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DISSERTATION

Expression and functionality of death receptors and death ligands in cultured melanoma cells

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I dedicate this piece of art to my mother, a goddess.

And to all the black women of her kind.

You would have done better,

If you got the chance.

Index

1. Introduction

1.1. Malignant melanoma

1.1.1. Epidemiology

Melanoma develops through malignant transformation of melanocytes, which are predominantly located in the skin. In rare cases, melanoma can also be found in the eyes, ears, gastrointestinal tract, leptomeninges, and oral and genital mucous membranes. Melanoma accounts for only 4% of all skin cancers; however, it is the most aggressive skin cancer type and is responsible for about 90 % of skin cancer related deaths worldwide (de Vries et al, 2003).

The incidence of melanoma worldwide is increasing, especially in light skinned people with sun exposure. In central Europe, the incidence is 10 to 15 cases per 100 000 head of population per year, and in the US, 15 to 25 cases. The highest incidence rates have been reported from Australia, at 50 to 60 cases per 100 000 per year (Garbe et al, 2001), (Garbe et al, 2008). Melanomas are rare in populations with more pigmented skin (Asians, Africans) and almost always located on the mucosa or on the palms of the hands or the soles of the feet. However, these populations are more likely to present the disease at advanced stages and suffer worse outcomes (Kabigting et al, 2009). The male/female ratio varies in melanoma databases in different countries. In countries with a high cutaneous melanoma (CM) incidence, such as Australia and the United States, a preponderance of men is observed (Marks, 2000), (Geller et al, 2002). In countries with a lower incidence, such as Great Britain, a higher ratio of women patients with melanoma can be found (Mackie et al, 2002).

1.1.2. Etiology and risk factors

The most important etiological factor for the development of melanoma is determined by the interplay between genetic factors and UV radiation (Jhappan et al, 2003). Case control studies on the risk of melanoma development revealed that melanoma was closely associated with the number of melanocytic nevi on the integument and additionally, to the occurrence of sunburns in childhood (Elwood et al, 1984), (Osterlind et al, 1988). With growing numbers of melanocytic nevi, the melanoma risk increases nearly linearly (Holly et al, 1987), (Garbe et al, 1994). Even in childhood, the influence of UV radiation results in the development of benign melanocytic neoplasms, in the form of melanocytic nevi (Bauer et al, 2003), (Wieker et al, 2003). Intermittent

sun exposure and sun burn in childhood and adolescence have therefore been identified as major risk factors for melanoma in epidemiologic studies (Gandini et al, 2005), (Elwood et al, 1997). Although it is widely accepted that sun exposure raises the total risk of melanoma development, the following clinical and epidemiologic features raise doubts about the effects of sunlight in melanoma development: (1) The anatomic distribution of CM does not closely match body areas of greatest sun exposure; unlike epithelial skin cancer, 80% to 90% of which is located on the head and neck, only 10% to 15% of CMs are located in this anatomic area. (2) CM is most common during the middle decades of life (except the subtype of lentigo maligna melanoma, which counts for 10% of all CMs) and not in older age, which is the time of highest cumulative sun exposure. (3) An elevated CM risk after higher cumulative sun exposure in adulthood and after sunburns during the years before melanoma diagnosis was not detected in most of the case control studies performed (Garbe et al, 2009). Moreover, it has been observed that people with more pigmented skin develop lesions predominantly located on sun-protected mucosal and acral sites (Swan et al, 2003), (Bellows et al, 2001), (Cress et al, 1997). Here, reported risk factors for melanoma include albinism, burn scars, immune suppression, radiation therapy and trauma (Reintgen et al, 1983). Melanoma development is therefore multifactorial and it appears that not all etiological factors have been fully described.

1.1.4. Tumour classification

Different types of melanomas can be identified clinically and histologically. Some tumours either represent mixed forms or are not classifiable.

Superficial spreading melanoma (SSM) starts with an intraepidermal horizontal or radial growth phase, starting as macule and slowly evolving into a plaque, often with multiple colours and pale areas of regression. Secondary nodular areas may also develop. A characteristic histological feature is pagetoid spread of clear malignant melanocytes throughout the epidermis. It is the most frequent form of CM and is diagnosed in about 65% of all melanoma cases.

Nodular melanoma (NM) in contrast is a primarily nodular, exophytic brown-black, often eroded or bleeding tumour, which has only short horizontal growth phase and then an aggressive vertical phase. Thus early identification in an intraepidermal stage is almost impossible.

Lentigo maligna melanoma (LMM) arises often after many years from a lentigo maligna (melanoma in-situ) almost exclusively on the face of elderly individuals. This clinical form accounts for about 10% of the melanoma cases.

Acral-lentiginous melanoma (ALM) is usually palmoplantar, or sub-/periungual. In its early intra epidermal phase, there is irregular, poorly circumscribed pigmentation; later a nodular region signals the invasive growth pattern. It is the most common type of melanoma found in populations with pigmented skin.

Examples of special forms are amelanotic melanomas, mucosal melanomas, and other extra cutaneous melanomas, which together account for about 5% of all melanomas.

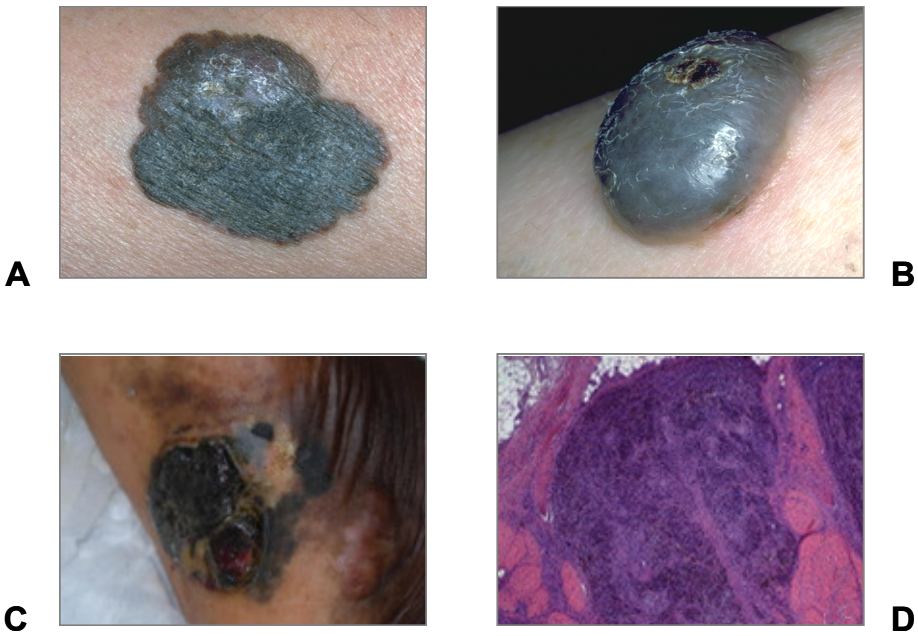

Figure 1.1 *Superficial spreading melanoma, SSM (A) and Nodular melanoma, NM (B) in light skinned persons; Eberle et al, Skin Cancer Centre, Charité, Berlin.*

Acral-lentiginous melanoma (ALM), plantar, in a person with pigmented skin as clinical presentation (C) and histological presentation (D);
Kabigting et al, Malignant melanoma in African-Americans, 2009

1.1.5. Diagnosis, staging and prognosis of melanoma

The A-B-C-D acronym was devised in 1985 by Kopf and colleagues to help patients recognize several clinical features useful in clarifying the differential diagnosis of pigmented lesions that are suspicious for melanoma and worthy of further investigation (Kaufmann et al, 1995), (Garbe, 1996).

1. A = asymmetry
2. B = border irregular
3. C = colour inhomogeneous
4. D = diameter > 5 mm

The acronym, however, is not very specific; seborrhoeic keratoses, which are very common in older patients, often exhibit A-B-C-D features. In addition, melanomas arising de novo (not in pre existing nevi) are often smaller than 5 mm. Also, amelanotic melanomas do not show these A-B-C-D features, which leads to late diagnosis.
Following requirements have been therefore described by Garbe and colleagues for the preoperative diagnosis and staging of malignant melanoma:
Dermatoscopy to enhance differential diagnosis of pigmented tumours. *Clinical evaluation of draining lymphatic pathways* to exclude the presence of a second melanoma or other melanoma precursors. *Lymph node sonography* for lesions thicker than 1 mm. *Excision biopsy* for confirmation of diagnosis. *Histopathology* should include type of melanoma, tumour thickness (Breslow depth), Clark level, and presence or absence of ulceration. *Sentinel lymph node biopsy* should be performed as part of routine staging in melanomas thicker than 1 mm. When the tumour is located on face, elective lymph node dissection or parotid gland excision should be considered. Laboratory evaluation includes LDH, alkaline phosphates and serum S100 protein for tumours thicker than 1 mm. *Chest X-ray* in two planes. *Sonography* of the abdomen including the pelvis and retro peritoneum. Useful in selected cases are *high-resolution sonography* to measure tumour depth preoperatively, *CT*, *MRT* or *PET* evaluation as alternative or supplement to above-mentioned staging examinations (Garbe et al, 2008).

The best prognosis indicator for malignant melanoma is the stage at first clinical presentation. Tumour staging in melanoma is reliant on vertical tumour thickness (Breslow depth), histological presence or absence of ulceration, the presence or absence of microscopic metastases and the number of regional lymph nodes involved (Ruiter et al, 2001). According to Breslow's depth, tumour thickness is measured from the granular cell layer downward using an ocular micrometer (Breslow, 1978). The Clark classification involves staging the primary lesion based on the anatomic 1evel of invasion into the dermis or subcutaneous fat rather than based on its metric depth (Clark et al, 1984).
The American Joint Committee of Cancer (AJCC) proposed a revised tumour-node-metastasis (TNM) staging system for melanoma in 2001, which has been approved by the International

Union Against Cancer (UICC) and the World Health Organisation (WHO). This new system forms the cornerstone for classifying melanomas and is summarized in tables 1.1 and 1.2.

Table 1.1 TNM classification of primary melanoma

T classification		N classification		M classification	
T1 ≤ 1.0 mm	a. no ulcer b. ulcer	N1 1 LN	a. micro metastasis b. macro metastasis	M1a	skin, LN, subcutaneous tissue; normal LDH
T2 1.01-2.0 mm	a. no ulcer b. ulcer	N2 2-3 LN	a. micro metastasis b. macro metastasis c. satellite metastasis	M1b	lungs; normal LDH
T3 2.01-4.0 mm	a. no ulcer b. ulcer	N3 ≥ 4 LN	satellite or in-transit metastasis	M1c	distant metastasis, normal LDH any distant metastasis
T4 > 4.0 mm	a. no ulcer b. ulcer	N0	no LN involvement	M0	no metastasis
Tis melanoma in situ					
Tx unknown primary tumour					

LN = lymph node LDH = Lactic dehydrogenase

Table 1.2 Staging of melanoma

Stage	Primary tumour (pT)	Regional LN metastases (N)	Metastases (M)
0	In situ tumour	None	None
1A	≤ 1.0 mm, no ulceration	None	None
1B	≤ 1.0 mm with ulceration or Clark Level IV or V	None	None
	1.01–2.0 mm, no ulceration	None	None
IIA	1.01–2.0 mm with ulceration	None	None
	2.01–4.0 mm, no ulceration	None	None
IIB	2.01–4.0 mm with ulceration	None	None
	> 4.0 mm, no ulceration	None	None
IIC	> 4.0 mm with ulceration	None	None
IIIA	Any tumour thickness, no ulceration	Micrometastasis	None
IIIB	Any tumour thickness with ulceration	Micrometastases	None
	Any tumour thickness, no ulceration	Up to three macrometastases	None
	Any tumour thickness ± ulceration	None but satellite and/ or in-transit metastases	None
IIIC	Any tumour thickness with ulceration	Up to three macrometastases	None
	Any tumour thickness ± ulceration	Four or more macrometastases, or lymph node involvement extending beyond capsule, or satellite and/or in-transit metastases with lymph node involvement	None
IV			Distant metastases

Modified: Garbe et al, 2008 & 2009

Melanoma progression is well defined in its clinical and histopathological aspects (Breslow's index, tumour size, ulceration, or vascular invasion), which also give hints to prognosis of the patient. Use of molecular markers gives additional information which cannot be determined by routine histopathology. Markers showing only a correlation to Clark level or tumour size are not useful. Several molecules influencing invasiveness and metastatic dissemination of melanoma have been identified. Expression of these molecules has been studied in primary melanoma and correlated with prognosis (Bosserhoff, 2006). Most of the melanoma markers used today are melanocytic markers or pigmentation pathway-associated genes driven by the microphthalmia transcription factor, MITF, and include among others, tyrosinase, dopachrome tautomerase, DCT, melan-A and S100B (Tímár et al, 2006).

1.1.6. Treatment of melanoma

The following therapeutic guidelines are consistent with the current interdisciplinary version of the German S2 guideline for melanoma (Garbe et al, 2007/2008/2009).
Curative surgical treatment: The gold standard in diagnosing malignant melanoma is the excision biopsy. The current German guideline recommends safety margins based on the risk of metastasis for excision and re-excision on melanomas (table 1.3).

Table 1.3 Recommended excision margins

Tumour thickness (Breslow)	Safety margin
In situ	0.5 cm
≤ 2 mm	1 cm
> 2 mm	2 cm

Palliative surgical treatment: When the patient has satellite and/or in transit metastases, complete excision of metastases is performed. For regional lymph node metastases, a radical lymph node dissection (or a modified or selective neck dissection) is a curative attempt.
Radiotherapy: This therapeutic option for malignant melanoma is only indicated for functionally inoperable tumours. The recommended radiation dose in macroscopic tumours is 70 Gy, five radiotherapy sessions per week with two individual doses of 2 Gy each. In metastases, radiotherapy is used for palliative purposes, most often for bone metastases. An indication for radiotherapy exists when patients are in pain and/or the vertebral column stability is threatened, or in patients with compression of the spinal canal with or without neurological symptoms.

Response rates for palliative radiotherapy, independent of the site, are 67% to 85% (Kirova et al, 1999). In patients with single brain metastases, operative resection or stereotactic single radiotherapy may be used. In a prospective study, the local control rate was improved after resection of a solitary brain metastasis by applying radiotherapy to the entire brain; the risk of dying from neurological complications was reduced simultaneously. The combination of stereotactic radiotherapy with irradiation of the brain can also increase the intracerebral control rate (Andrews et al, 2004).

Adjuvant therapy: Numerous prospective randomized studies have shown that systemic chemotherapy, for example, as monochemotherapy with dacarbazine, is not beneficial. A recent meta-analysis of more than 6000 patients from 13 randomized interferon protocols based on individual patient data has found not only a highly significant advantage for prolonging the recurrence-free survival interval but also for total survival. The risk of death was reduced by 3% after five years when interferon therapy was administered (Wheatly et al, 2007). Currently, the main distinction is made between low-dose treatment and high-dose treatment with interferon alpha. Pegylation enables once-weekly administration and avoidance of peak concentrations in the blood; the objective is to improve patients' quality of life and clinical efficacy.

Palliative chemo immunotherapy: The main indications for systemic chemotherapy and chemo immunotherapy are inoperable recurring tumours, inoperable regional metastases, and distant metastases (stage IV). Objective response rates can be improved by combining cytotoxic drugs with cytokines. However, none of the studies conducted so far has found a significantly prolonged overall survival period (Eigentler et al, 2003). The subjective and objective tolerability of monochemotherapy is worsened if interferon or interleukin-2 is added. Since poly chemotherapeutic regimens are potentially toxic, intensive supportive treatment is crucial for the patient's quality of life.

1.1.7. Drug resistance in melanoma

Advances in the therapy for metastatic melanoma to date have been rather disappointing. No new drugs have been approved in over two decades (Agarwala, 2009). Current systemic therapeutic approaches, either as mono or poly chemotherapy, and combination of chemo immunotherapy (bio chemotherapy) have produced low response rates while maintaining toxic side effects and high expenses. In fact, combination therapies are not superior to single agent chemotherapy, as proven by randomized trials.

Recent studies, however, have identified defects at multiple levels of the apoptosis program in melanoma, which provided new clues to drug resistance of this highly aggressive neoplasm. The process of apoptosis provides a conceptual framework to link melanoma genetics with the outcome of melanoma therapy. Hence, the genes and proteins that control apoptosis provide exciting new targets for rationally designed anti-melanoma therapeutic strategies.

1.2. Apoptosis

1.2.1. History

The word "apoptosis" (the second p is silent) comes from the ancient Greek απσπτόσισ, meaning the "falling off of petals from a flower" or "falling off of leaves from a tree in autumn". Apoptosis was first introduced by John Foxton Ross Kerr and colleagues in 1972 and refers to the unique morphological features of the cell during apoptosis. The phenomenon was first described by Carl Vogt more than a 100 years earlier in 1842 (Lawen, 2003), (Peter et al, 1997) while performing his seminal work on "resorption" of notochord cells in the toad, which included distinct changes in the nuclei (Gastman, 2001). Vogt's work was the first ever published account of the histological features of apoptosis. The first breakthrough in apoptosis research was the study of the genetic regulation of apoptosis in the nematode *Caenorhabditis elegans (C. elegans)* (Ellis et al, 1986). In this worm, three genes (ced-3, ced-4 and ced-9) are directly involved in controlling the execution of apoptosis during development. The ced-3 and ced-4 gene products were found to be proapoptotic, whereas the ced-9 product was found to be antiapoptotic (Ellis et al, 1991), (Xue et al, 1997). The importance of these discoveries is highlighted by the award of the 2002 Nobel Prize for Physiology/Medicine to Sydney Brenner, Robert Horvitz and John Sulston for their work concerning the "genetic regulation of organ development and programmed cell death" in *C. elegans.* This lies in the fact that apoptosis is evolutionarily conserved, albeit with an increase in complexity with continuing development.

1.2.2. Definition of apoptosis

Apoptosis defines a specific physiological form of cell death with a distinct set of morphological and biochemical changes involving the cytoplasm, nucleus and plasma membrane. Early in apoptosis, the cell rounds up, losing contact with neighbour cells, and shrinks. This led to Kerr's initial term "shrinkage necrosis" (Kerr, 1965). In the cytoplasm, the endoplasmic reticulum

dilates and the cisternae swell to form vesicles and vacuoles. In the nucleus, chromatin condenses and aggregates into dense compact masses, and is fragmented by endonucleases, which can be often analysed by the typical ‘‘DNA ladder’’ formation in apoptosis, for which DNA (either total or cytosolic) is extracted from the cells and separated in an agarose gel (Johnson et al, 1996). The cell breaks up in a florid manner leading to the ‘‘falling away’’ of several membrane spheres containing the ‘‘packaged’’ cellular contents identified as apoptotic bodies of various sizes (Kerr et al, 1994). Under physiological conditions, apoptotic bodies are recognized and engulfed by phagocytes or neighbouring cells. Since the apoptotic bodies are surrounded by an intact plasma membrane, apoptosis usually occurs without leakage of cell content and usually without inflammation. This form of physiological cell death is morphologically quite different from oncosis, in which the cell swells and disintegrates in an unordered manner, leading to the destruction of the cellular organelles, ruptures of the plasma membrane and leakage of the cell content (necrosis). Apoptosis is often used synonymously with programmed cell death, and is over 20 times faster than mitosis. Sightings of apoptotic cells in vivo are therefore rare.

1.2.3. Apoptosis in disease

For cell homeostasis to be maintained, a balance between the increase - by differentiation from precursors and by proliferation - and decrease - by further differentiation and cell death - in cell populations must be balanced. If mitosis proceeded without cell death, an 80-year-old person would have 2 tons of bone marrow and lymph nodes, and a gut 16 km long (Melino, 2001). Unregulated excessive apoptosis may be the cause of various degenerative and autoimmune diseases that are characterized by an excessive loss of normal or protective cells, such as in multiple sclerosis, type-I diabetes mellitus, Hashimoto thyroiditis (Giordano et al, 1997). On the other hand, an inappropriately low rate of apoptosis may promote survival and accumulation of abnormal cells that can give rise to tumour formation and prolonged autoimmune stimulation such as in cancers and autoimmune diseases. A number of diseases, in which deregulation of apoptosis has been described or is currently in discussion as being involved, is listed below in table 1.4.

Table 1.4 Apoptosis in disease

Increased apoptosis	Decreased apoptosis
AIDS	Autoimmune diseases:
Neurodegenerative diseases	- lupus erythematosus
Multiple sclerosis	- rheumatoid arthritis
Diabetes mellitus type I	- Graves disease
Hashimoto thyroiditis	Lymphoma
Ulcerative colitis	Leukaemia
Wilson disease	Solid tumours

1.3. Apoptotic pathways

The elucidation of the apoptosis pathways in *C. elegans* has been helpful to better understand apoptosis signaling pathways in higher eukaryotes. The understanding of cell death comes from the genetic studies of *C. elegans* and the finding that homologues of the genes directly involved in controlling the execution of apoptosis exist in mammals, only with more complexity. A broad array of external signals can trigger two major apoptotic pathways, namely the extrinsic pathway and the intrinsic pathway. Both apoptosis signaling pathways converge at the level of specific proteases, the caspases.

1.3.1. Caspases and their inhibitors

The activation of caspases is a hallmark of apoptosis. Caspases are cysteine proteases related to mammalian interleukin-1β-converting enzyme (ICE) and are homologues to the ced-3 gene product of *C. elegans* (Yuan et al, 1993). There are 14 mammalian caspases identified to date (Ashkenazi et al, 2002). Caspases are synthesized as inactive zymogens that can be cleaved to form active enzymes following the induction of apoptosis. Based on their function, the caspases can be classified into three groups: *Inflammatory caspases* include caspase-1, -4, -5, -11, -12, -13 and -14, and are involved in inflammation instead of apoptosis. *Apoptotic initiator caspases* contain a death effector domain (DED), caspase-8 and -10, or a caspase activation and recruitment domain (CARD), caspase-2 and -9, which mediate the interaction between upstream adaptor molecules. *Apoptotic effector caspases* contain a short prodomain, caspase-3, -6 and -7.

They are processed and activated by upstream caspases and perform the downstream execution steps of apoptosis by cleaving multiple cellular substrates (Degterev et al, 2003).

Inhibitor of apoptosis proteins (IAPs) may be the most important negative regulators of caspases. IAPs are able to inhibit apoptosis via direct binding and inactivation of certain caspases. Most human IAPs share common features including the Baculovirus IAP repeat (BIR) domains and, in some cases, a RING zinc-finger domain (Irusta et al, 2003). To date, eight mammalian IAPs have been identified, including c-IAP1, c-IAP2, NAIP, Survivin, X-linked IAP (XIAP), Bruce, ILP-2, and Livin (Salvesen and Duckett, 2002). The most powerful caspase inhibitor is XIAP, which is also the best characterized. XIAP contains three BIR domains ((Deveraux et al, 1997). The BIR3 domain of XIAP directly binds to caspase-9, -3 and -7, and prevents their active sites from binding with death substrates (Le Blanc, 2003).

The cellular FLICE-inhibitory proteins (c-FLIP) have sequence homology to caspase-8 and -10, but lack protease activity (Wang and El Deiry, 2003). The recruitment of FLIP in place of caspase-8 or -10 blocks the caspase activation and consequently, inhibits apoptosis downstream signaling. So far, two forms of c-FLIP (long and short) have been described (Krueger et al, 2001).

1.3.2. The intrinsic apoptotic pathway: Mitochondria and Bcl-2 family

One major pathway for the induction of apoptosis is the intracellular mediated intrinsic pathway. The most important turning point in the course of the intrinsic apoptotic pathway occurs in the mitochondria. The mitochondrial pathway is activated by a variety of extra- and intracellular stresses, including oxidative stress and treatment with cytotoxic drugs. The apoptotic signal leads to the release of cytochrome c from the mitochondrial inter membrane space into the cytosol, where it binds to the Apoptotic Protease Activating Factor-1 (Apaf-1), which is the mammalian homologue to the ced-9 gene product of *C. elegans*. Binding of cytochrome c to Apaf-1 triggers the formation of the apoptosome, an Apaf-1-containing complex that catalyses activation of caspase-9. The apoptosome contains each of seven molecules Apaf-1, cytochrome c, dATP and procaspase-9. Procaspase-9 acts as the initiator caspase of the apoptosome (Waterhouse et al, 2002). The apoptosome bound procaspase-9 is activated and can then activate an effector caspase (e.g., caspase-3), which then can cleave a large number of cellular substrates needed for the orchestration of apoptosis (see Figure 1.2).

