1. INTRODUCTION

1.1. Malignant melanoma

1.1.1 Epidemiology

Skin cancer is the most common type of cancer and accounts for half of all new cancers in Western populations. It occurs often in people with light complexion who had a high exposure to sunlight. Three types of cancer such as basal cell carcinomas, squamous cell carcinoma and malignant melanoma (MM) are often diagnosed cases in dermatological practice. Each of these three cancers begins in a different type of cell within the skin, and each cancer is named for the type of cell in which it occurs. The other skin cancers account for less than 1% of diagnosed cases and include Merkel cell carcinoma, dermatofibrosarcoma protuberans, Paget’s disease and cutaneous T-cell lymphoma.

Malignant melanoma is the most aggressive skin cancer, which develops through malignant transformation of melanocytes. The number of MM cases worldwide has increased faster than any other cancer in recent decades. In Australia, MM is the fourth most common cancer among males (after prostate, bowel and lung cancers) and the third among females (after breast and bowel cancers) and accounts for about 50 new cases per 100,000 population each year (Diepgen and Mahler 2002). In the U.S.A., the incidence of malignant melanoma has been steadily increasing for the past few decades. For instance, in 1935 the lifetime risk of developing MM was 1 in 1,500 individuals, while in 2002, the risk was 1 in 68 individuals (Rigel 2002). In Europe, MM is the 17th most commonly diagnosed cancer in males and eighth in females. Approximately 26,100 males and 33,300 females were diagnosed with MM in Europe in the year 2000 (de Vries and Coebergh 2004). The highest incidence rates have been reported in Scandinavia (about 15 per 100,000 inhabitants per year) and the lowest in the Mediterranean countries (about five to seven cases per 100,000 inhabitants per year) (Garbe and Blum 2001). The increased melanoma incidence is likely due to changes of lifestyle in terms of extreme exposure to sunlight (Brochez and Naeyaert 2000).

High mortality rates due to tumor progression (metastasis) are the major problem of MM. In parallel, mortality has been stably increasing throughout the world in recent decades (Diepgen and Mahler 2002). Mortality from MM increased both in young adults (20–44 years) and in middle-aged populations (45–64 years) in most European countries, North America, Australia and New Zealand, with a rate of increase of about 2–4% annually (Bosetti et al., 2004). Despite rising incidence of MM, a marked improvement in the 5-year survival rate, from approximately 40% in the 1940s to over 90% by now has been reported (Lens and Dawes...
The improvement in survival can be attributed to the earlier detection of melanoma. Active public education campaigns aimed at encouraging earlier detection of melanoma led to the diagnosis of thinner lesions which have a better prognosis (Lens and Dawes 2004).

1.1.2 Melanoma progression

The development of melanoma is multifactorial and appears to be related to multiple risk factors, including fair complexion (skin types I and II), excessive childhood sun exposure and blistering childhood sunburns, an increased number of common and dysplastic moles, a family history of melanoma, the presence of a changing mole or evolving lesion on the skin, and older age.

The sequence of events in which normal melanocytes undergo transformation into malignant melanoma ("melanomagenesis") is poorly understood. A multistep process of progressive genetic mutations is probably involve alteration of cell proliferation, differentiation, and death and may increase susceptibility to the carcinogenic effects of ultraviolet radiation. Melanoma development and progression pass through several distinct stages. Primary MM may develop from precursor melanocytic nevi (common, congenital, and atypical/dysplastic types), which is the first step, although more than 60% of cases are believed to arise de novo (i.e., not from a preexisting pigmented lesion). Radial growth phase (RGP) of primary melanoma is the next step of MM progression. The cells in this phase are locally invasive but they lack metastatic capacity. In the phase of radial growth, malignant cells can progress to the vertical growth phase (VGP) of primary lesions. In this step, melanoma cells infiltrate and invade the dermis as a large cluster of cells and exhibit metastatic potential. Metastasis to distant organs followed by overgrowth of tumor cells in affected sites is the last step of MM progression (Clark 1991).

1.1.3 Clinical characteristic of early melanoma and classification

Clinically, malignant melanoma can be diagnosed by the typical ABCD criteria:

A) Asymmetry – melanoma lesions cannot be easily divided into two halves with one half looking like the other (Fig. 1a)

B) Border irregularity - the borders of most early melanomas are irregularly shaped (Fig 1b)

C) Color variability - most early melanomas have differences in color ranging from subtle nuances of tans and browns, to areas of black and more rarely red, white (regression) and blue (deeper pigment). Amelanotic melanomas lack the color that is usually seen in pigmented melanomas (Fig. 1c)
D) Diameter - most early melanomas, when they are clinically identified, are more than 6 mm in diameter (Fig. 1d)

In the case of patients with histologically proven melanoma, the ABCD clinical features are found in 91% cases. The diameter parameter cannot be seen as an absolute criterion, as melanoma lesions smaller than 6 mm also occur frequently. In this regard, an important clue for diagnosis, regardless of the actual diameter of the lesion, is a change (increase) in the diameter of a given lesion over time.

Four main types of malignant melanoma have been described, based on clinical and histological criteria:

1. Superficial spreading melanoma (SSM). This is the most frequent form of MM in the Caucasian population and is diagnosed in about 65% of all MM cases. At the beginning, a lesion of SSM is flat and grows horizontally, subsequently its surface becomes irregular as circumscribed infiltrated papules or nodules develop, signaling vertical growth. The prognosis is relatively favorable in early phases (horizontal growth). The risk of metastasis significantly increases when vertical growth and dermal invasion occur.

2. Nodular melanoma (NM). This form of MM shows an early vertical growth with rapid invasion of the dermis, makes prognosis unfavorable even in the early phases.

3. Lentigo melanoma (LM). This clinical form accounts for about 10% of the MM cases and can growth for years or even decades until it develops malignant features. The prognosis of this type is more favorable, as the vertical growth occurs only late.

4. Acrolentiginous melanoma (ALM). This type of melanoma is rare in the white population (5%), but it is the principal form in the dark population. It primarily affects the skin of palms and soles.

5. Other malignant melanomas. These include melanoma of the retina and conjunctivae, as well as oral and genital mucosa. A special concern is the amelanotic MM, which raises great problems in diagnosis. It develops as pink or red nodule, usually on extremities. Its prognosis is worse than in other tumors, most probably due to its late recognition or misdiagnosis. And lastly, in some cases, melanoma metastasis can be diagnosed without evidence of a primary tumor.
1.1.4. Prognostic parameters

Unlike most other cancers, the physical thickness of the primary melanoma is directly related to its likelihood of metastases. Initially, it was observed that the extent of anatomic invasion by the primary tumor may predict the 10-year survival probability (Clark, Jr. et al., 1984) (Table 1). The Clark classification involves staging the primary lesion based on the anatomic level of invasion into the dermis or subcutaneous fat rather than based on its metric depth. According to Breslow's thickness, tumor depth is measured from the granular cell layer downward using an ocular micrometer. If the tumor is sphere shaped, the maximal thickness, as measured from the granular cell layer to the deepest component of the tumor, is mathematically related to the tumor volume (Breslow 1978). To date, tumor thickness remains the most powerful prognostic indicator that can be determined from evaluation of the primary melanoma itself.

