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Regulation of UV induced apoptosis in human melanocytes

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To my Family
with all my love

ABSTRACT

Malignant melanoma arises from the pigment producing melanocytes in epidermis and is the most aggressive type of skin cancer. The incidence of malignant melanoma is increasing faster than any other type of cancer in white population worldwide, with a doubling rate every 10-20 years. So far, the only identified external risk factor for malignant melanoma is UV exposure. Elimination of photodamaged cells by apoptosis (programmed cell death) is essential to prevent tumor formation. Melanocytes are considered relatively resistant to apoptosis, however, the regulation of apoptosis in melanocytes is still unknown.

The aim of this thesis was to investigate the apoptotic process following ultraviolet (UV) irradiation in primary cultures of human melanocytes. Focus was on regulation of mitochondrial stability by Bcl-2 family proteins and the possible participation of lysosomal proteases, cathepsins. UV irradiation activated the mitochondrial pathway of apoptosis, leading to cytochrome c release, caspase activation, and nuclear fragmentation. No change in protein expression of Bax and Bcl-2 was observed in response to UV. Instead, translocation of the Bcl-2 family proteins from cytosol to mitochondia was important in the regulation of survival and death of melanocytes. The findings further demonstrated permeabilization of the lysosomal membrane to occur early in the apoptotic process, resulting in cathepsin release into the cytosol. The cathepsins were potent pro-apoptotic mediators and triggered apoptosis upstream of Bax translocation and mitochondrial membrane permeabilization. In response to both heat and UV irradiation, there was a marked increase in expression of stress-induced heat shock protein 70 (Hsp70), which inhibited apoptosis by binding lysosomal and mitochondrial membranes and counteracting the release of cathepsins and cytochrome c. Furthermore, UV irradiation activated c-jun N-terminal kinase (JNK), which triggered apoptosis upstream of cathepsins release from the lysosomes. In addition, JNK mediated apoptosis through phosphorylation of pro-apoptotic Bim, which was released from anti-apoptotic Mcl-1, by UV induced Mcl-1 depletion.

This thesis illustrates that permeabilization of mitochondria and lysosomes and release of their constituents to the cytosol participates in UV induced apoptosis signaling in human melanocytes *in vitro*. The process is regulated by a complex network of pro- and anti-apoptotic proteins, exerting their effects through intracellular translocation and alteration of protein expression.

TABLE OF CONTENTS

ORIGINAL PUBLICATIONS

This thesis is based on the following papers, which will be referred to in the text by their Roman numerals (I-IV).

I. **Cecilia Bivik**, Eva Andersson, Inger Rosdahl
Wavelength specific effects on UVB induced apoptosis in melanocytes.
A study of the Bcl-2/Bax expression and keratinocyte rescue effects.
Melanoma Research 15(1):7-13, 2005

II. **Cecilia Bivik**[*], Petra Larsson[*], Katarina Kågedal, Inger Rosdahl, Karin Öllinger
UVA/B induced apoptosis in human melanocytes involves translocation of cathepsins and Bcl-2 family members.
Journal of Investigative Dermatology 126(5):1119-1127, 2006
[*]These authors contributed equally to this work.

III. **Cecilia Bivik**, Inger Rosdahl, Karin Öllinger
Hsp70 protects against UVB induced apoptosis by preventing release of cathepsins and cytochrome c in human melanocytes.
Carcinogenesis 28(3): 537-544, 2007

IV. **Cecilia Bivik**, Karin Öllinger
JNK acts pro-apoptotic upstream of lysosomal membrane permeabilization and Bim activation in UVB induced apoptosis in melanocytes.
Manuscript

ABBREVIATIONS

AIF	apoptosis-inducing factor
Apaf-1	apoptotic protease activating factor 1
BH	Bcl-2 homology domain
C_T	threshold cycle
DAPI	4',6-diamidino-2-phenylindole
DED	death effector domain
FADD	Fas-associated protein with death domain
GAPDH	glyceraldehyde-3-phosphate dehydrogenase
Hsp	heat shock protein
IAP	inhibitors of apoptosis protein
LDH	lactate dehydrogenase
JNK	c-jun N-terminal kinase
MAPK	mitogen activated protein kinase
MC1R	melanocortin-1 receptor
NAG	β-N-acetylglucoseaminidase
NGF	nerve growth factor
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PTP	permeability transition pore
RISC	RNA inducing silencing complex
siRNA	short interference RNA
tBid	truncated Bid
TNF	tumor necrosis factor
TRAIL	tumor necrosis factor related apoptosis-inducing ligand
UV	ultraviolet
UVA	ultraviolet irradiation 320-400 nm
UVB	ultraviolet irradiation 280-320 nm
UVC	ultraviolet irradiation 100-280 nm
VDAC	voltage-dependent anion channel

INTRODUCTION

THE SKIN

The skin is the largest organ in the body, with a mass of 4-5 kg, and the total surface area of about 1.5-2 m^2 in an adult individual. The primary function of the skin is to act as a protective shield against harmful environmental influence, such as irradiation, injury, chemical agents, heat, and infection. It also regulates body temperature and protects the body from excessive water loss. The skin can be divided into three layers (Figure 1). The deepest layer, the subcutis, mainly consists of adipocytes, which provides insulation and mechanical protection and also serves as energy storage. Blood vessels are situated at this level and the subcutis binds the skin to underlying structures. The next layer is the dermis, composed of fibroblasts, which produce an extracellular matrix with collagen and elastin. Nerves, blood vessels and sweat glands are located in this layer. The top layer of the skin is the epidermis, which mainly consists of keratinocytes, but also melanocytes and Langerhans cells. The keratinocytes differentiate as they migrate from the basement membrane, which separates epidermis from dermis, to the surface. Normally, this migration takes about one month. The cells loose their nuclei and organelles as they reach the top and will then constitute the corneocyte (horny) layer, which serves as the protective barrier to the external environment.

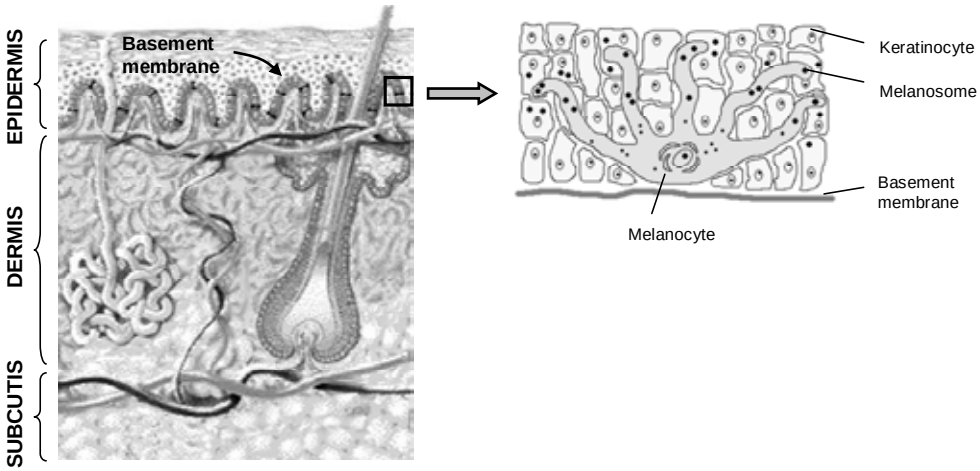

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Figure 1. The three layers of the skin and an epidermal melanin unit, located at the basement membrane of epidermis, are presented.

EPIDERMAL MELANIN UNIT

A central function of the skin is to protect the body from harmful ultraviolet (UV) irradiation. The UV induced damage is limited by chromophores in the skin, such as DNA and proteins, which absorb the irradiation. In addition, the skin contains the effective UV absorbing pigment, melanin, which in mammals exists in two types; the brownish-black eumelanin, which is the main pigment type in dark-skinned individuals and is considered to have the most effective protection against UV irradiation, and the reddish-yellow pigment in fair-skinned individuals (Vincensi et al., 1998). The melanin production, melanogenesis, takes place in the melanocytes, which originates from the neural crest and migrates during embryogenesis to various sites in the body, primarily to epidermis, eye and hair follicles (Goding, 2007). If all melanocytes in the body were assembled to one unit, this "melanocyte organ" would represent a total size of 1.0-1.5 cm^3 in an adult human, with the dominant part consisting of epidermal melanocytes (Rosdahl and Rorsman, 1983). In the skin, the melanocytes are located at the basement membrane in epidermis, with an average density of 1000-2000 melanocytes/mm^2 skin (Szabó, 1954). The pigment cells were first described in squid in 1819 by Sangiovanni, who called them "chromatophores", and Henle was first to identify these cells in human skin in 1837 (reviewed in Westerhof, 2006). The term "melanocyte" was introduced by Meyerson in 1889. Furthermore, the melanin is packed in organelles, called melanosomes, transferred, via the dendrites of the melanocyte, to neighboring keratinocytes, where they form protecting caps above the nuclei of the keratinocytes (Seiji et al., 1961; Pathak et al., 1971; Goding, 2007). Each melanocyte donates melanin to a group of approximately 36 keratinocytes, which together are termed an epidermal melanin unit (Fitzpatrick and Breathnach, 1963) (Figure 1). In the keratinocytes, the melanosomes are packed according to size, with several small melanosomes as complexes surrounded by a membrane and the larger ones as single units (Toda et al., 1973; Rosdahl and Szabó, 1976). Differences in skin color between Caucasians and Negroids are not primarily due to variation in the number of melanocytes (Szabó, 1967b). Instead it has been shown to depend on type and amount of melanin and the size, packaging, distribution and degradation of the melanosomes within the keratinocytes (Szabó et al., 1969).

UV IRRADIATION

UV irradiation from the sun contains UVA (320-400 nm), UVB (280-320 nm), and UVC (100-280 nm) (Figure 2). The atmospheric ozone layer absorbs all UVC rays and the majority of the UVB (90%), which means that UV irradiation reaching the surface of Earth is mainly in the UVA range and to a minor extent UVB. UVB irradiation is, however, much more potent in generating sunburns, tanning and DNA damage, than UVA (Abdulla et al., 2005). The amount of UV irradiation reaching the Earth varies depending on many factors, such as time of the day, season, ozone layer, solar zenith angle, clouds, air pollutions, and surface reflections (Godar, 2005). The depth of penetration through the skin and biological effects differ between UVA and UVB irradiation. UVB irradiation reaches epidermis and is directly absorbed by the DNA, which might cause formation of UV photoproducts, such as cyclobutane pyrimidine dimers and pyrimidine(6-4)pyrimidone photoproducts (Rosenstein and Mitchell, 1987; Sarasin, 1999). These photoproducts might give rise to C to T or CC to TT transition mutations (Sarasin, 1999). The more long-waved UVA penetrates deeper into the skin and reaches dermis (Kadekaro et al., 2003). In contrast to UVB, UVA first reacts with endogenous photosensitizers, which generate reactive oxygen species that in turn can cause DNA damage, such as single-strand breaks (Wenczl et al., 1998; Wang et al., 2001). Free oxygen radicals might also induce lipid peroxidation, which can result in protein and membrane damage (Kadekaro et al., 2003).

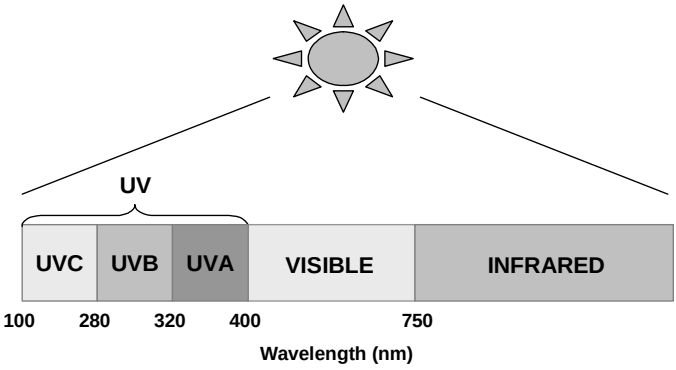

Figure 2. The irradiation spectrum of the sun light includes UV, visible, and infrared wavelengths.

SKIN CANCER

Skin cancer is the most common type of cancer in white population, living in countries with much sun irradiation and can be divided into cutaneous malignant melanoma, which originates from melanocytes, and non-melanoma skin cancers, involving basal and squamous cell carcinomas, which originate from keratinocytes (de Gruijl, 1999). Cutaneous malignant melanoma is the most aggressive tumor type appearing in the skin, with high capacity to metastasize. About 4% of all skin cancer cases in USA are represented by malignant melanoma, but this cancer causes as much as 79% of the skin cancer deaths (Abdulla et al., 2005).

UV irradiation is considered to be the major external factor in the development of skin cancer. The non-melanoma skin cancers have been associated with total lifetime UV exposure (Gilchrest et al., 1999; Perlis and Herlyn, 2004). In contrast, malignant melanoma has been considered to be linked to intense and intermittent sun exposure (Walter et al., 1999; Perlis and Herlyn, 2004), but contribution of chronic daily low dose UV cannot be excluded. In accordance, basal and squamous cell carcinomas are most often found in continuously sun exposed areas of the body, like the face and back of hands and forearms, while malignant melanoma most frequently occurs in sun-protected areas that receive intermittent exposure (Gilchrest et al., 1999).

MALIGNANT MELANOMA

Historical aspect

Archaeological findings of nine pre-Colombian Inca mummies from Peru, approximately 2.400 years old, showed diffuse melanoma metastases in the bones of the skull and extremities, as well as rounded melanotic masses in the skin (reviewed in Urteaga and Pack, 1966). The first known description of melanoma is from Hippocrates (460-375 B.C.). In several reports between 1650-1760, these pigmented malignant tumors were referred to as ”fatal black tumors with metastases and black fluid in the body”. René Laënnec used the word “la mélanose”, from the Greek word for black, “melas”, when describing this type of tumor in 1806 and Robert Carswell first utilized the medical term “melanoma” in 1838.

Incidence

Malignant melanoma is the most rapidly increasing type of cancer in white populations worldwide, with a doubling rate every 10-20 years (Diepgen and Mahler, 2002; Lens and Dawes, 2004). In USA, the cumulative lifetime risk for melanoma was 1:1500 in 1935, while it had increased to 1:68 in 2002 (Lens and Dawes, 2004). The highest incidence has been reported from Australia, where the lifetime risk is 1:25 (Diepgen and Mahler, 2002).

In Europe, the highest incidence is found in Scandinavia and the lowest in the Mediterranean countries (Lens and Dawes, 2004). In Sweden, the estimated risk for developing melanoma during life is 1:67 (Cancer incidence in Sweden 2005). Furthermore, melanoma affects about 2100 individuals and leads to 400 deaths each year in Sweden (Cancer incidence in Sweden 2005, Causes of death 2004).

Risk factors

Individuals exhibiting large amounts of pheomelanin and small amounts of eumelanin have the characteristic phenotype of fair skin, red or blond hair, and a tendency to sunburn, that is associated with a significant increased risk for melanoma development (Gilchrest et al., 1999). Excessive recreational exposure to sunlight (Westerdahl et al., 1992; Gilchrest et al., 1999; Lens and Dawes, 2004), childhood sunburns (Whiteman et al., 2001), increased number of nevi (Augustsson et al., 1991; Bauer and Garbe, 2003), use of sunbeds (Walter et al., 1999; Westerdahl et al., 2000a), and depletion of the ozone layer, resulting in that more UVB reaches the Earth (Mettlin, 2001), are factors that have been reported to contribute to higher skin cancer risk. Furthermore, some studies have found a protective effect of sunscreens, while others show an increased risk of melanoma development with the use of suncreens, due to prolonged duration of exposure to the sun (Espinosa Arranz et al., 1999; Westerdahl et al., 2000b; Bastuji-Garin and Diepgen, 2002).

Genetics

Of all melanoma cases, approximately 10% have been reported to have a hereditary predisposition for the disease (Platz et al., 2000). About 40% of individuals with familial melanoma, display mutations or deletions of the tumor suppressor gene CDKN2A (cyclin-dependent kinase inhibitor 2), which encodes the p16 protein (Hussussian et al., 1994; Piepkorn, 2000). p16 normally inhibits the cell cycle by interaction with cyclin-dependent kinases 4 or 6 (Piepkorn, 2000). Loss of its function by mutation, results in escape from cell cycle arrest, which might lead to incomplete DNA repair. Recently, BRAF, which primarily is implicated in growth regulation, was found to be mutated in 66% of the melanoma cell lines and tumors investigated (Davies et al., 2002). A melanoma specific V599E missense mutation was found to result in activation of BRAF kinase activity (Brose et al., 2002). This mutation was also detected in 82% of the melanocytic nevi tested (Pollock et al., 2003), suggesting BRAF mutation to have an early involvement in melanocyte transformation. Another melanoma susceptibility gene is the melanocortin-1 receptor (MC1R), which is highly polymorphic. A loss-of-function mutation of MC1R alleles has been shown to be associated with red hair phenotype and a higher risk for melanoma (Palmer et al., 2000; Rees, 2000).