Upon activation of the intrinsic pathway, a range of proapoptotic molecules in addition to cytochrome c are released from the mitochondria (van Loo et al, 2002). One such molecule is Smac/Diablo, an inhibitor of cellular IAPs (Du et al, 2000), (Verhagen et al, 2000), (De Laurenzi et el, 2000). Mitochondria can also release apoptosis inducing factor, AIF, which appears to induce an apoptosis-like cell death that is independent of caspases (Joza et al, 2001), (Hunot et al, 2001).

A key event in the mitochondrial pathway is the mitochondrial outer membrane permeabilization (MOMP). MOMP is mainly mediated and controlled by Bcl-2 (B cell leukemia/lymphoma 2) family members. Once MOMP occurs, it causes cell death either through the release of proapoptotic molecules, or the loss of mitochondrial functions essential for cell survival (Green et al, 2004).

Bcl-2 family members

Bcl-2 proteins are homologues to the ced-9 gene product of *C. elegans*. Like ced-9, Bcl-2 contains four so-called Bcl-2 homology domains (BH1–BH4), which are required for its survival functions. At present, three groups of the Bcl-2 family proteins can be distinguished: (1) the antiapoptotic proteins, most of which contain a C-terminal membrane anchor and the four BH domains, such as Bcl-2 and Bcl-xL, (2) the proapoptotic multidomain members, which lack some of the four BH domains, such as Bax and Bak and (3) the BH3-only proteins that, as the name suggests, only contain the third BH domain, and are all proapoptotic, such as Bad, Bik, Bid and Bim. The relative levels of pro- and antiapoptotic proteins determine a cell's susceptibility to apoptosis (Borner, 2003).

1.3.3. The extrinsic apoptotic pathway: death receptors and their ligands

The second major pathway for the induction of apoptosis is the receptor-ligand mediated extrinsic pathway. The receptors triggering this pathway are located in the plasma membrane of the cell that is to undergo apoptosis. Death receptors (DR) belong to the tumour necrosis factor receptor (TNFR) gene super family, which have several functions including initiating apoptosis (Baud et al, 2001). The TNFR super family is characterized by the presence of cysteine rich domains (CRDs) that mediate receptor and ligand binding. To date, 6 death receptors have been classified, which are TNF-R1, Fas (CD95/APO-1), DR3 (LARD/WSL-1/TRAMP/APO-3), TRAIL-R1 (DR4), TRAIL-R2 (DR5), and DR6 (Igney and Krammer, 2002).

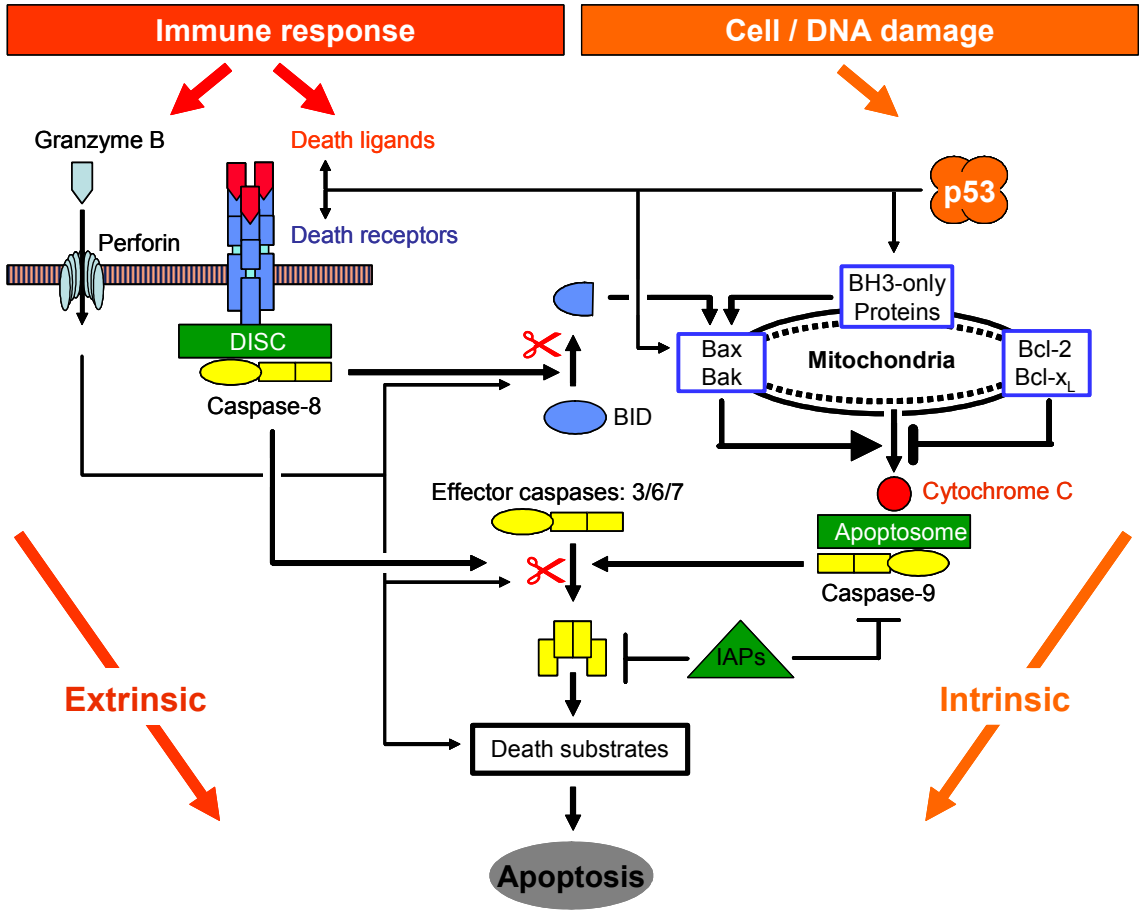

Figure 1.2 *Apoptosis pathways: Modified, Eberle and Kurbanov, 2006*
The intrinsic pathway can be initiated by DNA damage and p53; Stress signals such as hypoxia and radiation can activate p53, which induces the expression of transcription factors including proapoptotic members of the Bcl-2 family. The extrinsic pathway is mediated by the binding of death ligands to death receptors. Perforin and granzyme B mediated apoptosis is triggered by CD8 T cells and NK cells. Description of the pathways is found in the text.

DRs get activated by their cognate death ligands. TNF-α binds TNF-R1, FasL (Fas ligand) Fas, TL1A (TNF-like ligand 1A) DR3, TRAIL (TNF related apoptosis inducing ligand) TRAIL-R1 and TRAIL-R2, and APP (ß-amyloid precursor protein) DR6 (Krammer, 2000), (Zhaoyu and El-Deiry, 2005), (Nikolaev et al, 2009). Death ligands function in an autocrine (activation induced cell death, AICD) or paracrine manner, and upon binding, cause trimerization of their respective cell surface membrane receptors, which is essential for apoptosis signaling (Sheikh et al, 2000), (Daniel et al, 2001).

The best investigated death receptor pathways are that of TNF-R1, Fas and TRAIL-R1/2. Ligand binding leads to death receptor trimerization; adaptor proteins are recruited, which bind to the cytosolic death domain (DD) of the death receptor. Two types of adaptor proteins have been described, namely Fas associated death domain protein (FADD) in Fas receptor signaling, and

TNF receptor associated death domain protein (TRADD) in TNFR signaling. TRAIL recruits FADD, while DR3 recruits TRADD. The adaptor protein FADD contains a death effector domain (DED), to which the DED of procaspase-8 can bind. The complex comprised of death receptor, death ligand, adaptor protein and procaspase- 8 is called DISC, death inducing signaling complex. Active caspase-8 can then directly cleave caspase-3, -6 or -7, which may lead to apoptosis (Figure 1.2).

Decoy receptors (DcR) are members of the TNFR superfamily that bind death ligands without activating the apoptosis pathway. These receptors either lack a cytosolic DD, e.g. TRAIL-R3/DcR1, or have a defective DD, e.g. TRAIL-R4/DcR2 and EDAR. Others are soluble receptors that lack a transmembrane domain, e.g. TRAIL-R5 and DcR3. A schema of the known and described DRs, DcRs and their ligands is shown in Figure 1.3.

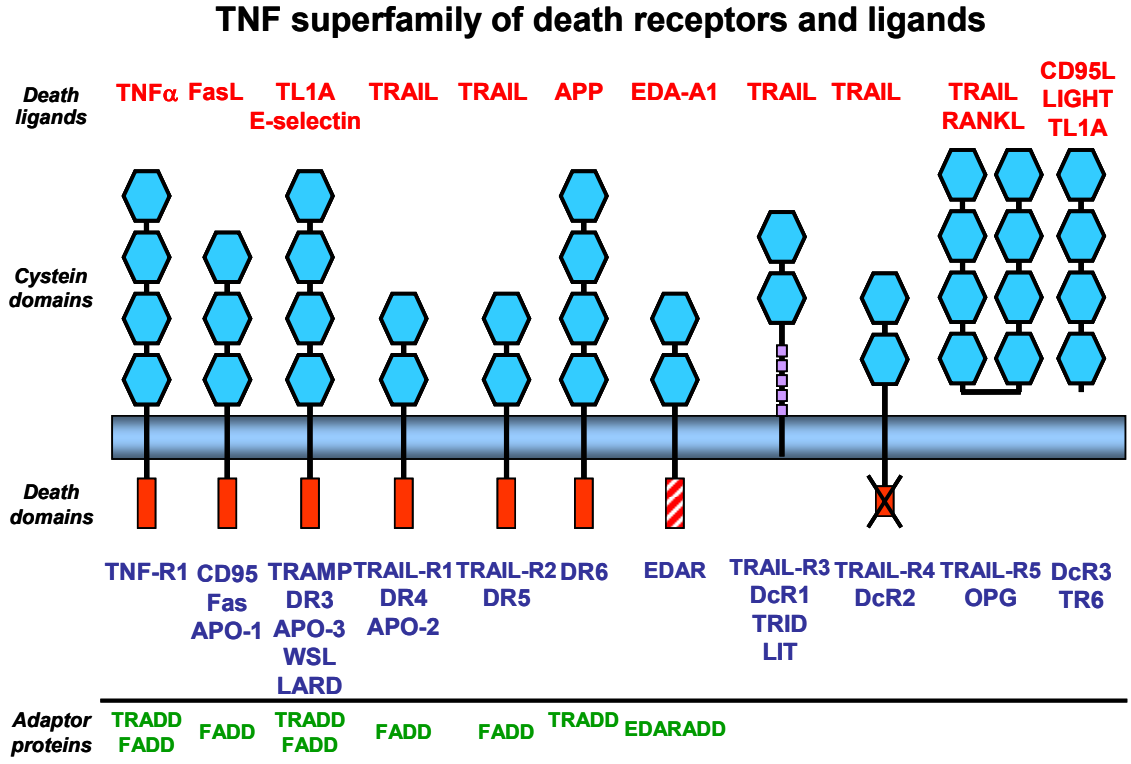

Figure 1.3 *Death receptors, decoy receptors and death ligands; Modified: Eberle 2003 FADD: Fas-associated death domain; TRADD: TNF receptor-associated death domain; EDAR: Ectodysplasin receptor. EDARADD: Ectodysplasin receptor-associated death domain.*

1.4. Melanoma resistance to apoptosis

The basis for drug resistance in melanoma is the deregulation of apoptosis, although other mechanisms including drug transport, detoxification, and enhanced DNA repair play additional roles. Several studies have revealed melanoma cell death control associated with the following three molecular changes: (1) activation of antiapoptotic factors, (2) inactivation of proapoptotic effectors and (3) reinforcement of survival signals (Soengas and Lowe, 2003).

Concerning the activation of antiapoptotic factors, an inhibitor of apoptosis, termed survivin, that is expressed in melanoma and required for maintenance of melanoma cell viability, has been identified (Grossman, 2001). Survivin is a single BIR protein of the IAP family which is also highly expressed in lymphoma and other cancer types. Another member of the IAP family, ML-IAP, has been detected in melanocytic nevi and shown to be over expressed in invasive and metastatic melanomas (Grossmann and Altieri, 2001), (Irmler et al, 1997), while remaining absent in normal human melanocytes (Vucic et al, 2000). Targeting of survivin and other apoptotic regulators increases the sensitivity of melanoma cells to cytotoxic drugs, and may provide a promising therapeutic approach to cancer (Ambrosini et al, 1997), (Kawasaki et al, 1998).

Concerning the inactivation of proapoptotic effectors, a decrease of the proapoptotic Bcl-2 proteins in SSM, Bax and Bak was significantly correlated with a poor prognosis: high Bax was associated with 10-year survival rates of 68%, whereas low Bax resulted in only 26% survival, and high Bak was associated with 10-year survival rates of 62%, whereas low Bak resulted in only 10% survival (Fecker et al, 2006). Apoptosis induced by immune responses is regulated by the Bcl-2 family of proteins. Many reagents have been developed against the Bcl-2 antiapoptotic proteins and clinical trials combining them with immunotherapy are awaited.

Immunotherapy based on T cell responses to the tumour is believed to involve killing of cancer cells by induction of apoptosis. The predominant mechanisms are death ligand-induced signaling mainly by TNF-related apoptosis-inducing ligand (TRAIL) mediated by CD4 T cells, monocytes and dendritic cells, and perforin/granzyme mediated apoptosis mediated by CD8 T cells and NK cells. Melanoma resistance against TRAIL involves loss of TRAIL death receptors, mainly TRAIL-R1/DR4 and down regulation of initiator caspases (Kurbanov et al, 2007) (Hersey and Zhang, 2009).

1.5. Objectives of thesis

So far, molecular studies have revealed several mechanisms of therapy resistance in melanoma, including deregulation of the apoptosis pathways in melanoma cells. Defects in both major apoptotic pathways have been described at multiple levels in melanoma. Knowledge of the precise molecular determinants responsible for apoptosis resistance in melanoma cells therefore represents a new way of devising unique therapeutic strategies to improve prognosis in late stages of the disease.
The main objective of this study was to investigate and describe new apoptosis regulatory molecules which have not yet been described in melanoma, but may play a role in apoptosis regulation. The following goals were coined at the initial stages of this study:

1. To determine the basic mRNA expression of CD95/Fas ligand, FasL and cellular FLICE inhibitory protein, cFLIP in melanoma cells compared to normal human melanocytes, NHM. The expression of FasL in melanoma cells has been controverted over the past years, while cFLIP has been suggested to enhance melanoma resistance to apoptosis.

2. To determine the basic mRNA expression of death receptor 3, DR3, death receptor 6, DR6 and decoy receptor, DcR3, as well as the ligand for DR3, TNF like ligand 1A, TL1A in melanoma cells compared to NHM. DR3 is probably the most interesting death receptor, since at least 12 distinct isoforms have been described. However, DR3 has not yet been described in melanoma cells or NHM. Simple or nested reverse-transcriptase polymerase chain reaction, RT-PCR should be used for determining mRNA levels in cell populations.

3. To verify the mRNA expression of the various mRNA products at the protein level using Western blots and immuno detection of proteins. Differences of expressions between the mRNA and protein levels could represent blockades in translation.

4. To determine the expression of the membrane bound, functional receptor in the cells using fluorescence-activated cell sorting, FACS analysis.

5. To investigate the functionality of DR3 by treating DR3 expressing cells with the natural DR3 ligand TL1A. Cell death can be detected using apoptosis and cytotoxicity assays.

In previous publications, the cellular investigations of the apoptosis signal ways in various cancerous cells, including melanoma, helped to understand the mechanisms of apoptosis resistance, leading to the formulation of new strategies against cancerous diseases.

The investigations of this doctoral thesis should assist in shedding more light on the expression and activity of new death receptors and ligands in melanoma cells compared to NHM, which in turn may benefit a deeper understanding of the pathological implications of this disease. This would help elucidate new strategies for the treatment of malignant melanoma in future.

In conclusion, combination of chemotherapy, immunotherapy and radiotherapy with agents that target apoptotic mechanisms in melanoma cells offers a new approach in the treatment of malignant melanoma.

2. Materials and Methods

2.1. Materials

2.1.1. Cell lines

Human melanoma cell lines

The established human melanoma cell lines A-375/CRL-1619 (Giard et al, 1973), Bro (Lockshin et al, 1985), JPC-298 (Aubert et al, 1993) and Mel-HO (Holzmann et al, 1988) originated from primary tumors, whereas Mel-2a (Bruggen et al, 1981), MeWo (Bean et al, 1975), M5 (Liao et al, 1975), SK-Mel-13, SK-Mel-19 and SK-Mel-23 (Carey et al, 1976) originated from metastases. Two melanoma cell populations, M186 and M221, were obtained from patients with histologically confirmed metastatic melanoma by surgical intervention (Raisova et al, 2000).

Normal human melanocyte cells

Several cultures of normal human melanocytes (NHM) were isolated from human foreskins after trypsin digestion (Eisinger and Marco, 1982), and were cultured as described by Eberle at al in serum free melanocyte growth medium (Eberle et al, 1999).

Jurkat cell line

Jurkat cells (Schneider et al, 1977) were established from the peripheral blood of a 14-year-old boy with acute lymphoblastic leukaemia (ALL) at first relapse in 1976. In this study, Jurkat cells were used as a control cell line since they represent a part of the human immune system. The cells were cultivated at 37°C with 5% CO_2 in a fully humidified atmosphere in RPMI 1640 medium, supplemented with 2 mM L-glutamine, 1% 10,000 IE penicillin / 10,000 µg/ml streptomycin and 10% FCS (Biochrom, Berlin, Germany). Passaging was done at a ratio of 1:3 every 2-3 days.

2.1.2. Cell culture media and solutions

The following media and solutions were used for cell culture:

Melanoma growth medium

1. 500 ml Dulbecco's modified Eagle's medium (DMEM) (4.5 g/l glucose; Invitrogen)
2. 50 ml Fetal calf serum (FCS) (Biochrom)
3. 5 ml 10,000 IE penicillin / 10,000 µg/ml streptomycin (Biochrom)

NHM growth medium

1. 500 ml MCDB 153 FX8105, Lot B054, specially manufactured for Dr. Eberle (Biochrom)
2. 147 mg CaCl2 (Calbiochem)
3. 250 µl Insulin (Sigma)
4. 1 µg BFGF (Boehringer Mannheim)
5. 2 ml BPE (Gibco)
6. 10 mg Apo-Transferrin (Calbiochem)
7. 250 µg Hydrocortison
8. 50 µl Cholera toxine (Calbiochem)
9. 5 ml 10,000 IE penicillin/ 10,000 µg/ ml streptomycin (Biochrom)

PBS, Ca^{2+}- und Mg^{2+}-free Phosphate buffered saline (Biochrom)

137 mM NaCl
2.7 mM KCl
10 mM Na_2HPO_4
2 mM KH_2PO_4 in Aqua dest., pH 7.4

Trypsin-EDTA-Buffer (Biochrom)

2.5% Trypsin
0.058% EDTA in Trypsin

2.1.3. Kits

1. RNeasy Midi Kit (isolation of total mRNA)	Qiagen
2. Ready-To-Go You-Prime-First-Strand Kit (Reverse transcriptase)	Amersham Biosciences
3. Taq DNA Polymerase 2.0 Master Mix Kit (PCR)	Biomol
4. Cell Death Detection ELISA Kit (quantification of apoptosis)	Roche
5. Cytotoxicity Detection Kit (quantification of cell toxicity)	Roche
6. BCA Protein Assay Kit (protein quantification)	Pierce

2.1.4. PCR primers

All primers used in the PCR were designed using PRIMER DESIGNER® version 1.01 after loading the nucleotide sequence of the various genes of interest.

Gene	*Primer sequence*	*Amplicon (bp)*
ß-actin	5' - ATC TGG CAC CAC ACC TTC TA - 3' 5' - GAT GTC CAC GTC ACA CTT CA - 3'	615
Fas-L	5' - CCT GTG TCT CCT TGT GAT GT - 3' 5' - TTG ACC AGA GAG AGC TCA GA - 3'	564
Fas-L	5' - TAC AGA AGG AGC TGG CAG AA - 3' 5' - TCC ATC ATC ACC AGA TCC TG - 3'	358
TLIA	5'- GGT CAC CAG TGG TCC AGT TAT T - 3' 5'- GAG TAG CCA GGA TTA CAG CCA T - 3'	666
LIGHT	5'- ATA CAA GAG CGA AGG TCT CAC G - 3' 5'- CTG AGT AGC TGG ATT ACA GGC A - 3'	735
EDA-A1	5'- AGG AGT CTC TGC CAG TTA CTT G - 3' 5'- GAA GAG TTC TGA TGA AGG CTG C - 3'	527
Granzyme-B	5'- CCT GAT ACA AGA CGA CTT CGT G - 3' 5'- CAC GCA CAA CTC AAT GGT ACT G - 3'	411
FLIP-L	5'- AGC TTC CCT AGT CTA AGA GTA GGA - 3' 5'- CCA TTA TGG AGC CTG AAG TTA TT - 3'	640
DR3	Ex2: 5' - GTG ACT GTG CCG GTG ACT TC - 3' Ex4: 5' - TAC TGC CAA CCA TGC CTA GA - 3' Ex9: 5' - TGC TGT CAG GAG GTG CTA GA - 3'	Ex2 / Ex4: 391 Ex2 / Ex9: 702
DR6	5' - GGC ATG AGA ATG GCA TAG AG - 3' 5' - CGG ATA CTG CAC ACC ACA AT - 3'	820
DcR3	5' - ACA CGC AGT TCT GGA ACT ACC T - 3' 5' - CTC TTG ATG GAG ATG TCC TGG A - 3'	450
Mannose-6-phosphate receptor	5'- ATG CAC TCT CTT CTT CTC CTG G - 3' 5'- GTC CTT ACA GCC TCC TTG TTC T - 3'	657
EDAR	5' - TGT AAC TGC CAA GAG CTC AGG A - 3' 5'- ATG GAA CAT GAG CTG ACA CTG G - 3'	618

2.1.5. Chemical substances

Acrylamid/Bisacrylamid (37.5 : 1)	BioRad
Agarose	Gibco BRL
Ammoniumpersulfat (APS)	BioRad

Bromphenolblue	BioRad
EDTA	Sigma
Ethanol	J.T. Baker
Ethidiumbromide	Sigma
Ficoll	Pharmacia
Formaldehyde	J. T. Baker
Glycerol	Sigma
Glycin	Serva
Isopropanol	J.T.Baker
Methanol	J.T. Baker
N-Acetyl-Leu-Leu-Nle-CHO (LLnL)	Calbiochem
Natriumchlorid	Merck
Natriumcitrate	Merck
Natriumhydroxide	Merck
Non-fat dry Milk	BioRad
Poly [d(I-C)]	Boehringer
Ponceau S	Sigma
Sodium dodecyl sulfate (SDS)	Amresco
TEMED	BioRad
Tris(hydroxymethyl-)aminomethan	Merck, Invitrogen
Tween-20	Calbiochem
Xylene cyanol	BioRad
β-Mercaptoethanol	Merck

2.1.6. Extraction buffers for cellular proteins

Lysis buffer (Bayer):

10 mM Tris-HCl, pH 7.5

144 mM NaCl

1% Nonidet P-40

0.5% SDS

Protease-Inhibiters

1 mM EDTA

2 mM PMSF

10 µg/ml Trasylol

20 µM leupeptin

10 µM pepstatin

2.1.7. Antibodies

Primary antibodies

Antigen	Source	Dilution	Company
DR3	rabbit	1:500	Santa Cruz Biotechnology
DR3	mouse	1:500	Santa Cruz Biotechnology
DcR3	rabbit	1:500	Santa Cruz Biotechnology
DcR3	goat	1:500	Santa Cruz Biotechnology
TL1A	rabbit	1:1,000	New England Biolabs
TL1A/VEGI	mouse	1:500	Santa Cruz Biotechnology
GAPDH	mouse	1:1,000	Sigma-Aldrich
β-actin	mouse	1:10,000	Sigma-Aldrich

Secondary antibodies:

horseradish peroxidase-labelled goat anti-rabbit (1:5,000) Dako Cytomation

horseradish peroxidase-labelled goat anti-mouse (1:5,000) Dako Cytomation

horseradish peroxidase-labelled mouse anti-goat (1:5,000) Dako Cytomation

Specific antibodies for Flow Cytometry (Alexis)

Antigen	Source	Dilution
DR3 (abcam)	mouse	1:100
IgG_1- (control)	mouse	1:100
mouse IgG_1 (PE-labelled)	goat	1:100

2.1.8. Antibiotics (stock solutions)

Ampicillin (Gibco)	50 mg/ml diluted in sterile water
Geneticin (Gibco)	100 mg Geneticin in 2 ml PBS

Hygromycin (Boehringer)	50 mg/ml in sterile filtered water
Doxycyclinhydrochloride (Sigma)	1 mg/ml diluted in sterile filtered water

2.1.9. Apoptosis stimulation agents

TL1A, (soluble, human, recombinant)	R & D Systems
TNF-α, (soluble, human, recombinant)	Sigma
Cycloheximide, CHX	Sigma

2.1.10. Solutions

10 x TBS (Tris-Buffered Saline), pH 7.6
24.2 g Tris-Base
80 g NaCl
In 1 l sterile water

4 x TBE (Tris-Borate EDTA), pH 7.4
0.356 M Tris-Base
0.356 M boric acid
0.01 M EDTA

10 x Klenow-Buffer, pH 7.6
0.5 M Tris HCl
0.1 M $MgCl_2$

50 x TAE (Tris-Acetat EDTA)
242 g Tris-Base
57.1 ml acetic acid
100 ml 0.5 M EDTA, pH 8.0
In 1 l sterile water

10% SDS, pH 7.2
100 g SDS
In 1 l sterile water

0.5 M EDTA, pH 8.0

186.1 g EDTA

In 1 l sterile water

2.1.11. Equipment

Centrifuges	Biofuge pico, Heraeus
	Biofuge fresco, Heraeus
	Laborfuge 400E, Heraeus
	Varifuge RF, Heraeus
	Centrifuge J2-21, Beckman Instruments
Imaging densitometer	Model GS-700, Bio-Rad
CO_2 -incubator	Heraeus
Drying oven	Heraeus
Electrophoresis -equipment	Mini Protean II, Bio-Rad
Electrophoresis-power supplies	Model 1000/500, Bio-Rad
ELISA photometer	Model 550, Bio-Rad
Flow cytometry system	FACS Calibur, BD Biosciences
Heating bath	1.002, GFL
Laminar flow bench	BSB 4A, Gelaire Flow Laboratories
Magnetic stirrer	MR 2000, Heidolph
Microscopes	Diavert, Leitz
	BX60F5, Olympus
pH- meter	pH 526, WTW
Precision balances	Kern 474, Gottl Kern& Sohn
	Type 2662, Sartorius
Western blot transfer equipment	Mini-Trans-Blot, Bio-Rad
BAS-1500 Phosphoimager	Fuji Photo

2.2. Methods

2.2.1. Cell culture

Human melanoma cell lines

Melanoma cells were grown in tissue culture flasks (75 cm^2) with 12 ml DMEM/10% FCS in a humidified atmosphere with 5% CO_2 at 37°C. All melanoma cell lines grew adherent at the bottom of the flasks. Growth medium was changed at least once in 3 days. Passaging was done for 70% - 80% confluent cells after washing with PBS. The cells were incubated with 1 ml trypsin solution at 37°C until detachment of the cells from the flask's bottom was seen (3-5 minutes). Cells were harvested in 10 ml medium with 10% FCS to stop the proteolytic activity of trypsin. The cells were then pelleted at 200 x g for 5 minutes. After aspiration of the supernatant, the cells were resuspended in DMEM/10% FCS, and distinct numbers of cells were distributed into new flasks. The first change of medium occurred after 36 hours.