The anatomic level of melanoma invasion has been reported to offer additional prognostic information in thin primary MM, as for example, tumors with less than 1 mm thickness but greater than Clark's level III have a worse prognosis than lesions of the same thickness with a Clark's level of III or less. Furthermore, it has been shown that a Clark's level of III or higher is an independent predictor of positive sentinel lymph node biopsy (Riegel D et al. 2005).
Table I. Clark’s anatomic level of melanoma invasion and survival

<table>
<thead>
<tr>
<th>Clark's level</th>
<th>Anatomic location of melanoma cells</th>
<th>10-year survival</th>
</tr>
</thead>
<tbody>
<tr>
<td>Level I</td>
<td>Confined to epidermis</td>
<td>99%</td>
</tr>
<tr>
<td>Level II</td>
<td>Penetrating the papillary dermis</td>
<td>96%</td>
</tr>
<tr>
<td>Level III</td>
<td>Filling the papillary dermis</td>
<td>90%</td>
</tr>
<tr>
<td>Level IV</td>
<td>Extending into the reticular dermis</td>
<td>67%</td>
</tr>
<tr>
<td>Level V</td>
<td>Invasion of the subcutis</td>
<td>26%</td>
</tr>
</tbody>
</table>

There are many other factors, which have been linked to melanoma prognosis, among them tumor ulceration, anatomic site, mitotic rate, tumor infiltrating lymphocytes, patient’s age, pregnancy, patient’s immune status, etc. (Riegel D et al. 2005).

The commission for Malignant Melanoma of the German Society of Dermatology (DDG) has proposed a TNM staging classification of MM (Orfanos, 2001; Table II):

Table II. Clinical staging and survival rate in MM

<table>
<thead>
<tr>
<th>Stage</th>
<th>Primary tumor</th>
<th>Lymph nodes</th>
<th>Distant metastases</th>
<th>Survival rate 10 years</th>
</tr>
</thead>
<tbody>
<tr>
<td>I a</td>
<td>pT1 (&lt; 0.75 mm)</td>
<td>N0</td>
<td>M0</td>
<td>97 %</td>
</tr>
<tr>
<td>I b</td>
<td>pT2 (0.76-1.5 mm)</td>
<td>N0</td>
<td>M0</td>
<td>90 %</td>
</tr>
<tr>
<td>II a</td>
<td>pT3 (1.5-4 mm)</td>
<td>N0</td>
<td>M0</td>
<td>67 %</td>
</tr>
<tr>
<td>II b</td>
<td>pT4 (&gt; 4 mm)</td>
<td>N0</td>
<td>M0</td>
<td>43 %</td>
</tr>
<tr>
<td>III a</td>
<td>in transit/satellite</td>
<td>N0</td>
<td>M0</td>
<td>28 %</td>
</tr>
<tr>
<td>III b</td>
<td>any</td>
<td>N1, N2</td>
<td>M0</td>
<td>19 %</td>
</tr>
<tr>
<td>IV</td>
<td>any</td>
<td>any</td>
<td>M1</td>
<td>3 %</td>
</tr>
</tbody>
</table>

1.1.5. Treatment of malignant melanoma

Detection of MM in an early stage is a highly important step of successful treatment. Complete surgical excision of the primary tumor has success rates over 95% at stages I/II and it can even substantially extend the long-term survival of patients with lymph node infiltrations (stage III) (Balch et al, 2001). Unfortunately, melanoma lesions can remain unnoticeable or asymptomatic for extended periods of time (Megahed et al, 2002) and are often detected only at stage IV (metastasis) (Vijuk and Coates 1998). Metastatic melanoma
cells tend to disseminate to multiple organs including brain, lung, liver or bone, rendering surgical interventions of limited use (Balch et al, 2001).

Chemotherapeutic drugs could theoretically target all metastatic sites, but current treatments do not provide a significant therapeutic benefit (Soengas and Lowe 2003). The alkylating agent dacarbazine (DTIC) allows remissions only in 5–10% of patients (Serrone et al, 2000). Temozolomide, a DTIC derivative with a higher permeability, has been shown to improve the response of brain metastasis, but it can not significantly increase overall survival (Soengas and Lowe 2003). Other chemotherapeutic agents that failed in large randomized studies include nitrosoureas (carmustine, lomustine), taxanes (taxol, docetaxel), vinca alkaloids (vincristine, vinblastine) and platinum-associated drugs (cisplatin, carboplatin).

The use of biotherapy or biochemotherapy is currently preferred as first line treatment in stage IV melanoma (Keilholz and Gore 2002). Various interleukin 2 (IL-2) dosing schedules and combinations with interferon alpha (IFN-alpha) or other chemotherapies have been tested in patients with advanced melanoma in phase I and II studies. Despite high response rate reported for the previous trials, combination of chemotherapeutic drugs and IFN-α with/ or without IL-2 had no clinically relevant activity in large phase III randomized trials (Keilholz et al, 2005).

The underlying cellular mechanisms involved in chemoresistance of melanoma have not yet been identified. Defects in drug transport, drug detoxification, mutation and increased activity of topoisomerase and apoptosis resistance have been proposed as mechanisms for chemoresistance (Rockmann and Schadendorf 2003).

Most chemotherapeutic drugs act through induction of apoptosis by activation of the transcription factor p53, and a defective apoptotic response may therefore be a main cause of chemoresistance. Apoptosis-related chemoresistance can be associated with three types of molecular changes: activation of antiapoptotic factors, inactivation of proapoptotic effectors, and reinforcement of survival signals (Soengas and Lowe 2003).

Recent advances in the understanding of melanoma biology may suggest new options for future therapeutic possibilities. In melanoma, numerous cellular pathways important to cell proliferation, apoptosis, resistance or metastases have been shown to be activated. Activation may occur through specific mutations (B-RAF, N-RAS, and PTEN) or changes in expression levels of various proteins (PTEN, Bcl-2, NF-kB, CDK2, and cyclin D1). Agents that block these pathways are entering clinical trials, including RAF inhibitors (sorafenib), mitogen-activated protein kinase inhibitors (PD0325901), mammalian target of rapamycin (mTOR) inhibitors (CCI-779), farnesyl transferase inhibitors (R115777) that inhibit N-RAS, and
proteasome inhibitors (PS-341, bortezomib) that block activation of NF-κB (Sosman and Puzanov 2006).

As melanoma may develop several defects that include loss of regulatory functions or the gain of proliferative or antiapoptotic functions, administration of drugs inhibiting several signaling pathways simultaneously may be highly promising. For instance, combined inhibition of NF-κB and the MAPK pathway, inhibition of MAPK pathways and the phosphatidylinositol 3-kinase (PI3K)/Akt pathways may offer therapeutic advantages over inhibiting a single pathway alone. Also, administration of traditional chemotherapies in combination with new drugs may be more effective (Rofstad and Halsor 2000).

Thus, high mortality rates of MM and inefficiency of traditional chemotherapeutic drugs may indicate the need for new, more effective therapeutic strategies.