MELANOCYTE HOMEOSTASIS

Several studies demonstrate that repeated UV irradiation induces a several folded increase in the population density of epidermal melanocytes in both mice (Sato and Kawada, 1972; Rosdahl, 1978; Rosdahl and Szabó, 1978) and humans (Quevedo et al., 1965; Stierner et al., 1989). In addition, repeated UV exposures result in a rise in the number of melanocytes in shielded unirradiated areas (Rosdahl, 1979; Stierner et al., 1989). A high mitotic activity might increase the risk for tumor development in both exposed and shielded skin (Stierner et al., 1989). After the initial increase, the cell population is reported to slowly revert to its original number (Szabó, 1967a; Rosdahl, 1979). With such a proliferative response and wide range in melanocyte population density in the skin, an efficient control system for homeostasis is essential. Apoptosis might therefore have a key function, but an apoptotic loss of melanocytes *in vivo* is seldom reported in response to UV exposure (Gilchrest et al., 1999). The general opinion is that melanocytes are resistant to apoptosis, suggesting that these cells have powerful anti-apoptotic mechanisms, triggering the survival.

APOPTOSIS

There are generally two principal mechanisms of cell death; necrosis and apoptosis (also called programmed cell death) (Figure 3). Cells undergoing necrosis swell, due to increased osmotic pressure, and will finally lyse, with release of cellular contents into the surroundings, which might induce inflammation (Hetts, 1998). Apoptosis is a more controlled process. Cells committing apoptotic cellular suicide rapidly shrink and lose their normal intercellular contacts. Other morphological changes associated with apoptosis include blebbing of the plasma membrane, nuclear condensation, DNA fragmentation, and segregation of the cell into a number of apoptotic bodies that are phagocytosed by macrophages (Kerr et al., 1972). Since nothing is released from the cell into the surrounding tissue, inflammation is not triggered. The word apoptosis is derived from the Greek word for "falling off" and was first described by Kerr and colleagues in 1972 (Kerr et al., 1972). This physiological cell death is important for the homeostasis of tissues and to eliminate potentially dangerous cells. In addition, apoptosis plays an essential role in the embryonic development. Dysregulation of the apoptotic process may result in a wide range of pathological conditions (Thompson, 1995). Cancer is one consequence of impaired apoptotic mechanisms that lead to an insufficient removal of damaged cells. In contrast, degenerative diseases are associated with inappropriate increase in apoptosis. Thus, it is highly important to understand the molecular mechanisms behind the regulation of apoptosis and which proteins that are involved in this process.

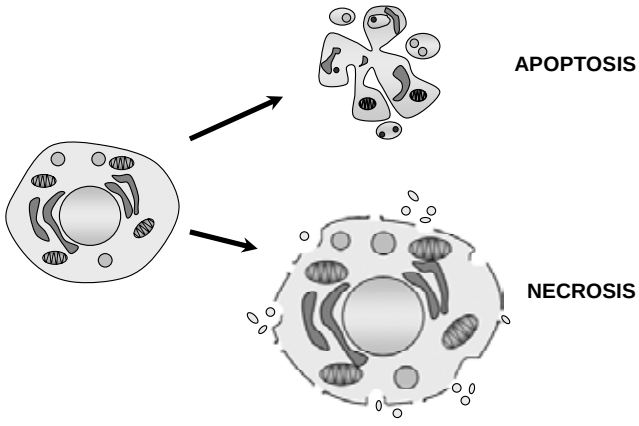

Figure 3. Cells die through either of two distinct processes; apoptosis or necrosis.

CAENORHABDITIS ELEGANS

The nematode *Caenorhabditis elegan*s has served as a model organism for investigating apoptosis. Genetic studies have identified gene products essential for the regulation and execution of apoptosis; CED-3, CED-4, and EGL-1, which are required for the death of cells during worm development, and CED-9 with anti-apoptotic capacity (Ellis and Horvitz, 1986; Hengartner and Horvitz, 1994; Conradt and Horvitz, 1998). EGL-1 triggers apoptosis by binding anti-apoptotic CED-9 and prevent it from sequester the CED-4 protein. Free CED-4 then binds and activates CED-3, which promotes apoptosis (Ellis and Horvitz, 1986; Chinnaiyan et al., 1997; Conradt and Horvitz, 1998) (Figure 4). Corresponding mammalian proteins have been identified, but the apoptotic pathways in mammals are more complex, with a larger amount of proteins involved.

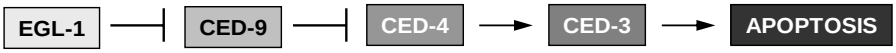

Figure 4. Pathway of cell death in *Caenorhabditis elegans*.

CASPASES

Most of the morphological changes observed during apoptosis are caused by caspases, which are homology proteins to CED-3 in *C. elegans* (Yuan et al., 1993). "c" in the term caspase stands for cysteine protease and "aspase" reflects its ability to cleave substrates after aspartic acid residues (Alnemri et al., 1996). The caspases are synthesized catalytically inactive and are usually converted to active enzymes by proteolytic removal of their prodomains (Thornberry and Lazebnik, 1998). Activated caspases then, in a cascade, cleave and activate each others' precursors. The constitutively expressed procaspases consist of three domains; an NH_2-terminal pro-domain and a large (~20 kDa) and a small (~10 kDa) subunit (Figure 5). When processed, the two subunits will associate and form a heterodimer. Caspase mediated cleavages of specific substrates might lead to both initiation and execution of apoptosis. The overall loss of shape in an apoptotic cell is, for instance, depended on cleavage of cytoskeleton proteins and nuclear shrinking is caused by cleavage of nuclear lamins (Hengartner, 2000).

In mammals, the apoptotic caspases can be divided into two groups, the initiator caspases (e.g. caspase-2, -8, -9, and -10) and the effector caspases (e.g. caspase-3 and -7) (Boatright and Salvesen, 2003). In response to an apoptotic stimulus, initiator caspases are first to be activated, which in turn activate effector caspases by cleavage. The initiator caspases are, in contrast to effector caspases, auto-activated through protein-protein interactions (Muzio et al., 1998; Boatright and Salvesen, 2003). As procaspases, they exist as inactive monomers, and become activated by dimerization, a process that might require involvement of specific adaptor molecules, which bring the caspases together (Stennicke

et al., 1999; Boatright and Salvesen, 2003; Donepudi et al., 2003). The exact regulation of initiator caspase auto-activation is, however, still not fully elucidated.

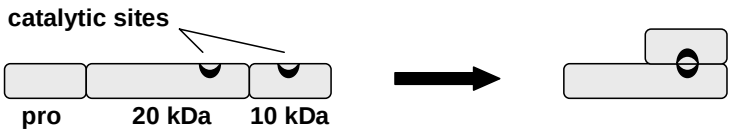

Figure 5. Procaspases consist of three domains; an NH_2-terminal pro-domain and a large (~20 kDa) and a small (~10 kDa) subunit. Upon cleavage and activation the two subunits will associate and form a heterodimer.

APOPTOTIC PATHWAYS

Apoptosis is generally described to be mediated through two major pathways, the death receptor (extrinsic) pathway and the mitochondrial (intrinsic) pathway (Figure 6).

Death receptor pathway

The death receptor pathway of apoptosis is involved in the elimination of cells during development and in the immune system (Osborne, 1996; Boatright and Salvesen, 2003). The pathway is initiated at the cell surface by activation of a trans-membrane death receptor of the tumor necrosis factor (TNF) receptor family (e.g. Fas, TNFR1) (Ashkenazi and Dixit, 1998) (Figure 6). When a ligand binds the Fas receptor (also called CD95 or Apo-1), which is one of the best characterized death receptors, it leads to clustering of the death effector domains (DEDs) of the receptors at the cell membrane, which allows recruitment and binding of the adaptor molecule FADD (Fas-associated protein with death domain) (Chinnaiyan et al., 1995; Ashkenazi and Dixit, 1998). FADD further recruits procaspase-8 molecules by interaction with their DEDs (Ashkenazi and Dixit, 1998) and in this death-inducing signaling complex (DISC), the initiator caspase-8 becomes activated and released into the cytosol, where it further will trigger the cell to apoptosis (Boatright and Salvesen, 2003).

Mitochondrial pathway

The mitochondrial pathway of apoptosis is involved in elimination of cells in response to stress stimuli, such as irradiation and chemotherapeutic drugs. Early events observed following a death signal are decreased mitochondrial membrane potential and changes in the mitochondrial permeability (Liu et al., 1996), leading to release of several pro-apoptotic proteins located in the mitochondrial intermembrane space, including cytochrome c, smac/DIABLO (second mitochondria-derived activator of caspase/direct IAP binding protein with low pl), Omi/Htra2, apoptosis-inducing factor (AIF) and endonuclease G (Tsujimoto, 2003) (Figure 6). Smac/DIABLO and Omi/Htra2 facilitate caspase activation by binding to and neutralizing the anti-apoptotic activity of inhibitors of apoptosis proteins (IAPs), which is a family of potent caspase inhibitors (Du et al., 2000; Verhagen et al., 2000; Suzuki et al., 2001; Hegde et al., 2002; Martins et al., 2002). AIF and endonuclease G induce caspase independent DNA condensation and fragmentation (Susin et al., 1999; Li et al., 2001). Once in the cytosol, cytochrome c binds the CED-4 corresponding protein Apaf-1 (apoptotic protease activating factor 1) and induces a conformational change and oligomerization of the protein (Li et al., 1997; Zou et al., 1999). This recruits and activates procaspase-9, which in turn cleaves caspase-3 and other key substrates in the apoptotic death process (Li et al., 1997; Pan et al., 1998; Zou et al., 1999). The complex formed, comprising cytochrome c, Apaf-1, procaspase-9, and ATP is known as the apoptosome (Zou et al., 1999). The mitochondrial pathway of apoptosis is controlled and regulated by members of the Bcl-2 protein family (see below).

Crosstalk between the apoptotic pathways

Activated caspase-8 processes the pro-apoptotic Bcl-2 family protein Bid, generating a truncated Bid (tBid) fragment (Li et al., 1998; Luo et al., 1998). This fragment translocates to the mitochondria, activates other pro-apoptotic Bcl-2 family proteins, which in turn trigger release of cytochrome c and downstream activation of effector caspases, ultimately leading to cell death (Desagher et al., 1999; Gross et al., 1999; Eskes et al., 2000). Thus, cleavage of Bid by caspase-8 constitutes a connecting link between the death receptor and mitochondrial pathways of apoptosis (Figure 6).

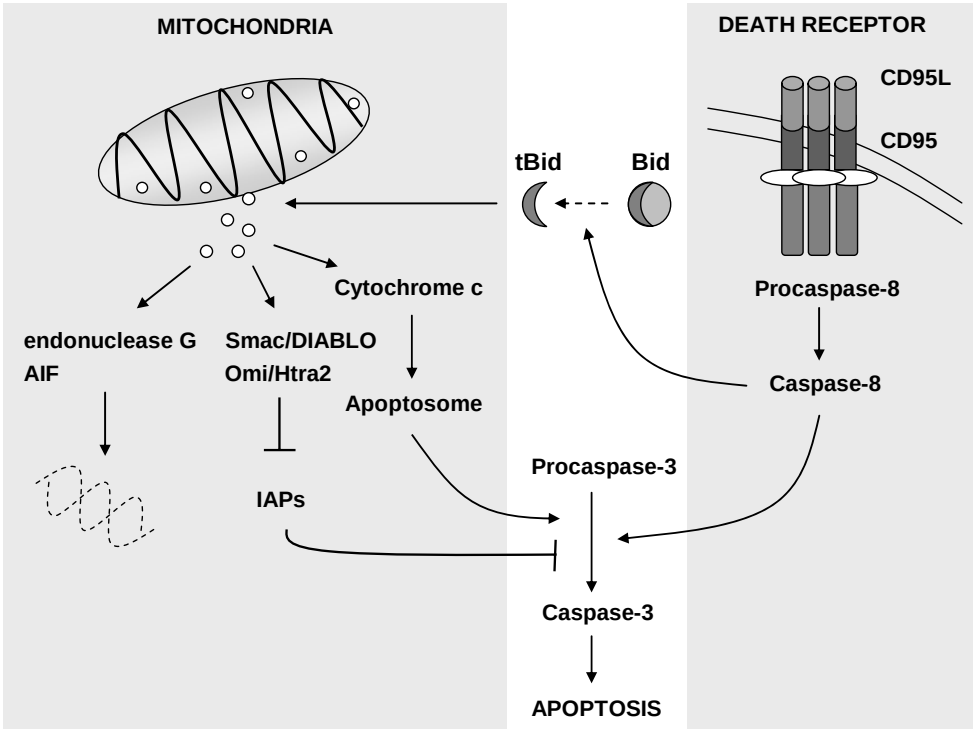

Figure 6. Apoptosis is generally reported to be mediated through two major pathways; the mitochondrial pathway and the death receptor pathway. Cleavage of the pro-apoptotic Bcl-2 family protein Bid constitutes a link between the two pathways.

BCL-2 FAMILY

The Bcl-2 family includes proteins that both induce and suppress apoptosis and they all contain one or several highly conserved domains, known as Bcl-2 homology domains (BH1-BH4) (Adams and Cory, 1998; Tsujimoto, 2003; Er et al., 2006). The Bcl-2 protein family can be subdivided into three main groups, based on their anti- or pro-apoptotic action and which BH domains they possess (Figure 7). The anti-apoptotic members (e.g. Bcl-2, Bcl-X_L, Mcl-1) contain all four domains (Er et al., 2006), of which BH4 seems to be important for their anti-apoptotic capacity (Huang et al., 1998). The pro-apoptotic proteins are further divided into two groups; the multidomain proteins, such as Bax, Bak, and Bok, which have BH1-3, and the BH3-domain-only proteins, such as Bid, Bim, Bad, and Bmf (Cory and Adams, 2002; Er et al., 2006). The Bcl-2 proteins appear to regulate each others function by forming homo- and hetero-complexes (Oltvai et al., 1993; Yin et al., 1994; Sedlak et al., 1995; Yang et al., 1995). Bcl-2 (B-cell lymphoma-2), the first member of the protein family to be identified, was initially defined as a proto-oncogene, located at the breakpoint of a translocation between chromosomes 18 and 14 in human follicular B-cell lymphomas (Tsujimoto et al., 1985). The anti-apoptotic Bcl-2 proteins are similar to CED-9 in *C. elegans* (Hengartner and Horvitz, 1994). Furthermore, the first pro-apoptotic member of the Bcl-2 family to be found was Bax (Bcl-2-associated protein X) (Oltvai et al., 1993).

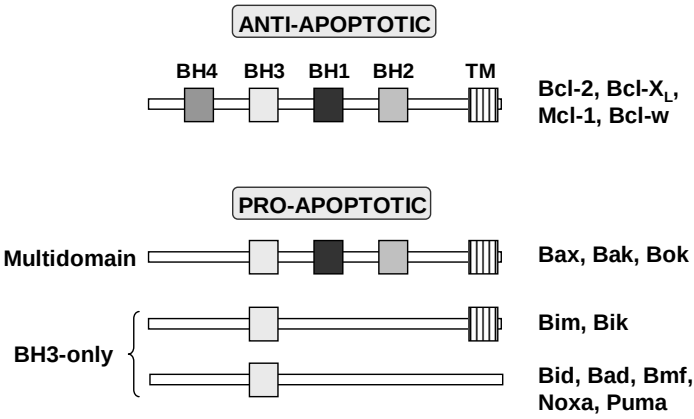

Figure 7. Classification of the members of the Bcl-2 protein family. Bcl-2 homology (BH) domains and transmembrane domains (TM) are presented.

Bcl-2 family proteins in the induction of cytochrome c release

The multidomain pro-apoptotic Bcl-2 family proteins, Bax and Bak play a crucial role in the mitochondrial pathway of apoptosis. Cells deficient in both Bax and Bak are completely resistant to apoptosis, induced by a wide range of stimuli, known to trigger mitochondrial-dependent apoptosis (Wei et al., 2001). Cells lacking one of these two proteins are, however, still sensitive for apoptotic signals.

Most of the Bcl-2 family members contain a hydrophobic transmembrane domain at the C-terminal (Cory and Adams, 2002; Er et al., 2006), which serves as an anchor that facilitates protein targeting and interaction with intracellular membranes (Nguyen et al., 1993), including the mitochondrial outer membrane, the endoplasmic reticulum, and the nuclear membrane (Hockenbery et al., 1990; Krajewski et al., 1993; Akao et al., 1994; Lithgow et al., 1994). Bax is predominantly located in the cytosol in healthy cells (Hsu et al., 1997), while Bak normally is integrated in the mitochondrial outer membrane (Griffiths et al., 1999). During healthy conditions, both C- and N- terminal domains of Bax are masked, allowing the protein to remain cytosolic (Lucken-Ardjomande and Martinou, 2005). Following an apoptotic signal, a conformational change of Bax and an unmasking of the domains occur. This induces Bax translocation to the mitochondria and insertion into the membrane (Hsu et al., 1997; Wolter et al., 1997). The normally monomeric forms of Bax and/or Bak now form oligomers and induce permeabilization of the mitochondrial outer membrane (Gross et al., 1998; Antonsson et al., 2000; Korsmeyer et al., 2000; Antonsson et al., 2001). The changes in the mitochondrial membrane permeability with cytochrome c release have been shown to be prevented by Bcl-X_L (Jürgensmeier et al., 1998; Narita et al., 1998; Desagher et al., 1999; Finucane et al., 1999; Gross et al., 1999) and Bcl-2 (Kluck et al., 1997; Yang et al., 1997; Narita et al., 1998; Desagher et al., 1999; Gross et al., 1999) in cell free apoptotic systems and in various cell types.

