maintained in a latent form until unshackled upon a death stimulus. Under normal conditions, phosphorylated Bad is sequestered by 14-3-3 scaffold protein (Downward et al., 1999). Several Bad kinases have been reported, such as Akt, protein kinase A (PKA), RSK1 (p90 ribosomal S6 kinase1), p70S6 kinase and p21 activated kinase (PAK1) (Datta et al., 1997; Harada et al., 1999 and 2001; Shimanura et al., 2000; Schürmann et al., 2000). Whereas phosphorylation at either Ser112 or Ser136 facilitates the formation of a complex between Bad and 14-3-3, phosphorylation at Ser-155 directly blocks BH3-dependent dimerisation with Bcl-xL (Tan et al., 2000). When the apoptotic program is triggered, Bad is dephosphorylated and translocates to the mitochondria, where it exerts its proapoptotic function through inhibiting Bcl-xL and probably also Bcl-2 (Yang et al., 1995; Adachi and Imai, 2002). It has also been proposed that cleavage of 14-3-3 protein during apoptosis could be an additional mechanism to release Bad from 14-3-3 (Won et al., 2003). Bad requires Bax/Bak expression for its proapoptotic function, however, Bad cannot directly interact with Bax or Bak (Ottilie et al., 1997, Adachi and Imai, 2002). Thus, an indirect activation of Bax or Bak via inactivation of Bcl-2 or Bcl-xL has been proposed (Zong et al, 2001). Since Bad can also be cleaved and activated during apoptotic processes similar to Bid (Condorelli et al, 2001), it can not be excluded that Bax could transiently bind to Bad and transport it to the mitochondria.

The proapoptotic Bid is normally present in an inactive form in the cytosol. Upon death receptor ligation, the N-terminus of Bid is cleaved to a truncated Bid (tBid), which is further modified by myristoylation (Zha et al., 2000). tBid translocates to the mitochondria, where it leads to oligomerisation of Bax and Bak (Wei et al, 2000; Eskes et al., 2000) which facilitates the release of cytochrome c and other apoptogenic proteins (Green DR, 2000). It is thought

that tBid is targeted to mitochondria through binding to cardiolipin at contact sites between outer and inner mitochondrial membrane (Lutter et al., 2000), and cardiolipin has been found to be critical for Bax membrane pore formation (Kuwana et al., 2002). In contrast, *in vitro,* tBid oligomers have been reported to facilitate cytochrome c release even in the absence of interaction with Bax or Bak (Grinberg et al., 2002). However, the presence of Bak may account for tBids ability to induce cytochrome c release (Wei et al., 2000). In addition to its cleavage by caspase-8, Bid can be cleaved by granzyme B (Barry et al., 2000) and calpains (Gil-Parrado et al., 2002). Furthermore, Bid has also been reported to be cleaved by caspase-3, independent of death-receptors (Blomgren et al., 2001) and in this context, Bid may amplify the apoptotic process (Degli Esposti et al., 2003). Mechanisms in addition to, or upstream of caspase cleavage, have been shown to regulate the mitochondrial action of Bid. Thus, phosphorylation of Bid has been shown to protect it from cleavage by caspase-8 and caspase-3 (Degli Eposti et al., 2003; Desagher et al., 2001).

2.5.3. Bax and Bak

In healthy cells, Bax exists as a monomer either in the cytosol or loosely attached to the outer mitochondrial membrane, and translocates to the mitochondria during apoptosis (Wolter et al., 1997). Its C-terminal membrane anchor is folded into a hydrophobic pocket and presumably becomes exposed and inserted into the mitochondrial membrane during apoptosis (Suzuki et al., 2000). In addition, the N-terminus may also regulate mitochondrial targeting (Cartron et al., 2002). At the mitochondria, Bax exposes its formerly buried N-terminal 6A7 epitope, before oligomerising, which is considered crucial for mitochondrial permeabilisation and cytochrome c release (Antonsson et al., 2000). Bax has been reported to be cleaved by calpain into a p18 Bax, which has increased proapoptotic function (Gao et al., 2000). It has been shown that p18 Bax needs the presence of full-length Bax and strongly binds Bcl-xL, which is inhibited when the p18 Bax dimerisation site to Bax is mutated (Cartron et al., 2004). In contrast to Bax, Bak is constitutively localised at the mitochondria. However, during apoptosis, Bak exposes its N-terminal epitope (Griffiths et al., 1999), in a similar manner to Bax, which has been shown to occur upon binding of Bid (Ruffolo et al., 2003) and prior to oligomerisation of Bax or Bak. Mice deficient in Bax or Bak have the normal ability to release cytochrome c, while mice deficient for both Bax and Bak are incapable of cytochrome c release, implying strong redundancy in their functions (Wei et al., 2000).

3. The Mitogen-Activated Protein Kinase Superfamily

The MAPK pathway is one of the major systems used by eukaryotic cells to transduce extracellular signals into intracellular responses. Conventional MAPKs consist of three family members: extracellular signal-regulated kinase (ERK), c-Jun NH2-terminal kinase (JNK) and p38. An additional MAPK, termed ERK5 or Big MAPK (BMK1) has been cloned and is a member of a larger MAPK family that also includes ERK7 and ERK 8 (reviewed in Bogoyevitch and Court, 2004). A common feature of all MAPK isoforms is that they are phosphorylated on both threonine and tyrosine residues in an activation motif designated "TXY" (X stands for Glu, Pro, and Gly in ERKs, JNKs and p38s, respectively) by a dual-specificity serine-threonine MAPK kinase (MAPKK). This MAPKK is in turn phosphorylated and activated by an upstream serine/threonine kinase MAPKKK (Fig. 4).

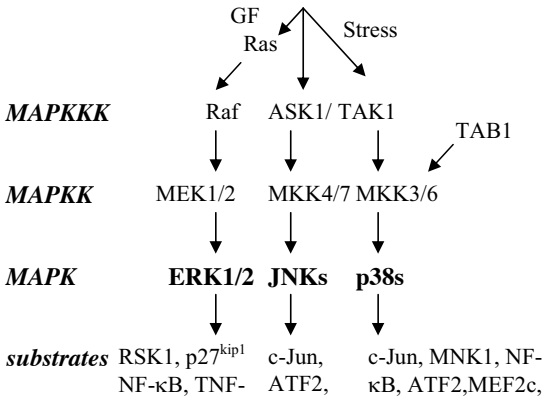

Fig. 4. MAP kinase pathways.

3.1. Stress activated protein kinases (SAPK)

The term stress activated protein kinases (SAPK) was first assigned by Kyriakis et al (1994) to a novel family of serine threonine kinases, i.e., JNK (Gupta et al., 1996). The SAPK/JNK enzymes were closely related in structure and mode of activation to the previously known MAPK. Later it became obvious that JNK and p38 are both activated in response to stress, thus both JNK and p38 are considered as SAPK (Nebrada and Porras, 2000). Although JNK and p38 are often activated by the same stress stimuli and can be activated by the same MKKK, such as apoptosis signal regulating kinase-1 (ASK-1) and TGF-β-activated kinase-1 (TAK1), JNK is activated by the upstream kinases MKK4 and

MKK7, whereas p38 is activated via MKK3 and MKK6 (Wada and Penninger, 2004). In addition, despite simultaneous activation of different MAPKs, selectivity is thought to be achieved by use of scaffold proteins. These proteins bind and sequester selected MAPK pathway components and thereby provide integrity and coordinated activation and function of MAPK components in response to specific stimuli (Garrington and Johnson, 1999). One such adapter protein has been described and is termed transforming growth factor-β-activating protein kinase-1 (TAB1), which can activate p38 in a MKK-independent manner in certain circumstances (Ge et al., 2002). Another scaffold protein called JNK interacting protein-1 (JIP-1) has been described for selective regulation of JNK activation (Garrington and Johnson, 1999).

3.1.1. p38 signalling

To date, there exist four isoforms of p38 (also called CSBP, mHOG1, RK and SAPK2): p38α, p38β, p38γ, and p38δ. Of these, p38α and p38β are ubiquitously expressed and best described because of the use of the p38 inhibitor SB203580 which only inhibits these isoforms (Kumar et al, 1997), while p38γ and p38δ are differentially expressed depending on the tissue (Hale et al, 1999). Each p38 MAPK isoform shares ~60% identity with the other isoforms of the p38 group but only 40-45% with the other MAPK family members (reviewed in Zarubin and Han, 2005). In endothelial cells p38α, p38β and p38δ isoforms are expressed. It was shown that p38α and p38β are expressed at comparable levels and p38δ to a less extent. Furthermore, stimulation with IL-1β induces >6-fold more p38α activity than p38β, suggesting that it could be the most activated isoform in cytokine-stimulated endothelial cells (Hale et al, 1999).