1.2. Apoptosis

1.2.1. Definition

The maintenance of cellular homeostasis is fundamental for tissue integrity in multicellular organisms. Programmed cell death (apoptosis) is a highly conserved mechanism that has evolved to maintain cell numbers and cellular positioning within tissues (Prindull 1995). Apoptosis was first described in 1972 by Currie and colleagues (Kerr et al., 1972). Apoptosis is delicately regulated and is balanced in a physiological context. Failure of this regulation often results in pathological conditions such as developmental defects, autoimmune, neurodegenerative diseases or cancer (Thompson 1995).

Characteristic apoptotic features include cell shrinkage, chromatin condensation and DNA fragmentation, finally ending with the phagocytosis of the rest by macrophages or neighboring cells, thereby avoiding an inflammatory response in surrounding tissues (Savill and Fadok 2000). Apoptosis is distinct from necrosis in which the cells suffer major damage, leading to loss of membrane integrity, swelling and disruption of the cells. During necrosis, the cellular contents are released uncontrolled into the cell’s environment, which results in damage of surrounding cells and a strong inflammatory response in the corresponding tissue (Leist and Jaattela 2001).

The original understanding of cell death has come from genetic studies in the nematode C. elegans. As part of the developmental process, exactly 131 somatic cells of the initial 1090 are eliminated by apoptosis. cell death depends on the presence of CED-3 (caspase homologue),
and CED-4 (Apaf-1 homologue) that binds to and activates CED-3. In healthy cells, CED-4 remains inactive by its association with CED-9 (antiapoptotic Bcl-2 homologue). The protein EGL-1 (BH-3 only protein homologue) was described to trigger cell death. EGL-1 binds to CED-9 displacing CED-4, which in turn activates CED-3 to induce apoptosis (Liu and Hengartner 1999).

1.2.2. Caspases as basal elements for proapoptotic pathways

Caspases are a hallmark of apoptosis. To date, 11 human caspases have been identified: caspases 1–10 and caspase-14 (Pistritto et al., 2002). The protein initially named caspase-13 was later found to represent a bovine homologue of caspase-4 (Koenig et al., 2001), and caspase-11 and -12 are murine enzymes that are most likely the homologues of human caspase-4 and -5. All caspases share a number of common features. They are synthesized as inactive zymogens containing a prodomain followed by 18-20 kDa (large) and 10-12 kDa (small) subunits. These zymogens can be cleaved to form active enzymes following the induction of apoptosis.

Based on their function, the caspases can be classified into three groups.

1) Inflammatory caspases (caspase-1, -4, -5, and -14) are involved in controlling inflammation. The deletion of caspase-1 in transgenic mice showed no functional disorders on animal development, however, it resulted in specific defects in processing and secretion of the proinflammatory cytokines IL-1β and IL-18. This caspase is originally identified as an IL-1β processing enzyme and also as caspase that controls the secretion of this cytokine (Thornberry et al., 1992; Kuida et al., 1995). Caspase-5 has been shown to play a critical role in the activation of caspase-1 (Martinon et al., 2002).

2) Initiator caspases (caspase-8 -10, -2, and -9) are well characterized caspases participating in the initiation of apoptotic signals. Caspase-8 and 10 possess long prodomains containing characteristic protein–protein interaction motif: the death effector domain (DED).

3) Effector caspases (caspase-3, -6, -7) characterized by a short prodomain are processed and activated by upstream caspases and perform the downstream execution steps of apoptosis by cleaving multiple cellular substrates (Degterev et al., 2003).

During apoptosis, effector caspases serve as signaling mediators that coordinate apoptotic execution pathways by cleaving a subset of cellular proteins (death substrates). The protein characterized best is poly-(ADP-ribose) polymerase (PARP), a nuclear protein implicated in DNA repair. PARP is one of the earliest proteins targeted for specific caspase cleavage, which abolishes the DNA repair activity (Vermeulen et al., 2005a). Cleavage and inactivation of
inhibitor of caspase-activated DNAse (ICAD) by effector caspases allow CAD (also known as DNA fragmentation factor (DFF45)) to translocate to the nucleus, where it is responsible for internucleosomal DNA cleavage, generating oligonucleosomal DNA fragments (Sakahira et al., 1998). Cleavage of lamins results in nuclear shrinkage, and cleavage of cytoskeletal proteins like fodrin and actin leads to cytosolic reorganization (Mashima et al., 1995; Orth et al., 1996).

1.2.3. Caspase inhibitors

Apoptosis inducing activity of caspases needs to be kept in control in order for healthy cells to survive. Several negative regulators of caspases have been identified in human cells as well as in viruses. Inhibitor of apoptosis proteins (IAPs) is a family of proteins defined by baculovirus repeat (BIR) domains and, in some cases, a RING zinc-finger domain (Irusta et al., 2003). To date, eight mammalian IAPs have been identified. Four of these proteins (cIAP1, cIAP2, X-linked IAP (XIAP) and neuronal apoptosis inhibitory protein (NAIP)) have three BIR domains. Other IAPs (ubiquitin conjugation domain (BRUCE), survivin, neuronal apoptosis inhibitory protein (NAIP) and Livin) have one BIR domain (LeBlanc 2003). XIAP, survivin, cIAP1 and cIAP2 block apoptosis by directly inhibiting caspases. XIAP is one of the most potent inhibitors of apoptosis through its ability to inhibit caspase-3, -7 and -9. The BIR3 domain of XIAP directly binds to caspase-9, -3 and -7, and prevents their active sites from binding with death substrates (Suzuki et al., 2001b).

In addition to direct inhibition of caspase activation, most of the IAP proteins have a carboxyl-terminal RING zinc-finger motif and exhibit E3 ligase activity that catalyses autoubiquitination. cIAP2 was shown to promote ubiquitination of caspase-3 and -7, and that XIAP catalyses the ubiquitination and this supports the degradation of caspase-3 (Suzuki et al., 2001b).

The activity of IAPs is downregulated during apoptosis. XIAP and cIAP-1 have been shown to be the target of activated caspases and that they undergo a specific cleavage by caspases to ensure the induction of apoptosis (Deveraux et al., 1999).

1.3. Intrinsic (mitochondria) apoptotic pathways

In mammals, a wide range of external signals may trigger two major apoptotic pathways, the intrinsic (mitochondrial) pathway and the extrinsic (death receptor) pathway. Mitochondrial outer membrane permeabilization is the important event occurring during the intrinsic apoptotic pathway. It results in cell death through two mechanisms including release
of soluble mitochondrial proteins, such as cytochrome c (cyt c) and disruption of mitochondrial functions essential for cell survival (Green and Kroemer 2004). Released cytochrome c binds to apoptotic-protease activating factor 1 (Apaf-1), which results in the formation of a protein complex known as the apoptosome (Liu et al., 1996). The apoptosome typically recruits procaspase-9 and thereby activates it through oligomerization. Caspase-9 subsequently activates the executioner caspase-3 (Zou et al., 1997).

Several other proteins were also found to be released from the mitochondria as the IAP antagonists Smac/DIABLO, HtrA2/Omi and GSPT1/eRF3 (Jin and El Deiry 2005). They can neutralize the inhibitory activity of IAPs and promote cytochrome c-dependent caspase activation (Suzuki et al., 2001a; Hegde et al., 2003).