Bid, known to become activated by caspase-8 cleavage during Fas induced apoptosis (Li et al., 1998; Luo et al., 1998; Gross et al., 1999), has been suggested to induce the conformational change of Bax, resulting in cytochrome c release from the mitochondria (Desagher et al., 1999; Eskes et al., 2000). Addition of Bax to Bax-deficient tumor cells significantly increases the Bid induced cytochrome c release (Desagher et al., 1999). Moreover, tBid also plays an important role in Bak induced mitochondrial permeabilization (Korsmeyer et al., 2000; Wei et al., 2000).

Mcl-1

Mcl-1 (myeloid cell leukemia-1) is an anti-apoptotic member of the Bcl-2 protein family, which has not been as thoroughly investigated as Bcl-2 and Bcl-X_L. It contains a C-terminal transmembrane domain, which localizes Mcl-1 to membranes, primarily the mitochondrial outer membrane (Michels et al., 2005). By alternative splicing, a second smaller protein isoform of Mcl-1, Mcl-1_S is produced, which in contrast to full length Mcl-1 promotes cell death. Decreased level of Mcl-1 by antisense oligonucleotides in human myeloblastic leukemia cells (U937) results in rapidly induced apoptosis (Moulding et al., 2000). During apoptosis, Mcl-1 expression has been found to be downregulated by many apoptotic stimuli, including UV irradiation and TRAIL (tumor necrosis factor related apoptosis-inducing ligand) (Nijhawan et al., 2003; Han et al., 2004; Han et al., 2006). Immunoprecipitation experiments demonstrate Mcl-1 to interact with Bak and the BH3-only proteins Bim and tBid, and neutralize their pro-apoptotic potential in HeLa and Jurkat T leukemic cells (Han et al., 2004; Willis et al., 2005; Clohessy et al., 2006).

Mitochondrial membrane permeabilization

The exact mechanism that leads to the mitochondrial membrane permeabilization is still not fully understood, but several hypotheses have been suggested (Figure 8). The three-dimensional structure of Bcl-X_L shows notable similarity with the ion pore-forming bacterial toxins colicin and diphtheria toxin (Muchmore et al., 1996). This observation has lead to studies examining the capacity of Bcl-2 family members to form ion channels. Indeed, Bcl-X_L, Bax, and Bcl-2 have pore forming activity in synthetic lipid membranes (Antonsson et al., 1997; Minn et al., 1997; Schendel et al., 1997; Schlesinger et al., 1997). However, Bax formed channels at physiological pH, while Bcl-X_L and Bcl-2 did it most efficiently at low pH (Antonsson et al., 1997; Minn et al., 1997; Schlesinger et al., 1997). Bax oligomers have been reported to form channels in liposomes (Antonsson et al., 2000), promote release of cathepsins from lysosomes (Kågedal et al., 2005), and trigger cytochrome c release from isolated mitochondria (Antonsson et al., 2000). Antonsson and coworkers showed, in experiments with liposomes, the pore-forming activity of Bax to be antagonized by Bcl-2 (Antonsson et al., 1997).

Other studies indicate that Bcl-2 family members might perturb or alter the activity of pre-existing channels in the membrane (Zoratti et al., 2005). Small solutes might enter the mitochondria, resulting in osmotic swelling and eventually rupture of the membrane. The mitochondrial changes are prevented by cyclosporine A, which closes a channel named the permeability transition pore (PTP), indicating that the mitochondrial permeabilization is mediated through opening of these pores (Narita et al., 1998). The PTP is a polyprotein channel that includes the voltage-dependent anion channel (VDAC), the adenine nucleotide translocator (ANT), and cyclophilin D (Zoratti et al., 2005). Yeast two-hybrid system and coimmunoprecipitation analysis have shown Bax and Bak to interact with the PTP (Marzo et al., 1998; Narita et al., 1998). By direct interaction with VDAC, Bax and

Bak triggered passage of cytochrome c out of liposomes, while the pore was closed by binding of Bcl-X_L (Shimizu et al., 1999).

In addition, during apoptosis mitochondrial lipids have been reported to be important for the membrane permeabilization (McMillin and Dowhan, 2002; Gonzalvez and Gottlieb, 2007). The phospholipid cardiolipin, present in the mitochondrial membrane, might interact with Bcl-2 family proteins. The lipid has been suggested to be required for tBid recruitment to the mitochondria (Lutter et al., 2000; Lutter et al., 2001; Wei et al., 2001) and it has been shown to coimmunoprecipitate with Bid during apoptosis, induced by the Fas death receptor (Sorice et al., 2004). In addition, Bax does not permeabilize artificial liposomes in the absence of cardiolipin (Kuwana et al., 2002; Terrones et al., 2004).

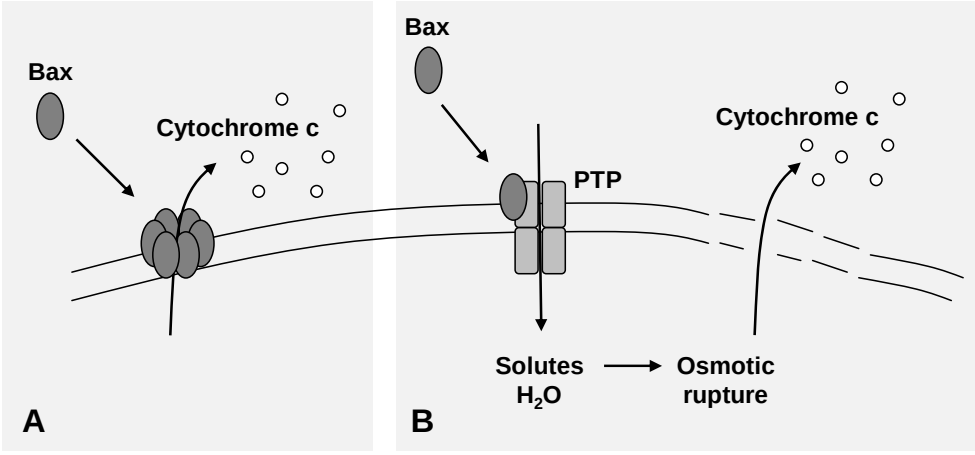

Figure 8. Two models for mitochondrial membrane permeabilization. (A) Bax might by oligomerization form a pore in the mitochondrial membrane and release cytochrome c. (B) Binding of Bax to permeability transition pore (PTP) might allow entrance of solutes, resulting in osmotic swelling and rupture of the mitochondrial membrane.

Sequestration of Bax in the cytosol

Several cytosolic proteins have been suggested to keep Bax in the cytosol and prevent its activation. Recent data implicate an apoptotic suppressing role for Ku70 (Sawada et al., 2003), which is essential for repair of DNA double strand breaks (Walker et al., 2001). Ku70 has been shown to coimmunoprecipitate with Bax in the cytosol and the interaction inhibits the conformational change and subsequent translocation of Bax (Sawada et al., 2003). 14-3-3θ is another protein that has been reported to play a role in the negative regulation of Bax activity (Nomura et al., 2003). Nomura *et al.* show this protein to interact with Bax in the cytosol in healthy cells, and following apoptotic stimulus, 14-3-3θ dissociates from Bax by both caspase dependent and independent mechanisms (Samuel et al., 2001; Nomura et al., 2003). The small (3 kDa) peptide humanin might also prevent Bax translocation from cytosol to mitochondria and subsequent cytochrome c release (Guo et al., 2003). In contrast to the above mentioned proteins, which bind to the inactive form of Bax, the glycoprotein clusterin interacts with conformational altered Bax in response to chemotherapeutic drugs and inhibits oligomerization of Bax (Zhang et al., 2005).

BH3-domain-only proteins

The pro-apoptotic BH3-only proteins (corresponding to EGL-1 in *C. elegans*; Conradt and Horvitz, 1998) seem to be key players in the activation of Bax and Bak. In response to an apoptotic signal, BH3-only proteins are transcriptionally upregulated and/or activated by post-translational modifications, such as proteolytic cleavage, phosphorylation or dephosphorylation (Puthalakath and Strasser, 2002; Willis and Adams, 2005). The BH3-only proteins seem to require Bax and/or Bak to be able to induce apoptosis (Zong et al., 2001) and these proteins include both "sensitizers" and "activators". The activators (e.g. Bid and Bim) are normally sequestered by anti-apoptotic Bcl-2 proteins, but following a stress stimulus, sensitizers (e.g. Bad and Bik) might bind to the pro-survival Bcl-2 proteins through their BH3 domain, allowing the activators to directly activate Bax or Bak and trigger their oligomerization and mitochondrial membrane permeabilization (Figure 9) (Letai et al., 2002; Kuwana et al., 2005; Willis et al., 2007). This was demonstrated with experiments showing that Bid and Bim could induce cytochrome c release from isolated mitochondria, whereas Bad and Bik only could do it in the presence of Bid or Bim (Letai et al., 2002). Further, Bim, Bid, Bad, and Noxa were all unable to induce apoptosis in Bax and Bak double-deficient cells (Cheng et al., 2001).

In response to death receptor ligation, caspase-8 cleavage of the inactive cytosolic form of Bid to tBid results in exposure of its BH3 domain , which allows it to translocate to the mitochondria (Li et al., 1998; Luo et al., 1998; McDonnell et al., 1999). Bid might target the mitochondria by binding to the mitochondria lipid cardiolipin (Lutter et al., 2000; Lutter et al., 2001) and is able to stimulate apoptosis by induction of Bax and/or Bak oligomerization and membrane insertion (Desagher et al., 1999; Eskes et al., 2000;

Korsmeyer et al., 2000; Wei et al., 2000). Bid has also been shown to be cleaved and activated by non-caspase proteases, such as lysosomal proteases (Stoka et al., 2001; Cirman et al., 2004; Heinrich et al., 2004), and granzyme B (Li et al., 1998; Barry et al., 2000).

Bim exists as three different splicing isoforms; Bim_S, Bim_L, and Bim_{EL} (O'Connor et al., 1998). Bim_S is constitutively pro-apoptotic, whereas Bim_L and Bim_{EL} normally are kept inactive by binding to the dynein motor complex in the microtubules (Puthalakath et al., 1999). Bmf is an additional BH3-only protein that is expressed in healthy cells, but inactivated by sequestration to the cytoskeleton, by binding to the myosin V actin motor complex (Puthalakath et al., 2001). UV irradiation can cause release of both Bim and Bmf from the cytoskeleton and when released they have been reported to either translocate to anti-apoptotic Bcl-2 proteins and neutralize their activity, or to directly activate Bax and induce cytochrome c release (Puthalakath et al., 1999; Puthalakath et al., 2001; Kuwana et al., 2005). In addition, Bim and Bmf might be transcriptionally upregulated in response to a stress stimulus (Ramjaun et al., 2007).

Noxa and Puma (p53 upregulated modulator of apoptosis) are induced by the transcription factor p53 and have been shown to trigger apoptosis by localizing to the mitochondria and inducing cytochrome c release and caspase-9 activation (Oda et al., 2000; Nakano and Vousden, 2001). Antisense oligonucleotides to Noxa and Puma inhibited the p53 induced apoptosis.

When Bad is phosphorylated, the protein is kept sequestrated in the cytosol by binding to 14-3-3 inhibitory proteins (Zha et al., 1996). In response to growth factor withdrawal, Bad becomes dephosphorylated and released. Free Bad heterodimerizes with $Bcl\text{-}X_L$ and Bcl-2, which leads to Bax displacement and apoptosis triggering (Yang et al., 1995). Bik is an additional BH3–only protein whose activity is regulated by phosphorylation (Verma et al., 2001).

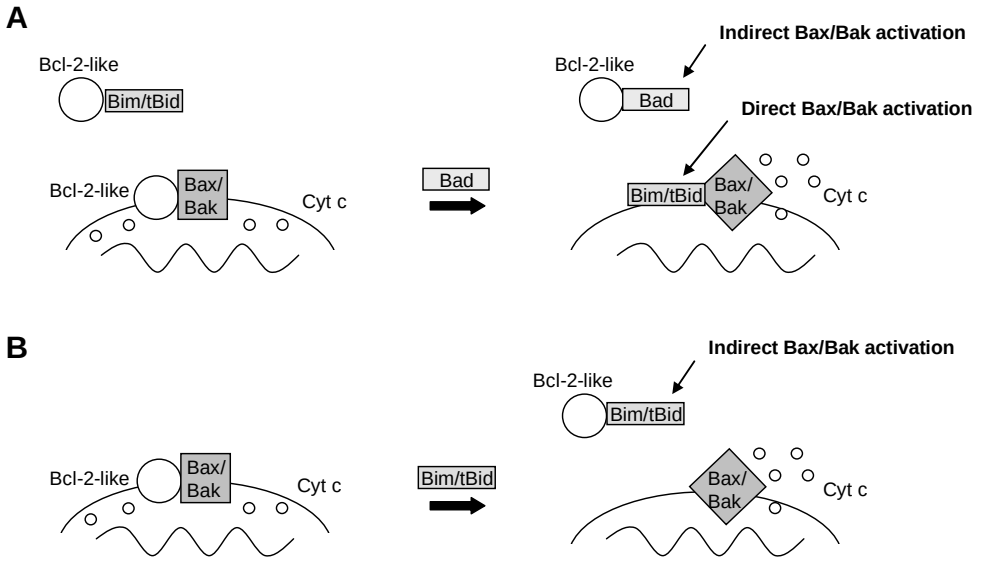

Figure 9. Two models of BH3-only protein activation of Bax. (A) Some BH3-only proteins, such as Bid and Bim, are able to directly activate Bax/Bak, leading to mitochondrial permeabilization. Other BH3-only proteins, such as Bad, instead activate Bax/Bak indirectly by binding to anti-apoptotic Bcl-2-like proteins, and displace BH3-only proteins, which can activate Bax/Bak. (B) BH3-only proteins might also activate Bax/Bak by binding anti-apoptotic Bcl-2-like proteins that sequester Bax/Bak.

LYSOSOMES AND LYSOSOMAL ENZYMES

The lysosomes were first described by de Duve and colleagues (de Duve, 1959). These acidic organelles (pH around 4.5) are present in all mammalian cell types except red blood cells (Tardy et al., 2006). The lysosomes (from the Greek for "digestive body") have long been referred to as "suicide bags" or "garbage disposals", since they represent the main site for degradation of intracellular macromolecules and long-lived proteins. This degradation is performed by a large group of lysosomal enzymes, comprising proteases, nucleases, lipases, glycosidases, and sulfatases. The cathepsins, which are the major group of lysosomal proteases, can be subdivided into three classes, according to the amino acid in their active site; serine (e.g. cathepsins A), cysteine (e.g. cathepsins B, L, and H), and aspartic (e.g. cathepsin D) cathepsins. The cathepsins are synthesized as inactive proenzymes in the endoplasmatic reticulum and following transfer to the lysosomes, they are activated by proteolytic cleavage (Gieselmann et al., 1983).

Increasing evidence now suggests that caspases are not the only proteases involved in apoptotic cell death. Cathepsins have also been shown to act as pro-apoptotic mediators in several different cell types (Deiss et al., 1996; Ishisaka et al., 1998; Roberg and Öllinger, 1998; Guicciardi et al., 2000; Foghsgaard et al., 2001; Kågedal et al., 2001a; Stoka et al., 2001; Bidère et al., 2003; Boya et al., 2003). Under normal physiological conditions, these proteases are localized inside the lysosomes, but after a variety of stress stimuli, such as TNF-α, staurosporine, oxidative stress, p53, and growth factor starvation, they have been described to translocate to the cytosol (Roberg and Öllinger, 1998; Brunk and Svensson, 1999; Guicciardi et al., 2000; Foghsgaard et al., 2001; Kågedal et al., 2001a; Yuan et al., 2002; Bidère et al., 2003; Johansson et al., 2003). The degree of lysosomal damage will determine the fate of the cell. Cellular damage, leading to an extensive release of lysosomal content, results in necrosis, while a partial permeabilization will trigger apoptosis (Brunk et al., 1997; Li et al., 2000; Kågedal et al., 2001b). Studies have shown the cathepsins, with an acidic pH optimum, to be active also at neutral pH, as in the cytosol (Roberg et al., 2002; Tardy et al., 2006). However, a cytosolic acidification has been observed to be accompanied by a lysosomal membrane permeabilization in TNF-α induced apoptosis (Nilsson et al., 2006).