3.1.1.1. p38 downstream targets

p38 has a wide range of substrates, and about half of those identified so far are transcription factors, among them are activating transcription factor (ATF)-1,2 and 6, myocyte enhancer factor 2C (MEF2C), ELK1 and p53. In addition to a regulation at transcriptional level, posttranscriptional regulation of inflammatory gene expression has also been linked with the p38 pathway. Thus, it has been suggested that the p38α/MAPK-activated protein kinase-2 (MK2) pathway regulates the stability of the messenger RNA for TNF and IL-1. These messenger RNAs contain AU-rich regions in the 3'untranslated region (UTR), which are normally occupied by AU-binding proteins, leading to a blockade of translation or

rapid turnover of transcripts. p38 has been shown to phosphorylate these AU-binding proteins, which results in their release and allows translation and secretion of TNF and IL-1 (Zarubin and Han, 2005). It has also been reported that p38α-dependent phosphorylation of histone H3 marks promoters for increased NF-κB recruitment, resulting in increased expression of several inflammatory cytokines and chemokines (Saccani et al., 2002). Additional kinase substrates of p38 are p38 regulated/activated kinase (PRAK) which is thought to regulate heat shock protein 27 (Hsp27) and MNK1, which is thought to function in translational initiation, since MNK1 and MNK2 can phosphorylate eukaryotic initiation factor-4e (eIF-4E).

3.1.1.2. p38 and apoptosis

The role of p38 in the regulation of apoptosis is both cell-type and stimulus-dependent. In addition, there are some reports suggesting that p38α and β isoforms play different roles in death and survival. Thus, p38α induced apoptosis in cardiomyocytes and Jurkat cells, whereas β enhanced survival (Nemoto et al., 1998; Wang et al., 1998b). p38 has been reported to be upstream as well as downstream from caspases (Zarubin and Han, 2005). In line with these findings, I observed a caspase-independent early p38 activation and a caspase-induced feedback stimulation of p38 MAPK at later time points of TNF treatment (Grethe et al., 2004).

There are several published reports on p38-mediated survival. We found that p38 protects neutrophils from Fas-induced apoptosis (Alvarado-Kristensson et al., 2001) and others found that p38 protects eosinophils from apoptosis induced by cytokine-deprivation (Kankaanranta et al., 1999).

Considering endothelial cells, several reports show that induction of apoptosis by treatment with thrombospondin-1 (Jimenez et al., 2001), high glucose (Nakagami et al., 2001) and oxLDL (Nihei et al., 2005) requires p38 activity. However, inhibition of apoptosis by estradiol and carbon monoxide has also been reported to be mediated via activation of p38 (Razandi et al., 2000; Zhangh et al., 2005).

In neutrophils, p38 signals survival via phosphorylation of caspase-8 and -3, resulting in their inhibition (Alvarado-Kristensson et al., 2004). It has also been suggested that upregulation of COX-2, probably via p38, mediates stabilisation of the COX-2 transcript, resulting in survival of cancer cells treated with photodynamic therapy (Hendrickx et al.,2003), and p38-mediated activation of the transcription factor MEF2 is necessary for the

survival of developing neurons (Mao et al, 1999). Furthermore, the lethal factor of *Bacillus anthracis* cleaves MKK3 and MKK6, the upstream kinases of p38, which was suggested to dismantle the p38 signal module in order to paralyse host innate immunity and result in macrophage apoptosis (Park et al., 2002).

Mechanisms of p38-mediated apoptosis have been reported to include upregulation of FasL in murine T cells (Zhang et al., 2000a) and phosphorylation and activation of p53 in doxorubicin-induced fibroblast apoptosis (Sanchez-Prieto, 2000). At the transcriptional level, expression of monoamine oxidase (DeZutter and Davis, 2001) or growth arrest and DNA damage (GADD)-inducible genes (Sarkar et al., 2002) have both been shown to mediate pro-apoptotic effects of p38. Furthermore, p38 mediates phosphorylation of Bcl-2 in growth factor-starved memory B lymphocytes, thus causing apoptosis (Torcia et al., 2001). In addition, p38-mediated phosphorylation of Bcl-xL results in degradation of Bcl-xL and endothelial apoptosis (Grethe et al., 2004). However, phosphorylation of Bcl-xL has also been linked to prostate cancer cell survival (Kazi et al., 2002).

3.1.1.3. Downregulation of p38 signalling

p38 activity can be regulated by dephosphorylation of the kinase, achieved by dual specificity phosphatases (DUSP) belonging to the MAP kinase phosphatase family (MKP), such as DUSP16 and DUSP10 (also known as MKP7 and 5, respectively) (Keyse et al., 2000; Tanoe et al. 2001). In addition, two protein tyrosine phosphatases (PTP), haemopoietic PTP (HePTP) and STEP-like phosphatase (PTP-SL) can dephosphorylate p38. Also the serine/threonine phosphatases PP2A and PP2C can dephosphorylate p38 (Keyse et al., 2000; Alvarado-Kristensson and Andersson, 2005), but Takekawa et al. (1998) suggested that PP2C is more important for negative regulation of the p38 pathway. p38 has also been reported to be inhibited either by Akt via phosphorylation and blocking of MEKK3 (Gratton et al., 2001) or by Akt2 through phosphorylation and inhibition of ASK1 (Yuan et al., 2003).

3.1.2. ERK signalling

The ERK pathway is activated by mitogenic stimuli, such as growth factors, cytokines and phorbol esters and plays an important role in regulating cell growth, differentiation and survival (Zhang and Liu, 2002). However, ERK signalling can also lead to apoptosis in cells treated with anthracyclines (Guise et al., 2001; Tang et al, 2002; Yeh et al.2004).

3.1.2.1. Regulation of ERK signalling

ERK1/2 is activated by a variety of receptor tyrosine kinases (RTK) and G-protein coupled receptors. Current understanding of the mechanisms by which cell surface receptors activate ERKs is based mainly on studies of epidermal growth factor receptor (EGFR). This receptor dimerises upon ligand binding allowing the transphosphorylation of tyrosines within the cytoplasmic domains (Pierce et al., 2001), which leads to recruitment of other signalling proteins containing phosphor-tyrosines such as phospholipase Cγ (PLCγ) and Src. In addition, tyrosine phosphorylation of EGFR results in phosphorylation of the adaptor protein SHC and the association of SHC and Grb2 with the receptor. Grb2 is associated with son of sevenless1 (SOS1), an exchange factor for Ras, that catalyses the exchange of GTP for GDP on Ras. Ras in turn initiates a phosphorylation cascade consisting of Raf (MAPKKK), MEK1/2 (MAPKK) and ERK1/2 (MAPK). ERK1 and 2 are 90% identical and commonly activated under the same circumstances by MEK1 and 2 (reviewed in Bogoyevitch and Court, 2004). However, knocking out ERK2 has been shown to be lethal to the embryo in contrast to ERK1, suggesting nonredundant functions (O'Neill et al., 2004).

ERK activation induced by TNF receptor ligation has been very recently reported to involve TRAF-2 binding to Src kinases, which in turn activate PLC-γ1, which can activate Ras and ERK (van Vliet et al., 2005). Furthermore, it has been proposed that TNF-induced ERK signalling requires lipid rafts (Doan et al., 2004).

Several phosphatases are involved in inhibition of ERK activity, such as PP2A (Alessi et al., 1995), haematopoietic tyrosine phosphatase (HePTP, Saxena et al., 1999) and the DUSP MKP3 (Kim et al, 2003).