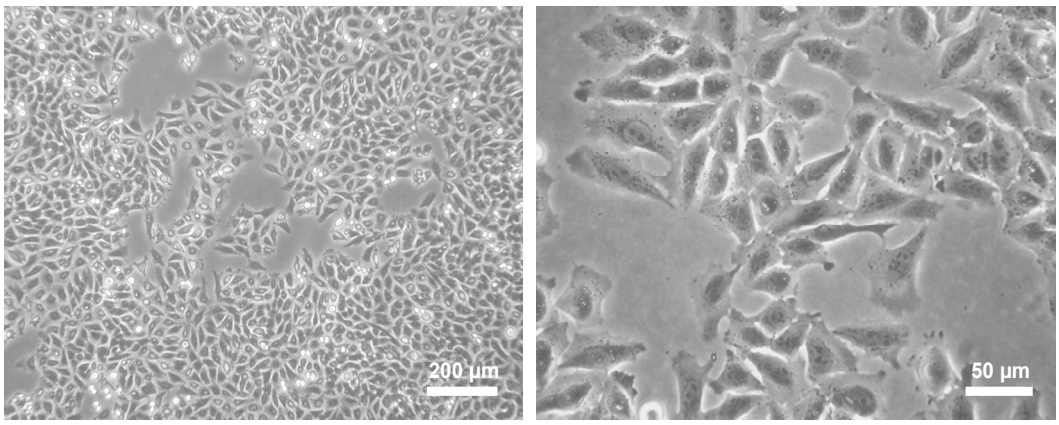

Figure 2.2.1 ***A-375***

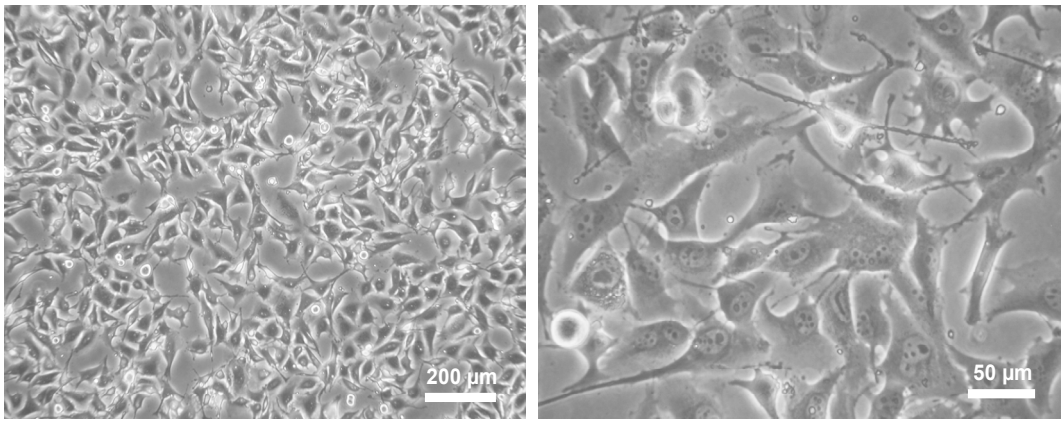

Figure 2.2.2 ***Mel-2a***

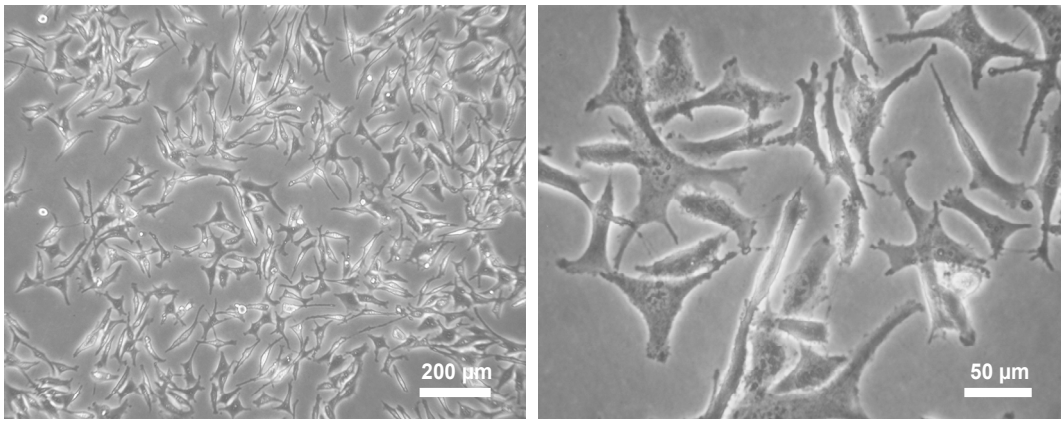

Figure 2.2.3 ***Mel-HO***

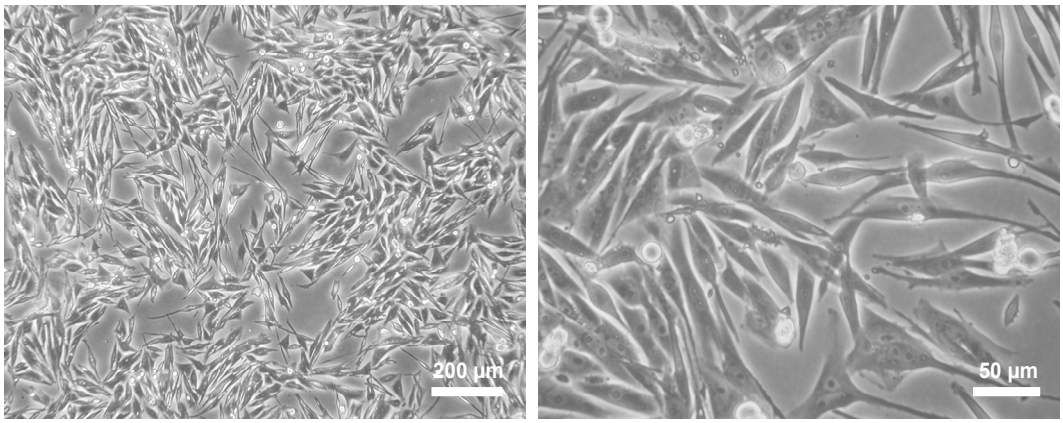

Figure 2.2.4 ***Me-Wo***

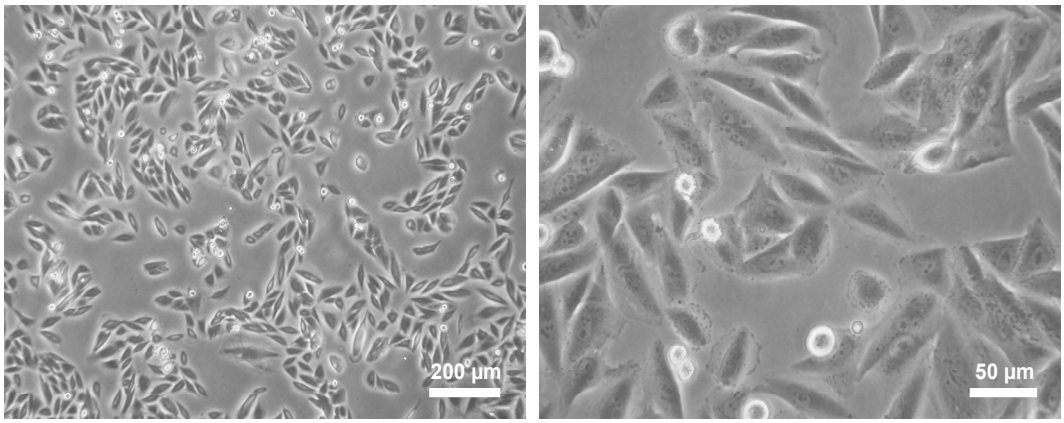

Figure 2.2.5 ***SK-Mel-13***

Normal human melanocytes, NHM

NHM cultures were established from human foreskins. The sterile foreskins were obtained from surgical circumcisions of babies and/or toddlers about 2 – 4 hours after surgery. They were prepared in a laminar flow hood using a pair each of sterile forceps, curved scissors and surgical scalpel blade in Ca^{2+}- und Mg^{2+}-free PBS with penicillin/streptomycin and amphotericin in a

sterile Petri dish. First, the skin ring was cut open and the fat and subcutaneus tissue trimmed off with scissors. The skin was cut into pieces of ca. 5 mm x 5 mm using the surgical scalpel blade. The skin pieces were transferred in a Petri dish containing Ca^{2+}- und Mg^{2+}-free PBS with 0.25% trypsin, and incubated overnight (16 – 18 hours) at 4°C in a refrigerator. On the next day, the samples were removed from the refrigerator and incubated at 37°C for 1 hour. The thin, translucent epidermis layers were separated from the dermis using the forceps. The harvested epidermal sheets were then transferred to a new Petri dish with NHM growth medium/10% FCS. Using the forceps, the cells were mechanically (beating and mincing) released from the epidermal sheets, and the rest epidermis pieces were discarded. The cell suspension was collected from the Petri dish with repetitive pipet motions and transferred into a centrifuge tube, then centrifuged at 200 x g for 5 minutes. The supernatant was carefully aspirated; the cell pellet was resuspended with 10 ml NHM medium/10% FCS, and the cell number determined by counting with the Neubauer haemocytometer. About 10 x 10^6 cells were seeded in Petri dishes with NMH medium/2% FCS + Geneticin (100 µg/ml) and incubated at 37°C in 5% CO_2 for 48 hours without disturbance. Medium change afterwards was done twice a week with FCS and Geneticin free NHM medium after washing the cells with Ca^{2+}- und Mg^{2+}-free PBS to remove non adherent cells. Passaging was done at about 70 – 80% confluence as described for melanoma cells previously (2.2.1); after each passage, the cells were seeded with NHM medium/2% FCS overnight, then medium change was performed with FCS free NHM medium.
In this study, an NHM cell line is defined as the melanocytes obtained from a distinct foreskin preparation, thus, the cells were not pooled from different foreskins.

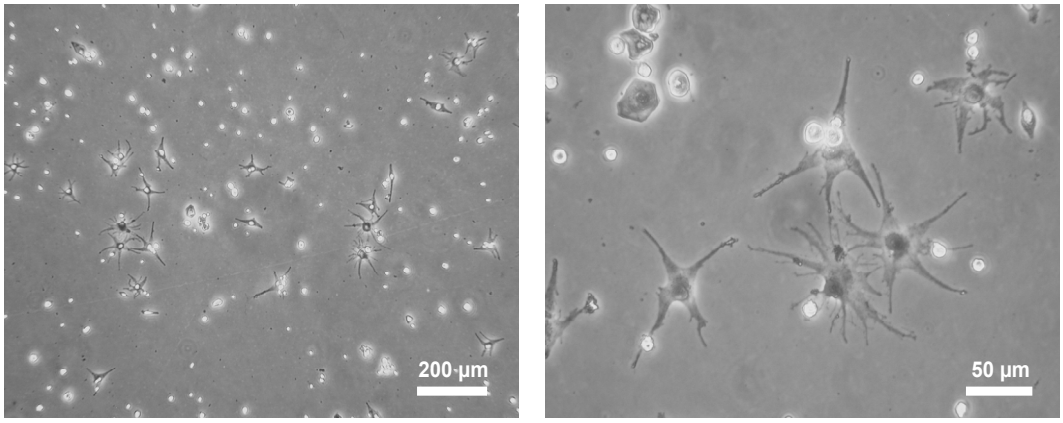

Figure 2.2.6 NHM primary culture

7 days after primary seeding, NHM are seen as multidentritic cells.

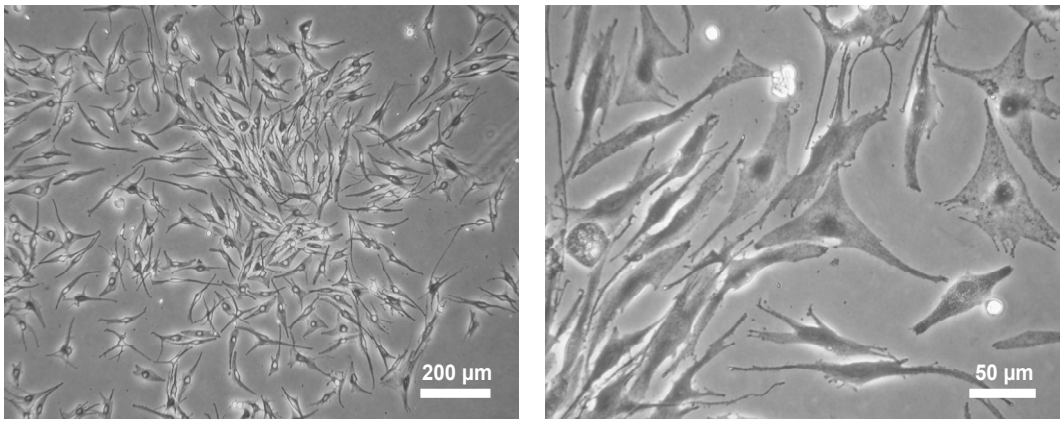

Figure 2.2.7 NHM primary culture

21 days after primary seeding, the NHM reach a confluence of 60 – 80%

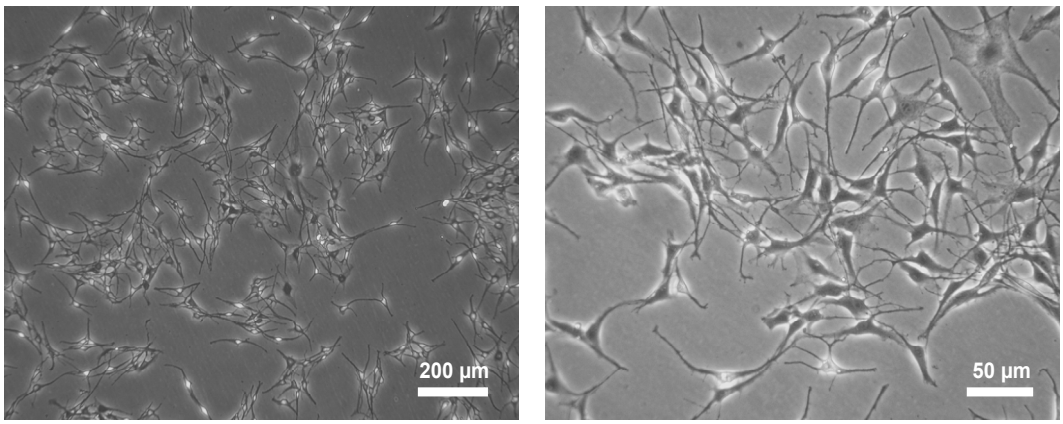

Figure 2.2.8 NHM primary culture

After the first passage, NHM are seen as oligodendritic cells

All cell culture procedures were carried out under sterile conditions using sterile or sterilised equipment under a laminar flow hood.

2.2.2. Freezing and thawing of cells

About 70% confluent cells with fresh growth medium overnight were washed with 10 ml PBS and trypsinized as described previously (2.2.1). The cells were then pelleted at 200 x g for 5 minutes. After aspiration of the supernatant, the cells were resuspended in a freezing solution containing 10% DMSO and 25% FCS. The cell suspension was transferred into 1 ml cryotubes. The presence of DMSO in the freezing solution prevents the formation of ice crystals within cells. Freezing of cells has to be a slow process; therefore the freezing tubes were wrapped up in thick soft tissue paper and kept for 2 hours at -20°C, then overnight at -80°C. The stock tubes were transferred to a container with liquid nitrogen at -196°C for long term storage.

Thawing of cells was done rapidly; conserved cells were thawed in a 37°C water bath and suspended in 10 ml of warm DMEM/10% FCS. The cells were then pelleted by centrifugation at 200 x g for 5 minutes. After aspiration of the supernatant, the cell pellet was resuspended in 12 ml culture medium and seeded in a culture flask (75 cm^2).

2.2.3. Isolation of total mRNA and quantification

Total mRNA was isolated from the cells using RNeasy® Midi kit (Qiagen) according to the protocol in the handbook. All steps were carried out at room temperature using sterile equipment in an RNase free laboratory area. 70 – 80% confluent cells which had had fresh growth medium overnight were washed with 10 ml PBS and harvested by trypsinization as described previously, and then pelleted at 200 x g for 5 minutes. After aspiration of the supernatant, the cells were resuspended in 10 ml PBS and counted with a Neubauer haemocytometer. About 6 x 10^6 cells were determined for total mRNA isolation. After pelleting the cells at 200 x g for 5 minutes, 600 µl buffer RLT with β-ME (β-Mercaptoethanol) (according to manufacturer's instructions, 10 µl β-ME must be added to 1 ml buffer RLT before use) were added to the cell pellet, the cells were disrupted by re suspension in the buffer. The cell suspension was then transferred to a QIAshredder® spin column and the cells were homogenized by centrifugation at maximum speed in a Biofuge® pico centrifuge for 2 minutes. 600 µl 70% ethanol was added to the sample and mixed thoroughly by vigorous shaking. The sample was then applied to an RNeasy midi column placed in the supplied collection tube and mRNA separation was done by centrifugation at maximum speed as described above for 2 minutes (mRNA is bound onto the RNeasy silica-gel membrane). The RNeasy midi column was then placed in the collection tube again and 700 µl buffer RW1 added. The tube was spun at maximum speed for 15 seconds to wash the column and the flow through discarded. 500 µl buffer RPE was added to the column, the tube was spun at maximum speed for 15 seconds to wash the column and the flow through was discarded. A last washing step was carried out by adding 500 µl buffer RPE on the column again and centrifugation for 2 minutes to dry the silica-gel membrane (to ensure that no ethanol is carried over during elution since ethanol is added to RPE before use).

To elute the total mRNA, the RNeasy midi column was placed in a fresh collection tube, 100 µl RNase-free water was directly pipetted onto the RNeasy silica-gel membrane, the tube was gently closed and let stand for 1 min, and then centrifuged at maximum speed in the Biofuge® pico for 2 min. The total mRNA of each cell line was thus extracted in about 100 µl RNase-free water.

Quantification and storage of total mRNA

The concentration of mRNA was determined by measuring the absorbance at 260 nm (A_{260}) in a spectrophotometer. To ensure significance, readings were carried out to be greater than 0.15. An absorbance of 1 unit at 260 nm corresponds to 40 µg of mRNA per ml (A_{260}=1: 40 µg/ml). This relation is valid for measurements in water, therefore the mRNA samples were diluted with water and water was used to zero the spectrophotometer. The cuvettes used for measurement were washed with 0.1M NaOH, 1 mM EDTA followed by RNase-free water to ensure they are RNase-free. Total mRNA yields varied between 8 – 14 µg/µl for melanoma cell lines, and 1.5 – 5 µg/µl for NHM.

Samples of mRNA were stored at -20°C over short periods of time (several weeks) or at -70°C over longer periods of time (for months/years).

2.2.4. Reverse transcriptase and polymerase chain reaction, RT-PCR

The polymerase chain reaction serves to copy DNA. It uses repeated cycles, each of which consists of three steps: (1.) the reaction solution containing DNA molecules is heated to 95°C. This causes the two complementary strands to separate, a process known as denaturing or melting. (2.) Lowering the temperature to 55°C causes the primers to bind to the DNA, a process known as hybridisation or annealing. The resulting bonds are stable only if the primer and DNA segment are complementary, i.e. if the base pairs of the primer and DNA segment match. (3.) Extension: The temperature is again increased, this time to 72°C. This is the ideal working temperature for the polymerases used, which add further nucleotides to the developing DNA strand.

Each time these three steps are repeated the number of copied DNA molecules doubles.

The polymerases can be used in the PCR to copy any nucleic acid segment of interest. Usually this is DNA; if RNA needs to be copied, it is usually first transcribed into DNA with the help of the enzyme reverse transcriptase, a method known as reverse transcription PCR (RT-PCR)

Reverse transcriptase, RT (First strand cDNA synthesis)

RT was performed using Ready-To-Go You-Prime First-Strand Beads® (Amersham Biosciences). Each tube of Ready-To-Go reaction mix beads contains two beads needed for a complete first strand reaction. First strand cDNA synthesis was carried out according to the protocol in the handbook supplied by the manufacturer. First, 5 µg of total mRNA was brought to a volume of 30 µl in a sterile, RNase-free microcentrifuge tube using DEPC-treated water. The RNA sample was then heated at 65°C for 10 min, and then chilled on ice for 2 min. The two

beads of the first strand reaction mix were brought to the bottom of the reaction tubes, e.g. by tapping. The RNA samples were transferred to the tube of first strand reaction beads. DEPC-treated water was added to the chosen primer, pd(N)6, to a concentration of 0.2 µg/l; 3 µl of the primer solution was added to the reaction mix and left at room temperature for ca. 1 min. The reaction mix was then vortexed, and then centrifuged to collect the contents at the bottom of the tube. The samples were incubated at 37°C for 60 min.