Lysosomal membrane permeabilization

The exact mechanisms for lysosomal permeabilization are still unknown, but several mechanisms have been proposed. TNF-α ligation stimulates the production of sphingosine (Chwieralski et al., 2006), which accumulates in lysosomes and might induce a detergent-like rupture of the membrane, with the release of lysosomal enzymes (Kågedal et al., 2001b; Werneburg et al., 2002; Chwieralski et al., 2006). In addition, generation of reactive oxygen species might contribute to lysosomal permeabilization (Brunk et al., 1997; Roberg and Öllinger, 1998; Antunes et al., 2001). H_2O_2 has been shown to induce release of cathepsin D to the cytosol in alveolar type II cells (Yin et al.,

2005). A third mechanism suggested for lysosomal leakage involves Bcl-2 family proteins. Bax and Bak form pores in the mitochondrial outer membrane and induce cytosolic relocation of cytochrome c. Similarly, in response to staurosporine treatment, Bax has been demonstrated to translocate to lysosomes and induce cathepsin release in human fibroblasts (Kågedal et al., 2005). Moreover, active caspase-8 and -2, have also been reported to cause cathepsin release from isolated lysosomes (Guicciardi et al., 2000).

Cathepsin B and D involvement in apoptosis

Cathepsins B, D, and L are the most abundant lysosomal proteases and they have all been found to be translocated from the lysosomal compartment to the cytosol in apoptotic human fibroblasts and T lymphocytes (Kågedal et al., 2001a; Bidère et al., 2003). Only inhibition of cathepsin D, with pepstatin A inhibitor or siRNA did, however, prevent the apoptotic process (Bidère et al., 2003; Johansson et al., 2003). Furthermore, microinjection of human fibroblasts with cathepsin D, but not cathepsin B, into the cytosol, triggered apoptosis (Roberg et al., 2002), implicating cathepsin D to be the major pro-apoptotic lysosomal enzyme. However, Guicciardi *et al.* found cathepsin B to contribute to TNF-α induced apoptosis in hepatocytes (Guicciardi et al., 2000) and inhibition of cathepsin B, but not cathepsin D, has been shown to inhibit p53 induced apoptosis in myeloid leukemic cells (Yuan et al., 2002). Thus, the role of the different cathepsins appears to be cell type and/or cell death stimulus specific.

The lysosomal pathway of apoptosis has been suggested to be mitochondria dependent (Roberg et al., 1999). In accordance, cathepsin D triggered Bax conformational change and Bax induced mitochondrial permeabilization in T lymphocytes (Bidère et al., 2003) and fibroblasts pretreated with pepstatin A displayed almost no cytochrome c release (Johansson et al., 2003). Cathepsin B has also been shown to trigger apoptosis through mitochondrial permeabilization (Guicciardi et al., 2000). Bid is generally known to be processed and activated by caspases, but recent reports demonstrate cathepsins to be able to cleave Bid as well (Stoka et al., 2001; Cirman et al., 2004; Heinrich et al., 2004). Specific Bid cleavage fragment, different from the one found by caspase-8 processing, was observed after incubation of full-length Bid with lysosomal extract, and this cleaved form of Bid induced cytochrome c release from mitochondria (Stoka et al., 2001). Studies have later demonstrated the cysteine cathepsins B, L, H, S, and K as well as the aspartic cathepsin D to be able to directly cleave Bid *in vitro* (Cirman et al., 2004; Heinrich et al., 2004). This suggests a possible link between cathepsins and the mitochondrial pathway of apoptosis.

Lysosomal participation in the apoptotic pathways

The lysosome might be important in the regulation of both the mitochondrial and the death receptor pathways of apoptosis (Figure 10). To make the apoptotic process as effective as possible, the two pathways might cooperate, by connecting links and feed-back-loops. The pathways involved might also vary depending on tissue and stress stimulus.

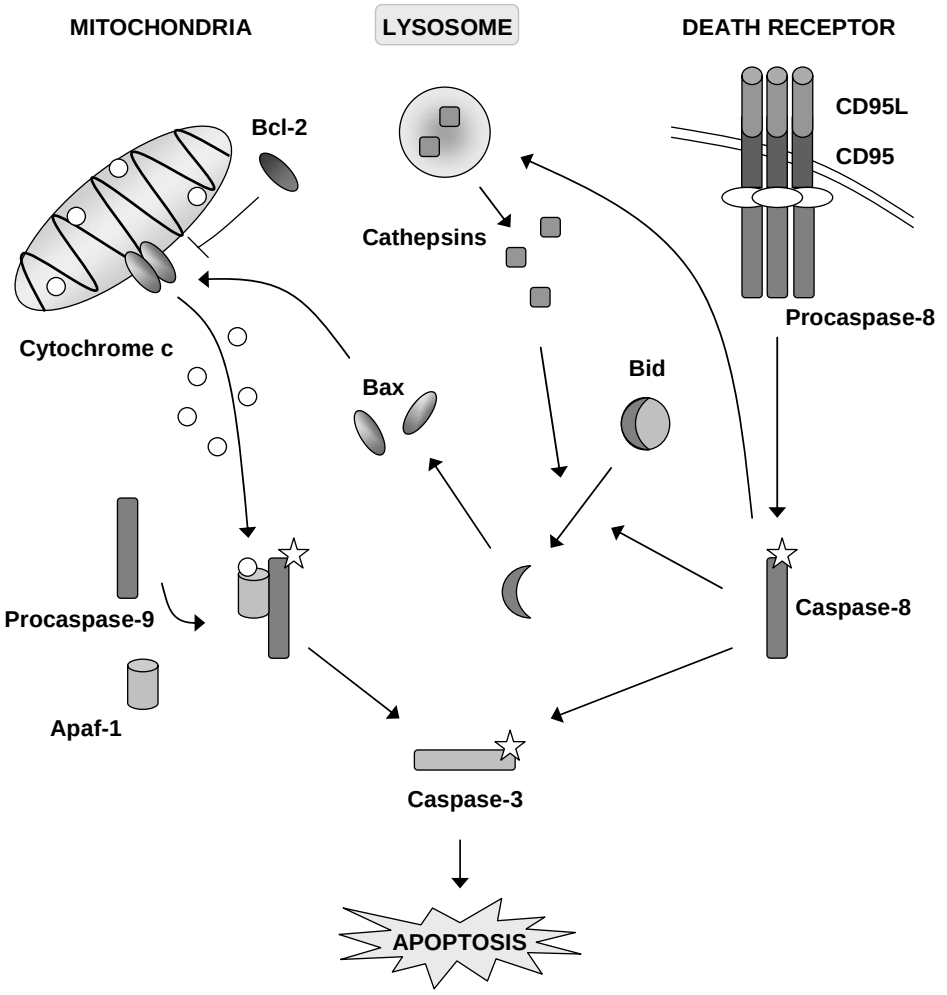

Figure 10. Participation of the lysosome and the lysosomal cathepsins in the pathways of apoptosis.
✯ Active caspase.

STRESS RESPONSE

Cells are continually challenged by different degrees of stress. To be able to cope with these environmental changes and various types of cellular damages, the cells possess a defense system, including stress proteins that detect and are able to minimize injury. If the damages are too extensive, the apoptotic machinery will be activated. Some proteins considered to be involved in cellular response to stress are heat shock proteins (Hsps), mitogen activated protein kinases (MAPKs), and p53.

HEAT SHOCK PROTEIN 70

Hsps are molecular chaperones, involved in folding of newly synthesized proteins, regulation of protein translocation and import, and control of activity of regulatory proteins (Parsell and Lindquist, 1993; Strub et al., 2000; Mayer and Bukau, 2005). They also limit cellular damage following stress by preventing protein aggregation and by binding denatured proteins and assist in their refolding or degradation (Parsell and Lindquist, 1993; Mayer and Bukau, 2005). A protective capacity of Hsps was first recognized by the observation that cells exposed to a mild hyperthermic shock decrease their susceptibility to a subsequent more severe and normally lethal heat shock (Gerner and Schneider, 1975; Moseley, 1997). This cellular adaptation is referred to as acquired thermotolerance.

The Hsps are divided into 6 major families according to their molecular size; Hsp100, Hsp90, Hsp70, Hsp60, Hsp40, and small Hsps (Jolly and Morimoto, 2000). The Hsp70 family represents the most conserved group (Beere and Green, 2001), consisting of both constitutively expressed members and of proteins that are induced in response to environmental, chemical, and physical stresses. The main stress-induced member Hsp70 (also called Hsp72 or Hsp70i) has been reported to effectively rescue various cell types from apoptosis in response to a wide range of stress stimuli, including heat shock, TNF-α, oxidative stress, irradiation, nitric oxide, and anti-cancer drugs (Jäättelä et al., 1992; Simon et al., 1995; Trautinger et al., 1995; Bellmann et al., 1996; Polla et al., 1996; Samali and Cotter, 1996; Mosser et al., 2000). The exact mechanisms for this protection are, however, still not elucidated. Hsp70 has been suggested to exert its anti-apoptotic function upstream of mitochondrial membrane permeabilization (Creagh et al., 2000; Gotoh et al., 2004; Steel et al., 2004; Stankiewicz et al., 2005), as well as, downstream of caspase-3 activation (Jäättelä et al., 1998). Moreover, increased Hsp70 expression has been found in many tumors, which might correlate with enhanced malignancy and resistance against therapy (Jäättelä, 1999; Garrido et al., 2003; Aghdassi et al., 2007).

C-JUN N-TERMINAL KINASE (JNK)

JNK is a subfamily of the MAPK superfamily (Hagemann and Blank, 2001). Three genes encode the JNK protein, *Jnk1*, *Jnk2*, and *Jnk3* (Davis, 2000). JNK1 and JNK2 are ubiquitously expressed, while JNK3 is restricted to brain, heart, and testis tissues. Besides, JNK1, 2, and 3 there also exist different JNK isoforms, due to alternatively splicing. JNK proteins phosphorylate and activate the transcription factors c-Jun, ATF2, Elk-1, p53, and c-myc (Liu and Lin, 2005). In response to apoptotic stimuli, JNK also has been reported to phosphorylate non-transcription factors involved in apoptosis, such as Bcl-2, Bcl-X_L, Bim, and Bad. Mouse embryos, deficient in *Jnk1* or *Jnk2*, survive normally, but *Jnk1* and *Jnk2* double knockout embryos die with severe dysregulation of neuronal cell death (Kuan et al., 1999; Sabapathy et al., 1999). There are contradictory data reported, concerning the role of JNK in regulation of apoptosis. An anti-apoptotic function of the protein has been proposed, since anti-sense JNK oligonucleotides inhibited growth and induced apoptosis in tumor cells (Potapova et al., 2002) and JNK has been suggested to suppress IL-3 withdrawal induced apoptosis by phosphorylation of Bad (Yu et al., 2004). Moreover, some tumor cells possess constitutively active JNK (Davis, 2000). On the other hand, plenty of reports support a pro-apoptotic function for JNK. UV irradiation did not cause mitochondrial cytochrome c release or apoptosis in *Jnk1*$^{-/-}$ *Jnk2*$^{-/-}$ fibroblasts, indicating the protein to be essential in the mitochondrial pathway of apoptosis (Tournier et al., 2000). JNK is also required for TNF-α induced apoptosis (Deng et al., 2003; Liu et al., 2004).

p53

p53 is a tumor suppressor transcription factor that is stabilized and activated by cellular stresses, such as the DNA damaging agents UV- and γ-irradiation and chemotoxic drugs (Slee et al., 2004). Upon cellular damage, an increased p53 level leads to apoptosis or cell cycle arrest with DNA reparation. Mice lacking p53 demonstrate a high susceptibility to tumor development (Donehower et al., 1992) and somatic mutations within p53 are found in more than 50% of all human tumors (Slee et al., 2004). The p53 protein can transcriptionally upregulate the expression of, among others, the pro-apoptotic proteins Bax, Noxa, Puma, and the death receptors DR5/KILLER and Fas (Miyashita and Reed, 1995; Oda et al., 2000; Nakano and Vousden, 2001; Slee et al., 2004). p53 is also able to suppress expression of anti-apoptotic proteins, such as Bcl-2 and survivin (Haldar et al., 1994; Hoffman et al., 2002) and to induce apoptosis directly from the cytosol through transcriptional independent mechanisms (Marchenko et al., 2000; Chipuk et al., 2004; Chipuk et al., 2005). Following an apoptotic stimulus, p53 translocates to the mitochondria and induces Bax dependent mitochondrial permeabilization and cell death.

AIMS OF THE THESIS

GENERAL AIM

The general aim of the thesis was to gain deeper insight into the so far unknown apoptotic process induced by UV irradiation in human melanocytes. The studies were performed in pure cultures of human epidermal melanocytes.

SPECIFIC AIMS

- To investigate how different wavelengths within the UVB spectrum affect the apoptotic potential and Bcl-2 and Bax mRNA and protein expressions.

- To study possible communication between keratinocytes and melanocytes during UV induced apoptosis.

- To examine the role and function of Bcl-2 family proteins in UVA and in UVB induced apoptosis, and the intracellular localization of these proteins.

- To investigate the involvement of lysosomal membrane permeabilization and lysosomal proteases, cathepsins, in the apoptotic process.

- To clarify the role of stress-induced Hsp70 in apoptosis triggered by UVB irradiation and to investigate the specific apoptotic regulatory function of the protein.

- To examine the role of JNK in the regulation of UVB induced apoptosis. To study the specific site of operation and function of the protein in the regulation of Bcl-2 family proteins.

MATERIALS AND METHODS

CELL CULTURE (PAPER I-IV)

Normal human melanocytes (papers I-IV) and keratinocytes (paper I) were isolated from foreskins obtained from Caucasian donors (0-2 years of age), and cultures were established as previously described (Andersson et al., 2001). In short, the skin was washed in penicillin/streptomycin, cut in small pieces, and incubated in dispase (2 mg/ml) for 18 h at 4°C. Epidermis was separated from dermis and incubated in trypsin/EDTA for 40 min at 37°C. Aspiration with a pipette every 10 min helped to dissociate the cells. Digestion was stopped by addition of Dulbecco's modified Eagle's medium (DMEM) supplemented with 5% serum, and the cell suspension was filtered through a 40 μm nylon cell strainer. Pure cultures were established by repeated differential trypsinization and the melanocytes were cultured in medium 199 with 2% fetal bovine serum, according to Gilchrest *et al.* (Gilchrest et al., 1984) (Figure 11A). The keratinocytes were cultured in DMEM-Ham's F-12 (3:1) with 10% fetal bovine serum, as described by Rheinwald *et al.* (Rheinwald and Green, 1975) (Figure 11B). The cells were grown at 37°C in a humidified atmosphere of 5% CO_2 in air, and culture medium was changed three times a week. Prior to experiments, cells were trypsinated and seeded at 2.5 x 10^4 cells/cm^2. The experiments were conducted between passage 2-7 and no cells were cultured for more than three weeks in total, after which they were analyzed or discharged. Untreated controls from the same donor were analyzed in parallel.

In paper I, a co-culture system was established (Figure 11C). Pure melanocytes were cultured in wells and keratinocytes from the same subject were grown in fitting inserts, with a pore size of 0.4 μm. The inserts were submerged into the wells with melanocytes after irradiation and melanocyte medium was then used.

In some experiments in paper II, the melanocytes were incubated with pepstatin A (100 μM, stock in DMSO) or E64d (10 μM, stock in DMSO) for 24 h before UV irradiation, to block cathepsin D and cysteine cathepsin (e.g. cathepsin B) activity, respectively. Controls for DMSO effects were analyzed and no interference with the experiments was noted.

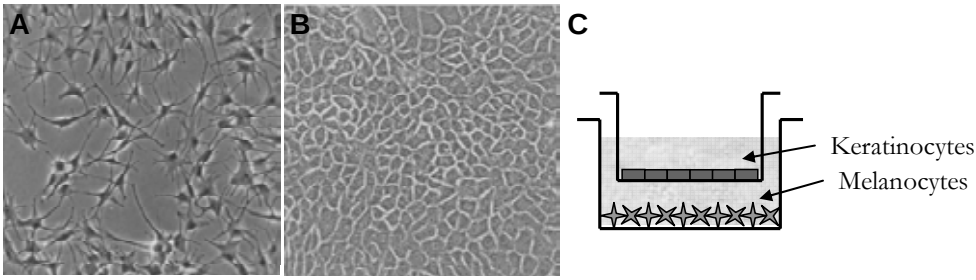

Figure 11. Pure cultures of (A) melanocytes and (B) keratinocytes, grown in Petri dishes for 12 and 8 days, respectively. (C) Co-culture system, with melanocytes growing in wells and keratinocytes from the same individual in fitting inserts.

UV (PAPER I-IV) AND HEAT (PAPER III) EXPOSURES

In papers I-IV, UV exposures of the cell cultures were performed. The UVB source was two Philips TL20W/12 tubes (Philips, Eindhoven, The Netherlands) emitting in the spectral range 280-370 nm, with a main output of 305-320 nm. For UVA, a Medisun 2000-L tube (Dr Gröbel UV-Elektronik GmbH, Ettlingen, Germany; 340-400 nm) was used. In most experiments, a Schott WG 305 cut off filter (50% absorption below 305 nm, Mainz, Germany) was used. The UV exposure was performed in culture dishes containing pre-warmed phosphate buffered saline (PBS) and no increase in temperature was noted during irradiation. Unirradiated control cells were handled identically, except for irradiation.