Bcl-2 family proteins play a pivotal role in the regulation of the mitochondrial pathway since they localize to intracellular membranes, in particular the mitochondrial membrane (Fulda and Debatin 2004). Bcl-2 proteins contain at least one of four conserved Bcl-2 homology domains (BH1-4) in mammals and can be subdivided into three groups: 1) Antiapoptotic proteins, including Bcl-2, Bcl-xL, Bcl-w, A1 and Mcl-1, which promote cell survival, 2) Two groups of members contributing to cell death. Proapoptotic “multi-BH domain” proteins Bax, Bak, and Bok share three domains (BH1, BH2 and BH3) with Bcl-2 (Hengartner 2000) and BH3-only proteins like Bid, Bad, and Bim have only the short BH3 interaction domain. Upon apoptosis, proapoptotic Bcl-2 proteins such as Bax translocate from the cytoplasm to the outer mitochondrial membrane, where they oligomerize to form a pore-like structure, thereby promoting cytochrome c release (Fulda and Debatin 2004).

Imbalances in this ratio between anti- and proapoptotic Bcl-2 members may turn the stability in favour of tumor cell survival or of cell death (Raisova et al., 2001).

1.3.1. The role of p53

Wild-type of p53 is found only at low levels in most cells because of its short half-life under normal conditions. DNA damage, hypoxia and inappropriate oncogene signaling can lead to stabilization and increase in the level of p53. The transcriptional factor p53 can bind to DNA in a sequence-specific manner and activate transcription of the targeted genes (Vousden and Lu 2002; Gudkov and Komarova 2003). p53 levels are regulated in large part by Hdm2 the human homolog (Mdm2) (Gudkov and Komarova 2003). Mdm2 is a RING finger-dependent ubiquitin protein ligase for p53 and for itself. It forms an autoregulatory loop with p53 by binding to its N-terminal domain, inhibiting its transcriptional activity and increasing its
degradation by the ubiquitin–proteasome pathway. On the other hand, transcription of the Hdm2 mRNA is activated by p53.

Important biological roles of p53 activation are growth arrest, which can be transient or permanent, and apoptosis. The number of physiological p53-responsive genes is likely to run into hundreds. A characteristic representative is cyclin dependent kinases (CDK)-inhibitor protein p21\textsuperscript{WAF1}, which mediates a G1/S arrest by blocking cyclin E-Cdk2-mediated phosphorylation of Rb. It is critical for the p53-mediated growth arrest (Meek 2004). Induction of apoptosis by p53 is based on the mitochondrial proapoptotic pathway (Wang 2001). The identification of p53 transcriptional targets revealed several proapoptotic proteins of the Bcl-2 family (Bax, PUMA, NOXA and Bid). Genetic evidence convincingly supports a major role for the BH3 proteins PUMA, NOXA and Bid, all of which have p53-responsive elements in their promotor regions (Schuler and Green 2005). In addition, p53 activates transcription of genes involved in the death receptor pathway, including DR4, DR5, FAS (de Stanchina et al, 2004).

1.4. **Extrinsic apoptotic pathways**

The extrinsic pathways are mediated by death receptors on the cell surface. Death receptors are members of the TNF (tumour-necrosis factor) receptor superfamily. This superfamily is characterized by a sequence of 2-5 cysteine-rich extracellular repeats (Schmitz et al, 2000). The death receptors have an intracellular death domain (DD), which plays a key role in the transmission of the death signal triggered by the interaction of death ligands with the receptor. Six death receptors are known so far, namely TNF-R1, CD95 (APO-1/Fas), DR3 (APO-3), TRAIL-R1 (DR4), TRAIL-R2 (DR5), and DR6 (Igney and Krammer 2002). Death receptors are activated through the interaction with their natural death ligands called the TNF family ligands (Fig. 3). Closely related to the death receptors are decoy receptors; they comprise TRAIL-R3 (DcR-1), TRAIL-R4 (DcR-2), OPG and DcR-3. The latter is bound by CD95L and LIGHT, the others, are bound by TRAIL (Vermeulen et al, 2005b).
Chemotherapy, irradiation and other stimuli can initiate apoptosis through the mitochondrial (intrinsic) pathway. Proapoptotic Bcl2 family proteins like Bax, Bid, Bad are important mediators of these signals. The apoptosome is formed by cytochrome c, Apaf-1 and caspase-9. Apoptosis through mitochondria can be inhibited on different levels by antiapoptotic proteins such as Bcl-2 and Bcl-XL.
Figure 3. TNF death receptors and ligands (Igney and Krammer 2002)
Ligands are shown at the top, receptors at the bottom. The receptors outside the box are the decoy receptors. The death domain is shown as a pink cylinder. Unknown ligand for DR6 (death receptor 6), and that the interaction between TWEAK (TNF-related protein with weak ability to induce cell death) and DR3 (death receptor 3) is not fully established.

TRAIL/APO-2L (TNF-related apoptosis-inducing ligand), DcR1 (decoy receptor 1), DR4 and DR5 (death receptor 4 and 5), LIGHT (a cytokine that is homologous to lymphotoxins, exhibits inducible expression and competes with herpes simplex virus glycoprotein D for herpesvirus entry mediator, a receptor expressed by T cells); LTα/ LTβ (lymphotoxin α/lymphotoxin β); OPG (osteoprotegerin); OPGL (osteoprotegerin ligand); RANK (receptor activator of NF-κB); RANKL (receptor activator of NF-κB ligand); TNF-α (tumour-necrosis factor-α); TNFR1 (tumour-necrosis factor receptor 1); TNFR2 (tumour necrosis factor receptor 2); TRANCE (tumour-necrosis-factor-related activation induced cytokine);

1.5. TRAIL and its receptors

Tumor necrosis factor-related apoptosis-inducing ligand (TRAIL/APO2L) was discovered by two independent groups, based on its sequence homology to TNF-α and CD95 (Wiley et al, 1995; Pitti et al, 1996). Like other members of the TNF ligand superfamily, TRAIL is primarily expressed as a type II transmembrane protein with the C-terminus located at the extracellular side and the N-terminus at the cytoplasmic side (Liabakk et al, 2002). The extracellular domain of the TRAIL molecule can be cleaved to release a soluble ligand. TRAIL displays 28% aminoacid sequence identity with CD95 and 23% identity with TNF-α (Bouralexis et al, 2005). Crystallographic analysis has shown that TRAIL, like other TNF
ligands, exists as a homotrimeric molecule. This ligand is stabilized by an internal Zn$^{2+}$ atom, which is essential for structural integrity (Bouralexis et al, 2005).

Besides binding to its agonistic death receptors (DR4 and DR5), TRAIL also binds to its three antagonistic decoy receptors DcR1(TRAIL-R3), DcR2 (TRAIL-R4) and OPG (osteoprotegerin) (Ashkenazi 2002). Decoy receptors are not able to transmit the death signal, and therefore they compete with DR4 and DR5 for TRAIL binding. DcR1 lacks a cytoplasmic domain while DcR2 contains a nonfunctional, truncated death domain. The third decoy receptor OPG, which is not located on the plasma membrane, binds to TRAIL in the bloodstream.