Polymerase chain reaction, PCR

PCR was performed using Taq DNA Polymerase 2.0 Master Mix RED (Biomol), which is a ready-to-use 2.0 x reaction mix containing 150 mM Tris-HCl pH 8.5; 40 mM $(NH4)_2SO_4$, 3 mM $MgCl_2$, 0.2% Tween 20®, 0.4 mM dNTPs; 0.05 units/µl Amplicon Taq DNA polymerase and an inert red dye. PCR reaction mixtures were set up in a separate area to avoid contamination using sterile equipment on an ice-bath. First, the Taq Master Mix RED was thawed on ice and briefly vortexed. 5 µl of the cDNA (from the first strand synthesis) was used for the PCR reaction. Specific primers were brought to final concentrations of about 1 µM in distilled water. After bringing the cDNA, specific primers and distilled water to a volume of 25 µl and then mixing the solution by gently vortexing, 25 µl of the Taq Master Mix RED was added to the solution to a total PCR reaction volume of 50 µl. The reaction samples were then placed in a thermal cycler, and the cycler was programmed according to the reaction conditions of PCR depending on specific primers in use. Nested PCR was carried out for DR3 and FasL.

PCR conditions

Gene	**PCR programme**
ß-actin	1 min 94°C, (1 min 94°C, 1 min 64°C, 1 min 72°C) 30 x, 10 min 72°C, 1 hr 4°C
TLIA	1 min 94°C, (1 min 94°C, 1 min 62°C, 1 min 72°C) 30 x, 10 min 72°C, 1 hr 4°C
DcR3	1 min 94°C, (1 min 94°C, 1 min 62°C, 1 min 72°C) 30 x, 10 min 72°C, 1 hr 4°C
DR3 *first reaction*	1 min 94°C, (1 min 94°C, 1 min 65°C, 1 min 72°C) 20 x, 10 min 72°C, 1 hr 4°C
DR3 *second reaction*	1 min 94°C, (1 min 94°C, 1 min 60°C, 1 min 72°C) 20 x, 10 min 72°C, 1 hr 4°C
DR6	1 min 94°C, (1 min 94°C, 1 min 60°C, 1 min 72°C) 30 x, 10 min 72°C, 1 hr 4°C
FLIP	1 min 94°C, (1 min 94°C, 1 min 60°C, 1 min 72°C) 30 x, 10 min 72°C, 1 hr 4°C
FasL *first reaction*	1 min 94°C, (1 min 94°C, 1 min 59°C, 1 min 72°C) 20 x, 10 min 72°C, 1 hr 4°C
FasL *second reaction*	1 min 94°C, (1 min 94°C, 1 min 61°C, 1 min 72°C) 20 x, 10 min 72°C, 1 hr 4°C

2.2.5. Agarose gel electrophoresis

For analysing PCR products, the samples are loaded into 1-1.4% agarose gel stained with EtBr (ethidium bromide), the gel is then run at 90-150 volts depending on size, and the DNA bands are visualised using a UV-transilluminator. For evaluating the DNA fragment's size, a DNA ladder mix is loaded on the gel and run parallel with the samples.
1.2% agarose gels were mostly used to analyse PCR products. The following substances were needed for the production of a 1.2% agarose gel:

Substance	**Quantity**
50 x TAE	2 ml
Water, bi-distilled	98 ml
Ethidium bromide, 10 mg/ml	2 µl
Agarose	1.2 g

Protocol:
First, 1.2 g agarose was weighed out and mixed with 1 x TAE in a conical flask. The mixture was placed in a microwave on full power for approx. 4 min or until it comes to a rolling boil. The hot solution was placed on a stirrer and allowed to cool to about 60-65°C, then EtBr was added. During cooling, the gel tray was carefully taped up at either end. The cooled agarose solution was poured into the gel tray, a comb of the desired number of lanes was inserted into the gel, and air bubbles were carefully removed using an RNase/DNase free pipette tip. The gel was left to harden for about 1 hour at room temperature.
After complete polymerisation of the agarose gel, the tapes were removed from the gel tray, which was then placed in the chamber so that the comb was at the cathode (negative terminal) and the samples could run through the gel towards the anode (positive terminal). The chamber was filled up with 1 x TAE so that the gel was completely covered with buffer and the comb carefully removed from the gel. A DNA ladder mix was loaded into the first well: MassRuler™ DNA Ladder Mix, ready-to-use (Fermentas #SM0403). The PCR samples, which already contained a red dye (Taq DNA Polymerase 2.0 Master Mix RED), were then loaded into the appropriate wells. The electrophoresis was run at 100 volts (150 mA) until the dye reached 2/3 of the gel length. The gel was photographed on a UV-transilluminator using a CCD camera (Polaroid).

2.2.6. Extraction of cellular protein and quantification

Extraction of cellular proteins was done after cells have grown to a confluence of about 70-80% using lysis buffer (Bayer) with freshly added protease inhibitors (Materials, 2.1.6).

Protocol:

70 – 80% confluent cells which had had fresh growth medium overnight were washed with 10 ml PBS and the PBS was completely aspirated. The cell culture flask was immediately placed on an ice bath and 500 µl lysis buffer containing protease inhibitors was pipetted onto the cells and distributed evenly on the cells. All steps were carried out on an ice bath using sterile equipment. Using a cell scraper, the cells were removed from the bottom of the culture flask and collected into an Eppendorf tube. The cell lysate was then passed 10 x through an insulin syringe, and then centrifuged at 10,000 x g for 10 min at 4°C to collect cell debris. The supernatant (containing cellular protein) was transferred into fresh Eppendorf tubes and quantified.

Quantification and storage of cellular proteins

Protein quantification was done with the BCA (Bicinchoninic acid)-kit from Pierce (Weiskirchen), which uses the principle of the biuret reaction, where the amino group of proteins reduces copper (II)-ions to copper (I)-ions in an alkaline medium. Copper (I)-ions then react with two molecules of BCA, producing a stable chelate complex (violet in colour), which exhibits maximal absorption at 562 nm. The kit contains solution A (#23223) and solution B (#23224).

Solution A

Components	Concentration	Quantity
disodium bicinchoninate	26 mM	10 g
disodium carbonate-monohydrate	161 mM	20 g
disodium tatrate-dihydrate	7 mM	1.6 g
NaOH	100 mM	4 g
sodium hydrogen carbonate	113 mM	9.5 g
water, double distilled		up to 1,000 ml

Solution B

Components	Concentration	Quantity
copper II sulphate-pentahydrate	160 mM	1 g
water, double distilled		up to 25 ml

Protocol:

First, a protein standard ranking was prepared by successive dilutions of BSA (bovine serum albumin) in cell lysis buffer (as used for protein extraction). Protein samples were also diluted in lysis buffer (1:5). 10 µl of the diluted protein sample and the standard ranking probes were transferred to a micro titer plate in triplicates. A working reagent was prepared by mixing fifty parts of Solution A to one part of Solution B. 200 µl of working reagent was added to each sample in the micro titer plate and the probes were incubated at 37°C for 30 min.

The absorption was then measured at 550 nm with an ELISA photometer. The protein concentration was calculated using the standard rank curve under consideration of the dilution factor of 1:5. Protein samples were then stored as aliquots of 50 µl at -70°C.

2.2.7. Sodium dodecyl sulphate polyacrylamide gel electrophoresis, SDS-PAGE

SDS-PAGE is a technique used to separate proteins according to their electrophoretic mobility. The solution of proteins to be analyzed is first mixed with SDS, an anionic detergent which denatures secondary and non–disulfide–linked tertiary structures, and applies a negative charge to each protein in proportion to its mass. Besides the addition of SDS, proteins are briefly heated to near boiling in the presence of a reducing agent, such as dithiothreitol (DTT) or 2-mercaptoethanol (ß-mercaptoethanol/BME), which further denatures the proteins by reducing disulfide linkages, thus overcoming some forms of tertiary protein folding, and breaking up quaternary protein structure (oligomeric subunits). This is known as reducing SDS-PAGE.

A tracking dye is added to the protein solution to allow the experimenter to track the progress of the protein solution through the gel during the electrophoretic run. Depending on their size, each protein will move differently through the gel matrix: short proteins will more easily fit through the pores in the gel, while larger ones will have more difficulty. Thus, after electrophoresis, smaller proteins will have travelled farther down the gel, while larger ones will have remained closer to the point of origin. Therefore, proteins may be separated roughly according to size (and therefore, molecular weight). Molecular markers of known molecular weight are run in a separate lane in the gel, in order to calibrate the gel and determine the weight of unknown proteins by comparing the distance travelled relative to the marker, which is measured in kilo Daltons, kDa.

The electrophoresis-equipment, Mini Protean II (Bio-Rad) was assembled to run two parallel gels according to manufacturer's instructions.

Working buffers and solutions

Separating gel buffer, pH 8.8	Stacking gel buffer, pH 6.8	5 x Electrophoresis buffer
0.64 M Tris	0.33 M Tris	0.125 M Tris
0.12 M Tris-HCl	0.2% SDS	1 M Glycin
0.2% SDS		0.5% SDS

Electrophoresis gels

Separating gel, 12%	Stacking gel, 5%
1.0 ml aqua bidistilled	1.6 ml aqua bidistilled
5.0 ml separating gel buffer	2.5 ml separating gel buffer
4.0 ml Acrylamid (30%)	0.84 ml Acrylamid (30%)
100 µl APS (10%)	50 µl APS (10%)
10 µl TEMED	5 µl TEMED

Protocol:

The components of the separating gel were first mixed in a test tube, noting that polymerisation begins upon addition of APS and TEMED. The gel was poured into the assembly of glass plates on the casting stand. About 500 µl isopropanol was pipetted onto the separating gel and the gel was left to completely polymerise for 30 min at room temperature. After polymerization, the isopropanol layer was completely removed with the edge of a Whatman 3 MM paper. The stacking gel was then directly poured onto the separating gel and a clean plastic comb was inserted immediately into the stacking gel, avoiding the formation of air bubbles, and left to polymerize for another 30 min. The comb was removed from the stacking gel and the wells in the gel were washed with electrophoresis buffer. The gel unit was then inserted into the electrophoresis chamber filled with 1 x electrophoresis buffer. The protein samples were prepared by adding three volumes of protein sample to one volume of sample buffer.

4 x Sample buffer
0.25 M Tris, pH 6.8
2% SDS
40% Glycerol
20% ß-MCE
0.04% Bromophenol blue
0.2 M DTT

About 30 µg of protein was mixed with sample buffer in the given ratio and the mixtures were incubated at 95°C for several minutes, and then loaded into the lanes of the stacking gel with a Hamilton syringe. Electrophoresis was performed at 75 V for 15 min, and then at 100 V. 5 µl of the protein marker Precision Plus Protein™ Standards, Dual Color (Bio-Rad) was loaded in the first lane of each gel to quantify molecular weights of proteins. When the bromphenol blue had reached the bottom of the running gel, electrophoresis was ended and the gel units disassembled for Western blotting.

2.2.8. Western blotting

In order to make the proteins accessible to antibody detection, they were moved from within the gel onto a membrane made of nitrocellulose. The membrane was placed on top of the gel, and a transfer stack of filter papers and sponges arranged as shown in figure 2.2.9. The entire stack was placed in a buffer solution and the proteins were transferred using electro blotting, i.e. an electric current was used to pull proteins from the gel into the nitrocellulose membrane. The proteins move from within the gel onto the membrane while maintaining the organization they had within the gel. As a result of this "blotting" process, the proteins were exposed on a thin surface layer for detection.

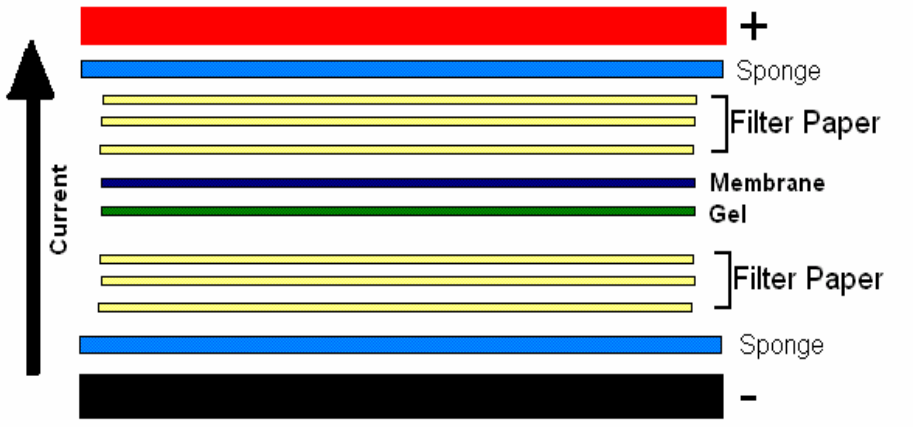

Figure 2.2.9 Transfer stack

A stack is put together in the above order from cathode to anode. The proteins are negatively charged (interactions with SDS) and are transferred from the gel onto the nitrocellulose membrane through electric current in a blotting buffer

Transfer (blotting) buffer, pH 8.3
48 mM Tris
30 mM Glycine
0.04% SDS
20% Methanol

Protocol:

After assembling the gel in the transfer stack as shown above in (figure 2.2.9), air bubbles were removed by rolling a glass pipette over the sandwich, which was then placed in the blotting chamber with the membrane towards the anode (positively charged). The chamber was filled with transfer buffer to completely cover the electrode panels. After placing a magnetic stirrer in the buffer, the chamber was placed in an ice bath and blotting was performed at 100 V for 1.5 hrs. The uniformity and overall effectiveness of transfer of protein from the gel to the membrane was checked by staining the membrane with Ponceau S dye by incubating the membrane for 2-5 min in Ponceau S upon gentle shaking at room temperature. Ponceau S was then washed off the membrane by washing the membrane several times in 1 x TBS.

2.2.9. Immunodetection of proteins

The blotted nitrocellulose membrane can be detected for various proteins of interest with modified antibodies linked to a reporter enzyme, either in a single or two step immunodetection procedure. Enhanced chemiluminescence (ECL) is then used to detect the antibody, which is bound to HRP (horseradish peroxidase). The enzyme complex catalyzes the conversion of the ECL substrate into a sensitized reagent in the vicinity of the molecule of interest, which on further oxidation by hydrogen peroxide, produces a triplet (excited) carbonyl which emits light when it decays to the singlet carbonyl. Enhanced chemiluminescence allows detection of minute quantities of a bio molecule. Proteins can thus be detected in quantities well below the detection limit for most assay systems.

Blocking

Blocking of non-specific binding is achieved by placing the membrane in a dilute solution of protein, either Bovine serum albumin (BSA) or non-fat dry milk with a minute percentage of detergent such as Tween 20. The protein in the dilute solution attaches to the membrane in all places where the target proteins have not attached. Thus, when the antibody is added, there is no

room on the membrane for it to attach other than on the binding sites of the specific target protein.

Protocol:

After washing off all traces of Ponceau S from the membrane, blocking was performed with 5% non-fat dry milk (Bio-Rad) in 1 x TBS (blocking buffer) for 1 hr at room temperature under constant gentle agitation. After blocking, the membrane was incubated in 10 ml dilute solution of primary antibody in blocking buffer either for 1 hr at room temperature or overnight at 4°C under gentle agitation. The membrane was then washed 3 x 5 min in TBST (1 x TBS/0.05% Tween 20), and then incubated in 10 ml dilute solution of secondary antibody in blocking buffer for 1 hr at room temperature under gentle agitation. The secondary antibody solution was discarded and the membrane was washed 3 x 5 min in TBST, then finally for 5 min in TBS only. For the chemiluminescence reaction, the membrane was incubated for 1 min in Super Signal working solution at room temperature, a luminol/enhancer solution and a stable peroxide solution purchased from Perbio. The membrane was then placed in a protective plastic sheet, excess substrate solution and air bubbles were removed by gentle smoothening. The membrane was placed with an X-ray film and enclosed in a film exposure cassette. The duration of the film exposure varied according to the intensity of the signal.

2.2.10. Apoptosis detection

Apoptosis can be assessed by various methods, among which the measurement of DNA fragmentation is highly reliable. DNA fragmentation, representing the cleavage of chromosomal DNA in oligonucleosome-sized fragments, is a biochemical hallmark of apoptosis. A specific endonuclease (CAD), which is activated at the end of the apoptosis signalling cascade, cleaves the nuclear DNA at accessible internucleosomal sites resulting in the release of mono- or oligonucleosomes in the cytoplasm. Released DNA fragments are therefore discrete multiples of a 180 bp subunit. They remain bound to histones and can be detected using a combination of anti-histone and anti-DNA antibodies. Detection of mono- and oligonucleosomes in the cytoplasm is possible due to occurrence of DNA fragmentation several hours before plasma membrane breakdown. Here, DNA fragmentation was measured using a commercially available kit "Cell Death detection ELISA plus" from Roche Diagnostics. The test was performed on streptavidin-coated microtiter plate. After transfer of cell lysates to the microtiter wells, a mixture of anti-histone biotin-antibodies and peroxidase-coupled anti-DNA antibodies was added. The anti-histone antibodies recognised the protein fraction of the nucleosomes and

immobilised it via streptavidin-biotin interaction on the microtiter plate. The DNA coiled around histones was recognised by the peroxidase-coupled anti-DNA antibodies. A chromogenic peroxidase substrate was added to measure the absorption by an ELISA photometer.

Reagents used (included in the kit):

anti-histone-antibodies, coupled with biotin
anti-DNA-antibodies, coupled with peroxidase
incubation buffer
lysis buffer
positive control
substrate solution (2,2'-azino-di[3-ethylbenzthiazoline-sulfonate])

immunoreagent solution for 30 probes:

120 µl of anti-histone-antibodies
120 µl of anti-DNA-antibodies
2160 µl of incubation buffer

Protocol:

Melanoma cells were treated either with TL1A at concentrations of 25 ng/ml, 50 ng/ml, 100 ng/ml and 200 ng/ml, with CHX (10 ng/ml) or with TNF-α (10 ng/ml) for different lengths of time. Cells were seeded in 6-well plates (2 x 10^5 cells per well). On the following day, growth medium was replaced by 1 or 2 ml medium containing treating agents. After the specific incubation period, plates were centrifuged at 200 x g for 5 min. The supernatant was removed, and cells were lysed in lysis buffer (1 ml/well) for 30 min at room temperature with moderate shaking. A cytosolic extract was prepared by centrifugating the plates at 300 x g for 10 min. Subsequently, 5 µl of the supernatant with 15 µl of lysis buffer was added to streptavidin-coated microtiter plates. The specimens were then incubated for 2 h with 80µl of the immunoreagent solution at room temperature with moderate shaking. After washing, each well received 100 µl substrate solution. Incubation at room temperature was performed until specific colour reaction appeared in the positive control (usually 10 min). Absorption at 409 nm was measured using an ELISA photometer. Relative apoptosis rates were determined by calculating substrates from treated cells with respect to untreated control cells, which were set to 1.

2.2.11. Cytotoxicity detection

Cell necrosis is characterised by plasma membrane damage and release of the intracellular content in the surrounding medium. This type of cell death can be evaluated by measuring the uptake or exclusion of vital dyes (such as trypan blue, eosin Y, nigrosine, propidium iodide or

ethidium bromide), the release of radioactive isotopes from previously labelled cells or the activity of cytoplasmatic enzymes which are released upon cell death. Lactate dehydrogenase (LDH) is a relatively stable cytoplasmatic enzyme, present in almost all cells, which is rapidly released in the culture supernatant after damage of the plasma membrane. LDH activity was evaluated with the "Cytotoxicity Detection Kit (LDH)" from Roche Diagnostics (Mannheim, Germany), by means of a two-step enzymatic reaction. In the first step, NAD^+ is reduced to $NADH/H^+$ by the LDH-catalysed oxidation of lactate to pyruvate. In the second step, the catalyst (diaphorase) transfers H/H^+ from $NADH/H^+$ to the tetrazoliumsalt 2-[4-iodophenyl]-3-[4-nytrophenyl]-5-phenyltetrazolium chloride, which is then reduced to red coloured formazan. The increase in LDH enzyme activity following plasma membrane cell damage correlates with the amount of formed formazan which can be measured by an ELISA photometer at 490 nm. The cell culture supernatant can be stored for a few days at 4°C without any changes of LDH enzyme activity. The increase in LDH activity in the supernatant is proportional to the quantity of damaged cells.

Reactive mixture:

catalyst-solution 2.5 µl
dye solution 118 µl

Protocol:

Cells were seeded at 2 x 10^5 cells per well in 6-well plates and allowed to adhere overnight. On the next day, cells were treated with apoptosis inducing agents for a certain period of time. The cell supernatant was then collected and centrifuged at 200 x g for 5 minutes. 100 µl of the supernatant was transferred into a microtiter plate and mixed with 100 µl of reaction buffer. The plate with samples was wrapped in light protecting paper (aluminium foil) and incubated for 30 min at room temperature. The absorption at 490 nm was measured by an ELISA photometer. Relative cytotoxicity rates determined after treatment with TL1A were calculated with respect to untreated control cells, which were set to 1.

2.2.12. Fluorescence-activated cell sorting, FACS

The concept of flow cytometry is the measurement of fluorescent cells flowing one at a certain point in time through a region of examination where multiple biophysical properties of each cell can be measured at rates of over 1,000 cells per second. To enable the measurement of biological properties, cells are usually stained with fluorescent dyes, which bind specifically to cellular

constituents. The dyes emit light at a long wavelength after excitation by a laser beam. The emitted light is picked up by detectors, and the signals are digitalized so that they may be stored for later display and analysis. Biological targets can be stained either directly by specific antibodies, which contain fluorescent dyes, or indirectly by using secondary labelled antibodies. This method is the choice for measuring surface expression of death receptors, such as DR3.

Solutions used:

Detaching solution

Components	Concentration	Quantity
EDTA	0.5 M	50 µl
PBS		50 ml

Washing solution

Components	Concentration	Quantity
BSA	1%	500 mg
PBS		50 ml

Fixation solution

Components	Concentration	Quantity
paraformaldehyd	1%	300 mg
PBS		30 ml

Protocol:

Cells were grown in culture flask (75 cm^2) for two days and received fresh medium a day before the FACS analysis. Cells were detached with EDTA/PBS for 10 min to avoid cell membrane damage, which can be caused by using trypsin/EDTA. Detached cells were collected with PBS in 50 ml tubes and centrifuged at 200 x g for 5 min. After washing with 1% BSA/PBS, aliquots of $5x10^5$ cells in 100 µl 1% BSA/PBS were incubated for 30 min with mAb against DR3 (Abcam, 1:100). The isotypic monoclonal mouse IgG_1 antibody (Alexis, 1:100) was used as a negative control. After incubation, cells were washed two times with 1% BSA/PBS. After that, they were incubated for 30 min with the secondary phycoerythrin-labeled goat anti-mouse IgG_1 antiserum (Alexis, 1:100). Cells were washed once with 1% BSA/PBS and with PBS and then fixed with 1% paraformaldehyde. Surface expression of DR3 was determined with a FACS-Calibur™ flow cytometer (Becton Dickinson). The mean fluorescence index was calculated by using WinMDI™ software version 2.9 (Joseph Trotter).

2.2.13. Statistics and general remarks

At least three independent experiments were performed for each analysis. Assays consisted of duplicate or triplicate values, and mean values and standard deviations were calculated from three independent experiments.
The research committee of the Charité-Universitätsmedizin Berlin has approved the described studies. The study was conducted according to the Declaration of Helsinki.

3. Results

3.1. Basic mRNA expression of DR3, DR6, DcR3, TL1A, FasL and FLIP in melanoma cell lines

To search for new targets in the treatment of malignant melanoma, the basic mRNA expression of death receptors (DR), decoy receptors (DcR) and death ligands that may regulate apoptosis in melanoma, and which have not yet been described in melanoma cells were investigated using RT-PCR. Independent samples of the established human melanoma cell lines A-375, Bro, JPC-298, MeWo, Mel-2a, Mel-HO, SK-Mel-13 and SK-Mel-19 were examined in comparison to several independent samples of primary cutaneous normal human melanocytes (NHM). The human T lymphoblastic leukaemia cell line Jurkat and the human colon adenocarcinoma cell line SW480 were used as controls.

3.1.1. Melanoma cell lines express DR6, DcR3 and TL1A mRNA

In this investigation, the basic mRNA expression of death receptor 6 (DR6), decoy receptor 3 (DcR3) and TL1A was determined using RT-PCR.

DR6 is expressed in most human tissues and abundant transcript has been detected in heart, brain, placenta, pancreas, thymus, lymph node and several non-lymphoid cancer cell lines (Pan et al, 1998). DR6 appeared as an 820 bp PCR amplicon as determined by selected primers. All 8 melanoma cell lines and 3 NHM in the analysis showed a constant DR6 mRNA expression (Figure 3.1). Compared to NHM, melanoma cell lines showed no difference in DR6 mRNA expression. The experiment was carried out for 2 independent samples of melanoma cell lines and repeated at least once for each sample.