In paper I, a UVB irradiation dose of 50 mJ/cm^2 was used, with or without the use of cut off filter, to study the effects of various spectral ranges within UVB. The UVB output was 0.96 mW/cm^2 (with filter) and 1.34 mW/cm^2 (without filter), measured with a PUVA Combi Light dosimeter (Leuven, Belgium). In the co-culture system, the melanocytes and keratinocytes were irradiated separately before the two cell compartments were brought together.

In paper II-IV, the irradiation doses were titrated to achieve an approximately 30% frequency of apoptosis with a minimum of necrotic cell contamination. This resulted in an experimental model using 60 J/cm^2 UVA and 500 mJ/cm^2 UVB. The output of UVA was 80 mW/cm^2 and 1.44 mW/cm^2 for UVB.

In paper III, some cultures were exposed to heat. Culture medium, pre-warmed to 42.5ºC, was added and the culture dishes were placed in an isolated box to keep the temperature stable at 42.5ºC during the incubation period of 1 h. The temperature was continuously controlled by a Testo 100 thermometer (Nordtec Instrument, Gothenburg, Sweden).

APOPTOSIS DETECTION (PAPER I-IV)

ANNEXIN V-FLUOS AND PROPIDIUM IODIDE STAINING (PAPER I)

As an early event in apoptotic cells, phosphatidylserine is transferred from the cytoplasmic surface of the cell membrane to an outer side location, where it serves as a signal to phagocytic cells (Fadok et al., 1992). By using annexin V, which has a high affinity for phosphatidylserine, cells in early and middle stages of apoptosis can be detected. A concomitant staining with the DNA binding dye propidium iodide, which only enters cells with permeabilized membrane, excludes cells that are necrotic.

Harvested melanocytes were resuspended in incubation buffer (10 mM Hepes/NaOH, pH 7.4, 140 mM NaCl, 5mM $CaCl_2$), containing annexin V conjugated with fluorescein (FLUOS) and propidium iodide and incubated in dark on ice for 15 min. 200 cells were, subsequently, counted on glass slides in a fluorescence microscope (Nikon, Tokyo, Japan). Cells binding annexin V, but excluding propidium iodide, were considered to be apoptotic, whereas cells with propidium iodide fluorescence with or without bound annexin V were considered to be necrotic or post-apoptotic necrotic, since late-stage apoptotic cells may enter secondary necrosis if not phagocytosed.

DAPI STAINING (PAPER II-IV)

Another characteristic event in apoptotic cell death is nuclear fragmentation and condensation. To investigate the frequency of apoptosis, melanocytes were fixed in 4% neutral buffered formaldehyde and mounted in Vectashield® Mounting Media, supplemented with 4',6-diamidino-2-phenylindole (DAPI). The nuclear morphology was evaluated in a fluorescence microscope (Nikon, Tokyo, Japan). In control cells, most nuclei were round in shape and glowed homogenously, while apoptotic cells were identified by either fragmented nuclei or by a condensed chromatin pattern gathered at the periphery of the nuclear membrane (Figure 12). Trypan blue exclusion test were performed in order to determine the fractions of necrotic cells after UV exposure. The cells were stained with 0.2% trypan blue solution in PBS for 1 min and examined in a light microscope.

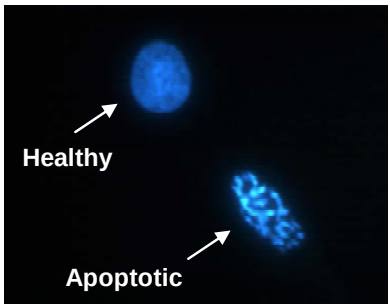

Figure 12. DAPI stained nuclei from a healthy and an apoptotic melanocyte.

IMMUNOCYTOCHEMISTRY (PAPER II-IV)

To study the localization and redistribution of apoptotic proteins, melanocytes were fixed in 4% paraformaldehyde for 20 min at 4°C and processed for immunocytochemistry (Brunk et al., 1997). After permeabilization with 0.1% saponin, the cultures were incubated with a primary antibody overnight at 4°C, followed by incubation with a secondary fluorescent conjugated antibody for 1 h at room temperature. The specimens were mounted in Vectashield® Hardset Mounting Media and the protein localization was analyzed in a Nikon Eclipse E600W fluorescence confocal microscope (Figure 13). Negative controls, incubated without primary antibody, showed no staining. For co-localization studies (papers III, IV), vital staining of mitochondria was accomplished by incubation of cells with Mitotracker® Red (200 nM) for 30 min at 37°C before fixation.

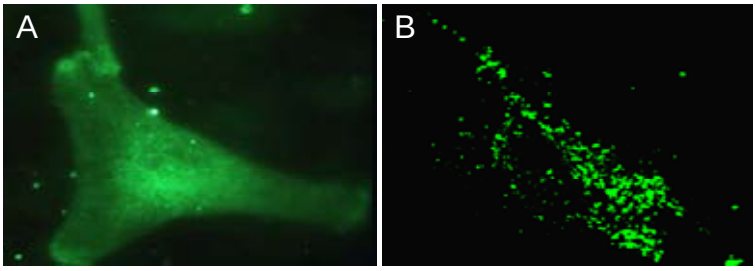

Figure 13. Immunostaining of Bax in (A) control (diffuse) and (B) UV exposed (punctate) melanocytes.

WESTERN BLOT ANALYSIS (PAPER I-IV)

Western blotting was introduced by Towbin *et al.* in 1979 and is a method for the transfer of proteins to a membrane using electrophoresis (Towbin et al., 1979). This method is used for identifying the presence and quantity of a specific protein.

The cells were harvested and incubated in lysis buffer (150 mM NaCl, 1% Triton X-100, 0.1% SDS 50, mM Tris pH 8.0, 5 mM EDTA) with protease inhibitors on ice for 30 min. The total protein concentration was determined, using the Bio-Rad D_C Protein Assay System, which is a colorimetric assay similar to the Lowry method (Lowry et al., 1951), and the absorption at 750 nm was analyzed, using bovine serum albumin as a standard. An equal amount of protein, mixed with Laemmli sample buffer with 5% β-mercaptoethanol, was denatured by heating, to disrupt inter- and intra-molecular bindings and thereby eliminate secondary and tertiary structures. The samples were then separated according to molecular weight by denaturing SDS-PAGE (sodium dodecyl sulfate polyacrylamide gel electrophoresis) and subsequently transferred to a polyvinylidene difluoride (PVDF) membrane. Blocking the membrane with 5% non-fat dry milk in PBS, supplemented with 0.05% Tween 20, at room temperature for 1 h, prevented nonspecific binding to the membrane. The immunodetection was performed using a primary antibody for 2 h at room temperature and after washing, a corresponding secondary antibody conjugated with horseradish peroxidase (HRP) was applied to the membrane for an additional hour. Specific proteins were detected with the chemiluminescent ECL-Plus Western blotting reagents and visualized on Hyperfilm™ ECL™ (Figure 14). The membranes were reprobed with glyceraldehyde-3-phosphate dehydrogenase (GAPDH) or β-actin antibodies to verify that an equal amount of the protein was loaded into each well.

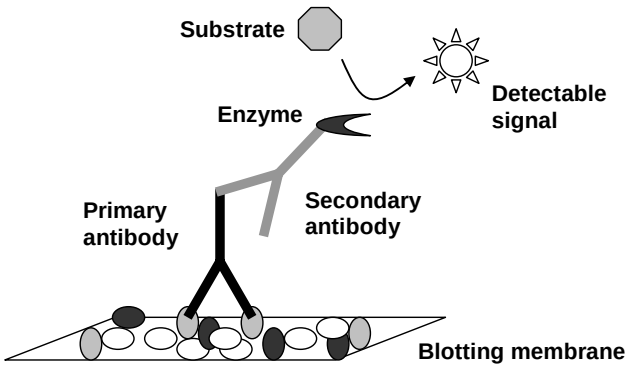

Figure 14. Immunodetection on Western blot membrane. A primary antibody binds the specific protein on the membrane. An enzyme conjugated secondary antibody then interacts with the primary antibody and then reacts with a substrate, which generates a detectable signal.

SUBCELLULAR FRACTIONATION EXPERIMENTS (PAPER III)

Subcellular fractions of cytosols, membranes and nuclei were collected by using ProteoExtract™ Subcellular Proteome Extraction Kit (Calbiochem, Darmstadt, Germany), according to the manufacturer's instructions. To verify the purity of each fraction, the following antibodies were used as markers in the Western blot analysis; lactate dehydrogenase (LDH) as a marker enzyme of the cytosolic fraction, cytochrome c oxidase subunit IV (COX IV) as a marker of the membrane fraction, and c-jun as a marker of the nuclear fraction.

CYTOSOLIC EXTRACTION (PAPER II-IV)

To analyze translocation of apoptotic proteins between different compartments within the cell, cytosols were extracted by adding digitonin in a buffer consisting of 250 mM sucrose, 20 mM Hepes, 10 mM KCl, 1.5 mM $MgCl_2$, 1 mM EGTA, 1 mM EDTA, 1 mM Pefabloc, and 8 mM dithiotriol (pH 7.5) to the melanocyte cultures for 12 min on ice. This procedure permeabilizes the cholesterol-rich plasma membrane, but leaves membranes of intracellular organelles intact, as determined by analysis of LDH and the lysosomal enzyme β-N-acetylglucoseaminidase (NAG) activities, respectively (Leaback and Walker, 1961; Vanderlinde, 1985). The digitonin concentration (10-20 μg/ml) was individually titrated for each melanocyte donor. Proteins of the extracted cytosol were precipitated in trichloric acid (50%), incubated on ice for 10 min, and subsequently pelleted by centrifugation. For Western blot analysis, the pellet was resuspended in urea-lysis buffer (6 M urea, 150 mM NaCl, 1% Triton X-100, 0.1% SDS, 50 mM Tris, pH 8.0, 5 mM EDTA), Laemmli sample buffer with 5% β-mercaptoethanol and 1 M NaOH.

CASPASE ACTIVATION (PAPER II-IV)

Active caspases cleave targets with a specific cleavage site, usually a four or five amino acid sequence ending with an aspartic acid (Cohen, 1997). The preferred sequence for the execution caspases -3 and -7 is DEVD. To study their activity, melanocytes were collected in lysis buffer (10 mM Tris-HCl pH 7.5, 130 mM NaCl, 1% Triton X-100, 10 mM sodium pyrophosphate, 10 mM sodium phosphate buffer), followed by incubation with the substrate Ac-DEVD-AMC for 1 h at 37°C. Intact Ac-DEVD-AMC is non-fluorescent, but when the AMC peptide is cleaved off by an active caspase, the resulting cleavage product will become fluorescent. The fluorescence of proteolytically released AMC (7-amino-4-methylcoumarin) was analyzed in a Shimadzu RF-540 spectrofluorometer ($\lambda_{ex}380/\lambda_{em}435$, Shimadzu Kyoto, Japan). Protein concentrations were analyzed with Bio-Rad D_C Protein Assay System and caspase activity was expressed as arbitrary units/μg protein/h.

In order to analyze the activity of initiator caspase-8, cells were instead incubated with the Ac-IETD-AFC substrate, and released AFC (7-amino-4-trifluoro-methylcoumarin) was measured in a VICTOR 1420 multiple counter ($\lambda_{ex}400/\lambda_{em}505$, Wallac Turku, Finland).

SIRNA TRANSFECTION (PAPER III-IV)

In 2006, Andrew Fire and Craig Mello received the Nobel Prize for their discovery that double-stranded RNA is able to trigger suppression of gene activity (Fire et al., 1998). This process is termed RNA interference. They described a new mechanism for gene regulation, where double-stranded RNA synthesized within the cell can reduce or abolish gene activity. In addition, RNA interference has been shown to protect against RNA virus infections, mainly in invertebrate animals and in plants. Today, this has become a powerful tool to investigate the function of specific genes in a cell. When synthetic double-stranded short interference RNA (siRNA, about 21 nucleotides long) is introduced into the cell, one of the strands incorporates into an endonuclease complex, called RNA inducing silencing complex (RISC), while the other strand becomes degraded (Hammond et al., 2000) (Figure 15). The siRNA sequence in the RISC complex serves as a probe to detect complementary cellular mRNA. When the mRNA is recognized, it is cleaved and degraded, leading to post-transcriptional gene silencing in the cell.

Human melanocytes were seeded one day prior to transfection. The cells were transfected with 1 μg (12-well plate) of siRNA together with 6 μl RNAiFect Transfection Reagent, according to the manufacturer's instructions (Qiagen, Germantown, MD, USA). Optimal transfection conditions were determined by transfection with Alexa Fluor 555 labeled non-silencing siRNA, consisting of a scrambled sequence with no homology to mammalian genes. This siRNA was used in the experiments as negative control and the positive control of siRNA targeting Lamin A/C was recommended by the manufacturer. The cells were incubated at standard culture conditions and the siRNA transfection medium was replaced with fresh medium after 8 h. A significant decrease in protein level was observed 36-48 h following siRNA addition. Silencing of the specific siRNA was confirmed by Western blot analysis.

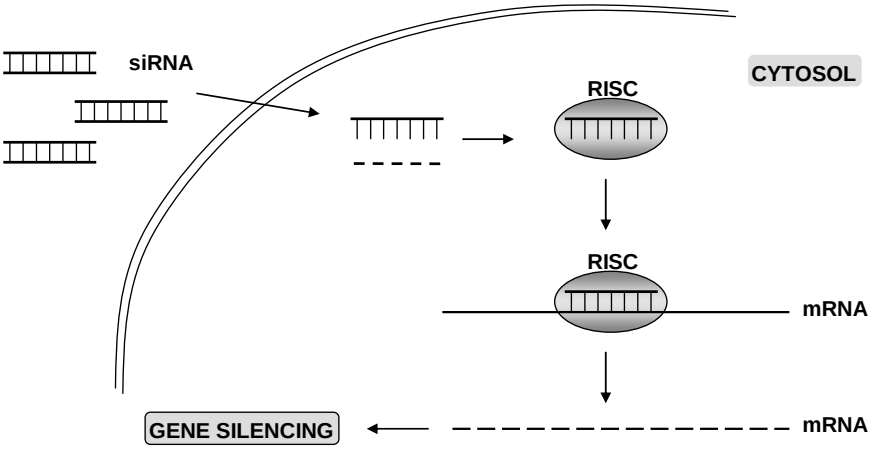

Figure 15. The principle of siRNA gene silencing.

REAL TIME POLYMERASE CHAIN REACTION (PAPER I)

In order to measure mRNA expression of Bcl-2 and Bax with real time polymerase chain reaction (PCR), RNA first needed to be extracted and converted to cDNA, in a PCR based procedure. The total RNA was extracted from the melanocytes using RNeasy Mini Kit (Qiagen, Hilden, Germany) and 400 ng total RNA was reversed transcribed to generate cDNA. RNA and random hexamers (5.7 μM) were incubated 3 min at 70°C, which allows the primers to randomly bind to the RNA, followed by 10 min at 5°C. Subsequently, reverse transcriptase (1.25 U/μl), dNTP (each 500 μM), RNase inhibitor (0.4 U/μl), $MgCl_2$ (5.5 mM), and TaqMan RT Buffer were included in the reaction, followed by incubation for 30 min at 48°C and 5 min at 95°C.

Real time PCR reactions containing cDNA (1 μl), primers (0.2 μM of each), TaqMan probe (0.4 μM), dNTP (200 μM of each), $MgCl_2$ (5 mM), AmpliTaq Gold (0.02 U/μl), and TaqMan buffer, were mixed and the amplification was carried out under the following conditions; 40 sequential cycles, each including 94 ºC for 15 sec and 60 ºC for 30 sec. The oligonucleotide probe was fluorescently labeled at the 5' end with the reporter dye FAM (6-carboxyfluorescein) and at the 3' end with the quencher dye TAMRA (6-carboxytetramethylrhodamine) (Figure 16). As long as the probe is intact, the reporter fluorescent dye is suppressed by the quencher dye. The probe anneals to the target sequence between the primers and during each PCR cycle, the probe is cleaved by the 5' → 3' exonuclease activity of Taq DNA polymerase. This results in separation of the reporter from the quencher, and the reporter dye will emit a fluorescent signal. The probe is removed and the extension of the strand can be continued. In each cycle additional probes are cleaved, leading to an exponential increase in fluorescence, which

intensity is monitored and directly related to the amount of input target DNA. The fluorescence intensity was, in this study, detected with an automated fluorometer (ABI Prism 7700 System, Applied Biosystems). A threshold was set, which determines the threshold cycle (C_T), defined as the number of cycles at which the fluorescence passes the threshold. A high amount of starting target results in an early detected fluorescence increase and a low C_T value. By using an endogenous control, quantification can be normalized for differences in the amount of total RNA added to each reaction. In our study, the Bcl-2 and Bax mRNA levels in each sample were normalized to the expression of β-actin. Control experiments using other housekeeping genes, such as GAPDH and beta-glucuronidase (GUS), were performed to confirm the stability of β-actin after UV irradiation. The quantification was done using the comparative C_T method (Leutenegger et al., 1999). The samples were run in triplicates from which a mean C_T value was calculated. The data, normalized to β-actin, was then presented as fold increase relative to corresponding non-irradiated control melanocyte sample.