3.1.2.2. ERK downstream targets

Activated ERK1/2 phosphorylates numerous substrates in various cellular compartments. Among the substrates are cytoskeletal proteins (neurofilaments and paxillin), several MKs and nuclear substrates (among them NFAT, ELK-1, STAT3, MEF2, c-Fos, c-Myc). In addition, ERK1/2 phosphorylates protein kinases such as RSKs, MSKs and MNKs; MSKs and MNKs can be phosphorylated by p38 as well as by ERK, but RSKs are exclusively activated by ERKs (reviewed in Roux and Blenis, 2004). RSK1 is a well described substrate of ERK1/2 and plays an important role in survival via phosphorylation of Bad (Shimumara et al., 2000) and activation of NF-κB and is also involved in cell cycle regulation via inhibition of the cyclin-dependent kinase inhibitor $p27^{kip1}$ (Roux and Blenis, 2004). Membrane proteins

can also be phosphorylated by ERK; one example is TNF-R1, the phosphorylation of which has been shown to result in accumulation of overexpressed TNF-R1 in the ER where it recruits Bcl-2 and protects against apoptosis in HeLa cells (Van Linden et al., 2000; Cottin et al., 2001).

Despite its well known function in survival and proliferation, ERK activation has also been shown to mediate apoptosis. Paclitaxel and doxorubicin-induced ERK activation resulted in phosphorylation of tau, a major helical filament in Alzheimers disease, and neuronal apoptosis (Guise et al., 2001). Furthermore, ERK2-mediated phosphorylation of p53 has been shown to be important in doxorubicin-induced apoptosis in MCF-7 breast cancer cells (Yeh et al, 2004).

4. PI3-K/Akt signalling

The serine/threonine kinase Akt/PKB (protein kinase B) was initially identified by three independent groups, based on its homology to protein kinase A (PKA) (Coffer et al., 1991) and C (PKC) (Jones et al., 1991) or as the cellular homolog to the retroviral oncogene viral akt (v-Akt) (Bellacosa et al., 1991). Akt has emerged as a central player in the signal transduction pathways activated in response to growth factors or insulin and is thought to be an important regulator of cell survival, cell growth and nutrient metabolism (Brazil et al., 2001). In mammals, three Akt genes have been identified, termed PKBα/Akt1 (Jones et al., 1991), PKBβ/Akt2 (Cheng et al., 1992) and PKBγ/Akt3 (Brodbeck et al., 1999), located at chromosomes 14q32, 19q13, and 1q44, respectively. The Akt signalling pathway has been shown to play an important role in survival of several tumours (Bao et al., 2004; Wendel et al., 2004).

4.1. Regulation of PI3-K/Akt signalling

Akt, the downstream effector of PI3-K, is activated by class 1A and class 1B PI3-K, which in turn are activated by tyrosine kinase and G-protein-coupled receptors, respectively (Wymann et al., 2003). Following recruitment to these receptors, PI3-K is activated and catalyses the phosphorylation of the inositol ring of phosphatidylinositol (PtdIns) lipids at the D-3 position producing PtdIns bisphosphate (PtdIns(3,4)P_2) and PtdIns trisphosphate (PtdIns(3,4,5)P_3). Direct binding of Akt through its pleckstrin homology (PH) domain to PtdIns(3,4,5)P_3 (Andjelkovic et al., 1997) recruits Akt to the plasma membrane and alters its confirmation to allow subsequent phosphorylation by a phosphoinositide-dependent kinase (PDK1, Stephens et al., 1998) at Thr308 in Akt1, Thr309 in Akt2 and 3, and at Thr305 in

PKBγ1. These phosphorylations stimulate the catalytic activity of the Akt isoforms, although phosphorylation at Ser473 (Ser474 in Akt2 and 3) is required for maximum activity (Datta et al., 1999; Marte et al., 1997). The mechanism of Ser473 phosphorylation is not completely understood. However, there is evidence suggesting that the site can be autophosphorylated (Toker et al., 2000) or phosphorylated by distinct serine kinases including the integrin-linked kinase (ILK) (Persad et al., 2001). However, it was suggested that ILK mediated phosphorylation at Ser473 could occur indirectly (Lynch et al., 1999), although other investigators could block this phosphorylation with an ILK-specific inhibitor (Persad et al., 2001). Furthermore, several recent reports have reported a role of tyrosine phosphorylation in Akt activation (Conus et al., 2002; Jiang and Qiu, 2003). Akt can also be activated in a PI3-K-independent manner by cAMP- elevating agents through PKA. For this activation, Akt phosphorylation at Thr308 is required, however the mechanism how PKA activates Akt is not fully clear (Filippa et al., 1999). It was also shown that Akt can be activated by Ca^{2+}/calmodulin-dependent kinase directly *in vitro* (Perez-Garcia et al., 2004) and another report (Konishi et al., 1997) showed that heat shock protein 27 (Hsp27) is involved in the activation of Akt after heat shock and superoxide treatment.

Activation of PI3-K is counteracted by the tumour suppressor phosphatase, PTEN (phospatase and tensin homolog deleted from chromosome 10) (Stambolic et al., 1998) by dephosphorylating the 3 position of PtdIns-3,4,5-P_3 to produce PtdIns-4,5-P_2. Loss of PTEN function or at protein level have been reported in several advanced human cancers, indicating that uncontrolled PI3-K signalling contributes to metastatic progression. Furthermore, the Src-homology 2 (SH2)-containing phosphatases (SHIP1 and SHIP2) dephosphorylate the 5 position of the inositol ring to produce PtdIns (3,4,5)P_3 (Cantley et al., 2002). Akt is also dephosphorylated and inhibited by PP2A (Andjelkovic et al., 1996; Tanaka et al., 2003)

4.2. Akt downstream targets

Activated Akt regulates survival via phosphorylation of the antiapoptotic Bcl-2 protein Bad, as described before. Similarly to its regulation of Bad, Akt–mediated phosphorylation of forkhead related transcription factors1 (FKHR-L1) creates a binding site for the 14-3-3 family of proteins (Datta et al., 1999) and the complex of 14-3-3 and FKHR-L1 is retained in the cytosol, where it blocks transcription of genes such as Fas ligand and TRAIL and the pro-apoptotic Bcl-2 member Bim (Bugering et al., 2003).

Akt has also been shown to phosphorylate human caspase-9 on Ser196, resulting in attenuation of its activity (Cardone et al, 1998). Since this site is not conserved in other lower

mammalian species such as mouse or rat, Akt-mediated phosphorylation of caspase-9 is specific for higher species (Datta et al., 1999; Fujita et al., 1999). Akt also controls cell proliferation by phosphorylating glycogen synthase kinase-3β (GSK-3β) resulting in increased levels of cyclin D_1 (Cross et al., 1995). Akt-mediated phosphorylation of GSK-3β is also known to regulate protein synthesis (Cantley et al., 2002). Furthermore, Akt enhances the degradation of IκBs and it cooperates with other factors to elicit NF-κB-mediated activation and along with that transcription of antiapoptotic genes for proteins such as A1 and c-IAPs (Datta et al., 1999). An additional regulation of antiapoptotic genes such as bcl-2 and mcl-1 is mediated by Akt via phosphorylation of cyclic AMP (cAMP)-response element binding protein (CREB) transcription factor on Ser133 (Du et al., 1998; Wang et al., 1999).

5. Protein Phosphatase 2A (PP2A)

Protein phosphatases are divided into three families designated phospho-protein phosphatases (PPP), protein phosphatase magnesium dependent (PPM) and protein tyrosine phosphatase (PTP). In contrast to PTP, which includes tyrosine phosphatases, PPM and PPP comprise serine/threonine phosphatases and the latter of which comprises PP1, PP2A (including PP2A-like PP4 and PP6), PP2B (also called calcineurin), and PP5 and PP7 subfamilies (Barford et al., 1998). PP2A is highly conserved during the evolution of eukaryotes, and PP2A has been implicated in the regulation of cellular metabolism, DNA replication, transcription, RNA splicing, translation, cell-cycle progression, morphogenesis, development and transformation (Millward et al., 1999). PP2A accounts for as much as 1% of total cellular proteins and for the major portion of serine/threonine phosphatases in most tissues and cells (Sontag, 2001). PP2A's importance in cellular homeostasis becomes obvious by the existence of many naturally occurring inhibitors, among them fostriecin and calyculin A, deriving from a marine sponge and bacterium *Streptomyces*, respectively. Furthermore, the deletion of the gene encoding the catalytic subunit is lethal in yeast and mouse (Millward et al., 1999).