DcR3 is a soluble receptor that is expressed in malignant cells of several tumour types and has been postulated to help tumour cells to gain survival advantage by inhibiting apoptosis and by interfering with immune surveillance (Wu et al, 2003). DcR3 appeared as a 450 bp PCR amplicon as determined by selected primers. Of the 8 melanoma cell lines analysed, all showed DcR3 mRNA in at least one RNA extraction. Also NHM showed DcR3 mRNA expression (Figure 3.2). The RT-PCR was carried out for 2 independent samples of melanoma cell lines and repeated at least once for each sample.

TNF-like cytokine 1A (TL1A) is a ligand for DR3 and DcR3. In DR3-expressing cell lines, TL1A induces apoptosis by interacting with DR3 (Migone et al, 2002). TL1A mRNA, a 666 bp amplicon in PCR, was detected in at least one RNA sample of 8 melanoma cell lines. Also NHM

showed TL1A mRNA expression (Figure 3.2). The analysis was carried out for 2 independent samples of melanoma cell lines and repeated at least once for each sample.

Thus, 8 melanoma cell lines and 3 NHM showed a basic mRNA expression of DR6, DcR3 and TL1A.

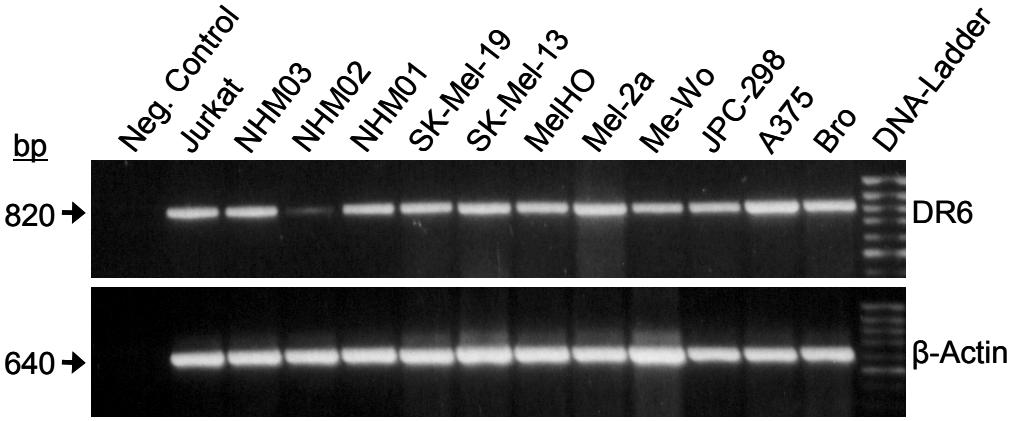

Figure 3.1 Melanoma cell lines and NHM express DR6 mRNA

DR6 appears as an amplicon of 820 bp. The RT-PCR was performed 2 times for 2 independent samples of each cell line with similar results.

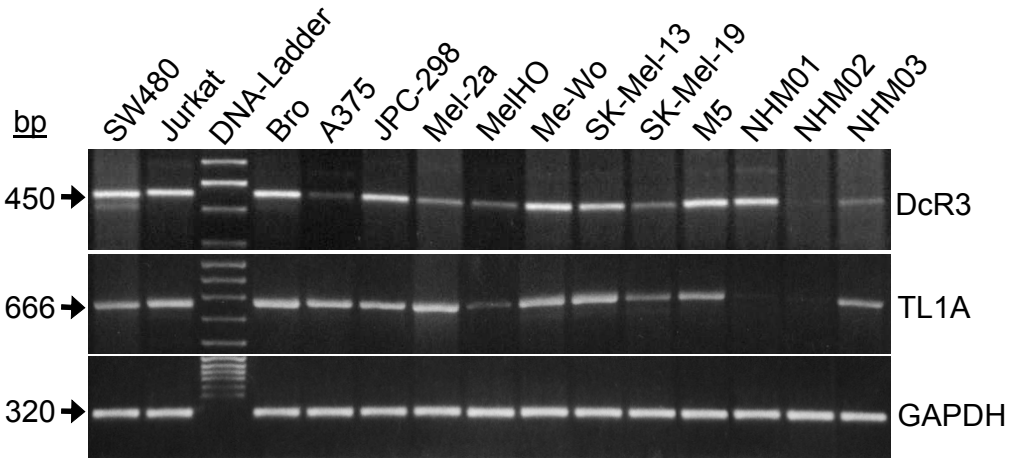

Figure 3.2 Melanoma cell lines and NHM express DcR3 and TL1A mRNA

DcR3 appears as a 450 bp amplicon and TL1A as a 666 bp. The RT-PCR was performed for 2 independent samples for each cell line and 2 times for each sample with similar results.

3.1.2. Lack of full length DR3 mRNA in melanoma cell lines

Death receptor 3 (DR3) is known to be predominantly expressed on lymphocytes (T and B cells) but not on macrophages or a number of transformed lymphocyte cell lines. Alternative pre-mRNA splicing generates at least 12 distinct isoforms of DR3. The full-length isoform, DR3-1, extends to include the transmembrane and death domains (Chinnaiyan et al, 1996), (Screaton et al, 1997), (Warzocha et al, 1998).

In this analysis, the expression of DR3 mRNA was analysed in 10 melanoma cell lines: A-375, Bro, JPC-298, MeWo, Mel-2a, MelHO, SK-Mel-13, SK-Mel-19, SK-Mel-23 and M5. The primers used were designed to produce full length DR3 mRNA, as well as DR3 isoforms as defined by alternative splicing. The expected amplicon sizes as specified by the selected primers used in the PCR are defined in table 3.1.

Table 3.1 DR3 mRNA isoforms shown in RT-PCR

DR3 mRNA isoform	12	4	11	1/2	3	10	9	5	6	7	8
Expected size (bp)	826	806	732	705	649	594	570	538	341	289	156

The expected amplicon size of the main DR3 mRNA isoform was 705 bp as specified by the selected primer pairs for PCR. Various DR3 mRNA isoforms were detected in all but one (SK-Mel-19) cell line after simple RT-PCR, as well as nested PCR. The analysis was carried out with 3 independent RNA extractions for each cell line and repeated once for each extraction. The positive control cell lines Jurkat and SW480, as well as NHM showed consistent reproducible expression of DR3 mRNA isoform 1, which is the full length transmembrane receptor (Figure 3.3). In contrast, the melanoma cell lines were negative for this isoform (results not shown). Instead, 8 of 10 melanoma cell lines showed an unexpected 480 bp amplicon after 35 cycles of simple RT-PCR, which was also shown in the NHM (Figure 3.3). However, the results of the PCR were not reproducible for melanoma cell lines in the second and third independent experiments and are therefore not shown.

The 700 bp amplicon corresponds to DR3 mRNA isoform 1/2, the 650 bp to isoform 3 and the 550 bp to isoform 5. The 480 bp amplicon, which the melanoma cell lines and NHM displayed, is undefined and could be a new, not yet classified isoform.

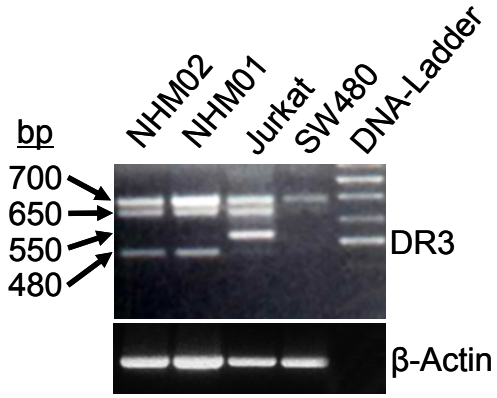

Figure 3.3 DR3 mRNA isoforms in NHM, Jurkat and SW480

NHM showed DR3 mRNA isoform 1 (700 bp), 3 (650 bp) and an undefined amplicon of 480 bp. Jurkat and SW480 also showed DR3 mRNA isoform 1. Jurkat displayed the isoforms 3 (650 bp) and 5 (550 bp) as well.

The 700 bp amplicon, DR3 mRNA isoform 1, was never detected in melanoma cell lines, but always in the control cell lines Jurkat and SW480 and frequently in NHM.

3.1.3. Melanoma cell lines express FLIP and FasL mRNA

FLIP (FLICE (FAS-associated death-domain-like IL-1beta-converting enzyme)-inhibitory protein) has been identified as an inhibitor of death receptor signaling. Overexpression of cellular (Medema et al, 1999) or viral (Djerbi et al, 1999) FLIP prevents the recruitment of procaspase 8. In this analysis, the basic mRNA expression of cellular FLIP was compared to that of NHM. FLIP mRNA was displayed as a 540 bp amplicon. The experiment was carried out for 2 independent RNA extractions of all cell lines and repeated at least once for each extraction.
The results showed no differences in the FLIP mRNA expression amongst melanoma cell lines, and between melanoma cell lines and NHM. All cell lines under investigation showed consistent expression of FLIP mRNA (figure 3.4).
The expression of Fas ligand (FasL) by human melanoma cells has remained unclear over the years. While several reports state that human melanoma cells do not express FasL (Chappell et al, 1999), (Eberle et al, 2003), the expression of FasL by melanoma cells has been reported as an important mechanism in the immune evasion by the tumour cells (Hahne et al, 1996). In this analysis, the basic expression of FasL mRNA in melanoma cell lines was compared to that of NHM using RT-PCR and nested PCR. The experiment was carried out for 2 independent RNA extractions of all cell lines and repeated at least once for each extraction.
After first reaction RT-PCR consisting of 30 cycles, only the positive control cell line Jurkat was positive for FasL mRNA, which was displayed as a 540 bp amplicon. All melanoma cell lines as well as NHM were negative for FasL mRNA after first reaction PCR.

Nested PCR was carried out using 10% of the sample from the first reaction PCR in a second PCR reaction consisting of 30 cycles. The FasL mRNA after nested PCR was displayed as a 340 bp amplicon. Melanoma cell lines showed inconsistent expression of FasL mRNA (Figure 3.4).

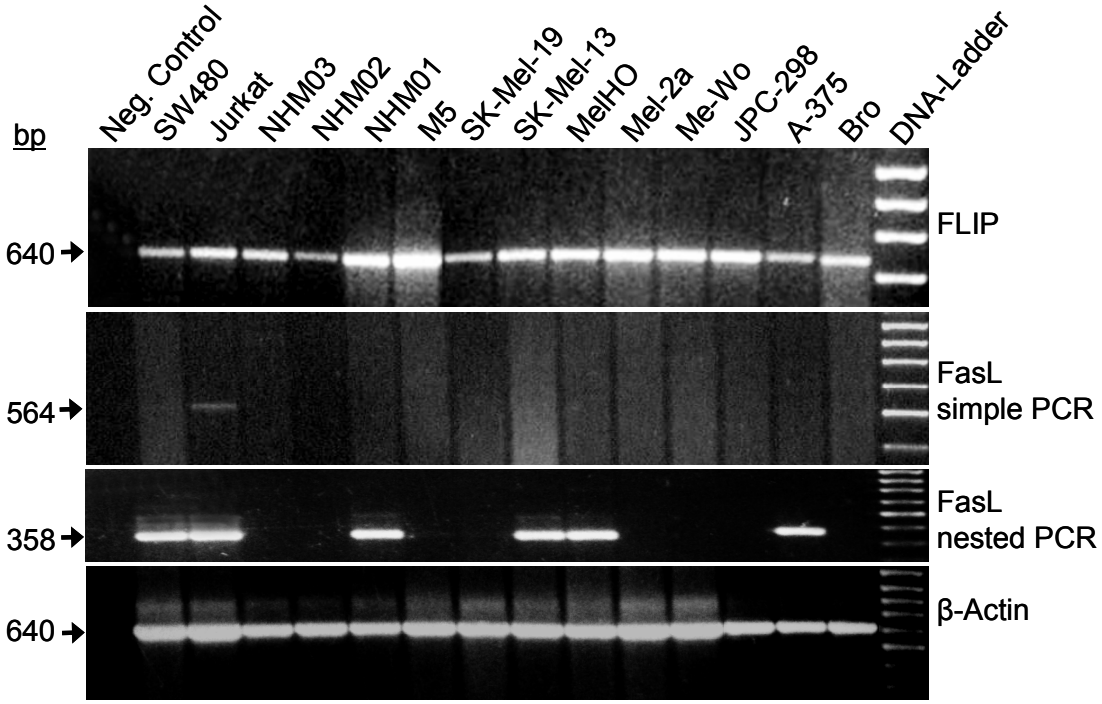

Figure 3.4 Melanoma cells express FLIP mRNA, but no FasL mRNA

All melanoma cell lines and NHM showed consistent expression of FLIP mRNA. After simple RT-PCR, all melanoma cell lines and NHM are negative for FasL mRNA; some melanoma cell lines and NHM were positive for FasL mRNA after nested PCR. The experiment was carried out for 2 independent RNA extractions with similar results.

Only one melanoma cell line, SK-Mel-13, showed consistent expression of FasL mRNA in all nested PCR reactions carried out in this analysis. The melanoma cell lines MeWo, SK-Mel-19 and M5 were always negative for FasL mRNA in all experiments. The melanoma cell lines Bro, A-375, JPC-298, Mel-2a and Mel-HO showed inconsistent expression of FasL mRNA, which was also the case with NHM. The positive control cell line Jurkat showed consistent expression of FasL mRNA in all PCR reactions after simple RT-PCR as well as nested PCR. The cell line SW480 was frequently positive after simple RT-PCR, and always showed FasL mRNA after nested PCR.

The results of FasL mRNA expression in melanoma cell lines and NHM after nested PCR are shown below in table 3.2.

Table 3.2 Expression of FasL mRNA after nested PCR

Cell line	Experiment I	Experiment II
Bro	-	+
A-375	+	-
JPC-298	-	+
MeWo	-	-
Mel-2a	-	+
Mel-HO	+	-
SK-Mel-13	+	+
SK-Mel-19	-	-
M5	-	-
NHM (3 x)	+/-	+/-
Jurkat	+	+
SW480	+	+

3.2. Protein expression of DR3, DcR3 and TL1A in melanoma cell lines

To prove the mRNA data, the protein expression of DR3, DcR3 and TL1A was analysed using Western blotting and immunodetection of proteins in melanoma cell lines and NHM.

3.2.1. Melanoma cell lines express a glycosylated DR3 protein band

The results of the RT-PCR had shown inconsistent, non-reproducible DR3 mRNA expression in melanoma cell lines. Western blot analysis was used to find out the protein expression of DR3 in melanoma cell lines compared to NHM. The expected size of the main DR3 isoform 1, which is the full length transmembrane receptor, is 47 kDa. However, due to glycosylation of DR3 proteins, protein bands of larger sizes between 60 kDa and 200 kDa may be expected in Western blot analysis (Kitson et al, 1996), (Marsters et al, 1998). The expression of DR3 in the positive control cell line Jurkat, the human mamma carcinoma cell line MCF-7 and the human cervix carcinoma cell line HeLa was first analysed as shown in figure 3.5.

In this analysis, Jurkat showed the expected 47 kDa protein band, which corresponds to the main DR3 isoform 1, a 66 kDa protein band. MCF-7 displayed a 44 kDa protein band which corresponds to DR3 isoform 9 and the glycosylated 66 kDa DR3 protein band. HeLa showed only the glycosylated 66 kDa DR3 protein band.

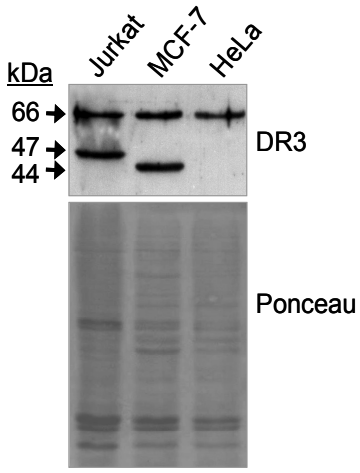

Figure 3.5 DR3 expression in Jurkat, MCF-7 and HeLa

The expected MW of DR3 is 47 kDa, which corresponds to DR3 isoform 1. The 66 kDa band is the glycosylated DR3. Jurkat, MCF-7 and HeLa show this 66 kDa band. Jurkat displayed the 47 kDa isoform 1. The 44 kDa band displayed by MCF-7 corresponds to DR3 isoform 9.

After verifying the expression of DR3 proteins in the control cell lines, 4 melanoma cell lines A-375, Mel-2a, MeWo and SK-Mel-13 and 4 NHM cultures were analysed for DR3 protein expression in comparison. All melanoma and NHM cell lines displayed only the 66 kDa protein band. The analysis was carried out with 2 independent whole cell lysates (protein extractions) for each cell line using 2 different DR3 antibodies of rabbit and mouse origin (Abcam).

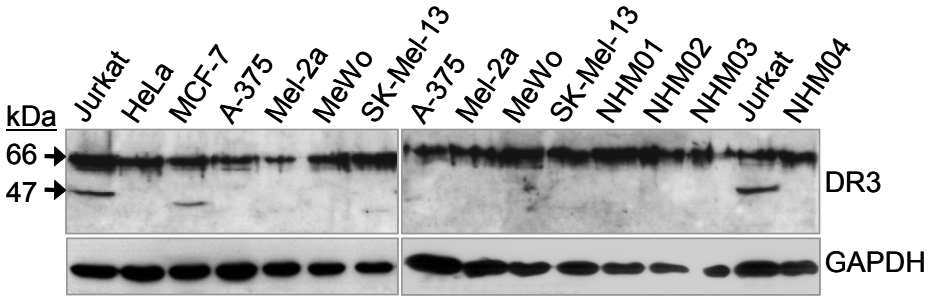

Figure 3.6 Expression of DR3 in melanoma cell lines compared to NHM and Jurkat

All melanoma cell lines and NHM analysed were positive for the glycosylated 66 kDa DR3 protein band. The analysis was done for 2 independent protein extractions of melanoma cell lines and 8 NHM cell lines with the same results. The positive control cell lines Jurkat, MCF-7 and HeLa displayed the previously described protein bands.

3.2.2. Consistent expression of DcR3 protein in melanoma cells

The results of the RT-PCR showed that melanoma cell lines consistently expressed DcR3 mRNA. DcR3 has been described as a soluble protein with a MW of about 35 kDa, which is abundantly expressed in several tissues such as the lung, spleen and colon, and especially in the colon carcinoma cell line SW480 (Pitti et al, 1998). DcR3 lacks a transmembrane domain, which indicates that it is a secreted protein, rather than a membrane associated molecule. Since DcR3 is a secreted protein which may bind to, and inhibits the activity of FasL and TL1A (Wroblewski et al, 2003), (Yang et al, 2004), protein extractions of cell supernatants were included in this analysis. The cells were maintained in serum free medium for 48 hr and 2 ml of supernatants were concentrated by 10 times using Centricon YM-10 (Millipore; Bedford, MA) and piling up to 0.2 ml. The analysis of DcR3 protein expression in cell culture medium (supernatant DcR3) was carried out once for 6 melanoma cell lines (A-375, JPC-298, Mel-2a, Mel-HO, MeWO and SK-Mel-13), 1 NHM and the positive control cell line SW480. The results showed all melanoma cell lines, the NHM and SW480 with a protein band migrating at 105 kDa (figure 3.7).

The analysis was then carried out using whole cell lysates of the melanoma cell lines A-375, Mel-2a, Mel-HO, MeWo and SK-Mel-13, and compared to Jurkat, SW480, MCF-7 and HeLa (figure 3.8). The 4 melanoma cell lines A-375, Mel-HO, MeWo and SK-Mel-13, as well as Jurkat, MCF-7 and HeLa displayed the 105 kDa protein band seen in the analysis of cell supernatant for DcR3 protein. Mel-2a and SW480, which showed a 105 kDa protein band in the cell supernatant, were only faintly positive for this protein band in the analysis using whole cell lysates.

As mentioned earlier, the expected protein size of DcR3 was about 35 kDa. Using 2 different antibodies for DcR3 of the sources rabbit and goat (Abcam), a protein band migrating at 105 kDa was consistently seen in whole cell lysates as well as in cell supernatant. Since DcR3 mRNA was consistently expressed by melanoma cell lines in RT-PCR analysis, it can be argued that the 105 kDa band may be the trimerized DcR3 protein. The analysis was carried out using whole cell lysates of a wide range of melanoma cell lines and NHM with similar results (figure 3.9).

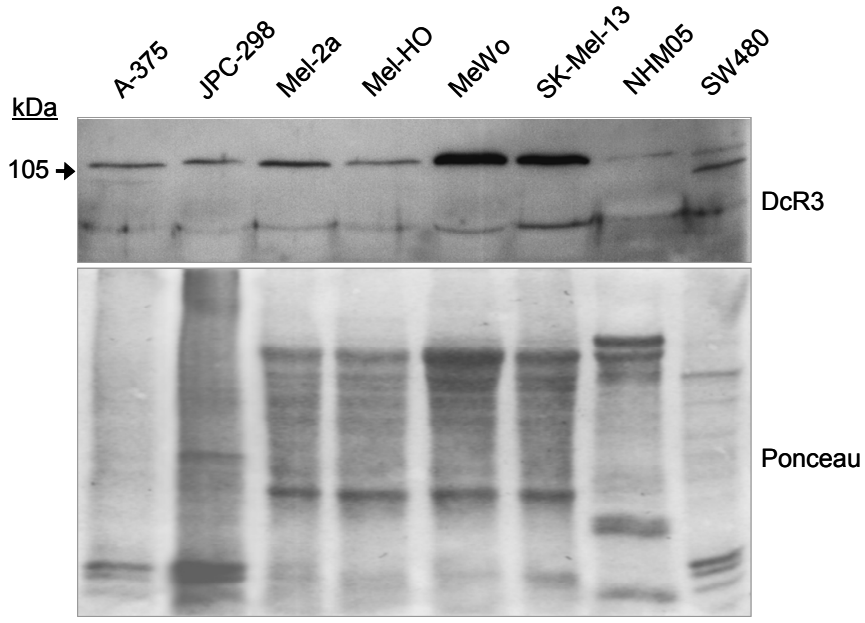

Figure 3.7 Expression of extracellular DcR3 protein in cell supernatant

Melanoma cell lines, NHM and the positive control cell line for DcR3, SW480, displayed a 105 kDa protein band in the analysis of cell supernatant. This analysis was carried out once.

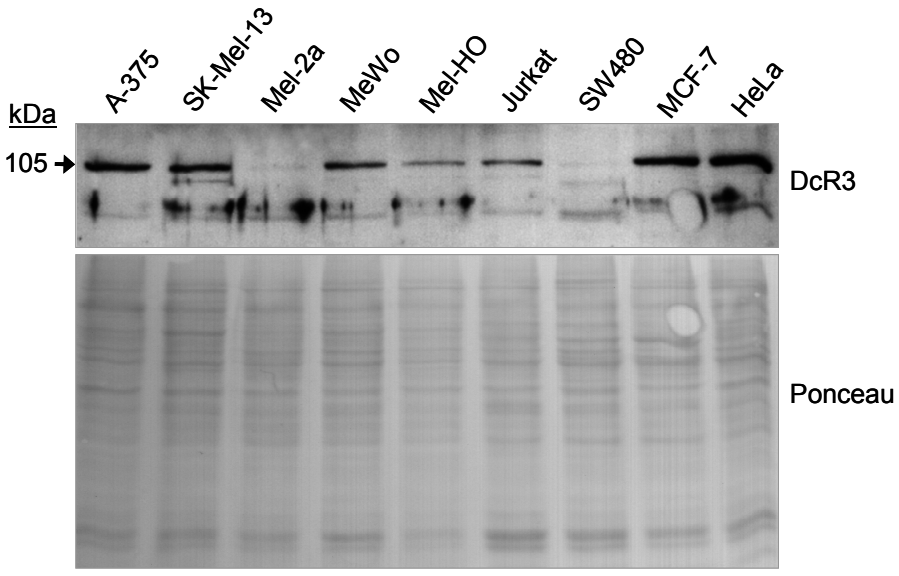

Figure 3.8 Expression of intracellular DcR3 protein in whole cell lysate

In the analysis of whole cell lysates, most cancer cell lines displayed the 105 kDa protein band seen in the cell supernatants. The analysis was carried out for 3 independent samples of each cell line using 2 independent antibodies with similar results.