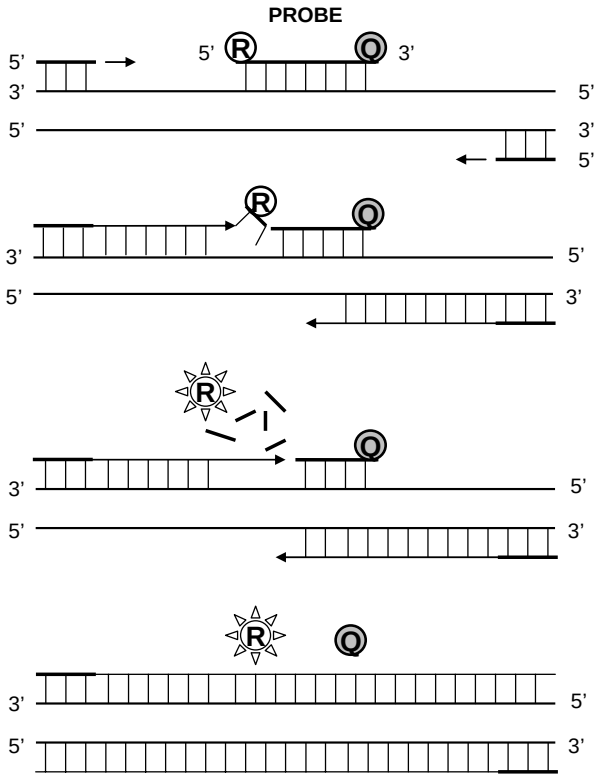

Figure 16. Principle mechanisms for mRNA quantification by using a fluorescent probe in real time PCR. R: reporter, Q: quencher

ISOLATION OF MITOCHONDRIA AND LYSOSOMES FROM RAT LIVER (PAPER III)

Purification of mitochondria from rat liver was performed as described before (Boutry and Briquet, 1982). Briefly, after homogenization, the sample was centrifuged at 1000 × g for 10 min and the mitochondria were isolated from the supernatant by centrifugation at 48 000 × g for 1 h in a sucrose gradient. Rat liver lysosomes were isolated adopting the method earlier described for purification of mouse liver lysosomes (Stoka et al., 2001). The mitochondria were disrupted with $CaCl_2$ and the integrity of lysosomes was verified by activity measurement of the lysosomal enzyme NAG (Leaback and Walker, 1961) in absence or presence of Triton X-100. All steps in the purification were carried out on ice and the procedure was only continued if more than 80% of the lysosomes were found to be intact. In lysosomal fractions, mitochondrial cross-contamination was excluded by analysis of p-iodonitrotetrazolium reductase activity and in mitochondrial fractions NAG activity was used. In addition, the purity of the lysosomal fractions obtained has been confirmed by the research group with electron microscopy (Kågedal et al., 2005).

PROTEIN INSERTION INTO LYSOSOMAL AND MITOCHONDRIAL MEMBRANES (PAPER III)

To investigate if Hsp70 was able to attach or to be inserted into lysosomal and mitochondrial membranes, 50 μg lysosomes or mitochondria were incubated with recombinant Hsp70 (0.75 μM, 2 μM) or Hsp70 diluents in Sucrose/Pipes buffer (250 mM sucrose, 20 mM Pipes, pH 7.2) for 1 h at 30ºC. The organelles were washed with Sucrose/Pipes buffer and pelleted by centrifugation at 18 000 × g for 10 min. Proteins attached, but not inserted into the membranes, were solubilized by incubation of the pellet in 0.1 M Na_2CO_3 in Sucrose/Pipes buffer on ice for 20 min, as described earlier (Antonsson et al., 2001). Following centrifugation at 100 000 × g for 45 min, the previously attached proteins were found in the supernatant. The pellet, containing membrane fraction with inserted proteins, was incubated in Sucrose/Pipes buffer supplemented with 2% CHAPS on ice for 1 h. These samples were subsequently sonicated and centrifuged at 100 000 × g for 30 min. The two samples of solubilized proteins were analyzed by Western blot of Hsp70. All steps were carried out on ice unless otherwise indicated. Sucrose/Pipes and Tris-HCl buffer served as negative controls for Hsp70. By incubating organelle fractions with GAPDH, cytochrome c, full-length Bid and caspase-8-cleaved Bid, our research group has previously demonstrated that the organelles are not generally adhesive to proteins (Kågedal et al., 2005).

IMMUNOPRECIPITATION (PAPER IV)

The immunoprecipitation method allows isolation of a protein and other proteins interacting with it, from a solution, such as a cell lysate with a mixture of proteins. An antibody against a specific antigen is added to the solution and will form an immune complex with the target protein (Figure 17). This is followed by incubation with agarose beads conjugated with protein G, where protein G will bind the Fc part of the antibodies. The complex is precipitated by centrifugation and the protein of interest are eluted and analyzed by Western blot.

Melanocytes were incubated in Chaps buffer (10 mg/ml Chaps, 10 µl/ml protease inhibitor cocktail, 150 mM NaCl, 10 mM Hepes, pH 7.4) on ice for 30 min. The lysate was pre-cleared by incubation with protein G-agarose beads at 4°C for 1 h on an orbital shaker. This procedure reduces non-specific binding of proteins to the agarose beads. The protein G-agarose beads were then removed by centrifugation (2700 × g for 5 min at 4°C). Protein concentration was analyzed by Bio-Rad D_C Protein Assay System and 25 µg total protein was diluted in Chaps buffer to a total amount of 200 µl. The proteins were subsequently incubated with Bim (2 µg) or Mcl-1 (1 µg) antibodies over night followed by incubation with protein G-agarose beads for 1 h. Both incubations were performed at 4°C on an orbital shaker. The beads were then collected by centrifugation (2700 × g for 5 min at 4°C) and washed four times in Chaps buffer. Subsequently, the beads were boiled in sample buffer (5% β-mercaptoethanol in Laemmli sample buffer) for 5 min to disrupt the bindings between proteins as well as between antibody-bead complexes and proteins. Coprecipitated proteins were visualized by Western blot. A negative control sample was handled in parallel in the same way, except from antibody addition.

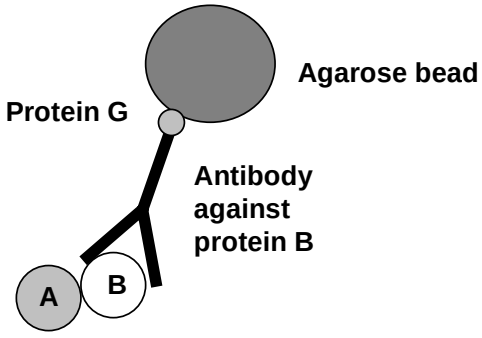

Figure 17. The immunoprecipitation complex.

MICROINJECTION (PAPER II)

To mimic translocation of cathepsins from lysosomes to cytosol following UV irradiation, microinjection of cathepsin B was performed on a stage of a Zeiss Axiovert inverted microscope (Zeiss, Gena, Germany), using a pressure injector from Eppendorf (model 5246; Eppendorf, Hamburg, Germany) and an Injectman micromanipulator (Eppendorf) (Roberg 2002). All injections contained 1 mg/ml dextran conjugated Alexa Fluor 488 (10 kDa) in PBS (pH 5.5). Freshly prepared Alexa Fluor 488 containing 0.25 mg/ml of cathepsin B, diluted in PBS (pH 5.5) was injected into the cytosol of the melanocytes (pressure 100 hPa, 1.5 seconds). In each experiment, approximately 100 cells were injected. All fluorescent cells, i.e. microinjected cells, were analyzed and the percentage of apoptotic cells was assessed based on nuclear morphology following DAPI staining.

STATISTICAL ANALYSIS (PAPER I, III, IV)

Statistical differences were evaluated by Kruskal Wallis Analysis of variance, which was followed by Mann-Whitney U-tests. A p value of <0.05 was considered significant.

ETHICAL CONSIDERATION

The Research Ethical Committee at Linköping University in Sweden has approved the use of residual skin after surgery for cell culture studies of UV effects on melanocytes and keratinocytes.

RESULTS

PAPER I

In the first paper, we investigated UVB induced apoptosis in human melanocytes *in vitro*. The mRNA and protein levels of the pro- and anti-apoptotic proteins Bax and Bcl-2 were analyzed following UVB irradiations of various wavelengths. In addition, we studied possible melanocyte-keratinocyte interplay regarding the regulation of apoptosis triggered by UVB exposure.

During standard conditions, melanocytes were observed to express high basal protein level of Bcl-2, compared to keratinocytes from the same subject. The protein expression of Bax was distinct in both melanocytes and keratinocytes and there was no noticeable difference in the protein level between the two cell types.

To clarify the significance of different wavelengths within the UVB range, melanocytes were irradiated with and without the use of a Schott WG 305 filter, with a cut-off at 305 nm. Exposure of mainly long wavelength UVB (>305 nm), did only induce a slight increase in apoptotic frequency, measured by annexin-V and propidium iodide staining of the cells. In contrast, when the shorter wavelength fraction was increased (280-305 nm), the number of apoptotic cells significantly increased. This concurred with an unaltered Bax mRNA expression, but upregulated Bcl-2 level. However, no change in protein expression of Bax and Bcl-2 were detected.

Furthermore, to study the interplay between melanocytes and keratinocytes with respect to UV induced apoptosis, a co-culture system was set up, in which the two cell types were cultured in the same media but physically separated. Measurements of the Bcl-2 and Bax mRNA and protein levels were performed in pure cultures of melanocytes and in melanocytes co-cultured with keratinocytes. Following UVB exposure (λ>280), a significantly lower rate of apoptosis was noted in melanocytes co-cultured with keratinocytes, compared to pure melanocyte cultures. This suggests keratinocytes to protect melanocytes from UVB induced apoptosis, possibly by the release of some yet unidentified substance(s). In our experiments, this rescue response concurred with a fast and significant increase in Bcl-2 mRNA level in the melanocytes.

Altogether, the shorter wavelengths in the UVB spectrum were significantly more powerful in inducing apoptosis than exposure of mainly long wavelength UVB. The results also demonstrate a pronounced melanocyte rescue effect from the keratinocytes.

PAPER II

In this study, we investigated the localization and translocation of pro- and anti-apoptotic members in the Bcl-2 protein family, following both UVA and UVB irradiation. The results indicate that the localization of the proteins within the various cell compartments is essential for life or death decision of the melanocytes. In apoptotic cells, exhibiting nuclear fragmentation, there was a shift in the staining pattern of the pro-apoptotic proteins Bax and Bid from diffused cytosolic into a punctate organelle-restricted pattern, as detected by immunocytochemistry. In the surviving cell population, the anti-apoptotic proteins Bcl-2 and Bcl-X_L were found to be translocated from the cytosol to organelles. The punctate staining pattern was similar to what was achieved when mitochondria were stained with Mitotracker Red®, suggesting the proteins to be redistributed to mitochondria or mitochondria-like structures. In parallel with this translocation, Western blot analysis showed decreased protein levels of Bcl-2, Bcl-X_L, Bax, and Bid in cytosolic fractions of both UVA and UVB exposed melanocytes.

Studies on several other cell types have demonstrated lysosomes to be involved in the apoptotic process. In this investigation, we showed lysosomal enzymes, cathepsins, to be potent apoptotic inducers in melanocytes. Following both UVA and UVB irradiation lysosomal membrane permeabilization was detected, resulting in release of cathepsins to the cytosol. In order to mimic the cathepsin release, melanocytes were microinjected with cathepsin B into the cytosol. These cells underwent apoptosis. To verify the role of various classes of cathepsins, the aspartic and cysteine cathepsin inhibitors pepstatin A and E64d, respectively were used. These inhibitors markedly decreased the number of apoptotic cells after both UVA and UVB exposure, indicating both aspartic and cysteine cathepsins to exert pro-apoptotic signaling after UV exposure. In addition, when using the cathepsin inhibitors, translocation of Bax to mitochondria was inhibited, suggesting the cathepsins to be operating upstream of the mitochondria.

To assess a possible involvement of the death receptor pathway in UVA or UVB induced apoptosis, the activation of caspase-8 was examined. Caspase-8 is known to be activated by cleavage upon death receptor stimuli. We did, however, neither detect caspase-8 cleavage fragments by using Western blot analysis, nor did we monitor activity of the enzyme in melanocytes exposed to UVA or UVB irradiation. This lead to the conclusion that the death receptor pathway is not involved in UV induced apoptosis in melanocytes.

Taken together, translocation of anti- and pro-apoptotic Bcl-2 family proteins is important in the regulation of UV induced apoptosis in melanocytes. Lysosomal membrane permeabilization occurs early in the apoptotic pathway, and results in release of cathepsins. In the cytosol, the cathepsins are potent pro-apoptotic mediators and trigger apoptosis upstream of Bax translocation and mitochondrial membrane permeabilization (Figure 18). No differences in the apoptotic response between UVA and UVB exposure were observed.

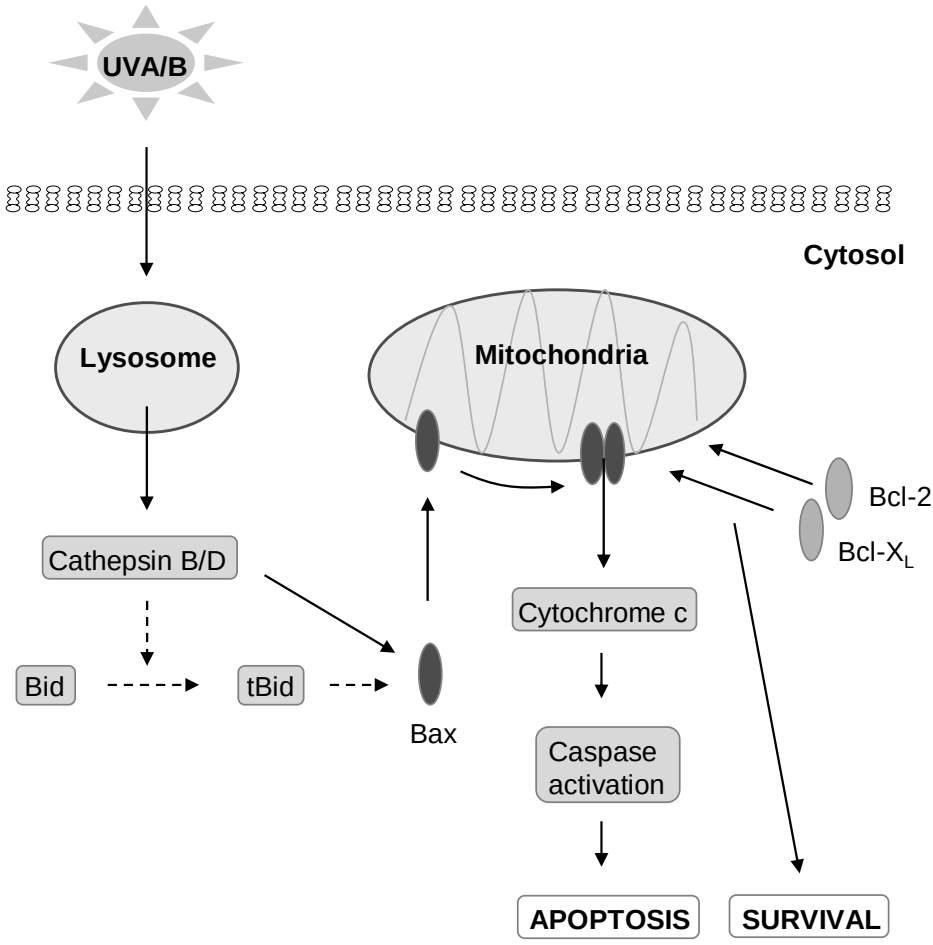

Figure 18. Schematic model of pro- and anti-apoptotic events in human melanocytes exposed to UVA or UVB irradiation, based on the results from paper II (arrows). Both UVA and UVB irradiation induce lysosomal membrane permeabilization, leading to cathepsin release. Recent studies have suggested cathepsins to cleave and activate Bid, which in turn activates Bax (dashed arrows). Bax translocates to the mitochondria and triggers cytochrome c release, leading to caspase activation and apoptosis. In contrast, translocation of Bcl-2 and Bcl-X_L to the mitochondria inhibits apoptosis.

PAPER III

In this study, we investigated the role and apoptotic function of stress-induced Hsp70 (also called Hsp72) in UVB induced apoptosis. Both exposure to heat and UVB irradiation induced Hsp70 protein expression in human melanocytes. Heat treated cells displayed a prominent resistance to apoptosis induced by UVB exposure. Transfection with Hsp70 siRNA, that silenced Hsp70 expression, abolished the protective effect observed by the heat pre-exposure. This suggests Hsp70 to have an effective anti-apoptotic function in melanocytes.