5.1. Structure of PP2A

PP2A has been shown to have a myriad of substrates *in vitro*, and is involved in a lot of cellular activities. This diversity of action can be explained by its structure. The core enzyme of PP2A comprises an approximately 36-kDa catalytic subunit C that is associated with a 65-kDa scaffolding subunit A (also called PR65). Distinct classes of B-type regulatory subunits, termed B, B′, and B′′ that are encoded by different genes, bind to this core enzyme and build

heterotrimers, the prevalent form of PP2A *in vivo*. Whereas two isoforms (α and β) of the A and C subunits have been described, there is an enormous, ever-growing number of B-(regulatory) subunit isoforms, which modulate PP2A activity and influence substrate specificity and location (Sontag et al., 2001).

5.2. Regulation of PP2A

It has been shown that theoretically more than 50 PP2A heterotrimers can exist because of the enormous diversity of B subunits (Millward et al., 1999). PP2A is regulated at the translational level of the mRNA coding for the catalytic subunit of PP2A via a mechanism involving auto-inhibition during translation. Presumably, PP2A dephosphorylates a specific phosphoprotein(s) from the translational apparatus leading to the inhibition of the enzyme synthesis (Baharians and Schönthal, 1998). It has been shown that distinct holoenzymes are differentially expressed and distributed in tissues and cells.

PP2A activity is also regulated by post-translational modifications. The catalytic subunit C is highly conserved between species and contains two potential phosphorylation sites (Thr304, Thr307) and a methylation site (Leu309). Thr304 is phosphorylated by "autophosphorylation-activated protein kinase", and Thr307 can be phosphorylated by receptor- and non-receptor tyrosine kinases such a Lyn, Fyn, Src and Jak2. PP2A inactivation requires phosphorylation at both threonine residues, and reactivation occurs through the unique ability of PP2A to catalyse intramolecular autodephosphorylation (Lechward et al., 2001). The level of PP2A activity has been shown to be relatively high in resting cells, so activation of extracellular signal-regulated cascades require transient deactivation of PP2A. This could occur via stimulus-dependent phosphorylation or methylation, but also fluctuations in the phosphorylations and/or methylation levels of C could promote subunit exchange, resulting in changes in PP2A targeting and substrate specificity (Sontag et al., 2001).

In addition, protein interactions between PP2A and other intracellular components play an important role in the functional specificity of PP2A signalling: Such interactions can direct PP2A to discrete cellular domains and/or determine PP2A's function. For example, proteins encoded by the genomes of DNA viruses, such as small t-antigen of simian virus (SV40) may function as variable regulatory subunits in order to subvert the signal transduction machinery of a host cell and to promote its survival and replication (Lechward et al., 2001; Millward et al., 2001).

5.3. PP2A downstream targets

It has been shown that PP2A directly regulates several major transcription factors such as signal transducer and activator of transcription (STAT), CREB and c-Jun (Millward et al., 2001). However, protein kinases have emerged as the main target group of PP2A. Most of these are inactivated by dephosphorylation, with the exception of GSK-3β, mammalian sterile 20-like1 (MST1) and Wee1 kinases. PP2A controls particularly those protein kinases belonging to the AGC subgroup (which includes Akt, protein kinase C and p70 S6 kinase), as well as calmodulin-dependent kinases, MEK/ ERK, the cyclin-dependent kinases and the IκB (Millward et al., 2001). PP2A can dephosphorylate and inactivate MEK1 and ERK, and its inhibition activates MEK and ERK (Alessi et al., 1995; Grethe and Pörn-Ares, 2005), although ERK dephosphorylation is also regulated by MKP-1. It has also been suggested that PP2A can regulate MEK/ERK signalling via Raf-1 (Abraham et al., 2000), but expression of SV 40 small t antigen in cultured cells had no effect on Raf-1 (Sontag et al, 1993). Transient expression of SV40 small t-antigen activates MEK1 and ERK, which might explain how it promotes transformation (Sontag et al., 1993). Furthermore, PP2A dephosphorylates Akt (Andjelkovic et al., 1996; Tanaka et al., 2003).

5.4. PP2A and apoptosis

PP2A has been implicated in the regulation of apoptosis and appears primarily proapoptotic. Thus, IL-3 withdrawal-induced apoptosis was shown to be mediated by PP2A-dependent dephosphorylation of Bad or Bcl-2 (Chiang et al., 2003; Deng et al., 1998). It has also been reported that the A subunit of PP2A can be cleaved by caspase-3 during apoptosis, resulting in increased activity (Santoro et al., 1998). Ceramide, a lipid messenger generated by sphingomyelinase-dependent pathways, can directly activate the subunit A of PP2A (Chalfant et al, 1998), and PP2A-mediated dephosphorylation and subsequent inactivation of Bcl-2 was involved in ceramide-induced apoptosis (Ruvolo et al., 1999). Furthermore, inhibition of PP2A protected U937 cells from apoptosis mediated by TNF, Fas and TRAIL (Härmälä-Braskén et al., 2003). My results show that PP2A mediates the downregulation of MEK/ERK and PI3K/Akt survival pathways, resulting in Bad dephosphorylation in TNF and doxorubicin-induced endothelial apoptosis, respectively (Grethe and Pörn-Ares, 2005; Grethe et al., 2005).

6. Death Receptor signalling

The death receptors belonging to the TNF superfamily characterized by the presence of a cysteine-rich domain (CRD) in the extracellular portion, include among others the following: Fas (CD95/Apo-1), TNF-R1 (CD120a), DR3 (Apo-3/LARD, TRAMP, WSL-1, LARD), TRAIL-R1 (DR4, Apo-2), TRAIL-R2 (DR5, KILLER, TRICK2) and DR6. As described before (chapter 2.3), ligation of these receptors, which lack any enzymatic activity, initiates the extrinsic pathway of apoptosis via recruitment of adapter proteins.

Endothelial cells express both Fas and Fas-L. The function of endothelial Fas-L is to inhibit leukocyte extravasation by inducing apoptosis in Fas-expressing mononuclear cells invading the vessel wall in the absence of normal inflammatory stimuli (Sata and Walsh, 1998a). Endothelial cells themselves are resistant to Fas-mediated apoptosis under normal conditions, but can be sensitised to Fas-mediated apoptosis by the atherogenesis promoter oxLDL (Sata and Walsh, 1998b), which occurs via downregulation of the well known caspase-8 inhibitor c-FLIP (cellular FLICE-inhibitory protein, Irmler et al., 1997; Sata and Walsh, 1998c). Furthermore, detachment-induced apoptosis (anoikis) in endothelial cells occurred via Fas/Fas-L interaction and FLIP downregulation (Aoudjit and Vuori, 2001). In addition, suppression of PI3-K/Akt signalling has been shown to downregulate FLIP and sensitise endothelial cells to Fas-mediated apoptosis (Suhara et al., 2001).

Endothelial cells are known to express both TRAIL-R1 and –2, and ligation of these receptors has been shown to result in apoptosis, occurring in a FADD-dependent manner and involving caspase activation. TRAIL-mediated cell death is enhanced by cotreatment with cycloheximide (CHX), similarly to TNF-induced apoptosis (Li et al., 2003). In contrast, other researchers reported that endothelial cells are normally resistant to TRAIL, but can be sensitised to TRAIL-induced apoptosis by inhibition of PI3-K/Akt (Secchiero et al. 2003) or inhibition of TRAIL-R3 (Zhang et al., 2000b).

6.1. TNF and TNF-Receptors

TNF is the prototypic member of a large cytokine family, the TNF ligand family, consisting of 19 members (reviewed by Aggarwal, 2003). TNF was originally identified 1975 as a factor that leads to rapid haemorrhagic necrosis of transplantable tumours in mice (Carswell et al., 1975), and its protein sequence determined by Aggarwal's group in 1984 (reviewed in Aggarwal, 2003). This proinflammatory cytokine is produced mainly by activated macrophages or monocytes, but also by endothelial cells, fibroblasts and neuronal tissues, the main target of TNF being the endothelium. Today it is known that TNF has

pleiotropic functions in immunity, inflammation, cell proliferation, differentiation and apoptosis (Ashkenaki and Dixit, 1998). Primarily produced as a type II transmembrane protein arranged in stable homotrimers, this membrane-integrated form (memTNF) can be cleaved by the metalloprotease TNF α converting enzyme (TACE). Subsequently this releases the soluble form of the protein (sTNF). MemTNF, sTNF, but also lymphotoxin-α, bind to death receptor TNF-R1 (also known as CD120a or p55/60) and TNF-R2 (CD120b; p75/80), but the function of lymphotoxin-α is largely undefined (reviewed in Wajant et al., 2003).