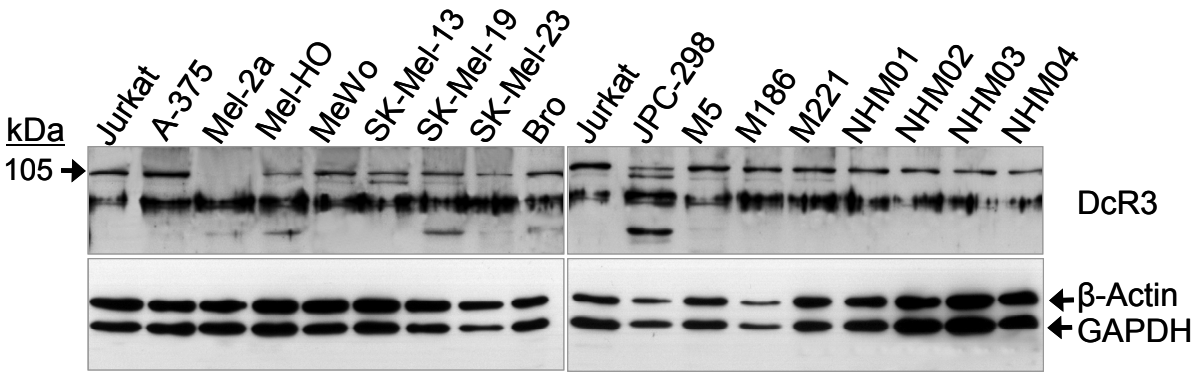

Figure 3.9 Melanoma cell lines and NHM express a 105 kDa DcR3 protein band

Using 2 different DcR3 antibodies of rabbit and goat origin (Abcam), melanoma cell lines and NHM consistently expressed a 105 kDa protein band in whole cell lysates. Jurkat was used as a positive control in this analysis, which was performed for 2 independent protein extraction of each cell line with similar results.

3.2.3. Melanoma expression of TL1A protein remains unclear due to unspecific antibodies

The RT-PCR analysis of melanoma cell lines for TL1A mRNA showed that melanoma cells consistently expressed TL1A mRNA. TL1A is a type II transmembrane protein (Tan et al, 1997) also known as vascular endothelial growth inhibitor (VEGI) due to its activity in endothelial cells (Zhai et al, 1999). TL1A, like TNF, is also presumed to circulate as a homotrimeric soluble form (Kim et al, 2005). TL1A is a ligand for DR3 and DcR3, and may induce NF-kappaB activation and apoptosis in DR3 expressing cell lines, while DcR3-Fc protein may antagonize these signaling events (Migone et al, 2002).

In this analysis, whole cell lysates of 10 melanoma cell lines and 4 NHM were tested for TL1A protein expression using Western blotting. Jurkat and peripheral blood mononuclear cells, PBMC (supplied by manufacturer, Abcam) were used as positive controls. The expected protein size was 22 kDa. The analysis was carried out for 2 independent protein extractions of the cell lines. The results showed unspecific protein binding of the antibody in use (Abcam). No protein band of the expected 22 kDa was seen in the positive control cell lines. Instead, several protein bands of higher MW were seen in all cell lines analysed. The results are therefore not shown.

In a further attempt to verify the protein expression of TL1A in melanoma cells, the melanoma cell lines A-375, Mel-2a, MeWo and SK-Mel-13 were analysed for membrane bound TL1A using FACS analysis. Jurkat cells were used as a positive control. Again all cell lines, including the positive control cell line Jurkat, were negative for membrane TL1A. Since Jurkat cells were

negative in the FACS analysis for TL1A, it can be concluded that the antibody in use was non specific for TL1A protein. The protein expression of TL1A in melanoma cells and NHM remained unclear in this analysis.

3.3. Functional activity of TL1A in melanoma cell lines

Although several studies have verified that TL1A is a ligand for DR3 (Migone et al, 2002), (Bamias et al, 2006) the physiological signal activated by DR3 through binding of its cognate ligand has been scarcely studied in relation to apoptosis.

The results of the Western blot analysis in this study showed that DR3 is endogenously expressed in melanoma cell lines; therefore, the activity of its ligand, TL1A, was investigated in functional assays. The apoptosis induction was quantified by measuring DNA fragmentation and cytotoxicity determined by Lactate dehydrogenase (LDH) release into the supernatant.

In a first experiment, 2 melanoma cell lines, A-375 and SK-Mel-13, were treated with 100 ng/ml TL1A for 6 and 24 hours. High LDH release was measured in A-375 after 6 hr treatment. LDH release was also seen in SK-Mel-13 after 6 and 24 hr treatment with TL1A. A-375 showed no DNA fragmentation after treatment with TL1A. In SK-Mel-13, DNA fragmentation was measured after 24 hr treatment with TL1A (figure 3.10a). The experiment was performed three times; twice with triple samples and once with double samples with similar results. The results of the functional analysis of the effects of TL1A in A-375 and SK-Mel-13 showed that TL1A induced cytotoxicity in both cell lines, and late apoptosis in SK-Mel-13.

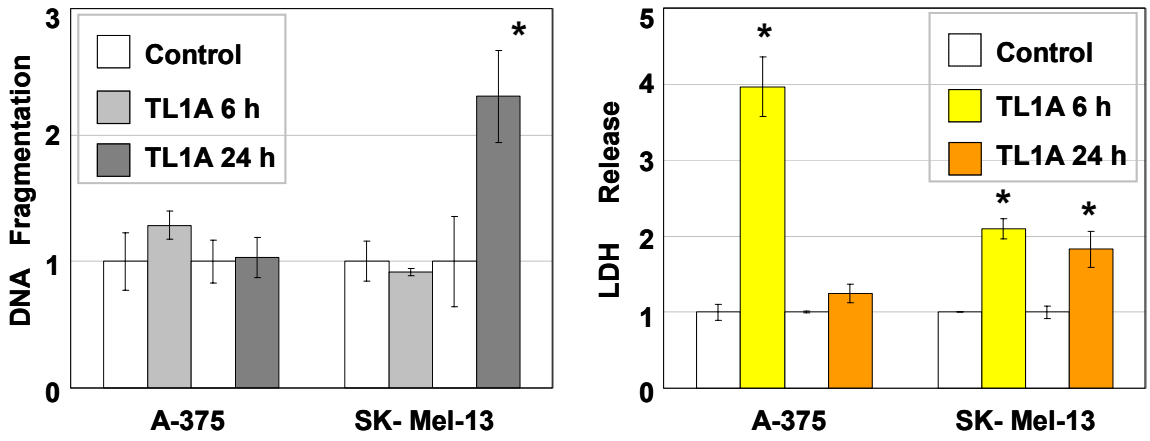

Figure 3.10a TL1A was cytotoxic in A-375 and SK-Mel-13, and apoptotic in SK-Mel-13

A-375 and SK-Mel-13 were treated with 100 ng/ml TL1A and compared with controls (white). DNA fragmentation reflects apoptosis, and LDH release reflects cytotoxicity. Data shows mean values of triple samples, the statistical significance ($p < 0.05$) is marked with asterisks (*).

Dose dependent effects of TL1A

In a further experiment, the dose dependent effects of TL1A were analysed by treating A-375 and SK-Mel-13 with TL1A at concentrations of 25 ng/ml, 50 ng/ml, 100 ng/ml and 200 ng/ml for 24 hours (figure 13.10b). No effects were measured at 25 ng/ml TL1A in both cell lines. LDH release and DNA fragmentation in A-375 and SK-Mel-13 were similar at concentrations of 50 and 100 ng/ml TL1A. These effects could not be enhanced at a higher concentration of 200 ng/ml. These results indicated that the effects of TL1A in the cell lines analysed could be measured at concentrations between 50 ng/ml and 100 ng/ml ligand.

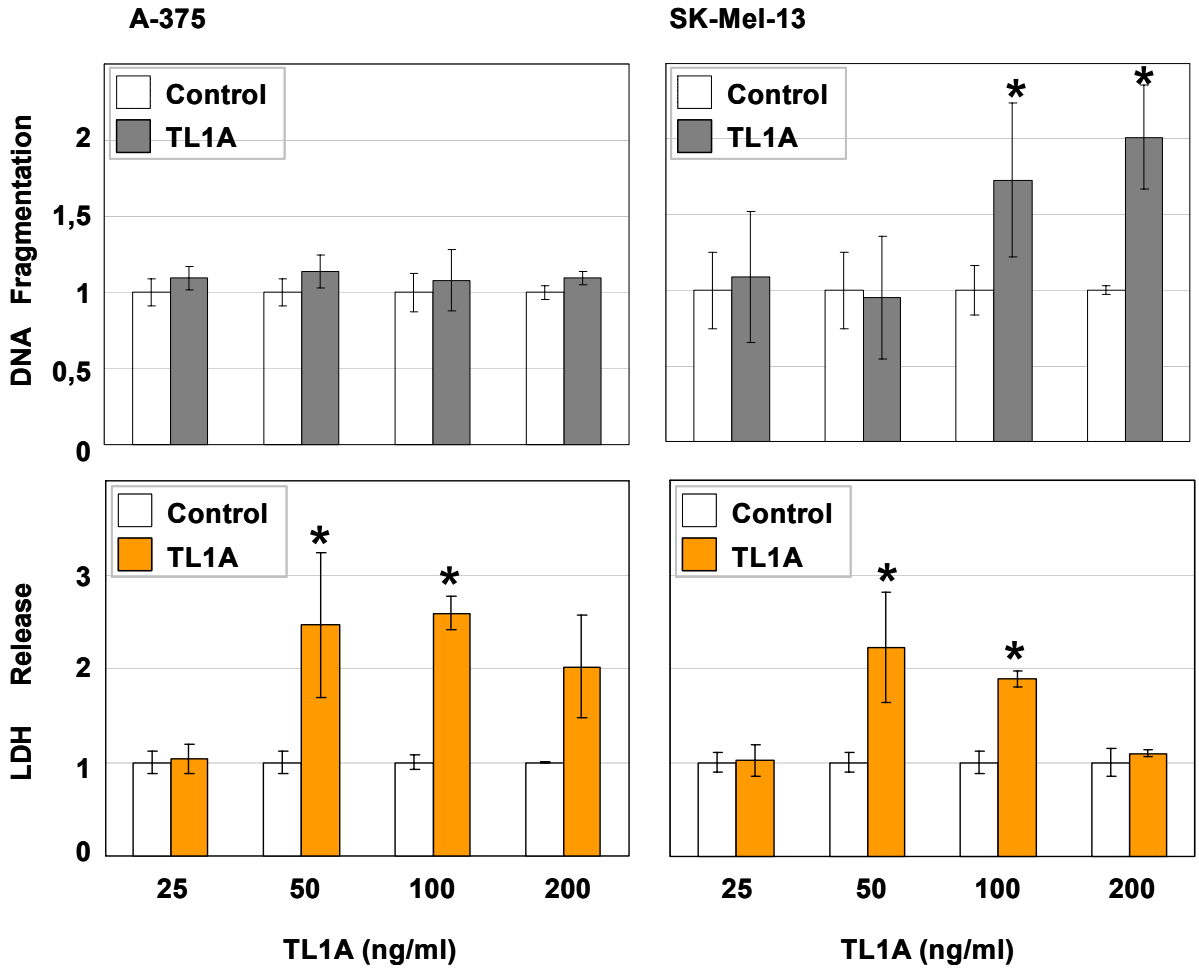

Figure 3.10b Dose dependent of TL1A in A-375 and SK-Mel-13

A-375 and SK-Mel-13 were treated with 25, 50, 100 and 200 ng/ml TL1A and compared with controls (white). DNA fragmentation reflects apoptosis, and LDH release reflects cytotoxicity. Data shows mean values of triple samples, the statistical significance ($p < 0.05$) is marked with asterisks (*).

3.3.1. TL1A induced cytotoxicity in melanoma cells

To further verify the effects of TL1A in melanoma cells, melanoma cell lines A-375, Mel-2a, Mel-HO, MeWo and SK-Mel-13 were treated with 100 ng/ml TL1A for 6, 24 and 48 hours. The experiment was performed 2 times, once with triple samples, once with double samples for Mel-2a, Mel-HO and MeWo, and at least 4 times with A-375 and SK-Mel-13.
TL1A induced cytotoxicity in 4 out of 5 melanoma cell lines (figure 3.11). The cytotoxic effect of TL1A was significantly higher after 6 hr treatment in A-375, Mel-HO, MeWo and SK-Mel-13, whereas effect in Mel-2a was not significant.

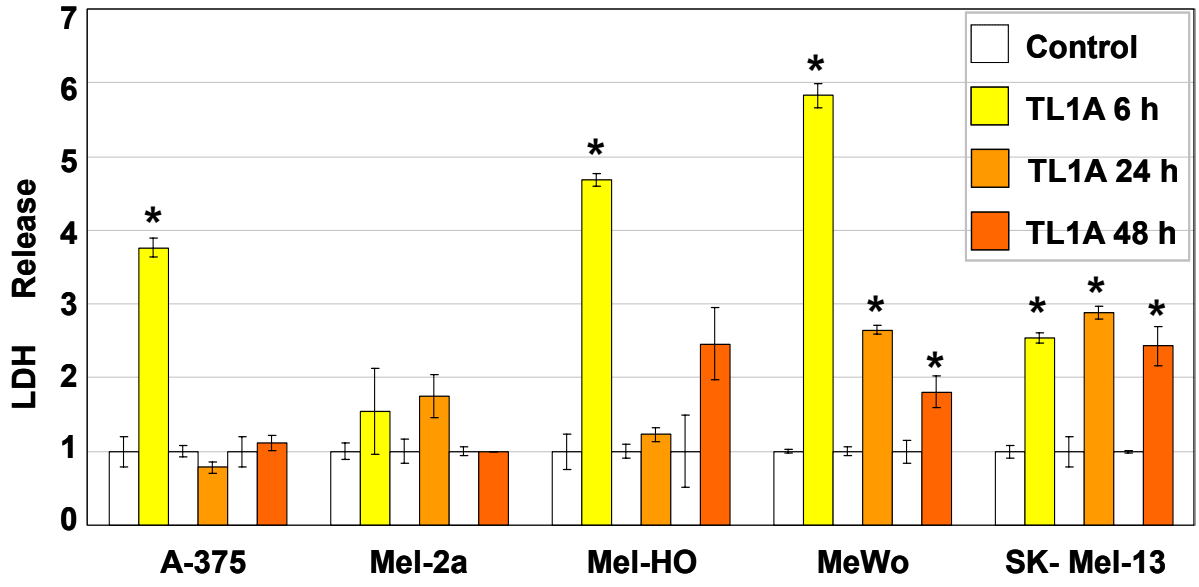

Figure 3.11 TL1A induced cytotoxicity in melanoma cell lines

Melanoma cell lines were treated with 100 ng/ml of TL1A for 6, 24 and 48 hours and compared with controls (white). LDH release reflects cytotoxicity. Data shows mean values, the statistical significance ($p < 0.05$) is marked with asterisks (*).

3.3.2. Apoptosis induction by TL1A was restricted to SK-Mel-13

TL1A induced apoptosis only in SK-Mel-13 after 24 and 48 hours, whereas no significant effects were seen in A-375, Mel-2a, Mel-HO and MeWo (figure 3.12).

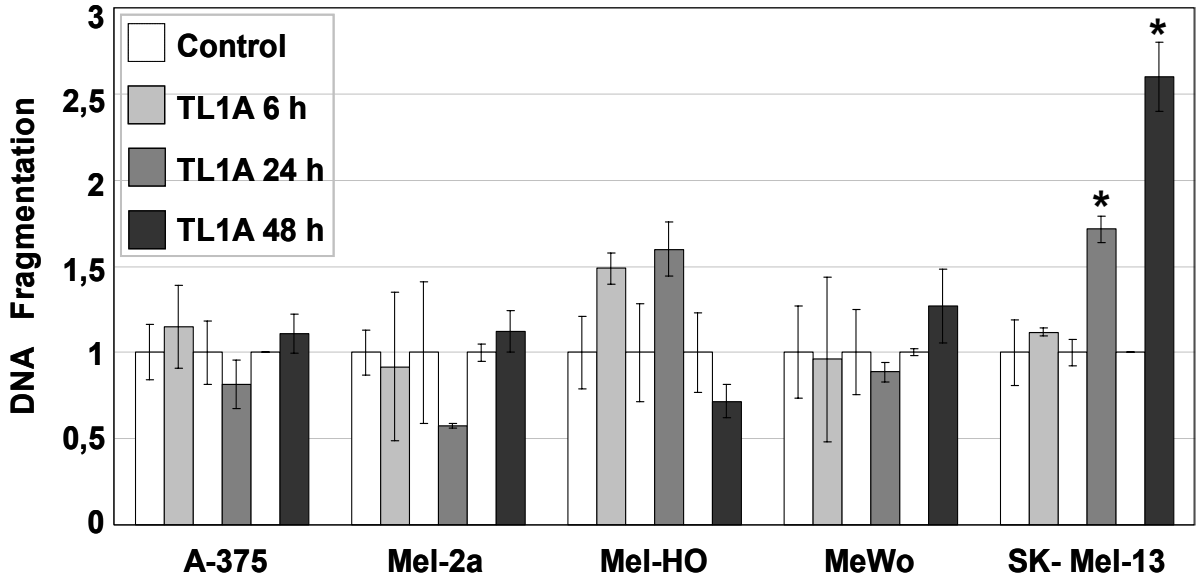

Figure 3.12 TL1A induced no apoptosis in melanoma cell lines, except in SK-Mel-13

Melanoma cell lines were treated with 100 ng/ml of TL1A for 6, 24 and 48 hours and compared with controls (white). DNA fragmentation reflects apoptosis. Data shows mean values, the statistical significance ($p < 0.05$) is marked with asterisks (*).

3.3.3. TL1A induced apoptosis but not cytotoxicity in NHM

The effects of TL1A in NHM were analysed by treating 3 NHM cell lines (an NHM cell line is defined as NHM isolated from one specific foreskin) with 100 ng/ml TL1A for 6, 24 and 48 hours. After treatment for 6 hours, no significant effects of TL1A were measured in NHM (results not shown). TL1A induced significant apoptosis of factor 1.5 - 2.0 in all 3 NHM cell lines after 24 and 48 hr treatment, whereas no cytotoxic effects were seen in two of three NHM cultures. Significant LDH release was measured in one NHM cell line tested at 24 hr treatment (figure 3.13).

To summarize, TL1A induced primarily cytotoxicity in melanoma cell lines, as measured by LDH release. The cytotoxicity was highest at 6 hours after treatment and was significant in 4 out of 5 melanoma cells with factors ranging from 2.0 - 6.0. This early cytotoxic effect was measured in A-375, Mel-HO, MeWo and SK-Mel-13. The LDH release in Mel-2a after TL1A treatment was measured with factor 1.5 - 2.5, however, this was not statistically significant. After 24 hours' treatment with TL1A, LDH release was significant in A-375, MeWo and SK-Mel-13, and after 48 hours, only SK-Mel-13 showed cytotoxic effects of TL1A.

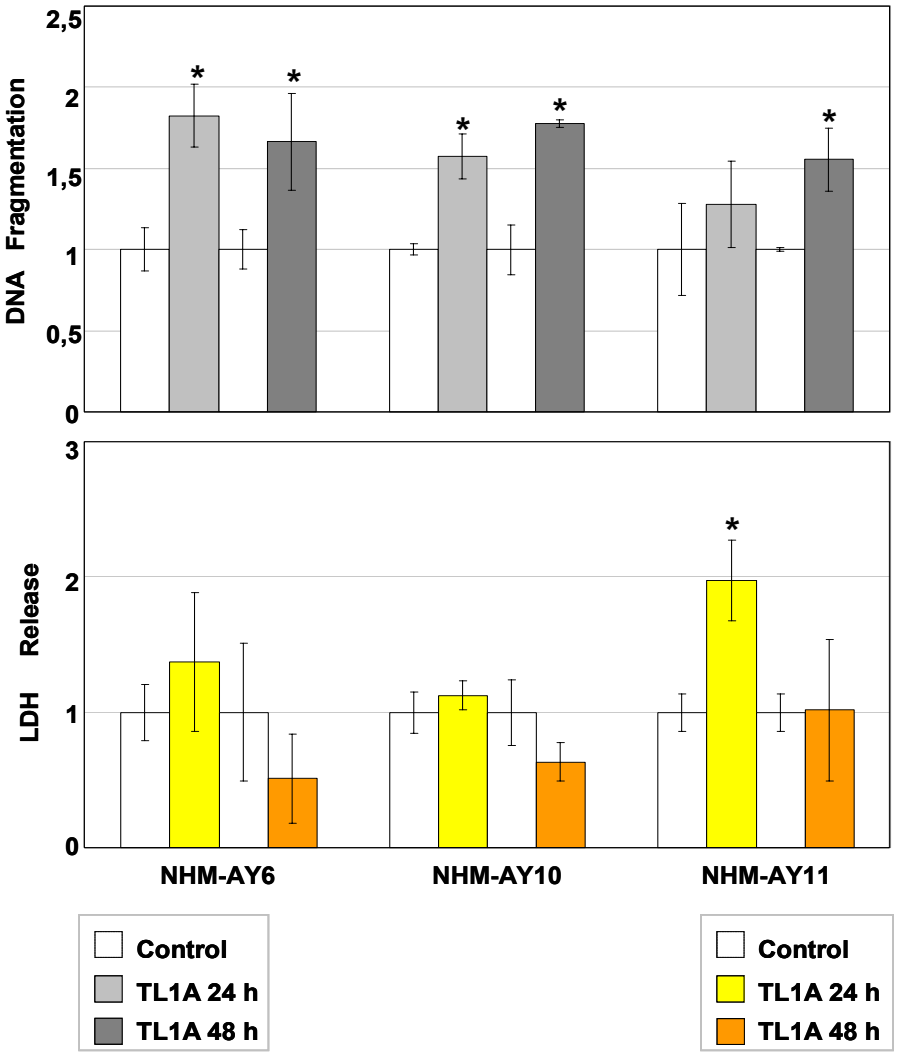

Figure 3.13 TL1A induced moderate apoptosis in NHM, but no cytotoxicity

3 NHM were treated with 100 ng/ml TL1A for 24 and 48 hours and compared with controls (white). DNA fragmentation reflects apoptosis, LDH release reflects cytotoxicity. Data shows mean values, the statistical significance ($p < 0.05$) is marked with asterisks (*).

TL1A showed no apoptotic activity in 4 out of 5 melanoma cell lines, as measured by DNA fragmentation. Apoptosis induction by TL1A only occurred in SK-Mel-13, and was measured with factor 1.5 - 2.5 after 24 hours' treatment. Apoptosis through TL1A was highest in SK-Mel-13 after 48 hours' treatment with factor 2.0 - 2.7.

In comparison, TL1A had no cytotoxic effects in NHM; only 1 NHM out of 3 showed significant cytotoxicity after 24 hours' treatment with TL1A with factor 2.0. On the other hand, significant DNA fragmentation induced by TL1A treatment was measured in all 3 NHM. This apoptotic effect of TL1A in NHM was significant in 2 of 3 NHM after 24 hours' treatment with factor 1.5

- 2.0, and in all 3 NHM after 48 hours' treatment with factor 1.5 - 2.0. No cytotoxicity or apoptosis was measured in NHM after 6 hours' treatment with TL1A (results not shown).

3.4. Analysis of DR3 in melanoma cell lines

In the functional analysis of TL1A in melanoma cells shown in the results above, no apoptotic effects could be measured in melanoma cell lines, excepting SK-Mel-13, which responded with moderate apoptosis at 24 and 48 hours' treatment. Instead, TL1A induced primarily early cytotoxicity in melanoma cells. NHM showed only moderate apoptosis after treatment with TL1A.
Using FACS analysis therefore, the surface expression of DR3 (membrane associated DR3) was analysed in melanoma cell lines to find out whether the protein expression in the Western blot analysis correlates with the surface expression of the death receptor.

3.4.1. Lacking DR3 surface expression of melanoma and NHM

The human melanoma cell lines A-375, Mel-2a, Mel-HO, MeWo, Bro and SK-Mel-13, as well as 3 NHM cell lines were analysed together with Jurkat for membrane bound DR3. The analysis consisted of 2 independent experiments, both showing similar results. The surface expression of CD95/Fas in A-375 and SK-Mel-13 was used as method control, and was carried out once.
Neither the 6 melanoma cell lines in the FACS analysis (A-375, Mel-2a, Mel-HO, MeWo, SK-Mel-13 and Bro) nor 3 NHM showed surface expression of DR3. In contrast, Jurkat cells showed a significant DR3 surface expression. The surface expression of CD95 in A-375 and SK-Mel-13 was verified in the control experiment.