After heat and UVB exposure, immunocytochemistry analysis of Hsp70 displayed a punctate organelle-restricted staining pattern. To further investigate the localization of Hsp70, the melanocytes were double-stained for Hsp70 and the lysosomal specific protein Lamp-2 or exposed to the mitochondria visualizing dye Mitotracker® Red before Hsp70 immunostaining. In pre-heated UVB exposed melanocytes, we found Hsp70 to co-localize with both lysosomes and mitochondria in the surviving cell population. Furthermore, incubation experiments with recombinant Hsp70 and purified lysosomes or mitochondria, respectively, clearly demonstrated Hsp70 to be able to bind to membranes of both these organelles. In paper II, we reported an increased lysosomal and mitochondrial membrane permeabilization to occur during apoptosis. This was accompanied with release of pro-apoptotic cathepsins and cytochrome c. When melanocytes were pre-exposed to heat followed by UVB irradiation, we found a reduced level of cathepsin D and cytochrome c in the cytosolic fraction, compared to cells only exposed to UVB. Furthermore, the Bax translocation from a diffuse to a punctate immunostaining pattern, observed in apoptotic cells, was inhibited by heat pre-treatment. This effect was abolished by Hsp70 siRNA transfection, demonstrating Hsp70 to be responsible for the reduced Bax redistribution. In addition, UVB irradiation induced cleavage of Bid, which was reduced in melanocytes pre-treated with heat.

This investigation demonstrates that Hsp70 translocates to lysosomes and mitochondria in response to UVB irradiation (Figure 19). At the membranes, the protein counteracts the membrane permeabilization of the organelles and thereby prevents cathepsins and cytochrome c to be released to the cytosol, resulting in an effective protection of the melanocytes from UVB induced apoptosis.

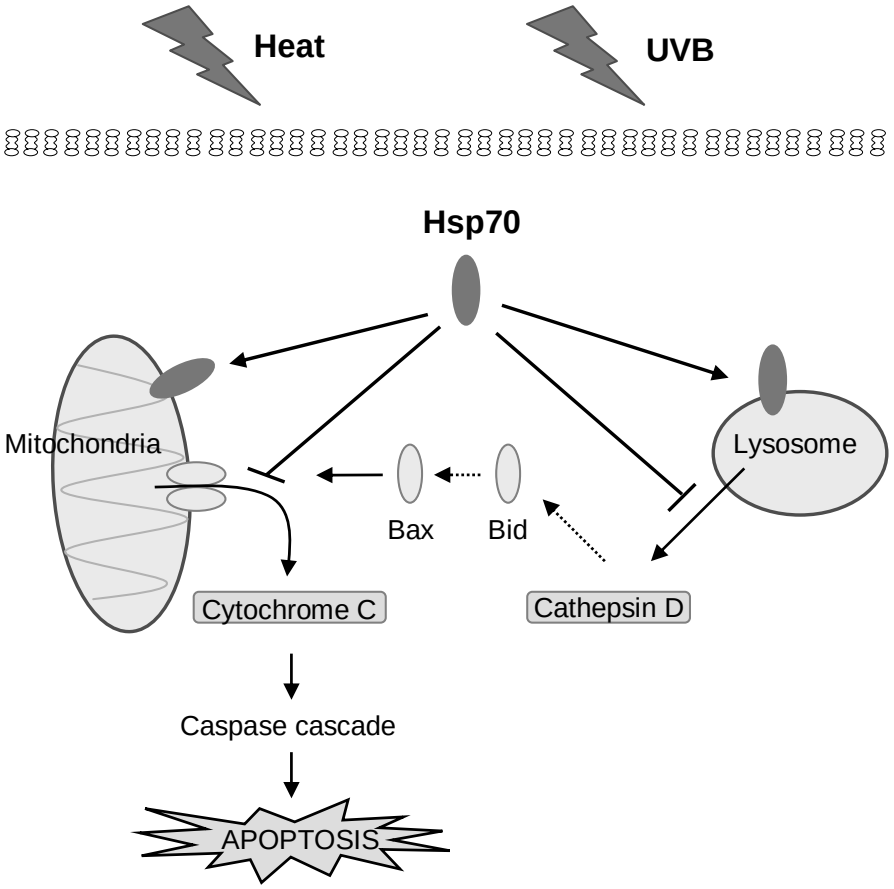

Figure 19. Proposed model for the anti-apoptotic action of Hsp70 following heat and UVB exposure in melanocytes, based on data presented in paper III (arrows). Both UVB irradiation and heat induce Hsp70 expression. Following apoptosis triggering, Hsp70 localizes to both lysosomal and mitochondrial membranes. At the membrane, Hsp70 counteracts lysosomal and mitochondrial membrane permeabilization, preventing cathepsins and cytochrome c to be released. If released, cathepsins might be able to cleave and activate Bid, which in turn mediates Bax activation (dashed arrows). Bax translocates to the mitochondria and induce membrane permeabilization with release of pro-apoptotic proteins from the intermembrane space, such as cytochrome c, which through activation of the caspase cascade finally results in apoptosis.

PAPER IV

In this investigation, we further examined the regulation of UV induced apoptosis in human melanocytes. Here, we focused on the function of the JNK protein. Activation of the protein, detected as phosphorylation by Western blot analysis, was observed following UVB irradiation. Transfection of the melanocytes with JNK siRNA significantly decreased the frequency of apoptosis, as well as caspase-3 activity after UVB exposure, which demonstrates JNK to exert a pro-apoptotic function in UV induced apoptosis. As demonstrated in paper II and III, cathepsins are released early in the apoptotic process, upstream of the mitochondria. With immunocytochemistry, we showed a change in cathepsin B staining pattern, from punctated lysosome-localized to a diffused cytosolic localization. This shift was repressed when cells were transfected with JNK siRNA. Furthermore, a reduced lysosomal permeabilization with decreased cathepsin D release to the cytosol was demonstrated when JNK was silenced by Western blot analysis. Altogether, these results demonstrate JNK to exert its activity upstream of the lysosomes and cathepsin release. After UVB irradiation, melanocytes, in which the JNK protein expression was inhibited with JNK siRNA, showed a marked lower amount of Bax translocation than non-transfected cells. These results suggest JNK to be involved in the regulation of Bax redistribution and mitochondrial pathway of apoptosis.

In addition, JNK was found to regulate the phosphorylation of the BH3-only protein Bim. Immunoprecipitation and immunocytochemistry analyses revealed that Bim, under normal conditions, co-localizes and interacts with the Mcl-1 protein. Mcl-1 was, by using siRNA transfection, demonstrated to exert an anti-apoptotic function. The siRNA experiments further suggested Mcl-1 to be operating upstream of the mitochondria, since Mcl-1 silencing inhibited Bax translocation. In response to UVB irradiation, the Mcl-1 protein expression significantly decreased, suggesting Bim to be released and free to induce apoptosis.

In summary, JNK is activated by UVB irradiation and is able to induce apoptosis in melanocytes in at least two ways, i.e. by inducing lysosomal release of cathepsins to the cytosol, which in turn activates Bax and mitochondrial pathway of apoptosis, and through phosphorylation of Bim, which normally is sequestered by the anti-apoptotic Mcl-1, but becomes released and free to trigger apoptosis, following a UV dependent reduction of the Mcl-1 protein level (Figure 20).

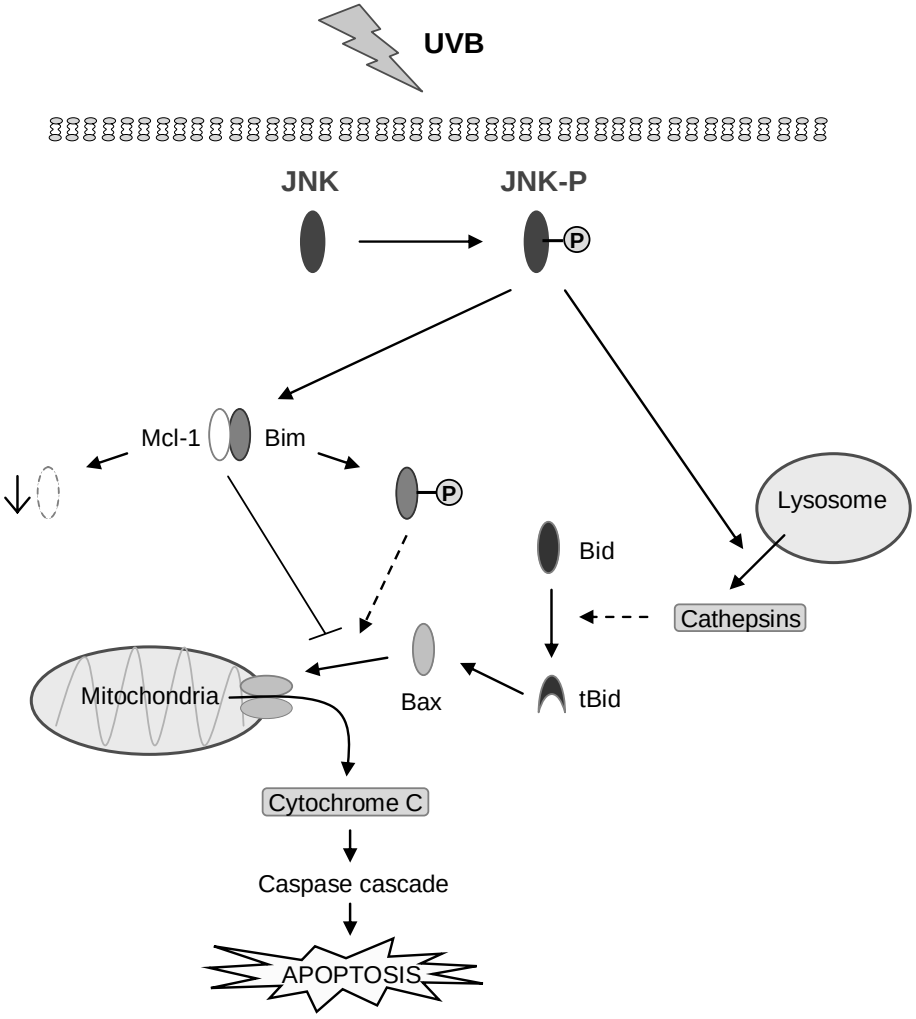

Figure 20. Proposed model of the mechanisms by which JNK induces apoptosis in melanocytes, based on the observations in paper IV (arrows). JNK triggers apoptosis upstream lysosomal membrane permeabilization, inducing release of pro-apoptotic cathepsins to the cytosol. The cathepsins might, by activation of Bid and Bax, cause cytochrome c release from mitochondria, which leads to caspase activation and apoptosis. In addition, JNK might induce apoptotic cell death by regulating phosphorylation of the BH3-only protein Bim. Bim is under normal conditions sequestered by the anti-apoptotic Mcl-1. In response to UVB irradiation, Mcl-1 is downregulated, resulting in release of activated Bim, which might provoke activation of Bax, mitochondrial membrane permeabilization and finally apoptosis.

DISCUSSION

The regulation of apoptosis in human melanocytes is poorly understood. In the studies included in this thesis, we have clarified several steps in the apoptotic pathway induced by UV irradiation.

APOPTOSIS RESISTANCE IN MELANOCYTES

The major external risk factor for malignant melanoma is UV exposure. UVB irradiation is able to direct generate DNA photoproducts, while UVA induced DNA damage mainly appears to be caused through generation of reactive oxygen species. When DNA damages are not repaired and accumulate, the epidermal melanocytes might become potential tumor precursors. In addition, the ability of UV irradiation to stimulate melanocytic mitosis (Quevedo et al., 1965; Stierner et al., 1989) might accelerate the carcinogenic effect of UV. Following the increased rate of mitosis, the number of cells slowly returns to its original number (Szabó, 1967a; Rosdahl, 1979). Apoptosis might thus be essential in the regulation of the melanocyte homeostasis. In addition, apoptosis has an important role by eliminating potentially pre-cancerous cells and prevent a clonal expansion of damaged cells. However, apoptotic melanocytes are rarely found in the skin following UV exposure, while apoptotic keratinocytes, i.e. sunburn cells, are frequently detected (Young, 1987; Gilchrest et al., 1999). Bowen *et al.* reported normal melanocytes to be markedly resistant to UV induced apoptosis, as compared to keratinocytes (Bowen et al., 2003). In paper I, we reported very high basal level of Bcl-2 protein expression in melanocytes in comparison to keratinocytes, which is in agreement with other studies (Klein-Parker et al., 1994; Olie et al., 2002) and might be one factor that provide the apoptosis-resistance characteristic of the melanocytes. Downregulation of Bcl-2 and Bcl-X_L expression with antisense oligonucleotides induced apoptosis in melanoma cells (Olie et al., 2002). Bowen *et al.* found the melanocytes to express relatively high levels of multiple anti-apoptotic proteins, including Bcl-2, Bcl-X_L, Mcl-1, and IAP proteins, whereas keratinocytes in contrast were relatively deficient in these proteins (Bowen et al., 2003).

SHORT WAVELENGTH UVB EFFECTIVELY INDUCES APOPTOSIS

In paper I, we examined the effectiveness in causing melanocyte apoptosis *in vitro* of two different spectra within the UVB range, achieved by the presence or absence of a cut off filter (50% absorption below 305 nm) (λ>280 nm/ λ>305 nm). The main output of the UVB source was 305-320 nm. Following exposure to longer UVB wavelengths, there was only a minor rate of apoptosis observed in the melanocytes. However, by increasing the

fraction between 280 and 305 nm, we obtained a significant increase in apoptosis. This shorter UVB wavelength spectrum has been described to be more effective than longer wavelengths in causing cells death in HeLa×skin fibroblast hybrid cells (Bettega et al., 2001). Most UVB sunscreens are a mix of different UV absorbing ingredients, each absorbing in narrow bands. When exposed to UV irradiation, a number of these compounds have been reported to become unstable, leading to wavelength-specific fenestrations and insufficient photoprotection (Tarras-Wahlberg et al., 1999). More knowledge about specific differences in melanocytic response to narrow spectral bands within UV might shed new light on the genesis of melanoma. A better understanding of how different spectra within the UV range affect melanocytes is also important in order to develop new more effective sunscreens.

KERATINOCYTES PROTECT MELANOCYTES FROM APOPTOSIS

The interplay between melanocytes and keratinocytes was investigated by using a co-culture system, which allowed the melanocytes to be studied separately from the keratinocytes. Melanocytes co-cultured with keratinocytes showed significant lower frequency of apoptosis in response to UVB irradiation, when compared to pure melanocyte cultures. These results suggest that the keratinocytes protect melanocytes from UV induced apoptosis, not by direct contact, but by release of substance(s) from the keratinocytes. Human keratinocytes at the basal layer of epidermis have been described to synthesize and secrete nerve growth factor (NGF) (Tron et al., 1990; Di Marco et al., 1991; Yaar and Gilchrest, 1991). NGF has also been found to be present in melanocytes *in vivo* (Stefanato et al., 2003), implying either that melanocytes are capable to synthesize NGF as well or that NGF might be secreted to melanocytes from neighboring keratinocytes. Zhai *et al.* showed pure melanocyte cultures supplemented with NGF to be protected from UV induced apoptosis (Zhai et al., 1996). Furthermore, NGF was shown to upregulate the Bcl-2 level in the UV irradiated melanocytes. In addition, UVB irradiation caused a decrease in Bcl-2 and Bcl-X_L expression in keratinocytes, but not in NGF overexpressed keratinocytes (Marconi et al., 1999). Our findings showed a potent increased in Bcl-2 mRNA expression in melanocytes co-cultured with keratinocytes after UVB exposure. Thus, we suggest NGF to be one possible candidate substance to be secreted by keratinocytes and to have an apoptosis rescue effect on surrounding melanocytes.

PROTEIN TRANSLOCATION REGULATES APOPTOSIS

In order to study the molecular mechanisms in the apoptotic process in detail, a 30-40 % frequency of apoptosis was considered to be necessary (paper II-IV). As an indication of the apoptosis resistant nature of the melanocytes, the UV doses required to reach this goal were well above physiological levels. In addition, melanocyte growth factors in this experimental system might have reduced the apoptotic response to UV and supported survival. This has to be considered when evaluating the results from these *in vitro* studies of UV induced pathways of apoptosis in human melanocytes.