TNF-R1 and TNF-R2 contain four cysteine-rich repeats in their extracellular domains and form elongated shapes, which interact with the lateral grooves of the trimeric ligands. Both receptors can be cleaved, yielding soluble receptors with potential neutralising capacities, although they have low affinities compared to the membrane-integrated forms. TNF-R1 is constitutively expressed in most tissues, whereas TNF-R2 is typically found in cells of the immune system (Wajant et al., 2003), but is also present in endothelial cells (Slowik et al., 1993). TNF-R1 appears to be the key mediator of TNF signalling in the vast majority of cells, including endothelial cells (Loetscher et al., 1993). An important difference between the two receptors is that TNF-R1 contains a DD which recruits other DD-containing proteins and couples the death receptor to caspase activation and apoptosis (Schulze-Osthoff et al., 1998). Ligand-dependent trimerization was long considered a key event for signal initiation, but it has been reported that preligand binding assembly domains (PLAD) could keep TNF-R1 and TNF-R2 in a silent, homomultimerised status (Chan et al., 2000) and antagonise spontaneous autoactivation.

TNF-R1 activation leads to many diverse responses, depending on cell type and environmental factors. The receptor can trigger cellular activation via NF-κB or apoptosis via activation of caspases. In most instances, TNFR-1 signalling results in NF-κB activation and expression of proinflammatory proteins, such as E-selectin, ICAM-1 and IL-8. However, TNF-R1 signals apoptosis in conditions where new protein synthesis is blocked or where NF-κB is inhibited prior to TNF stimulation (Wallach, 1997; Kreuz et al., 2001; Varfolomeev and Ashkenazi, 2004). However, NF-κB can also regulate proapoptotic signals through the regulation of death receptors (DR1-6) and death receptor ligands such as FasL, TNF and TRAIL as well as via upregulation of p53 and cMyc (Aggarwal, 2003).

CHX is not only required for sensitisation of cells to TNF-induced apoptosis. Zen et al (1999) have shown that endothelial cells undergo IL-1β- and LPS-induced apoptosis upon cotreatment with CHX. Others reported that inhibition of mRNA or protein synthesis

sensitises renal cell carcinoma cells to TRAIL-induced apoptosis via downregulation of FLIP or upregulation of TRAIL-Rs (Griffith et al., 2002; Brooks and Sayers, 2004) and CHX enhanced Fas-induced endothelial apoptosis after sensitisation with IFN-γ (Li et al., 2002).

6.1.1. TNF-R1 signalling

TNF-R1-mediated apoptosis requires FADD and caspase-8, but no direct association of these molecules with endogenous TNF-R1 has been seen (Harper et al., 2003). In contrast to Fas signalling, where Fas binds directly to FADD, TNF-R1-induced proapoptotic signalling requires the formation of two distinct signalling complexes (Fig 5).

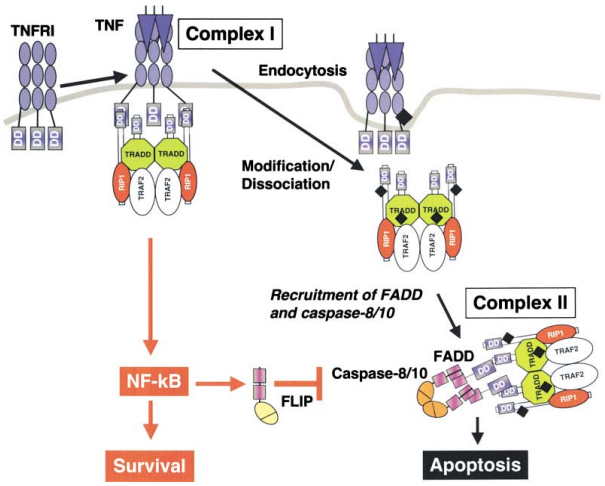

Fig.5. Model of TNFR1-Mediated Apoptosis. After binding of TNF to TNFR1, rapid recruitment of TRADD, RIP1, and TRAF2 occurs (complex I). Subsequently TNFR1, TRADD, and RIP1 become modified (♦) and dissociate from TNFR1. The liberated death domain (DD) of TRADD (and/or RIP1) now binds to FADD, resulting in caspase-8/10 recruitment (forming complex II) and resulting in apoptosis. If NF-κB activation triggered by complex I is successful, cellular $FLIP_L$ levels are sufficiently elevated to block apoptosis and cells survive. (Adapted from Micheau and Tschopp, 2003).

TNF-R ligation results in a very quick and transient assembly of complex I, which seems to occur in lipid rafts (Legler et al., 2002) and contains the TNF-R1 itself, the serine/threonine kinase receptor-interacting protein kinase-1 (RIP1), TNF-R associated factor 2 (TRAF2) and TNF-R associated DD (TRADD). Complex I transduces signals leading to

NF-κB activation through recruitment of the I-κB kinase "signalosome" complex (Poyet et al., 2000; Zhang et al., 2000c). NF-κB activation results in the expression of antiapoptotic proteins such as c-FLIP (Kreuz et al., 2001), A20 (Krikos et al., 1992), cIAP1, cIAP2, TRAF1 and TRAF2 (Wang et al., 1998a). In addition, A1 and Bcl-xL are regulated by NF-κB (Zong et al., 1999; Chen et al., 2000). After prolonged (>2h) TNF-R1 signalling, possibly after receptor internalisation, RIP1, TRAF2 and TRADD dissociate from the receptor in complex I, and recruit FADD and caspase-8 and –10 into the new complex II. Since caspases have to be brought into close proximity for their activation (Boatright et al., 2003), it has been suggested that TRADD remains oligomerised upon dissociation from TNF-R1 and thus brings caspase-8/10 into closed proximity after recruitment of FADD (Micheau et al., 2003).

$FLIP_{L,}$ is a well known caspase-8 inhibitor (Irmler et al., 1997), which is structurally similar to procaspase-8 in that it contains two DED domains and a caspase-like domain lacking the critical active site cysteine residue essential for catalytic activity. $FLIP_L$ and $FLIP_{s,}$, a splice variant of $FLIP_{L,}$ containing only two DED domains, bind to FADD within the DISC and interfere with caspase-8 processing and activation (Irmler et al., 1997). The availability of $FLIP_{L,}$ at the moment complex II is formed, is dependent on complex I-triggered NF-κB signalling. NF-κB activation promotes FLIP expression, whereas if complex I–triggered NF-κB activation is not productive, FLIP levels decrease and allows procaspase-8 to induce apoptosis.

6.1.2. Crosstalk between TNF-R1 and TNF-R2

Apoptosis induced through TNF-R1 might be regulated via TNF-R2. It has been observed that TNF-R2 can potentiate the apoptotic response to TNF (Chan and Lenardo, 2000; Lucas et al., 1998; Fotin-Mleczek et al., 2002) and several mechanisms have been proposed for this. One such mechanism is as a TNF-R2-mediated depletion of TRAF2 and IAPs (Fotin-Mleczek et al., 2002) or a TNF-R2-mediated sensitisation to other death receptors such as TRAIL1 and TRAIL2 or DR5 (Chan and Lenardo, 2000). The participation of TNFR-2 in TNF responses has been attributed to the ability of TNF-R2 to raise the virtual TNF concentration in the proximity of TNF-R1 and therefore to enhance signalling of the later receptor (the model of ligand passing by Tartaglia et al., 1993). Grell et al (1995) showed that TNF-R2 can be strongly stimulated by memTNF rather than by sTNF, resulting in cooperative signalling. Regarding endothelial cells, actinomycin D sensitised microvascular endothelial cells to TNF-induced apoptosis via TNF-R1, whereas TNF-induced apoptosis by

prolonged treatment with TNF alone required both receptors (Lucas et al., 1998). In addition, Slowik and coworkers (1997) showed that engagement of TNF-R1 was sufficient to signal apoptosis in HUVEC mediated by TNF in combination with CHX or ceramide. In this study, no killing was seen when TNF selectively binding to TNF-R2 was used, although it had reduced affinity for TNF-R2 compared to wt-TNF. Together, these findings suggest a more important role of TNF-R1 in TNF-induced endothelial apoptosis upon sensitisation with CHX.