In humans, decoy receptors are widely expressed in different normal tissues as in ovary, testis, prostate, thymus, spleen, liver, colon, lung, placenta, heart, kidney, and bone marrow. It is generally believed that DcR1 or DcR2 may protect normal cells and tissues from TRAIL-induced apoptosis (Sheridan et al, 1997; Pan et al, 1997; Daniels et al, 2005). Decoy receptors have also been detected in several cancer cells, however, no correlation has been found so far between their expression and TRAIL resistance (Zhang and Fang 2004), thus suggesting another mechanism of resistance in cancer cells rather than a resistance caused by decoy receptor expression.

The two agonistic receptors DR4 and DR5 share significant similarities in the gene structure and expression pattern in human cells. Both are type I transmembrane receptors. The mature DR4 protein consists of a 445 amino acids and is generated through the cleavage of a signal sequence of 23 amino acids from precursor protein (Pan et al, 1997). DR5 consists of a 411 amino acid that includes a 51 amino acid-long signal peptide (Chaudhary et al, 1997). DR4 and DR5 share identity in the cysteine-rich extracellular domain (66%) and in the death domain (64%) (Sheridan et al, 1997). DR5 exists in two forms, which result from alternative splicing: DR5A (short) and DR5B (long). They differ by the presence of a 23 amino acid extension between the transmembrane domain. These two isoforms do not appear to have distinct functions (Screaton et al, 1997). DR4 does not contain this additional sequence and is therefore more closely related to DR5A.

Various agents may modulate the levels of TRAIL death receptors. Agents that induce DNA damage, such as antitumor chemotherapies, UV irradiation or X-ray radiation have been shown to upregulate DR4 and DR5 through p53- and p63-dependent mechanisms (Liu et al, 2004; Gressner et al, 2005).
1.5.1. **TRAIL activates several pathways**

Upon binding to DR4 and DR5, TRAIL can recruit and activate the apoptosis-initiating proteases caspase-8 and caspase-10 through the death-domain (DD)-containing adaptor molecule Fas-associated death domain (FADD). In type I cells, activated caspase-8 and -10 may cause direct cleavage of effector caspase-3, which then lead to cleavage of death substrates. In type II cells, cleavage of effector caspases is through engagement of the cell intrinsic (mitochondrial) apoptosis pathway (Kelley and Ashkenazi 2004). In this case, caspase-8 or caspase-10 cleaves and activates the pro-apoptotic Bcl-2 family protein Bid, which then interacts with Bcl-2 family member, Bax and Bak to induce the release of cytochrome c and Smac/Diablo from mitochondria.

Upon binding to DR4 and DR5, TRAIL can also mediate the activation of the transcription factor NF-κB (nuclear factor kappa B) and of mitogen-activated protein (MAP) kinases (Di Pietro and Zauli 2004). Activation of these two pathways by TRAIL may be mediated via TRADD (TNF-R1-associated death domain protein), TRAF2 (TNF receptor-associated factor 2), and RIP (receptor-interacting protein) and occurs independently of caspase-8/-10 activation (MacFarlane 2003). Supporting this hypothesis, dominant-negative TRADD as well as TRAF2 deletion mutant were shown to block the NF-κB activation induced by TRAIL (Hu et al., 1999; Zhang and Fang 2004).

TRAIL is also known to activate the JNK kinase pathway. A possible signaling pathway leading to TRAIL receptor-induced JNK activation has been suggested due to dominant negative mutants of TRAF2, MEKK1, MKK4, which were capable of blocking TRAIL-induced JNK activation. On the other hand, dominant negative mutants of NIK and IKKα, which are involved in NF-κB activation, had no inhibitory effect on TRAIL-induced JNK activation (Hu et al., 1999). These data suggest that TRAIL-induced JNK activation is mediated by a TRAF2-MEKK1-MKK4-dependent pathway and is independent of the NIK-IKKα/β cascade (Fig. 4).

TRAIL can also lead to activation of the MAP kinases Erk1/2 (extracellular signal-regulated kinase). The Erk1/2 pathway appears to be of particular importance as it can protect cells against apoptosis induced by a variety of stimuli. Recent reports have indicated that activation of ERK1/2 by TRAIL induces the expression of Bcl-2 and Bcl-xL, and thus promotes the survival of a variety of human tumor cells (Tran et al., 2001). Specific inhibition of activated ERK1/2 resulted in the down-regulation of the expression levels of anti-apoptotic Bcl-2 protein (Lee et al., 2006).
Figure 4. Activation of different pathways by TRAIL

The extrinsic apoptosis signaling pathway (engaged by TRAIL though DR4 and DR5) is represented. Engagement of the extrinsic pathway by TRAIL is sufficient to trigger apoptosis in some cell types (type I cells), whereas amplification through the intrinsic pathway is needed in other cells to induce apoptosis (type II cells). Crosstalk between these two pathways involves cleavage of Bid and subsequent activation of Bax and Bak. In parallel to this apoptosis signal, TRAIL induces also NF-κB and MAP kinase activation.

1.5.2. Targeting tumors by TRAIL and its possible clinical application

Death ligands are potentially useful for cancer therapy and unlike many conventional cancer therapeutic agents, they can trigger tumor cell apoptosis independently of the p53 tumor suppressor gene, which is inactivated in more than half of human cancers (Wang and El Deiry 2003). Thus, death receptor ligands may also be effective against tumors that have acquired resistance to chemotherapy or radiotherapy.

Despite these potential advantages, clinical application of ligands like TNF-α and CD95L has been hampered by their high toxicity to normal tissue cells, which led to animal death in experimental models (Ashkenazi 2002). Intravenous administration of TNF-α caused a systemic inflammatory syndrome that resembled septic shock by activating the pro-
inflammatory NF-κB in vascular endothelial cells and macrophages. Injection of agonistic CD95 antibodies induces hepatocyte apoptosis and lethal hepatic failure in mice (Kelley and Ashkenazi 2004). The ability of TRAIL to induce apoptosis in a wide variety of cancer cell lines, while having only little toxicity in many types of normal cells, suggests that this molecule may be highly useful for cancer therapy (Table III). Many cancer cell lines from different origin such as colon, lung, breast, prostate, pancreas, kidney, central nervous system, thyroid cancer, as well as lymphoma, leukemia and multiple myeloma have been found highly sensitive to the proapoptotic effects of TRAIL (Ashkenazi 2002).