The balance between Bax and Bcl-2 has been suggested to decide the fate of the cell (Oltvai et al., 1993; Raisova et al., 2001). UVB irradiation induced downregulation of Bcl-2 and maintained levels of Bax in human skin (Isoherranen et al., 1999). Kim *et al.* instead showed that UVB exposure caused no change in Bcl-2 expression, but did upregulate Bax protein level in melanocytes (Kim et al., 2000). However, in our experimental system both Bcl-2 and Bax protein level remained unchanged after UVB exposure, despite an increased apoptotic frequency (paper I). This encouraged us in paper II to investigate the intracellular location of the protein. In control melanocytes, Bax was situated in the cytosol, but during apoptosis, induced by UV exposure, the protein translocated to mitochondria or mitochondria-like organelles, observed as a punctate immunostaining pattern. In apoptotic cells, Bid was observed to be translocated in a similar pattern as Bax in response to UV irradiation. The significance of translocation of proteins seems to be true for other Bcl-2 family proteins as well. Accordingly, in the surviving cell population, Bcl-2 and Bcl-X_L were observed to be redistributed to the mitochondria. We showed these Bcl-2 family proteins to exert their apoptotic regulatory actions by translocation between different compartments within the cell, rather than changing the protein balance between anti- and pro-apoptotic proteins in melanocytes. In accordance, other groups have reported translocation of Bax, Bid, and Bcl-X_L from the cytosol to mitochondria following many different apoptosis inducers, such as γ-irradiation, staurosporine, serum deprivation, and TNF-α in thymocytes, kidney epithelial cells, fibroblasts, hepatocytes, and HeLa cells (Hsu et al., 1997; Wolter et al., 1997; Desagher et al., 1999; Gross et al., 1999). However, all Bcl-2 family proteins do not regulate their actions by translocation. The anti-apoptotic function of Mcl-1 was, for instance, demonstrated to be regulated by UV induced elimination of the protein (see below). Furthermore, it is generally thought that Bcl-2 is stationary and attached to membranes, in particularly to the mitochondria membrane (Hockenbery et al., 1990; Hsu et al., 1997). We have for the first time demonstrated Bcl-2 to have a cytosolic location in melanocytes. The cytosolic distribution of Bcl-2 and the translocation of the protein upon UV irradiation were confirmed with both immunocytochemistry experiments and Western blot analysis of cytosolic fractions. The diversity between different studies, regarding the location of Bcl-2, can not be explained but might be due to differences between cell types.

PRO-APOPTOTIC SIGNALING BY LYSOSOMAL CATHEPSINS

The lysosomes have been reported to be involved in the apoptotic process (Deiss et al., 1996; Brunk et al., 1997; Ishisaka et al., 1998; Roberg et al., 1999; Guicciardi et al., 2000; Johansson et al., 2003). To our knowledge, no studies before have investigated the role of this organelle in the regulation of cell death in melanocytes. We clearly demonstrated the lysosomal cathepsins to be essential pro-apoptotic mediators in UV induced apoptosis (paper II-IV). Shortly after UV exposure, cathepsins were detected in the cytosol with both immunocytochemistry and Western blot analysis on digitonin-extracted cytosolic fractions. Increased lysosomal membrane permeabilization, following a wide range of apoptotic stimuli, has been reported in a variety of cell types, such as fibroblasts, T lymphocytes, hepatocytes, and myeloid leukemic cells (Roberg et al., 1999; Guicciardi et al., 2000; Foghsgaard et al., 2001; Werneburg et al., 2002; Yuan et al., 2002; Bidère et al., 2003). In melanocytes, we found the aspartic cathepsin D and the cysteine cathepsins B and L to be able to trigger apoptosis with cytochrome c release from the mitochondria and subsequent caspase-3 activation. The groups of lysosomal proteases involved in apoptosis might, however, be cell type- and/or stimulus-specific, since in fibroblasts and T lymphocytes exposed to staurosporine, only cathepsin D is pro-apoptotic (Bidère et al., 2003; Johansson et al., 2003). In contrast, cathepsin B is involved in TNF-α and p53 induced apoptosis in hepatocytes, and myeloid leukemic cells (Guicciardi et al., 2000; Yuan et al., 2002). We further reported the aspartic cathepsin D inhibitor pepstatin A and the cysteine cathepsin inhibitor E64d to markedly reduce the amount of Bax translocation from cytosol to mitochondria following UV irradiation, indicating the enzymes to exert their effect upstream of Bax activation. In accordance, it has been reported that cathepsin D silencing using siRNA in human T lymphocytes resulted in suppressed Bax activation (Bidère et al., 2003). The conclusion that lysosomal membrane permeabilization precedes cytochrome c release has been described by several groups (Roberg et al., 1999; Guicciardi et al., 2000; Yuan et al., 2002).

Both UVA and UVB irradiation were found to induce apoptosis with lysosomal and mitochondrial membrane permeabilization, cytochrome c release and caspase-3 activation. We did not observe any differences between these two wavelength spectra in the regulation of apoptosis.

BID ACTIVATION BY CATHEPSINS

In keratinocytes, UV irradiation has been shown to induce apoptosis directly through activation of death receptors (Aragane et al., 1998). Caspase-8 is strongly associated with the death receptor pathway of apoptosis and is established to cleave and activate Bid (Li et al., 1998; Luo et al., 1998). However, in melanocytes we excluded an initial involvement of the death receptor pathway, as neither proteolytic processing of

procaspase-8 nor caspase-8 activity were detected after UV exposure (paper II). Despite the lack of caspase-8 activity, Bid was relocalized to mitochondria after UVB exposure and Bid cleavage fragment was found (paper II, III). Recently, cathepsins have been reported to be alternatively cleavers of Bid (Stoka et al., 2001; Cirman et al., 2004; Heinrich et al., 2004), suggesting Bid to be a possible link between the lysosome and the mitochondrial apoptotic pathway by activating Bax.

JNK ACTS PRO-APOPTOTIC UPSTREAM OF THE LYSOSOME

The initial signaling pathways that might trigger the lysosomal membrane permeabilization are still largely unknown. In paper IV, we reported the JNK protein to effectively trigger UVB induced cell death in melanocytes and that JNK operates upstream of cathepsin release. The role of JNK is debated. The protein has been suggested to have both anti- and pro-apoptotic functions. JNK might, through phosphorylation and inactivation of Bad, suppress apoptosis induced by IL-3 withdrawal (Yu 2004). Moreover, JNK2 silencing of prostate carcinoma cells resulted in apoptosis (Potapova et al., 2002). In contrast, following UV irradiation JNK has been suggested to induce cytochrome c release and to trigger apoptosis in murine embryonic fibroblasts (Tournier et al., 2000). The study further showed *Jnk1*$^{-/-}$ *Jnk2*$^{-/-}$ fibroblasts to be almost completely resistant to UV induced apoptosis. In melanocytes, we demonstrated JNK to be pro-apoptotic and that JNK inhibition by siRNA transfection stabilized the lysosomes and prevented release of cathepsins to the cytosol. JNK dependent apoptosis has been shown to require Bax activation (Lei and Davis, 2003; Putcha et al., 2003; Tsuruta et al., 2004; Papadakis et al., 2006) and in addition JNK has been demonstrated to control Bak oligomer formation (Ihrlund et al., 2006), suggesting the protein to regulate the mitochondrial pathway of apoptosis. In accordance, our findings showed JNK siRNA transfection to inhibit Bax translocation to the mitochondria.

UV INDUCED REGULATION OF BIM AND MCL-1

In paper IV, we found Bim to be phosphorylated in a JNK dependent manner, which suggests an additional pathway of JNK to initiate apoptosis. Normally, Bim is sequestered to the dynein motor complex in the microtubule cytoskeleton (Puthalakath et al., 1999), but exposure to UV irradiation has been reported to detach the protein by phosphorylation (Lei et al., 2002; Putcha et al., 2003). We found Bim to interact with the Mcl-1 protein, but in response to UVB irradiation, we observed a distinct reduction of Mcl-1 expression. Nijhawan *et al.* reported the synthesis of Mcl-1 to be blocked after UV irradiation in HeLa cells and that the existing Mcl-1 rapidly was degraded by the proteasome (Nijhawan et al., 2003). Other studies demonstrate that caspase or granzyme B mediated cleavage might contribute to the Mcl-1 elimination in HeLa, Jurkat T

leukemic, and colon cancer Hct116 cells (Clohessy et al., 2004; Han et al., 2004; Weng et al., 2005; Han et al., 2006). It has further been suggested that the caspase-3 generated fragments of Mcl-1 lose their anti-apoptotic potential and instead become pro-apoptotic (Michels et al., 2004; Weng et al., 2005). We did, however, not detect any Mcl-1 cleavage fragments in the melanocytes following UVB irradiation.

Mcl-1 antisense therapy sensitized human melanoma to chemotherapy in xeno-transplantation model in mice (Thallinger et al., 2003). The combination of Mcl-1 antisense oligonucleotides and the melanoma therapy agent dacarbazine resulted in enhanced tumor cell apoptosis and reduced tumor weight. Except from interacting with Bim, Mcl-1 also, through direct binding of tBid, counteracts the pro-apoptotic potential of Bid and thereby inhibit the activation of Bax and Bak during TRAIL and TNF-α induced apoptosis (Clohessy et al., 2006). In addition, Mcl-1 has been demonstrated to coimmunoprecipitate with Bak in HeLa cells (Cuconati et al., 2003). Upon apoptosis, induced by adenovirus infection, there was a loss of the Mcl-1-Bak complex, and Bak was shown to bind to Bax. This represents an alternative model for the pro-survival function of Mcl-1, where Mcl-1 in healthy cells sequesters Bak and keeps it in an inactive state. The loss of Mcl-1-Bak complex might be caused by Noxa dependent displacement from Mcl-1 (Gélinas and White, 2005). Moreover, we found Mcl-1 to have an anti-apoptotic function in melanocytes by preventing Bax translocation. Mcl-1 depletion after UV exposure might eliminate the anti-apoptotic effect and result in release of phosphorylated Bim.

BH3-only proteins seem to be able to induce apoptosis by several different mechanisms. Some BH3-only proteins (Bid and Bim) can directly activate Bax and Bak, which results in cytochrome c release (Letai et al., 2002; Kuwana et al., 2005). Other BH3-only proteins (e.g. Bad, Bik, and Noxa) are not capable of activating Bax directly but instead bind anti-apoptotic Bcl-2-like proteins, which results in displacement of activator BH3-only proteins from the pro-survival proteins. Free activators then induce Bax dependent cell death. A recent study suggests an alternative indirect model, where the BH3-only proteins trigger apoptosis by binding anti-apoptotic proteins that sequester Bax and Bak (Willis et al., 2007).

HSP70 EFFECTIVELY PREVENTS UVB INDUCED APOPTOSIS

In paper III, we showed Hsp70 to efficiently rescue melanocytes from UVB induced apoptosis. Hsp70 has been reported to inhibit apoptosis following different stress stimuli, including heat shock, TNF-α, anti-cancer drugs, oxidative stress, and nitric oxide (Jäättelä et al., 1992; Simon et al., 1995; Trautinger et al., 1995; Bellmann et al., 1996; Polla et al., 1996; Samali and Cotter, 1996; Mosser et al., 2000). Microinjection of fibroblasts with Hsp70 antibodies rendered the cells sensitive to heat shock, resulting in cell death, while fibroblasts injected with control antibodies survived (Riabowol et al., 1988). The apoptosis-regulatory mechanism for Hsp70 is not fully understood, but there might be several different ways by which the protein is able to block apoptosis. We demonstrated that Hsp70 prevents apoptosis by translocation to lysosomal and mitochondrial membranes following UVB irradiation. At the membranes, Hsp70 then prevents permeabilization and release of cathepsins and cytochrome c to the cytosol. An additional mechanism for the anti-apoptotic action of Hsp70 is that the protein might inhibit stress induced apoptosis by inhibiting activation of JNK (Gabai et al., 1997; Mosser et al., 1997). This remains to be studied in melanocytes. Furthermore, Gabai *et al.* showed Hsp70 to be able to block Bid activation and cytochrome c release from mitochondria via inhibition of JNK (Gabai et al., 2002). In accordance, we observed in paper III heat pre-treated melanocytes to display suppressed Bid cleavage following UVB irradiation. Our findings from paper IV propose that the JNK-dependent Bid cleavage might be accomplished by JNK induced release of cathepsins, which has been shown to be able to cleave and activate Bid (Stoka et al., 2001; Cirman et al., 2004; Heinrich et al., 2004). We further demonstrated inhibited Bax translocation in melanocytes transfected with Hsp70 siRNA, suggesting Hsp70 to operate upstream of Bax activation. Gotoh and colleagues recently showed Hsp70 to interact with Bax (Gotoh et al., 2004). Stankiewicz *et al.* reported Hsp70 to inhibit oligomerization and membrane insertion of Bax, although no direct interaction between the two proteins was found (Stankiewicz et al., 2005). Notable, Hsp70 has also been suggested to act at a level downstream of mitochondrial membrane permeabilization (Jäättelä et al., 1998). In addition, the protein might by binding Apaf-1 prevent its oligomerization and thereby restrain the recruitment of procaspase-9 to the apoptosome (Beere et al., 2000; Saleh et al., 2000).

CONCLUSIONS

GENERAL CONCLUSIONS

In this thesis, we have mapped several steps in the signaling pathways of UV induced apoptosis in human melanocytes *in vitro*. UV irradiation activates the mitochondrial pathway of apoptosis, leading to cytochrome c release, caspase activation, and nuclear fragmentation. The findings illustrate that permeabilization of mitochondrial and lysosomal membranes and release of their pro-apoptotic constituents are important events in UV induced apoptosis in these cells. The process is regulated by a complex network of pro- and anti-apoptotic proteins, exerting their effects through intracellular translocation and alteration of protein expression.

SPECIFIC CONCLUSIONS

- Melanocytes express high protein level of pro-survival Bcl-2, as compared to keratinocytes. The shorter wavelengths of the UVB spectrum are significantly more powerful in inducing apoptosis than the longer UVB wavelengths.

- When co-cultured, keratinocytes release factors to the culture medium that protect melanocytes from UV induced apoptosis

- The localization and translocation of Bcl-2 family proteins are important in the regulation of UVA and UVB induced cell death. In apoptotic melanocytes, the pro-apoptotic proteins Bax and Bid were found to be redistributed to the mitochondria, while in the surviving population the anti-apoptotic Bcl-2 and Bcl-X_L were localized at the mitochondrial level.

- UV irradiation induces lysosomal membrane permeabilization with release of cathepsins B and D from the lysosomes to the cytosol. The cathepsins act as potent pro-apoptotic mediators upstream of Bax activation and mitochondrial membrane permeabilization.

- Upregulation of stress-induced Hsp70 effectively inhibits apoptosis in melanocytes following UVB irradiation. Hsp70 prevents release of cathepsins from lysosomes and cytochrome c from mitochondria by attaching to their respective membranes.

- JNK is activated by UVB irradiation in melanocytes and exerts its effect upstream of lysosomal membrane permeabilization. In addition, JNK induces apoptosis through phosphorylation of pro-apoptotic Bim, which becomes released from anti-apoptotic Mcl-1, by UV induced Mcl-1 degradation.

SIGNIFICANCE OF THE STUDY AND FUTURE PERSPECTIVES

Melanocytes are generally considered relatively resistant to UV induced apoptosis. Avoidance of apoptosis following UV exposure might entail the survival of potential melanoma precursor cells and risk for malignant melanoma development. Improved knowledge of apoptotic responses in UV exposed melanocytes is essential to understand and prevent the formation of pigment nevi and melanoma and might give opportunity to develop more effective sunscreens. Identification of apoptotic key proteins involved, might also lead to findings of new prognostic markers for malignant melanoma. Furthermore, this type of skin cancer is often reported to poorly respond to cytotoxic drugs. To map the complex mechanisms in the regulation of apoptosis is of high importance to find new specific apoptosis inducing targets for melanoma treatment.

We demonstrate Hsp70 to exert efficient anti-apoptotic properties in melanocytes *in vitro*. In the next project, we intend to investigate the role of Hsp70 induction in UV exposed human skin *in vivo*. Hsp70 protein has been reported to be upregulated in human skin following both heat and UV exposure (Muramatsu et al., 1992; Wilson et al., 2000), but a specific study of such effects in melanocytes *in vivo* has not been performed. The heat production in combination with UV damage from the sun has not been considered in relation to melanoma development. When sunbathing, the heat production in the skin might induce Hsp70 expression, which in turn might result in survival of DNA damaged melanocytes. This issue would be of relevance even in the clinical setting, where UV irradiation constitutes an important therapeutic tool for various inflammatory skin diseases. Consequently, repeated UV treatments have been associated with an increased risk for development of cutaneous squamous cell carcinoma and basal cell carcinoma (Stern and Lange, 1988; Bruynzeel et al., 1991; Lindelöf et al., 1999; Pasker-de Jong et al., 1999). Although less frequently reported, some studies have also described an increased incidence of malignant melanoma among psoriasis patients who have received phototherapy (Stern et al., 1997; Wolf et al., 1998). Furthermore, many tumors display elevated levels of the Hsp70 protein, which has been shown to correlate with enhanced malignancy (Garrido et al., 2003; Aghdassi et al., 2007). Interestingly, a Hsp70 neutralizing peptide, ADD70, delayed tumor growth and reduced the metastatic potential of mouse melanoma *in vivo* (Schmitt et al., 2006). The study further demonstrated the peptide to make the cells more sensitive to the cytotoxic drug cisplatin. This suggests Hsp70-targeting as a strategy for cancer therapy. More knowledge regarding the role of Hsp70 in melanocytes and melanoma therefore would be of importance.

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