7. Doxorubicin

7.1. Historical background

The anthracycline doxorubicin is one of the most effective and widely used anti-neoplastic agents in cancer therapy (Weiss 1992). It is an analog to the the original anthracycline daunorubicin (DNR), which was initially isolated from cultures of *Streptomyces peucetius* in the early 1960s by two different groups (Di Marco et al., 1964; Dubost et al., 1964). Doxorubicin was developed by subjecting the Streptomyces that produced DNR to the mutagenic effects of N-nitroso-N-methyl urethane. The new anthracycline, differing from DNR only by a single hydroxyl group, was originally named Adriamycin (Arcamone et al., 1969), but was later renamed, since Adriamycin was a registered trade name. Doxorubicin was approved for marketing in the United States in 1974 and still remains the agent with the widest spectrum of antitumour activity, although more that 2000 anthracycline analogs are have been synthesised in the last 25 years in order to develop an anthracycline which could be orally absorbed and show less cardiotoxicity than doxorubicin (Weiss, 1992).

7.2. Mechanism of doxorubicin-induced apoptosis

Doxorubicin consists of a napthacenequinone coupled to an aminosugar daunosamine (Fig.6). The anthracycline ring is lipophilic, whereas the saturated end of the ring system contains abundant hydroxyl groups adjacent to the amino sugar, producing a hydrophilic center. The molecule is amphoteric, with acidic functions in the ring phenolic groups, and a basic function in the sugar amino group, which facilitates doxorubicin binding to cellular membranes.

The cytotoxic mechanism of doxorubicin is complex and not fully understood. Its intracellular effects include inhibition of topoisomerase II and DNA intercalation, resulting in inhibition of DNA replication and strand break-related DNA damage (Gerwitz 1999). However, the concentration of doxorubicin required to intercalate into the DNA and to cause

inhibition of DNA synthesis cannot be achieved *in vivo* without excessive toxicity. Another form of DNA damage, independently from DNA interaction, occurs via generation of reactive oxygen species (ROS). However, also ROS-induced DNA damage occurs only at very high doxorubicin levels upon treatment (Potmesil et al., 1984). Treatment of endothelial cells and cardiomyocytes with clinically relevant concentrations of doxorubicin also involves formation of ROS and results in apoptosis (Kotamraju et al., 2000; Wang et al., 2002 and 2004). Furthermore, ROS can activate MAPK and my results along with others have shown that p38, ERK and JNK are important regulators of doxorubicin-induced apoptosis (Grethe et al., 2005, manuscript submitted; Panaretakis et al., 2005).

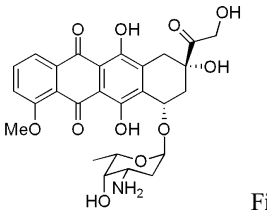

Fig.6. Structure of doxorubicin.

Increasing evidence firmly suggests that the underlying mechanism for anthracycline cytotoxicity is the induction of apoptosis through intracellular signalling pathways. Studies with agarose bead-immobilized doxorubicin by Maestre and coworkers (2001) showed that cellular internalisation of doxorubicin is required for apoptosis and ceramide-mediated signalling. Ceramide and its downstream effector sphingosine have been reported to be involved in doxorubicin-induced apoptosis in cardiomyocytes (Delpy et al., 1999).

Doxorubicin has been shown to stimulate both, the extrinsic (Fas/Fas-L-mediated) and intrinsic pathway of apoptosis in cellular and *in vivo* models (Kotamraju et al., 2000; Nakamura et al., 2000; Lorenzo et al., 2002) but it is unclear if these pathways are linked or independent of each other. There are contrary reports about the importance of Fas signalling in doxorubicin-dependent death in several cancer cells, some studies showing a significant role of the Fas-mediated pathway (Friesen et al., 1999), whereas others reported the opposite (Villunger et al., 1997; Gamen et al., 1997).

Another important protein is p53, which is thought to play a crucial role in some types of doxorubicin-induced tumour cell apoptosis (Wu et al., 2002a; Wang et al., 2004), whereas doxorubicin-induced endothelial apoptosis has been reported to occur in p53-dependent as well as –independent ways (Lorenzo et al., 2002; Wang et al., 2004).

The Present Investigation

Aims

The main purpose of the present studies was to examine p38 signalling involved in the regulation of endothelial apoptosis. More specifically, the aims were as follows:

- To evaluate the role of p38 in TNF-induced endothelial apoptosis
- To elucidate the mechanism of p38's proapoptotic action in TNF-induced apoptosis by studying its effect on Bcl-2 proteins
- To investigate a potential crosstalk between p38 and the MEK/ERK pathway and their effect on Bad phosphorylation in TNF-induced endothelial apoptosis.
- To study the role of p38 in doxorubicin-induced endothelial apoptosis and its possible regulation of Bcl-2 proteins.

Results

p38 mediates TNF-induced apoptosis in endothelial cells via phosphorylation and downregulation of Bcl-xL (paper I)

In earlier studies, we observed (Alvarado-Kristensson et al., 2001), as did others (Roulston et al., 1998; Kankaaranta et al., 1999), that p38 can protect some cell types from apoptosis. Considering endothelial cells, apoptosis induced by thrombospondin-1(Jimenez et al., 2000) or high glucose (Nakagami et al., 2001) were reported to require p38 activity, whereas inhibition of apoptosis by estradiol was mediated via p38β (Razandi et al., 2000). Since the role of p38 appears to be cell-type and stimulus-dependent, the objective of this study was to elucidate the role of p38 in TNF-induced endothelial apoptosis. In contrast to TNF treatment alone, combined treatment with TNF and CHX resulted in downregulation of FLIP expression and initiation of TNF-induced endothelial cell apoptosis. This agrees well with previous studies that CHX induced sensitization to death-receptor-mediated apoptosis via downregulation of FLIP (Wallach et al., 1997; Kreuz et al, 2000).

A novel finding in this study was that p38 mediates TNF-induced endothelial apoptosis. This is indicated by several results: A prolonged activation of p38 appeared to be crucial for execution of the apoptotic program, since the p38 inhibitor SB203580 significantly attenuated TNF-induced caspase-3 activity and cell death in endothelial cells. Furthermore, treatment with TNF resulted in a dose-dependent activation of p38 and cell death, and activation of p38 preceded activation of caspase-3. However, at later time points (2-4h of TNF treatment), the caspase inhibitors z-Val-Ala-Asp(Ome)-fluoromethylketone (zVAD-fmk) and Boc-

Asp(Ome)-fluoromethylketone (BAF) did impede p38 phosphorylation. This suggests the existence of caspase-induced feedback stimulation of p38, which is in agreement with reports by other investigators (Zarubin et al., 2005).

I observed a time-dependent increase in active p38 in the mitochondrial subfraction of cells exposed to TNF. Furthermore, p38 MAPK mediated a decrease in Bcl-xL protein in cells undergoing TNF-induced apoptosis, whereas the levels of other antiapoptotic Bcl-2 family proteins, Bcl-2 and A1, and proapoptotic Bcl-2 family proteins, Bax and Bak were not affected. Immunoprecipitation experiments revealed a p38-mediated serine-threonine phosphorylation of Bcl-xL in apoptotic cells. Furthermore, exposure to the proteasome inhibitor lactacystin prevented both the downregulation of Bcl-xL and activation of caspase-3, which implies that degradation of Bcl-xL is of great importance for TNF-induced apoptosis. In conclusion, TNF-induced p38 activity mediates endothelial apoptosis via phosphorylation of Bcl-xL which is then degraded by proteasomes.

p38 MAPK regulates phosphorylation of Bad via PP2A-dependent suppression of the MEK1/2- ERK1/2 survival pathway in TNF-α-induced apoptosis (paper II)

In this study I wanted to extend myprevious finding that p38 mediates TNF-induced endothelial apoptosis. Accordingly, the objective was to study a possible crosstalk between p38 and the MEK/ERK pathway and their effect on Bad phosphorylation. Results revealed that p38 mediates downregulation of the MEK/ERK pathway during TNF-induced endothelial apoptosis at the level of MEK, as treatment with SB203580 significantly increased the TNF-stimulated activity of MEK prior to its downstream kinase ERK in cells undergoing apoptosis. We showed that the MEK/ERK pathway is an important survival signal in TNF-induced apoptosis, since inhibition with PD98059 increased caspase-3 activity and cell death, whereas ERK activation by short term treatment with PMA nearly totally abolished caspase-3 activity and apoptosis. These results agree with a report by Tran et al. (2001), showing that ERK signalling exerts an important survival factor and can, when overexpressed, override Fas, TRAIL and TNF-induced apoptosis in HeLa cells.