3.5. Induction of DR3 expression in melanoma cells

In a series of experiments, the melanoma cell lines A-375 and SK-Mel-13 were analysed in their growth behaviour and their DR3 protein expression compared to that of Jurkat.
Firstly, it was reasoned that the different growth media of the cell lines, namely DMEM for melanoma and RPMI for Jurkat, could influence cell activity of the different cell lines, probably causing the difference in protein expression. A-375, SK-Mel-13 and Jurkat were therefore cultured under the same conditions and DR3 protein expression was analysed in Western blotting.

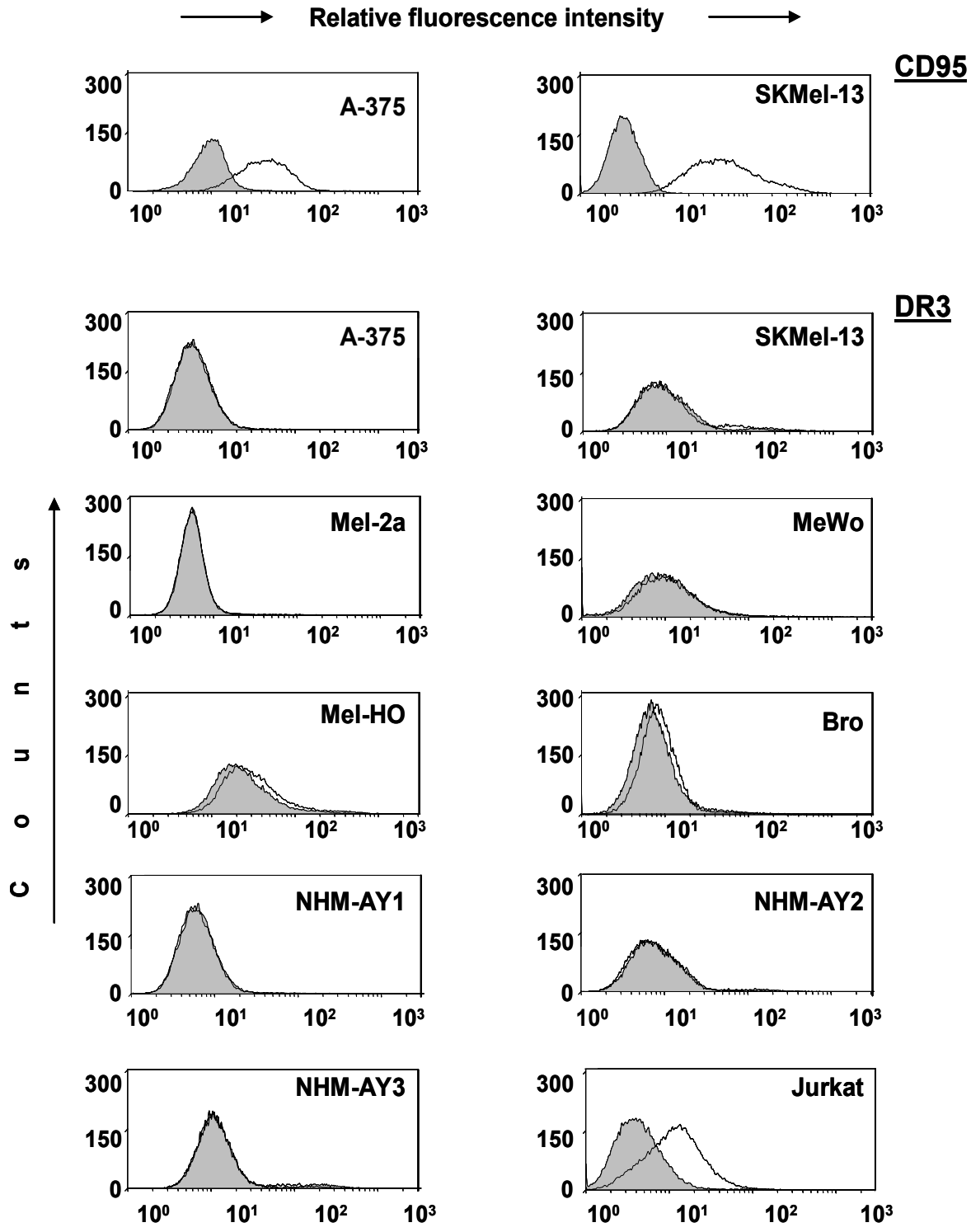

Figure 3.14 Melanoma cells and NHM showed no surface expression of DR3

Data reflect the number of counts plotted against the relative fluorescence intensity in FACS analysis. The grey curve represents unspecific IgG binding; a shift of the open graph to the right shows a positive surface expression of the receptor. All 6 melanoma cell lines tested and 3 NHM were negative for DR3 surface expression compared to Jurkat. The analysis was done at least twice for all cell lines.

The results showed A-375 and SK-Mel-13 with the 66 kDa DR3 protein band, and Jurkat with two DR3 protein bands of 66 kDa and 47 kDa (similar results already shown in 3.2).
Secondly, melanoma cell lines were cultured in RPMI and in DMEM. The result of the DR3 expression under these conditions in the Western blot analysis was unaltered (figure 3.15).
Thirdly, it was reasoned that Jurkat cells could probably induce their own expression of surface DR3 whilst melanoma cells could not. Melanoma cell lines were therefore cultured with Jurkat supernatant, which was defined as follows:
10 million Jurkat cells are left in 30 ml growth medium and incubated at 37°C under 5% carbon dioxide overnight. The melanoma cell lines were cultured with 50% and 75% of the Jurkat supernatant for 24 hours and 48 hours. Protein extraction of whole cell lysates followed, then Western blotting was performed to analyse DR3 protein expression. Samples of the cell lines were also treated with 10 ng/ml TNF-α to answer the question whether the effects of Jurkat supernatant in A-375 and SK-Mel-13 were mediated by TNF-α.

3.5.1. Jurkat supernatant induced DR3 protein expression in melanoma cells

The Western blot analysis of A-375 and SK-Mel-13 after 24 and 48 hours' treatment with 50% and 75% Jurkat supernatant showed induced expression of DR3 protein band of 47 kDa (figure 3.15). TNF-α did not mimic the effect of Jurkat supernatant in the 2 melanoma cell lines.
The induction of DR3 in melanoma cells after treatment with Jurkat supernatant can be seen at two levels: (1) the quantitative increase of the 47 kDa DR3 protein and (2) the induced expression of DR3 protein in A-375 and SK-Mel-13 increased at a higher level of Jurkat supernatant after 24 hours' treatment.

To verify the induction of DR3 in melanoma cells, the cell lines Mel-2a and MeWo were also treated with Jurkat supernatant. The cells were treated with rising concentration of Jurkat supernatant at 10%, 25%, 50% and 75% for 24 hours. RPMI and DMEM growth media were used as controls. This treatment of Mel-2a and MeWo with Jurkat supernatant was done once.
The results of the Western blot after treating Mel-2a and MeWo with Jurkat supernatant verified that Jurkat supernatant effectively induced DR3 expression in melanoma cells (figure 3.16).

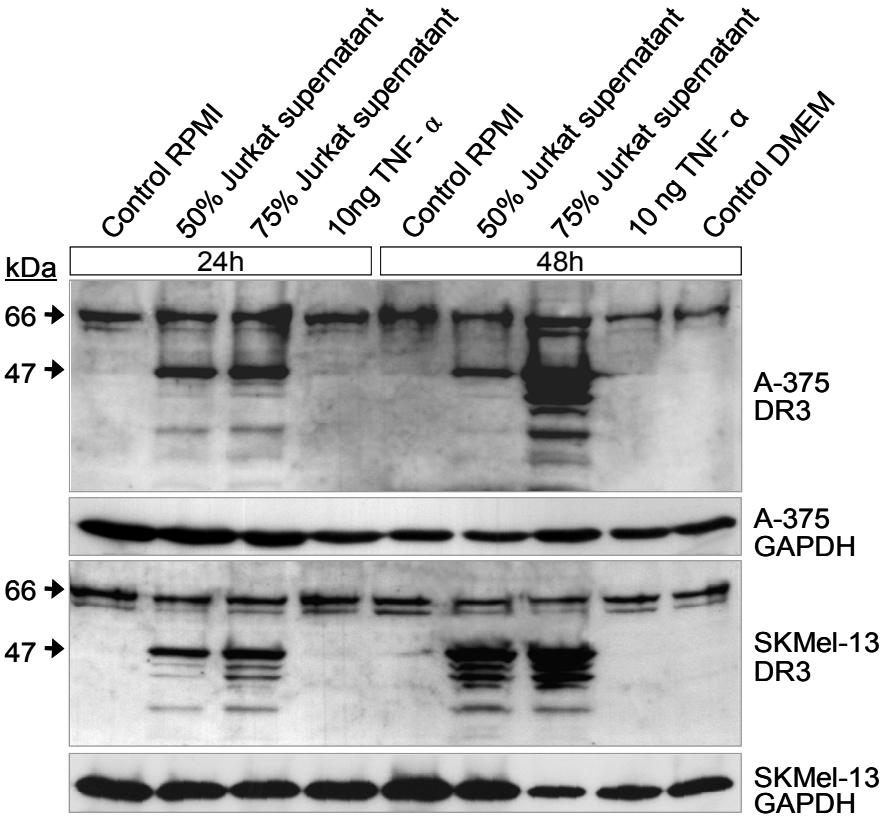

Figure 3.15 Jurkat supernatant induced DR3 protein in A-375 and SK-Mel-13

A-375 and SK-Mel-13 were treated with Jurkat supernatant and TNF-α for 24 and 48 hours. RPMI and DMEM growth media were used as controls. Induced DR3 protein expression is shown by the 47 kDa band.

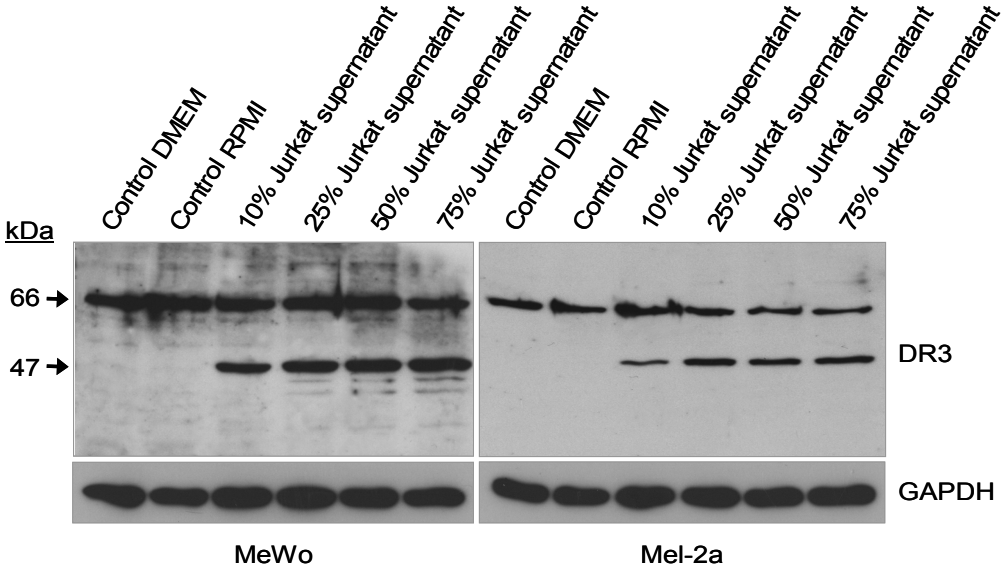

Figure 3.16 Jurkat supernatant induced DR3 expression in MeWo and Mel-2a

Mel-2a and MeWo were treated with Jurkat supernatant at concentrations of 10%, 25%, 50% and 75% for 24 hours. RPMI and DMEM growth media were used as controls. Induced DR3 protein expression is shown by the 47 kDa protein band.

3.5.2. The induction of DR3 in melanoma cells is neither mediated by TNF-α nor TL1A

The results of the Western blot analysis after treating melanoma cell lines A-375, Mel-2a, MeWo and SK-Mel-13 with Jurkat supernatant showed that Jurkat supernatant could induce DR3 protein expression in melanoma cells, while TNF-α could not mimic this effect in A-375 and SK-Mel-13.

In a further experiment, A-375 and SK-Mel-13 were treated with rising levels of Jurkat supernatant at 10%, 25%, 50% and 75% for 24 hours. At the same time, the cell lines were treated with 10 ng/ml TNF-α. The results of the Western blotting verified once again the induced DR3 in melanoma cells (figure 3.17). This induced expression is shown by the 47 kDa protein band, which represents the main major isoform of DR3 (isoform 1). Again TNF-α did not induce DR3 in the melanoma cell lines, indicating that the induction of DR3 in melanoma cells by treatment with Jurkat supernatant is not mediated by TNF-α.

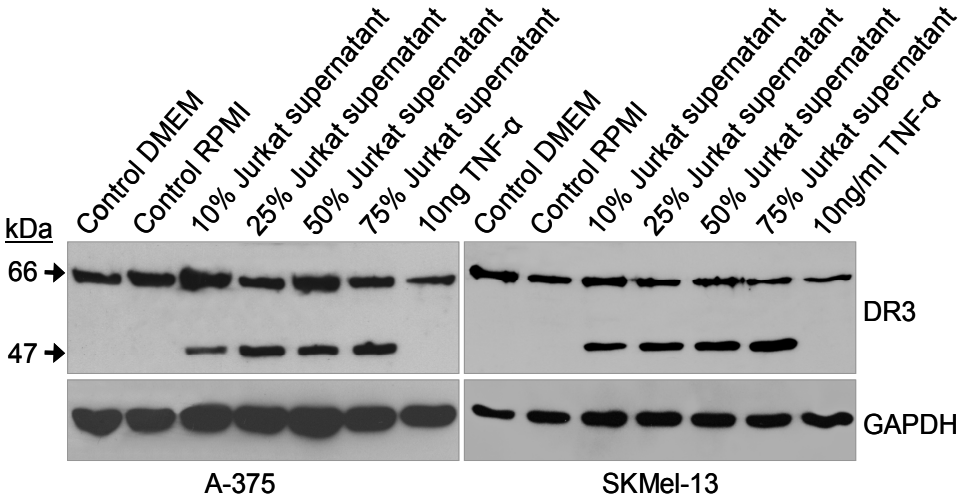

Figure 3.17 Jurkat supernatant induced DR3 expression in MeWo and Mel-2a

A-375 and SK-Mel-13 were treated with Jurkat supernatant at concentrations of 10%, 25%, 50% and 75% and TNF-α for 24 hours. RPMI and DMEM growth media were used as controls. Induced DR3 protein expression is shown by the 47 kDa protein band.

To answer the question whether TL1A could induce DR3 expression in melanoma cells, A-375 and SK-Mel-13 were treated with 100 ng/ml TL1A for 24 hours. At the same time, the cell lines were treated with Jurkat supernatant and TNF-α. After protein extraction, Western blot analysis was performed. The treatment of A-375 and SK-Mel-13 with TL1A was carried out in 2 independent experiments.

The results showed that TL1A, like TNF-α, could not induce DR3 protein expression in melanoma cells (figure 3.18).

In summary, Jurkat supernatant effectively induced the expression of DR3 protein in melanoma cells, as shown above in A-375, Mel-2a, MeWo and SK-Mel-13. This effect of Jurkat supernatant in melanoma cells was shown by the induction of the main DR3 isoform 1, which was displayed as a 47 kDa protein band in Western blot. The DR3 protein induction in melanoma cells by Jurkat supernatant increased at higher levels of Jurkat supernatant. Neither TL1A, nor TNF-α could mimic the effect of Jurkat supernatant in melanoma cells, indicating that the DR3 protein induction was not mediated by these two ligands.

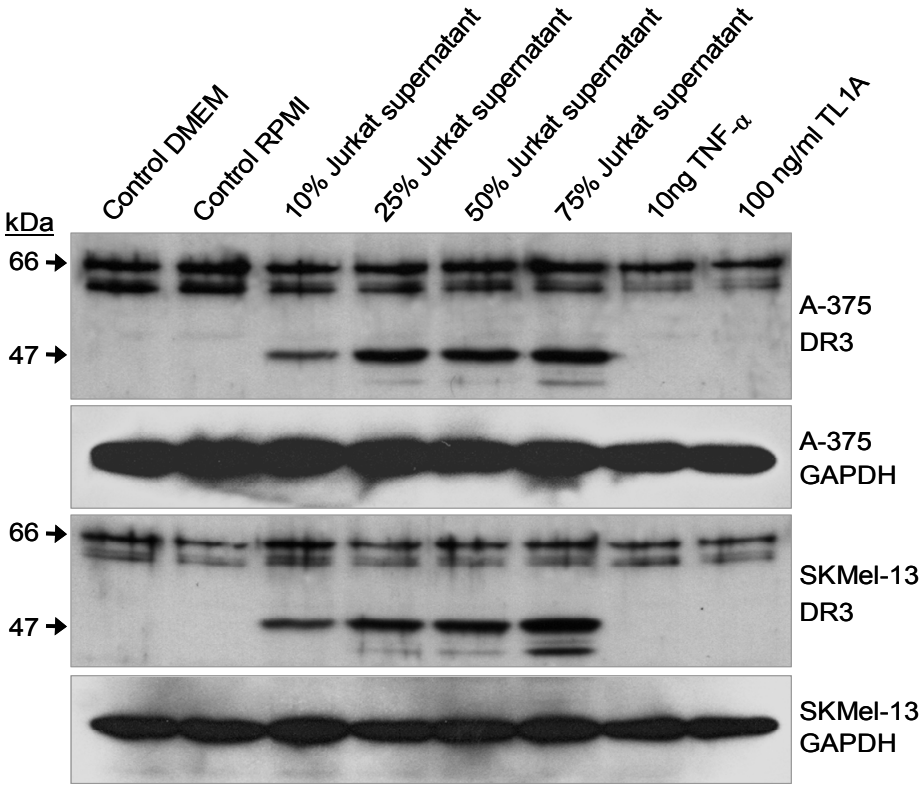

Figure 3.18 TL1A and TNF-α did not induce DR3 expression in melanoma cells

A-375 and SK-Mel-13 were treated with Jurkat supernatant, TL1A and TNF-α for 24 hours. RPMI and DMEM growth media were used as controls. Induced DR3 protein expression is shown by the 47 kDa protein band.

3.6. Induction of 47 kDa DR3 protein correlated with DR3 surface expression

To address the question whether the 47 kDa band correlated with surface DR3 expression, FACS analysis was carried out with the treated A-375 and SK-Mel-13 to find out whether the induced protein is membrane bound. A-375 and SK-Mel-13 were treated with rising concentration of 10%, 25%, 50%, 75% and 100% Jurkat supernatant for 24 hours and 48 hours. After treatment with 100% Jurkat supernatant, only A-375 could be analysed (24 hours) because the cells were no longer viable for the FACS analysis. This analysis was carried out 3 times with similar results. A-375 and SK-Mel-13 showed significant surface expression of DR3 already after treatment with 10% Jurkat supernatant, which increased with higher concentrations (figures 19 and 20). The experiment was carried out once in Mel-2a and MeWO with similar results.

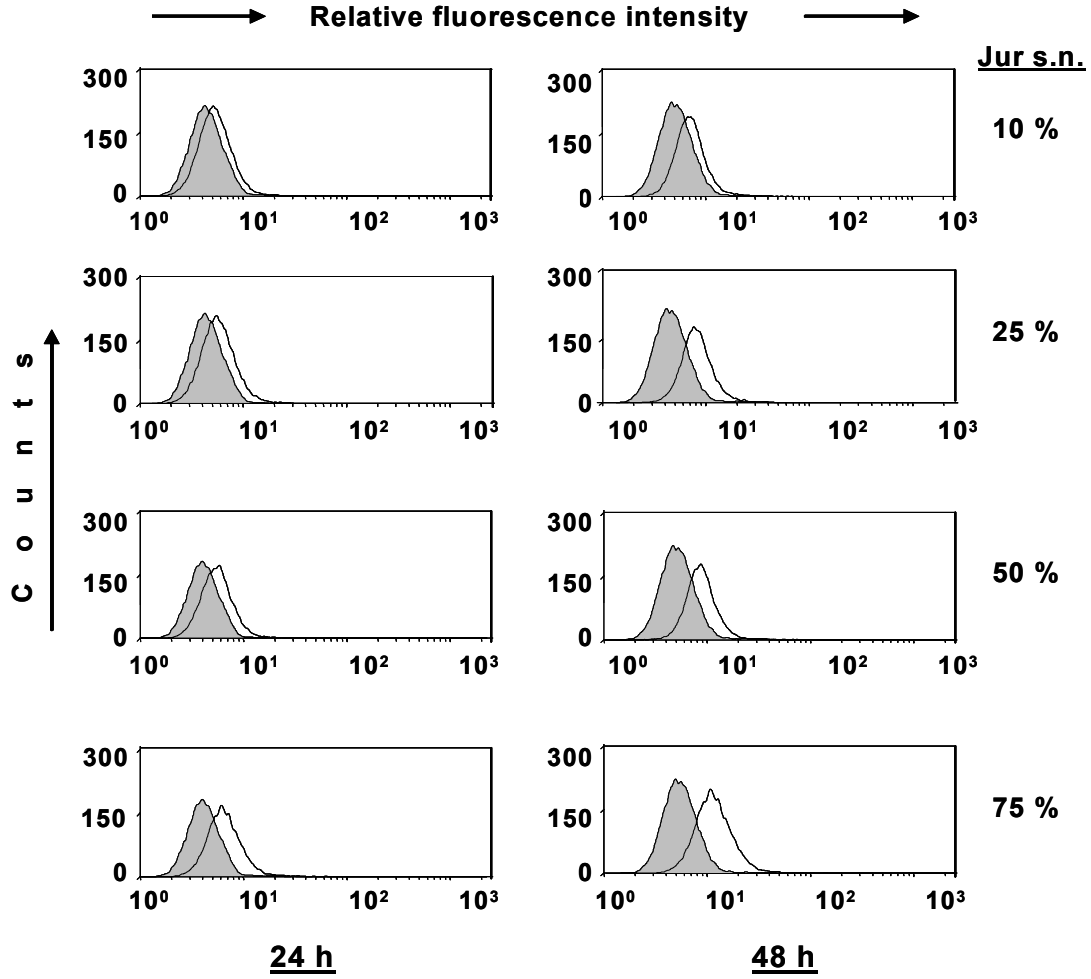

Figure 3.19a DR3 surface expression in A-375 after treatment with Jurkat supernatant

Data reflect the number of counts plotted against the relative fluorescence intensity in FACS analysis. The grey curve represents unspecific IgG binding; a shift of the white curve to the right shows a positive surface expression of DR3. Jur s.n. = Jurkat supernatant.

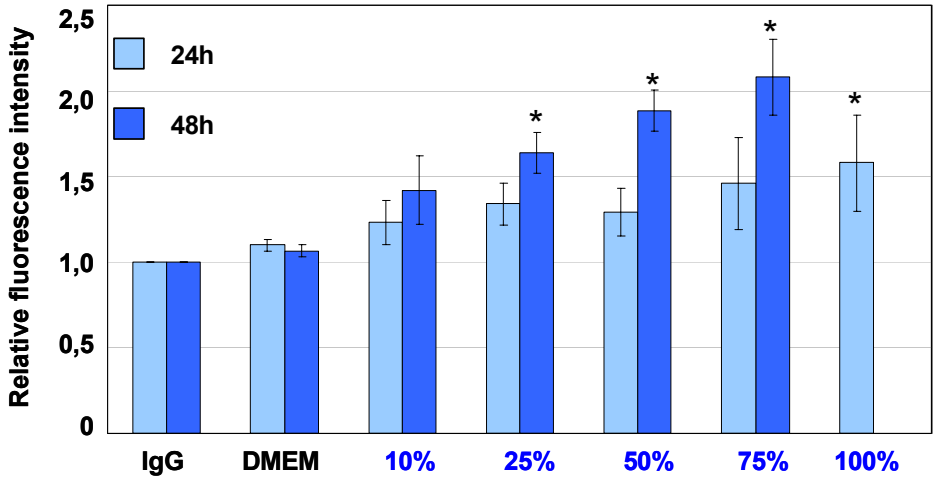

Figure 3.19b DR3 surface expression in A-375 after treatment with Jurkat supernatant

A-375 was treated with Jurkat supernatant and compared to IgG and melanoma growth medium as controls. Data shows mean values of the FACS analysis, the significance ($p < 0.05$) is marked with asterisks (*).