**Table III. Side effects of death ligands in mouse models**

<table>
<thead>
<tr>
<th>Ligand</th>
<th>Death receptor</th>
<th>Apoptosis in vitro</th>
<th>Side effects in vivo</th>
<th>Animal death</th>
</tr>
</thead>
<tbody>
<tr>
<td>FasL</td>
<td>CD95</td>
<td>yes</td>
<td>Hepatic toxicity</td>
<td>yes</td>
</tr>
<tr>
<td>TNFα</td>
<td>TNFR1</td>
<td>yes</td>
<td>Septic shock</td>
<td>yes</td>
</tr>
<tr>
<td>TRAIL</td>
<td>DR4, DR5</td>
<td>yes</td>
<td>no</td>
<td>no</td>
</tr>
</tbody>
</table>

Administration of TRAIL either as a single agent or in combination with chemotherapy or with radiotherapy has shown substantial antitumor activity in mouse models with tumor xenografts from many human origins. For instance, tumor xenografts such as colon cancer (Kelley et al, 2001; Ashkenazi 2002; Naka et al, 2002b; LeBlanc and Ashkenazi 2003), breast cancer (Walczak et al, 1999), glioma (Roth et al, 1999; Fulda et al, 2002b), prostate cancer (Ray and Almasan 2003), multiple myeloma, and lung cancer (Mitsiades et al, 2001a). Significant apoptosis in tumors, partial or complete tumor shrinkage and delayed tumor progression indicated the high activity of TRAIL in vivo. The synergistic effect of TRAIL with anticancer therapies is particularly compelling. Apoptosis induced by TRAIL has been increased upon combination with IFN-γ (Shigeno et al, 2003), genotoxic agents (e.g. doxorubicin, cisplatin or etoposide) (Arizono et al, 2003; Bouralexis et al, 2003; Miao et al, 2003; Jones et al, 2003) and X-ray radiation (Ramp et al, 2003). The underlying mechanisms behind this synergy vary between therapies but include TRAIL death receptor upregulation, modulation of Bcl-2 family members, inhibition of IAP family members and c-FLIP.

Specific targeting of cancer cells by DR4 and DR5 selective agonistic antibodies may be even more effective than using TRAIL itself to eradicate tumors, which may be protected from TRAIL-induced apoptosis by the expression of decoy receptors. Antitumor effects of
agonistic DR4 and DR5 mAbs were synergistically enhanced in vitro by combination with chemotherapeutic drugs such as adriamycin and cisplatin (Yagita et al., 2004).

Currently, there are several clinical studies underway investigating the therapeutic potential and safety of TRAIL and death receptor agonists as anticancer agents (Table IV). Genentech (South San Francisco, CA) and Amgen (Thousand Oaks, CA) have started clinical phase 1 trials with soluble TRAIL. Human Genome Sciences Inc. has recently enrolled patients in phase 2 clinical trials with the DR4-specific human monoclonal antibody HGS-ETR1 for the treatment of non-small cell lung cancer, colorectal carcinoma, and non-Hodgkin’s lymphoma. So far, there were no indications of hematological or hepatic toxicity after treatment with HGS-ETR1 antibody in these patients. Six of 57 patients enrolled in a preliminary trial even reached stable disease.

**Table IV. Clinical therapeutics targeting TRAIL death receptors** (Fischer and Schulze-Osthoff 2005c)

<table>
<thead>
<tr>
<th>Molecular Target</th>
<th>Reagent</th>
<th>Principle</th>
<th>Company/Reference</th>
<th>Clinical Trial</th>
</tr>
</thead>
<tbody>
<tr>
<td>DR4</td>
<td>HGS-ETR1</td>
<td>Agonistic DR4 mAb</td>
<td>HGS/CAT</td>
<td>Phase 2</td>
</tr>
<tr>
<td>DR5</td>
<td>HGS-ETR2</td>
<td>Agonistic DR5 mAb</td>
<td>HGS/CAT</td>
<td>Phase 1</td>
</tr>
<tr>
<td>DR5</td>
<td>HGS-TR2J</td>
<td>Agonistic DR5 mAb</td>
<td>HGS/I KNBWY</td>
<td>Phase 1</td>
</tr>
<tr>
<td>DR4 and DR5</td>
<td>PRO1752</td>
<td>Soluble human TRAIL</td>
<td>Amgen/Genentech</td>
<td>Phase 1</td>
</tr>
</tbody>
</table>

**1.5.3. Resistance to TRAIL**

Resistance to TRAIL, however appears as a major barrier for the development of efficient therapies (Wang and El Deiry 2003). The analysis of the molecular mechanisms underlying TRAIL resistance of tumor cells has led to the identification of some characteristic defects in the apoptotic mechanisms of cancer cells.

First and foremost, TRAIL resistance may be the result of functional dysregulation, mutation or low expression of DR4 and DR5. Mutations of DR4 have been shown to occur in 35% to 44% of cells of ovarian, breast, bladder, lung and head cancers. Mutations in the death domain DR5 gene have been identified in 1% to 10% of head, neck, non-small-cell lung and breast cancers, non-Hodgkin’s lymphoma, and hepatocellular carcinoma (Zhang and Fang 2004).

Functional expression of DR4 and DR5 has been shown to correlate with TRAIL resistance in cells from lung, colon and breast carcinomas (Kim et al., 2000; Jin et al., 2004a). On the other
hand, no clear correlations between expression of death receptors and TRAIL sensitivity has been found in cancer cell lines and primary tumours (Petak et al, 2000; Nimmanapalli et al, 2001), which may be indicative for the other factors regulating TRAIL sensitivity.

Initiator caspases

Several studies have shown that caspase-8 is a key and irreplaceable molecule in TRAIL-induced apoptosis as well as in FasL- and in TNF-α-induced apoptosis (Bodmer et al, 2000; Seol et al, 2001; Fulda et al, 2001b). Several types of cancer cells, including Ewing’s tumor, neuroblastoma, malignant brain tumors, small-cell lung cancer and Jurkat cells showed high resistance to TRAIL-induced apoptosis that correlated with downregulated or missing caspase-8 expression. Importantly, restoration of caspase-8 expression in some resistant cell lines rendered them sensitive to TRAIL (Eggert et al, 2001; Fulda et al, 2001b; Ehrhardt et al, 2003). In a few cases, loss of caspase-8 expression resulted from gene deletion. In most cases, however, absence of caspase-8 expression resulted from gene silencing by DNA methylation in its promoter region (Eggert et al, 2001; Fulda et al, 2001b).

The role of the initiator caspase-10 in TRAIL-induced apoptosis, however, is less well understood. In lung and breast cancer cells, caspase-10 has been shown as an initiator caspase in TRAIL death receptor signaling and it may induce apoptosis in the absence of caspase-8 (Kischkel et al, 2001), however it could not substitute for caspase-8 in Jurkat cells (Sprick et al, 2002).

cFLIP

The cellular FLICE-inhibitory proteins (c-FLIP) have sequence homology to caspase-8 and -10, but lack protease activity (Wang and El Deiry 2003). Therefore, the recruitment of FLIP to the DISC in place of caspase-8 or -10 blocks their activation and consequently may confer TRAIL resistance. So far, two forms of cFLIP (long and short) have been detected (Krueger et al, 2001). cFLIP_L contains two DEDs and a caspase-like domain, but it cannot activate caspase cascades because this domain lacks a cysteine residue essential for catalytic activity (Krueger et al, 2001). Expression of cFLIP was shown to correlate with the malignant potential in colonic adenocarcinomas, hepatocellular carcinoma and Hodgkin lymphoma (Bullani et al, 2001; Okano et al, 2003). Increase of the cFLIP/caspase-8 ratio has also been reported to correlate with TRAIL resistance in several tumor types such as Burkitt’s lymphoma and B-cell chronic lymphocytic leukemia (Tepper and Seldin 1999; Okano et al, 2003). Downregulation of cFLIP expression by using antisense RNA or siRNA may therefore
be a valuable strategy for overcoming TRAIL resistance in types of cancer cells in which cFLIP overexpression is a key determinant of TRAIL resistance.