We further found that p38-mediated downregulation of MEK activity occurred via the serine/threonine phosphatase PP2A. Two different inhibitors of PP2A, fostriecin and calyculin A, stimulated MEK activity, and SB203580 had a similar effect. Importantly, fostriecin and calyculin A inhibited caspase-3 activity and cell death, indicating that PP2A activity was necessary for execution of the apoptotic program. These findings agree well with a report by Westermarck et al. (2001) showing a PP1/PP2A-dependent, p38-mediated

inhibition of MEK/ERK signalling in fibroblasts. In contrast to that study, I used the very specific PP2A inhibitor fostriecin, which inhibits PP2A activity >10,000 fold more potently than it inhibits PP1 activity (Walsh et al., 2001). Furthermore, immunoprecipitation experiments revealed the existence of a complex of p38 MAPK, PP2A and MEK in endothelial cells, and binding of PP2A to MEK and p38 was strengthened in cells treated with TNF. Furthermore, results from a phosphatase assay and from Western blotting against active p38 showed that PP2A was a downstream target of p38 MAPK, which is in agreement with other reports (Avdi et al., 2002; Liu and Hoffmann, 2004).

The most interesting finding in this study was that p38 regulates phosphorylation of Bad at Ser112 via stimulation of PP2A and downregulation of MEK/ERK signalling. TNF-induced p38 activation results in decreased phosphorylation of Bad, which then becomes pro-apoptotic. In summary, these findings together with results from paper I show that p38 mediates TNF-induced endothelial apoptosis via degradation of the antiapoptotic Bcl-xL and inhibition of the MEK/ERK survival pathway as well as a decrease in phosphorylated Bad.

p38 downregulates Akt-mediated Bad phosphorylation in doxorubicin-induced apoptosis (paper III)

In this study I wanted to investigate the role of p38 in endothelial apoptosis induced by doxorubicin, which is a widely used chemotherapeutic drug against a broad spectrum of tumours (Weiss et al. 1992). Treatment with doxorubicin resulted in a dose- and time-dependent activation of p38 and was observed before caspase activation and cell death. Expression of dominant negative p38 by lentiviral transfection and pharmacological inhibition of p38 greatly reduced doxorubicin-induced caspase activity and apoptosis. Virtually all DNp38-expressing cells were resistant to death induced by doxorubicin, indicating that p38 MAPK plays a crucial role in doxorubicin-induced endothelial apoptosis. In contrast to TNF-induced apoptosis, MEK/ERK signalling appeared to be proapoptotic in this system, since PD98059 inhibited caspase-3 activity and cell death, and this has also been reported by other investigators (Guise et al., 2001; Yeh et al., 2004). In addition, results from experiments with SP600125 indicated that JNK seemes to be involved in doxorubicin-induced apoptosis, in contrast to endothelial apoptosis induced by TNF.

Furthermore, PI3-K/Akt signalling represents an important survival pathway in doxorubicin-treated cells, since the PI3-K inhibitor LY294002 greatly increased caspase-3 activity and cytotoxicity. We observed that p38 downregulated Akt signalling, which in turn resulted in a decrease in phosphorylated Bad. Such crosstalk between p38 and Akt has not

been shown before, and results from a phosphatase assay suggested that p38 MAPK-mediated inhibition of Akt is probably mediated by PP2A. Furthermore, p38 also downregulated Bcl-xL in doxorubicin-induced apoptosis, similarly as in TNF-induced cell death. To conclude, p38 is able to transduce a strong doxorubicin-induced apoptosis signal by simultaneously decreasing the level of antiapoptotic Bcl-xL and increasing the level of proapoptotic Bad.

Discussion

In paper I and II, I studied endothelial apoptosis induced by TNF, an inflammatory cytokine, the main target of which is the endothelium, and in particular, the objective was to elucidate the role of p38. I observed that a key event for the initiation of TNF-induced endothelial apoptosis seems to be the downregulation of FLIP, a downstream target of NF-κB. This agrees with a report by Kreuz et al. (2001), showing that FLIP is the most important CHX-sensitive inhibitor of TNF- and TRAIL-R signalling. Furthermore, it has also been shown that FLIP can be downregulated by IL-2 (Du et al., 2005), and others have reported that FLIP downregulation mediated by adenoviral E1A sensitises tumour cells to TNF-induced apoptosis (Perez and White, 2003). In addition, cotreatment of TNF and IFN-γ induces endothelial apoptosis *in vitro* and *in vivo* (Rüegg et al., 1999; Yamaoka et al., 2002), and IFN-γ has been reported to upregulate both TNF-Rs in HL-60 and HeLa cells (Pandita et al., 1992). The afore mentioned upregulation of TNF-Rs and the downregulation of FLIP observed in paper I could initiate the TNF-induced death pathway *in vivo* as a result of an increased TNF-R/FLIP ratio. The FLIP degradation upon treatment of TNF in combination with CHX was followed by p38-mediated caspase-8 activation and Bid cleavage (paper I). However, p38-mediated endothelial apoptosis seems to occur at the mitochondrial level. Evidence for this is shown by the fact that p38 is not involved in regulating FLIP levels and that caspase-8 activity can be amplified via the mitochondrial pathway (Cowling and Downward, 2002).

Furthermore, I found an increase in active p38 at the mitochondria in cells undergoing TNF-induced apoptosis, and this correlated with p38-mediated phosphorylation- and proteasome-mediated degradation of Bcl-xL (paper I). It is plausible that p38-mediated phosphorylation of Bcl-xL takes place at the mitochondria and could occur at Thr47, Thr115 and Ser62, phosphorylation sites which can be recognised by this kinase. p38 has been shown to phosphorylate Bcl-2 and to induce apoptosis in B-lymphocytes in response to NGF withdrawal (Torcia et al., 2001). In addition, JNK-mediated phosphorylation of Bcl-xL at

Thr47 and Thr115 has been involved in ionising radiation-induced cell death in U937 cells, and a mutant Bcl-xL protein with the threonines substituted by alanines was a more potent inhibitor of apoptosis (Kharbanda et al., 2000). In line with my finding that Bcl-xL is an important regulator of endothelial apoptosis, overexpression studies have shown that Bcl-2 and Bcl-xL protect bovine aortic endothelial cells from TNF-induced apoptosis (Badrichani et al. 1999).

Although activation of JNK and p38 occurred with similar kinetics in TNF-treated cells, JNK does not appear to play an important role in this system, since its inhibition with SP600125 had no effect on caspase-3 activity (paper II). JNK signalling was not affected by p38 or ERK, as it remained unchanged by SB203580 and PD98059 in cells undergoing TNF-induced apoptosis. In contrast, SP600125 is a potent inhibitor of doxorubicin-induced apoptosis (paper III), but compared to doxorubicin-induced apoptosis, endothelial cells undergo a rapid death when treated with TNF, which might not require the involvement of proapoptotic JNK. An earlier report showed that JNK activation can be a caspase-dependent, secondary effect in Fas-induced apoptosis in Jurkat cells (Lenczowski et al., 1997), which could also be the case in TNF-treated cells.

In paper II, I observed that TNF-induced apoptosis entailed functional crosstalk between p38 and the MEK/ERK pathway, in which p38 limits the activation of MEK/ERK and phosphorylation of Bad. I showed that the MEK/ERK pathway plays an important role in protection of endothelial cells from TNF-induced apoptosis, demonstrated by the nearly complete survival of PMA-pretreated cells (without affecting JNK and p38) and increased caspase-3 activity and cell death in cells cotreated with TNF and PD98509. These findings agree with a report by Tran et al. (2001) who showed that ERK signalling protected HeLa cells from TNF, Fas and TRAIL-induced apoptosis. Together, this suggests a particular role for ERK as survival factor in death-receptor-mediated apoptosis. In contrast, although TNF treatment resulted in Akt activation, inhibition of the PI3-K/Akt pathway with LY294002 did not influence TNF-induced caspase-3 activity and cell death (unpublished observation). The observed p38-mediated downregulation of MEK/ERK signalling occurs via PP2A, which agrees with a report by Westermarck et al. (2001), although in their report it was not possible to discriminate between PP1 and PP2A. Furthermore, my results were the first to show that TNF-induced p38/PP2A-mediated downregulation of the MEK/ERK pathway involves decreased phosphorylation of Bad at Ser112. This could occur through decreased activity of RSK1, a well known downstream target of ERK which has been reported to phosphorylate Bad at Ser112 (Bonni et al., 1999) or directly via PP2A, which has been suggested as the

main Bad phosphatase (Chiang et al., 2003). In concurrence with our results, shear stress-induced ERK5 activation and indirect phosphorylation of Bad at Ser112 has been shown to protect endothelial cells from growth factor withdrawal-induced apoptosis (Pi et al., 2004). In addition, bFGF was reported to inhibit radiation-induced endothelial apoptosis partly via Ser112 and Ser136 phosphorylation of Bad (Gu et al., 2004a,b). To conclude, the results from paper I and II show that p38 can create a strong apoptosis signal via downregulation of Bcl-xL and suppression of the MEK/ERK survival pathway and Bad phosphorylation.