**Bcl-2 family proteins**

Overexpression of Bcl-x\(_L\) or Bcl-2 can protect some types of cells against TRAIL-mediated apoptosis, suggesting that the mitochondrial pathway predominates in these types of cells. Bcl-x\(_L\) expression correlated highly with resistance to TRAIL-induced apoptosis in pancreatic adenocarcinoma cells (Hinz *et al.*, 2000). Colon cancer cells became resistant to TRAIL after Bcl-x\(_L\) overexpression. In another study, overexpression of Bcl-2 conferred protection against TRAIL in neuroblastoma, glioblastoma, and breast cancer cell lines (Fulda *et al.*, 2002a). Mutational inactivation of the genes for Bax or Bak can render cancer cells resistant to apoptosis induced by TRAIL or chemotherapy. The importance of both molecules in apoptosis was demonstrated by the discovery that TRAIL could induce cytochrome c release and apoptosis in wild-type, Bax-/- or Bak-/- mouse embryonic fibroblasts, but not in double-knockout Bax-/-Bak-/- cells (Kandasamy *et al.*, 2003) indicating substitution of Bax and Bak for each other in these cells.

**Inhibitors of apoptosis (IAP) proteins**

High expression of IAPs in cancer cells can confer resistance to TRAIL-induced apoptosis as shown for prostate cancer cells (Ng *et al.*, 2002). Sensitivity of glioma cells to TRAIL-mediated apoptosis has been increased by downregulation of survivin and XIAP (Kim *et al.*, 2005a). The activity of IAPs can be blocked by Smac/Diablo, a mitochondrial protein that is released into the cytosol during the apoptotic cascade, where it promotes cell death by eliminating IAPs inhibition of caspases (Du *et al.*, 2000).

**1.6. Nuclear factor – kappa B (NF-κB)**

The NF-κB family of heterodimeric transcription factors plays an important role in determining cell survival during immune, inflammatory, and stress responses (Karin and Lin 2002). NF-κB comprises closely related transcription factors that include five protein subunits NF-κB\(_1\) (p50/p105), NF-κB\(_2\) (p52/p100), RelA (p65), c-Rel and RelB. NF-κB proteins share a highly conserved N-terminal Rel homology domain (Karin and Greten 2005). This domain is responsible for DNA binding, dimerization and interaction with specific inhibitors. Five NF-κB subunits could be further subdivided into two types depending on their function (Karin
and Lin 2002): 1) p65, c-Rel and RelB are synthesized in their mature forms and contain a transactivation domain, which interacts with the transcriptional apparatus; and 2) NF-κB1-p105/p50 and NF-κB2-p100/p52 that are synthesized in a precursor form. The precursor forms (p100 and p105) contain C-terminal ankyrin repeats that are proteolysed by the proteasome resulting in the production of the mature (p50 and p52) proteins. Both p50 and p52 contain the DNA binding domain but lack a transactivation domain.

In most types of cells, NF-κB dimers are predominantly cytoplasmic due to their interaction with the inhibitors of NF-κB (IκBs) and therefore remain transcriptionally inactive. The IκBs (IκBα, IκBβ, IκBγ and Bcl-3) include a family of proteins, which are responsible for interaction with the Rel homology domain of NF-κB proteins and for their retention in the cytoplasm. Upon phosphorylation, IκBs undergo proteasome-dependent degradation and this releases NF-κB proteins (Karin and Ben Neriah 2000).

Activation of NF-κB may result from different signalling pathways triggered by a variety of cytokines, growth factors and tyrosine kinases. Also, TNF-related death ligands are known to activate NF-κB. Several combinations of NF-κB proteins (p65-p50, c-Rel-p50, RelB-p52, c-Rel-P65, c-Rel-c-Rel) or homodimers (p50–p50 and p52–p52) are known to be released upon NF-κB activation, however the most common dimer is comprised of p65 and p50 (Karin and Greten 2005). Each NF-κB dimer is likely to have distinct regulatory functions. In normal cells, NF-κB becomes active only after the appropriate stimuli, and upregulates the transcription of its target genes. Afterwards, regulatory mechanisms return NF-κB to its inactive state by re-expression of IκB proteins. NF-κB activation is therefore an inducible and transient process (Karin and Ben Neriah 2000). In tumor cells, different types of molecular alterations may result in an impaired regulation of NF-κB. It loses its inducibility and becomes constitutively active. This leads to deregulated expression of genes under NF-κB control.

NF-κB exerts a leading role in immune response and inflammation but has also been unmasked as supporting tumor progression, angiogenesis, metastasis and apoptosis resistance (Ravi and Bedi 2004). NF-κB may activate the transcription of several genes involved in the suppression of cell death by both mitochondrial (intrinsic) and death receptor (extrinsic) pathways. Among NF-κB targets are c-FLIP (Kreuz et al, 2001), IAP’s proteins: (c-IAP1, c-IAP2, XIAP, survivin) (Ravi et al, 2001; Chen et al, 2003), Bcl-xL (Chen et al, 2000). In addition to promoting expression of antiapoptotic proteins, NF-κB may also attenuate expression of the proapoptotic protein BAX (Bentires-Alj et al, 2001).
 NF-κB also is known to regulate cell cycle progression, by regulating the expression of several genes such as cyclins D1, D2, D3 and cyclin E, c-myc (Dolcet et al, 2005). NF-κB-induced cyclin D1 expression appears to be a key element in mammary gland development and breast carcinogenesis. NF-κB induces the expression of cell adhesion molecules (ICAM-1, E-selectin) and proteins involved in invasion (matrix metalloproteinases). Several angiogenic factors, including vascular endothelial growth factor (VEGF), are also promoted by NF-κB (Dolcet et al, 2005).

1.7. Melanoma and TRAIL

The finding that TRAIL can induce apoptosis in cancer cells but not in most normal cells suggested that TRAIL may also be a promising therapeutic agent for patients with malignant melanoma. The large number of papers related to a search “melanoma and TRAIL” reflect an increased interest in this burning issue.

Sensitivity to TRAIL-induced apoptosis of cancer cells depends on the expression of two agonistic receptors TRAIL-R1/DR4 and TRAIL-R2/DR5. In almost all melanoma cell lines, DR5 has been found consistently expressed, but expression of DR4 was often lost (Zhang et al, 2000a). In this regard, TRAIL sensitivity of melanoma cell lines has been speculated to correlate with expression of DR5 (Zhang et al, 1999; Hersey and Zhang 2001; Zhang et al, 2004b). Melanoma cell lines with high levels of DR5 were sensitive to TRAIL-mediated apoptosis and cells with low levels were often resistant (Zhang et al, 2000a). However, the biological significance of DR4 has not been investigated.

Freshly isolated melanoma cells of primary tumors were found relatively resistant to TRAIL-induced apoptosis, and it has been suggested that this is a result of low DR5 expression (Nguyen et al, 2001). Continuous culturing of these cells was associated with an increase in DR5 expression and increased sensitivity to TRAIL-induced apoptosis. The low expression of DR5 in vivo and its re-expression in vitro have been suggested to result from selection of TRAIL resistant tumor cells in vivo (Nguyen et al, 2001). It has been further shown that melanoma cells selected under permanent TRAIL treatment acquired resistance to TRAIL and, as cells from primary tumors, revealed reduced DR5 expression (Zhang et al, 2004b). Posttranscriptional regulation of DR5 expression has been shown as a possible mechanism underlying this phenomenon (Zhang et al, 2000a; Zhang et al, 2004b).