In paper III, I showed that p38 is a predominant proapoptotic factor in doxorubicin-induced endothelial apoptosis. Doxorubicin is a widely used antitumour agent against a broad range of tumours (Weiss et al., 1992) and can exert its effect via directly targeting the tumour vasculature (Bocci et al., 2002; Schiffelers et al., 2003). Endothelial apoptosis is also implicated in doxorubicin-mediated cardiotoxicity (Kalyanaraman et al., 2002; Wu et al., 2002b), an undesirable side effect of the cancer therapy.

In addition to pharmacological inhibition, lentiviral vector transfection of EA.hy926 cells was used to express a dominant negative mutant Flag-p38 MAPK harboring T180A and Y182 F amino acid substitutions (Han et al., 1994). Since FACS analysis showed that 60% of the EC^{DNp38} expressed the DNp38, this means that virtually all DNp38-expressing cells were resistant to death induced by doxorubicin. These findings indicate that p38 MAPK plays a crucial role in doxorubicin-induced endothelial apoptosis. In contrast to TNF-induced cell death, the ERK and JNK pathways appeared to be proapoptotic in doxorubicin-treated cells. This function is at least partly independent of p38, and these findings are in good agreement with other reports showing that ERK signalling is proapoptotic in doxorubicin-treated neuroblastoma, MCF7 breast cancer cells and in NIH3T3 fibroblasts (Guise et al., 2001; Tang et al., 2002; Yeh et al, 2004). Regarding JNK signalling, Panaretakis et al. (2005) have recently shown its importance in doxorubicin-induced apoptosis in mouse embryonic fibroblasts.

Interestingly, p38-mediated apoptosis induced by doxorubicin also involved the inhibition of an important survival pathway, PI3-K/Akt. In line with our results that PI3-K/Akt protects endothelial cells from doxorubicin-induced apoptosis, O'Gorman et al (2000) reported that inhibition of PI3-K/Akt sensitised HL-60 leukaemic cells to doxorubicin-induced cell death.

Although it has been shown that Akt negatively regulates p38 signalling (Gratton et al., 2001; Yuan et al., 2003), there were no previous reports about cross talk originating from p38. PP2A is known to dephosphorylate Akt (Andjelkovic et al., 1996; Tanaka et al., 2003) and the

results from a phosphatase assay suggested that p38-mediated downregulation of Akt occurs indirectly via PP2A. It is plausible that doxorubicin-induced p38 activity in endothelial cells maintains PP2A activity at a basal level and thereby prevents phosphorylation of Akt and Bad. However, p38 could perhaps also directly inhibit Akt by phosphorylation at p38 consensus sites found in Akt at Ser64, Ser67 and Ser368. As discussed before, PP2A can dephosphorylate Bad at Ser112 (Chiang et al., 2003). Therefore, p38-mediated activation of Bad seen during doxorubicin-induced apoptosis may occur directly via PP2A and/or through inhibition of PI3-K/Akt activity.

In summary, p38 exerts a strong proapoptotic action in endothelial cells treated with TNF or doxorubicin by simultaneously decreasing the level of anti-apoptotic Bcl-xL and increasing the level of pro-apoptotic Bad.

Conclusions

- p38 plays an important proapoptotic role in TNF- induced endothelial apoptosis.
- In TNF-induced cell death, p38 mediates phosphorylation of Bcl-xL, probably at the mitochondrial level, which is followed by its degradation in the proteasomes.
- p38 mediates suppression of the MEK/ERK survival pathway and phosphorylation of Bad, thus creating a strong apoptosis signal.
- p38 activity is crucial for endothelial apoptosis induced by doxorubicin.
- In addition to p38, JNK and ERK signalling appear to be proapoptotic in doxorubicin-treated endothelial cells.
- p38 signalling in doxorubicin-induced apoptosis also involves the downregulation of Bcl-xL and inhibition of the PI3-K/Akt survival pathway leading to decreased Bad phosphorylation.

Popularised Summary in Swedish/Populärvetenskaplig Sammanfattning

Endotelceller bildar ett skikt på insidan av alla blodkärl och har många specifika funktioner, som till exempel att reglera blodflöde, blodtryck och förhindrar uppkomsten av trombos. Dessutom spelar endotelceller en viktig reglerande roll vid inflammation: de producerar så kallade cytokiner som lockar och aktiverar inflammatoriska celler. Dessa passerar genom blodkärl in i infekterade vävnader och eliminerar de mikroorganismer som orsakade inflammationen. Olika faktorer säkrar endotelcellernas överlevnad, så som olika tillväxt- och överlevnadsfaktorer och adhesion till extracellulärt matrix (ECM). ECM är ett nätverk av proteoglykaner och proteiner som celler omges av i vävnaden. I en vuxen människa delar sig normala endotelceller i genomsnitt bara 2 gånger per livstid, i raka motsatsen till tumörendotelceller som delar sig 1-2 gånger per vecka.

Apoptos är en reglerad form av celldöd och sker som en naturlig process i organismers liv, exempelvis tillbakabildas simhuden mellan tår och fingrar under fosterstadiet genom apoptos. Apoptos förekommer under hela organismens liv, och allvarliga konsekvenser uppstår om balansen mellan celldöd och tillväxt rubbas; alltför mycket apoptos kan orsaka Alzheimers sjukdom och andra degenerativa sjukdomar, medan nedsatt apoptosfrekvens kan leda till cancer.

Endotelcellsapoptos har visats spela en viktig roll i ibland annat ateroskleros och bakteriell sepsis, och induktion av apoptos i tumörendotelceller verkar vara en lovande typ av cancerterapi. Syftet med mina doktorandstudier var därför att studera vilka signalmolekyler och signalvägar som deltar i endotelcellsapoptos.

Jag har studerat apoptos inducerad via en speciell dödsreceptor, kallad tumör-nekros faktor (TNF) receptor, en mottagare som binder TNF, som är en inflammatorisk cytokin vars huvudsakliga målvävnad är endotelet. Dessutom har jag studerat signalvägar som leder till apoptos efter behandling med doxorubicin, ett cytostatikum som används i behandling av flera olika tumörtyper. Huvudsyftet var att klarlägga vilken roll proteinkinaset p38 MAPK, ett protein som fosforylerar andra signalproteiner och därigenom ändrar deras aktivitet och funktion, spelar. Jag har huvudsakligen studerat effekten av p38-aktivitet på Bcl-2-familjen av proteiner, som innefattar både anti-apoptotiska (Bcl-xL) eller pro-apoptotiska (Bad) medlemmar.

Jag har visat att p38 MAPK-aktivering leder till apoptos efter behandling med både TNF och doxorubicin. p38 MAPK skapar en stark apoptos-signal via nedreglering av anti-

apoptotiskt Bcl-xL och ökning av pro-apoptotiskt Bad (genom att hämma fosforyleringen av Bad). Vidare visar mina studier att detta sker via hämning av överlevnadssignaler, som till exempel MEK/ERK- och PI3-K/Akt-signalvägarna, i celler som är behandlade med TNF respektive doxorubicin. Jag har också sett att fosfataset PP2A (som genom att ta bort fosfatgrupper från sina substrat ändrar deras aktivitet), är inblandat i p38 MAPK-medierad hämning av dessa signalvägar.

Sammanfattningsvis kan sägas att jag har klarlagt viktiga signalvägar mellan p38 MAPK och Bcl-2 proteiner, via olika kinaser och fosfataser, och mina studier har bidragit med att belysa signaler för endotelcellsapoptos som kan ha en stor betydelse för cancerterapi.

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