These negative findings on the role of TRAIL in melanoma cells somehow did not support TRAIL applications in patients with malignant melanoma and may explain why clinical trials
with TRAIL or agonistic monoclonal antibodies have been initiated for some solid tumors (Fischer and Schulze-Osthoff 2005a), however, so far excluding melanoma. Some melanoma cell lines were found resistant to TRAIL-induced apoptosis in spite of sufficient TRAIL death receptor expression (Zeise et al, 2004), suggesting a high significance of intracellular signaling mechanisms for controlling TRAIL resistance (Hussein et al, 2003). Thus, it has been suggested that TRAIL resistance melanoma cells is result of high expression of antiapoptotic proteins as Bcl-2, c-FLIP, XIAP and Survivin (Chawla-Sarkar et al, 2004). Since c-FLIP has already been found to play important roles in TRAIL sensitivity in several tumor models, its anti-apoptotic effect has also been investigated in melanoma cells. Strong expression of the short splice variant of c-FLIP (FLIPs), which exerts high anti-apoptotic activity, was found in TRAIL resistant melanoma cells, and its exogenous overexpression in sensitive melanoma cells rendered them resistant (Zeise et al, 2004). On the other hand, no correlation has been found in other melanoma cell models (Zhang et al, 1999).

Melanoma cells have been mainly described as type II cells, where death receptor signaling is mediated through the mitochondria pathway(Raisova et al, 2001; Eberle et al, 2003; Hossini et al, 2003). Thus, antiapoptotic factors at this level may contribute to TRAIL-resistance. Overexpression of Bcl-2 has been shown to block TRAIL-induced apoptosis by preventing the release of cytochrome c and SMAC/DIABLO from mitochondria (Thomas et al, 2000). However, the nature of the antiapoptotic effect of Bcl-2 is still unclear, as no correlation between TRAIL-resistance and Bcl-2 expression levels has been found in untransfected melanoma cells (Hersey and Zhang 2001).

Activation of caspase-3 by TRAIL was also found not to be related to the level of apoptosis response induced by TRAIL in melanoma cells. Cells with high level of activated caspase-3 induced by TRAIL revealed different response levels to TRAIL. However, caspase-3 substrates, such as ICAD or PARP, were not cleaved in the resistant cells. The caspase-3 inhibitor XIAP has been suggested as a main factor leading to TRAIL resistance as its overexpression was shown to cause inhibition of caspase-3 activation (Zhang et al, 2001). However, many melanoma cells had high constitutive expression of XIAP, irrespectively of their sensitivity to TRAIL-induced apoptosis.

MAP kinases, such as extracellular-regulated kinases (ERK1 and ERK2), have also been discussed as pro-survival factors inhibiting TRAIL-induced apoptosis. ERK1 and ERK2 inhibitors were shown to increase TRAIL-sensitivity of melanoma cell lines (Zhang et al, 2003b). However, the mechanism how MAP kinesis may block TRAIL sensitivity has not
been elucidated. The dominant effect of TRAIL-induced ERK1/2 activation in sensitive melanoma cell lines is still unclear.

NF-κB, a prominent candidate with broad anti-apoptotic features, has also been investigated in melanoma cells. NF-κB activation induced by TRAIL was found to be higher in TRAIL-resistant melanoma cells, and inhibition of its activity was able to increase sensitivity to TRAIL (Franco et al., 2001; Chawla-Sarkar et al., 2003). Dose-response studies showed that NF-κB was activated at lower TRAIL concentrations than required for the induction of apoptosis. This suggested that low levels of TRAIL might induce resistance and might even protect against TRAIL-induced apoptosis (Franco et al., 2001). However, some sensitive melanoma cell lines revealed high levels of constitutively activated NF-κB, and the role of antiapoptotic factors induced by NF-κB was not clarified.

Recent studies on the possible mechanisms of TRAIL resistance made use of resistance models of TRAIL-selected melanoma cells positive only for DR5. Here, selection caused downregulation of DR5, initiator caspases and proapoptotic Bcl-2 proteins (Zhang et al., 2006) as well as a high proliferation activity that possibly related to reduced p53 and p21 expression and increased activation of Erk1/2 and Akt (Wu et al., 2005).

However, the role of DR4 in TRAIL-mediated apoptosis in melanoma cells has not been investigated. In particular, it was unclear how melanoma cells expressing DR4 would respond to selective pressure of TRAIL and what the mechanisms of TRAIL resistance in DR4 positive melanoma cells are.

### 1.8. Objective of the study

Malignant melanoma is a highly aggressive skin cancer with high mortality rates. Surgical excision is effective when applied at an early stage of melanoma development, however, conventional chemotherapies of metastatic disease have archived only limited effect. Given these limitations, there is a great need for the development of alternative therapeutic strategies based on new drugs that specifically target tumor cells and have a relatively low toxicity.

In this context, new biologic therapies are particularly promising. Among the new biologic agents, TRAIL seems to offer a promising anti-tumor therapeutic potential. Apoptotic effect of TRAIL is based on its capacity to selectively kill a wide variety of cancer cells while sparing most normal cells. A number of in vivo investigations have described TRAIL as a highly effective agent against tumor xenografts essentially without systemic toxicity.

Targeting tumors by TRAIL or agonistic antibodies depends highly on the functionality of the respective death receptors. Unlike other tumors with predominant significance of DR4,
TRAIL sensitivity of melanoma was so far assumed to depend mainly on DR5, whereas the biological significance of DR4 in melanoma was neglected. The aim of this study is to investigate the functionality of DR4 and DR5 for TRAIL-induced apoptosis in melanoma cells. In this regard, melanoma cell lines and cultures of normal human melanocytes were investigated in terms of TRAIL sensitivity, expression and functionality of death receptors and activation of the engaged pathways. Immunohistochemical staining for DR4 and DR5 has been investigated in tumor sections from patients with malignant melanoma.

Apoptosis resistance is a major problem of cancer therapies. In several tumor models, resistance to TRAIL-induced apoptosis was suggested to correlate with high expression of antiapoptotic proteins. Also for melanoma cells, endogenous overexpression of several antiapoptotic factors has been suggested to contribute to TRAIL-resistance. On the other hand, the role of proapoptotic proteins as initiator caspases was not investigated. Thus, the mediators of TRAIL resistance in melanoma cells are still poorly understood. In the present study, the nature of TRAIL resistance has been addressed in melanoma cell lines positive and negative for DR4 expression. In this regard, cell culture model of TRAIL resistant cells, which were cultivated permanently in the presence of TRAIL and their recovery after TRAIL withdrawal have been investigated.

NF-κB is a well-known transcription factor mediating apoptosis resistance and it has been shown to contribute also to TRAIL resistance in several tumor models. Also in melanoma cells, a role of TRAIL-induced NF-κB activation has been suggested, however, controversial results have been published and the way how NF-κB may contribute to TRAIL resistance was largely unclear. Thus, to investigate the nature of TRAIL-induced NF-κB activation and its possible contribution to TRAIL-resistance, parental and resistance model of melanoma cell lines were applied in the present study.