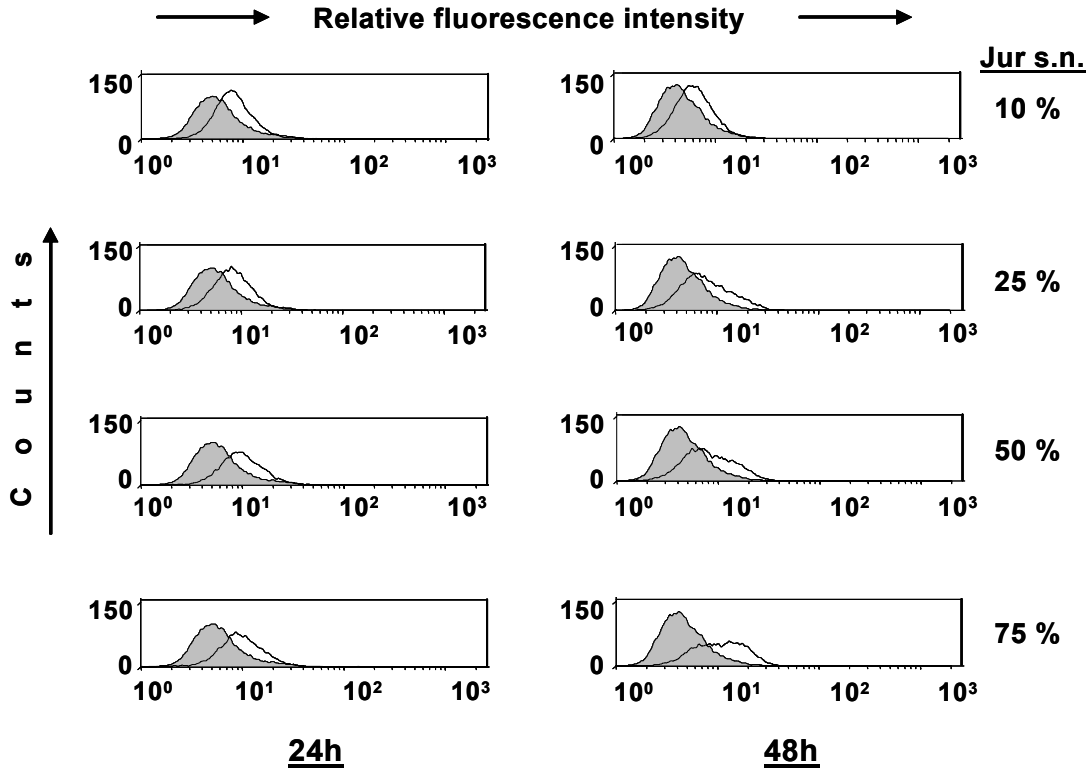

Figure 3.20a DR3 surface expression in SK-Mel-13 after treatment with Jurkat supernatant

Data reflect the number of counts plotted against the relative fluorescence intensity in FACS analysis. The grey curve represents unspecific IgG binding; a shift of the white curve to the right shows a positive surface expression of DR3. Jur s.n. = Jurkat supernatant.

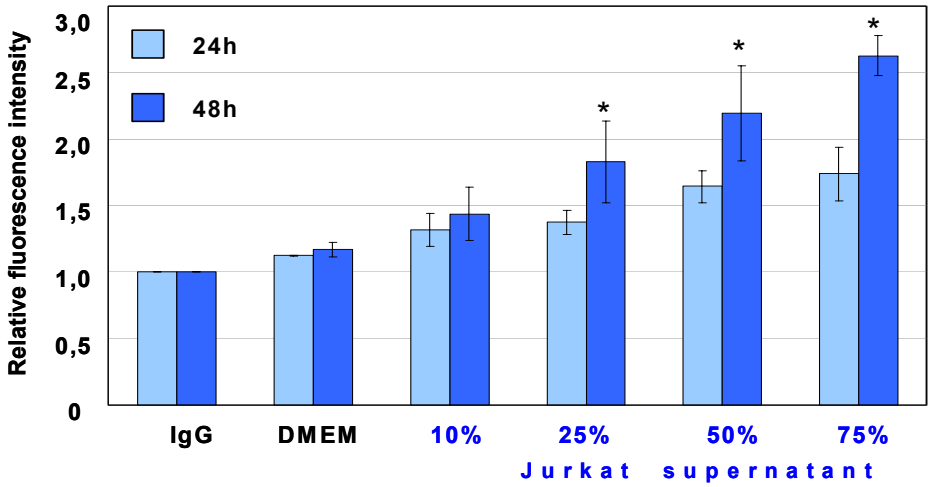

Figure 3.20b DR3 surface expression in SK-Mel-13 after treatment with Jurkat supernatant

SK-Mel-13 was treated with Jurkat supernatant and compared to IgG and melanoma growth medium as controls. Data shows mean values of the FACS analysis, the significance ($p < 0.05$) is marked with asterisks (*).

3.7. Upregulation of DR3 by Jurkat supernatant correlated with increased apoptosis

To address the question whether the expression of DR3 alone could induce apoptosis in melanoma cells, A-375 and SK-Mel-13 were treated with 10% Jurkat supernatant for 6 and 24 hours, after which DNA fragmentation was measured. In the same analysis, A-375 and SK-Mel-13 were treated simultaneously with 10% Jurkat supernatant and 100 ng/ml TL1A for 6 hours and 24 hours to answer the question whether Jurkat supernatant could sensitize melanoma cells for the effects of TL1A. The analysis was performed twice for the 24 hour treatment, first with triple samples, and then with double samples, showing similar results. The analysis was carried out once for the 6 hour treatment. The treatment with Jurkat supernatant alone triggered apoptosis in A-375 with factor 3.0 - 4.0 after 6 hours as quantified by DNA fragmentation in treated cells compared to non treated cells. The apoptotic effect of Jurkat supernatant in A-375 was stronger with factor 5.0 at 24 hours. Jurkat supernatant also sensitized for TL1A in A-375 at 6 hours' treatment. No apoptosis was measured in SK-Mel-13 after 6 hours' treatment with Jurkat supernatant. After 24 hours' treatment, significant apoptosis was measured in SK-Mel-13 with only factor 1.7. Jurkat supernatant also sensitized for TL1A effects (figure 3.21).

These results reflect the previous data of the FACS analysis, which show the DR3 surface expression of both cell lines after treatment with Jurkat supernatant; the induction of surface DR3 in A-375 took place earlier and was much stronger than in SK-Mel-13 (figures 3.19 and 3.20).

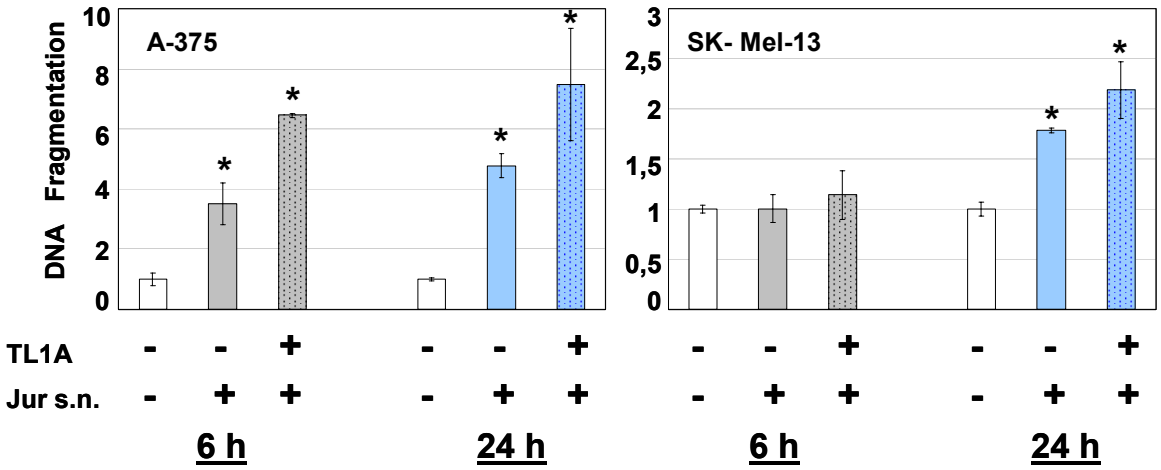

Figure 3.21a Jurkat supernatant alone triggered apoptosis in A-375 and SK-Mel-13

A-375 and SK-Mel-13 were treated with 10% Jurkat supernatant alone and with a combination of 10% Jurkat supernatant and 100 ng/ml TL1A for 6 hours and 24 hours. The analysis was done twice. Relative apoptosis is shown by DNA fragmentation. Data shows mean values. Significance is marked with asterisks (*). Jur s.n. = Jurkat supernatant.

The LDH release was also measured to mark cytotoxic effects of Jurkat supernatant in A-375 and SK-Mel-13. Jurkat supernatant showed early cytotoxicity in A-375 but had no cytotoxic effects in SK-Mel-13 after treatment with Jurkat supernatant, and Jurkat supernatant combined with TL1A (figure 3.21b). The cytotoxic response in A-375 was not statistically significant.

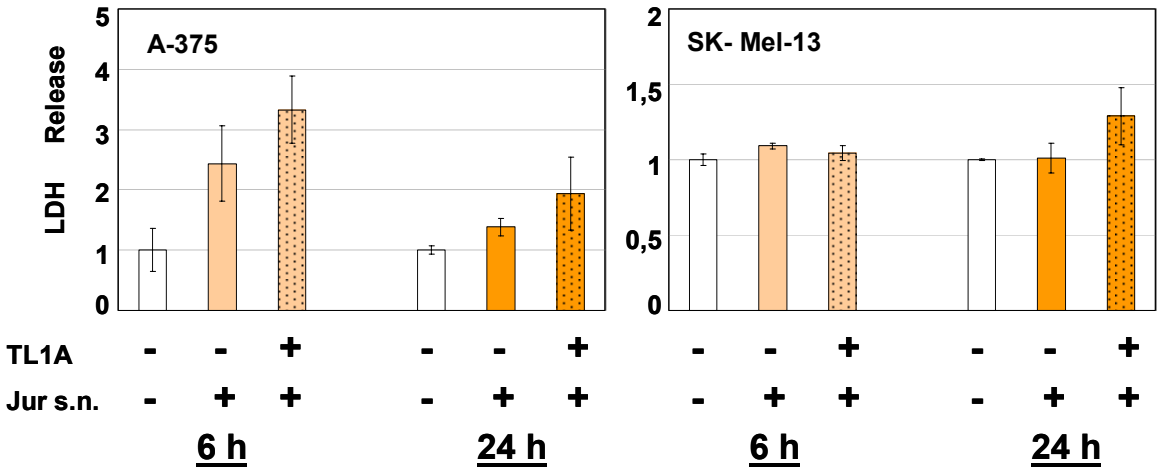

Figure 3.21b Jurkat supernatant triggered early cytotoxicity in A-375, but not in SK-Mel-13

A-375 and SK-Mel-13 were treated with 10% Jurkat supernatant alone and with a combination of 10% Jurkat supernatant and 100 ng/ml TL1A for 6 hours and 24 hours. The analysis was done twice. Relative cytotoxicity is shown by LDH release. Data shows mean values. Jur s.n. = Jurkat supernatant.

4. Discussion

4.1. Significance of FasL, FLIP, TL1A, DcR3, DR6 and DR3 expression

The interplay of death receptors, decoy receptors and their cognate ligands regulate apoptosis in different cell systems. To understand the apoptosis resistance of malignant melanoma, the basic expression of the death receptors and ligands that have not yet been fully described in melanoma cells, but may play a role in apoptosis regulation in malignant melanoma, was investigated in this study. In an initial stage of experiments, the basic mRNA expression of FasL, FLIP, TL1A, DcR3, DR6 and DR3 was analysed in melanoma cell lines using RT-PCR and nested PCR. In a second stage, the protein expression of DR3, DcR3 and TL1A in melanoma cell lines was investigated using Western blot and immuno detection of proteins.

FasL

Fas ligand (FasL) is the natural ligand for the death receptor Fas; the binding of FasL to Fas triggers apoptosis in Fas expressing cells (Krammer, 1999). FasL, which plays a very important role in the immune system, is expressed in a more restricted way than the receptor Fas. Cytotoxic T lymphocytes (CTL) for example, remove virus-infected cells via expression of FasL and interaction with the Fas receptor (Golstein, 1997), (Medema et al, 1997) on target cells. The death inducing function of FasL is best documented in the context of apoptosis in T cells (Janssen et al, 2003). Outside the lymphocyte system, FasL expression has been associated with the establishment of immune privilege and tumour survival (Restifo, 2000).

The expression of FasL by human melanoma cells has remained unclear over the years. While several reports state that human melanoma cells do not express FasL (Chappell et al, 1999), (Eberle et al, 2003), the expression of FasL by melanoma cells has been reported as an important mechanism in the immune evasion by the tumour cells (Hahne et al, 1996). In this analysis, the basic expression of FasL mRNA in melanoma cell lines was investigated using RT-PCR and nested PCR. After first reaction RT-PCR consisting of 30 cycles, only the positive control cell line Jurkat was positive for FasL mRNA. All melanoma cell lines as well as NHM were negative for FasL mRNA after first reaction PCR, thus showing no significant expression.

Nested PCR was carried out using 10% of the sample from the first reaction PCR in a second PCR reaction consisting of 30 cycles. 5 out of 9 melanoma cell lines then revealed some weak expression of FasL mRNA, which was however largely inconsistent. Similarly, NHM showed inconsistent expression of FasL after nested PCR. These results suggest that melanoma cells have low FasL mRNA expression. In fact, transfection of FasL proved to be a highly efficient

tool against melanoma cells in vitro and in vivo, showing that targeted expression of CD95L may even represent a suitable strategy for melanoma therapy (Eberle et al, 2003). The results of this analysis, therefore, falls in line with the view that FasL expression plays a less significant role in the apoptosis resistance and immune escape of melanoma cells.

FLIP

FLICE (FAS-associated death-domain-like IL-1beta-converting enzyme)-inhibitory protein, FLIP has been identified as an inhibitor of death receptor signaling. Overexpression of cellular (Medema et al, 1999) or viral (Djerbi et al, 1999) FLIP prevent the recruitment of procaspase 8, which is an initiator caspase in the apoptotic pathway. In this analysis, the basic mRNA expression of cellular FLIP was compared to that of NHM. All melanoma cell lines showed a consistent expression of FLIP. There were no differences of FLIP expression in melanoma cell lines compared to NHM.

It has been reported that resistance to Fas mediated apoptosis may be partly due to the constitutive expression of FLIP in chondrocytes. Activation of caspase 8 was minimal in cultured chondrocytes because of high FLIP expression. Correspondingly, surface Fas expression has been shown in melanoma cell lines (Eberle et al, 2003) however, malignant melanoma remains a highly aggressive tumour with early infiltration abilities. The results of this analysis suggest that FLIP expression may play a role in resistance to Fas mediated apoptosis in melanoma cells. Since FLIP expression in melanoma cells did not differ from that in NHM, the role of FLIP in the apoptosis resistance in melanoma cells may be of lesser importance.

TL1A

Melanoma cell lines showed a basic mRNA expression of TL1A, the ligand for DR3. This ligand was first identified on HUVECS and characterized by its function as an angiogenesis inhibitor of the TNF family. TL1A acts by directly inhibiting endothelial cell proliferation, hence the synonym, vascular endothelial growth inhibitor, VEGI, (Zhai et al, 1999a). In a later report, Zhai et al examined the anticancer potential of TL1A in a breast cancer xenograft tumour model in which the cancer cells were co-injected with cells overexpressing a soluble TL1A. This treatment resulted in potent inhibition of xenograft tumour growth, consistent with the view that TL1A is an endothelial cell specific negative regulator of angiogenesis (Zhai et al, 1999b). Thus, for the first time, this study describes expression of TL1A in melanoma cells.

DcR3:

Decoy receptors are known to bind ligands and block the biological functions mediated by these ligands. DcR3 is reported to bind to at least three different ligands: FasL (Pitti et al, 1998), LIGHT (Yu et al, 1999) and TL1A (Migone et al, 2002). Studies on DcR3 describe it as a proteolytically processed, soluble receptor (Wroblewski et al, 2003) with clinical implications for cancer growth and progression as well as modulation of the immune system. Its expression in malignant cells of several tumour types has been postulated to help tumour cells to gain survival advantages by inhibiting FasL and LIGHT mediated apoptosis. To date, DcR3 has not yet been described in the melanoma.

DcR3 mRNA was detected in all melanoma cell lines and NHM analysed. Using two independent DcR3 antibodies of goat and rabbit origin, a 105 kDa protein band was detected in medium supernatants of melanoma cell lines as well as in whole cell lysates. Although the expected protein size of DcR3 as defined by the antibodies was 33 kDa, the 105 kDa protein band could be DcR3 trimer, since (1) this band was detected by two independent antibodies and (2) TNFR family members are known to build trimers upon activation. The protein expression of DcR3 in melanoma cell lines may be of high significance because of the following findings:

In gastric carcinomas, serum DcR3 levels were closely correlated with tumour differentiation status and TNM classification. Tumour mass was the source of serum DcR3 since its levels decreased drastically after curative tumour resection (Wu et al, 2003). Overexpression of DcR3 was found in B-cell lymphoma cell lines and diffuse large B-cell lymphoma (DLBCL) patients. DcR3 overexpression was associated with a worse prognosis in DLBCL patients ($p=0.05$). An in vitro study showed that neutralization of DcR3 increased the percentage of doxorubicin-mediated apoptosis in two B-cell lymphoma cell lines, which indicated the possibility of DcR3 mediated chemo-resistance in B-cell lymphomas (Chang et al, 2008). Moreover, DcR3 is highly expressed in many pancreatic cancers and endogenous DcR3 blocks the growth inhibition signals mediated by FasL. DcR3 reduced growth inhibition when added exogenously. Regression analysis showed that the DcR3 expression significantly correlated with the sensitivity to FasL, and not to CH-11, an agonistic antibody for Fas receptor (Tsuji et al, 2003). Through neutralization of TL1A with soluble DcR3, Yang et al demonstrated that DcR3 could induce angiogenesis in human umbilical vein endothelial cells (HUVECs) (Yang et al, 2004).

DcR3 has been shown to play a significant role in modulating immune responses. It can inhibit T-cell costimulation by blocking the one-way signaling from TL1A to DR3. It also inhibited actin polymerization of T cells upon mitogen stimulation, and suppressed T-cell pseudopodium

formation, which is known to be important for cell-cell interaction. Consequently, T-cell aggregation was suppressed by soluble DcR3 (Wan et al, 2003).

All the above-mentioned activities and effects of DcR3 may suggest a high significance in diagnosis, prognosis and therapy options by targeting this receptor in future. Indeed, Wu et al (2007) demonstrated that gastric cancer patients with high DcR3 expression presented more advanced disease than those with low DcR3 expression. Hence, preoperative control of serum DcR3 might be an additional approach to imaging modalities for evaluating stages in malignant melanoma to help guide treatment procedures in future.

DR6

The expression of DR6 has not yet been described in malignant melanoma. All melanoma cell lines and NHM analysed showed a basic DR6 mRNA expression. Melanoma cell lines showed no differences in their basic expression in DR6 mRNA amongst the cell lines, or in comparison to the NHM and Jurkat/SW480 positive controls. These results are in line with a study of DR6 in human tissues and cancer cell lines by Pan et al: DR6 was expressed in most human tissues and abundant transcript was detected in heart, brain, placenta, pancreas, thymus, lymph node and several non-lymphoid cancer cell lines. Among human cancer cell lines, transcript levels were detected in the melanoma cell line G361, the colorectal adenocarcinoma cell line SW480, the lung carcinoma cell line A549 and the cervical carcinoma cell line HeLa S3 (Pan et al, 1998). Furthermore, ectopic expression of DR6 has been shown to induce apoptosis in the cervical carcinoma cell line HeLa, but not in the mammary carcinoma cell line MCF7, suggesting cell type differences in DR6 signaling to induce apoptosis. Despite the ability of exogenous DR6 expression to induce apoptosis, DR6 mRNA and protein were found to be elevated in prostate tumour cell lines and in advanced stages of prostate cancer. Here, the report suggested that NF-κB regulated survival proteins may protect from DR6 induced apoptosis and that DR6 is a target of NF-κB regulation. TNF-α is able to induce expression of DR6 (Kasof et al, 2001). Lastly, a regulatory role of DR6 in the activation and function of B cells was demonstrated by observations in DR6(-/-) B cells upon stimulation, which showed themselves more effective as antigen-presenting cells (Schmidt et al, 2003). A specific ligand for DR6 has been recently described by Nikolaev et al, who reported that β-amyloid precursor protein (APP) is a regulated DR6 ligand, and that APP and DR6 binding triggers neuronal cell death. The results also indicated that APP and DR6 are components of a neuronal self-destruction pathway that contributes to Alzheimer's disease (Nikolaev et al, 2009).

Here, a basic mRNA expression of DR6 is described for the first time in melanoma cell lines and NHM.

DR3

In all 10 melanoma cell lines and 6 NHM, as well as the positive control cell lines Jurkat, SW480 and MCF-7, DR3 mRNA was very weak after 35 cycles of simple RT-PCR. Strong amplicon signals could only be detected after nested PCR (2 x 30 PCR cycles). This shows a low intracellular DR3 mRNA and implies that either the cell lines have a low basic transcription of the DR3 gene, or high rates of DR3 mRNA decay.

DR3 is one of the genes that undergo alternative mRNA splicing, producing 12 distinct mRNA variants to date (Screaton et al, 1997; SWISS-PROT entry Q99831/Q93038). The products of the RT-PCR correspond to the various mRNA splice forms as predicted by the chosen PCR primers. In the first RNA extractions, 8 of 10 melanoma cell lines showed an unexpected cDNA (480bp) which did not correspond to any of the 12 described splice forms; this could be a new, not yet defined DR3 splice form. This is not surprising because while investigation the DR3 mRNA expression in tumour tissues obtained from newly diagnosed follicular lymphoma patients, Warzocha et al characterized a new DR3 isoform differing from the original 417aa DR3, termed DR3β with 426aa, suggesting that there could still be undefined isoforms in different tissues and/or tumours depending on type and/or stage (Warzocha et al, 1998).

In the second, independent RNA extraction, the cell lines displayed a change of alternative splice pattern. These results are similar to the findings of Screaton et al (1997) in a study of differential expression of DR3 in lymphocytes, namely, the pattern of alternative splicing changed in the lymphocyte subsets according to activation and/or proliferation rate.

It was first suggested that DR3 expression was restricted to tissues enriched in lymphocytes such as the spleen, thymus, intestines, lymph nodes and peripheral blood lymphocytes (Chinnaiyan et al, 1996; Kitson et al, 1996; Screaton, 1997). Recent studies, however, show that DR3 could be found in different organs exhibiting various activities depending on the system.

Here, for the first time, DR3 mRNA expression in malignant melanoma cells was shown by nested RT-PCR. However, compared to NHM, Jurkat and SW480, the main DR3 mRNA isoform was not detected in melanoma cell lines.

The results of the Western blots showed that melanoma cell lines and NHM expressed a glycosylated DR3 protein of 66 kDa. Jurkat showed two DR3 protein bands of 66 kDa and 47 kDa. The two different polyclonal antibodies (rabbit and mouse) used in the Western blotting defined an expected protein size of about 62 kDa. The full length, functional DR3 has been

defined as a 47 kDa protein. However, Kitson et al (1996) characterized a 54 kDa protein by Western blotting of total spleen protein using a polyclonal antibody. Furthermore, using two different polyclonal antibodies, DR3 proteins of 47 kDa, 55 kDa, 66 kDa, 97 kDa and 180 kDa have been identified in colon cancer cell lines HT29 and LoVo. The DR3 proteins of high molecular weight were identified as having glycosylated rests (Gout et al, 2006).
Therefore, the 66 kDa DR3 protein band seen in melanoma cell lines, NHM, Jurkat, MCF-7 and HeLa may correspond to the glycosylated DR3, whilst the 47 kDa band very likely represents full length, non-glycosylated DR3 protein.
These results describe the expression of DR3 protein in melanoma cells for the very first time. The expression of DR3 in melanoma cells is important because DR3 could be a target for understanding the pathogenesis of malignant melanoma, which would in turn help in the development of new strategies in treatment.

4.2. TL1A induced early cytotoxicity in melanoma cells

The physiological signal activated by DR3 through binding of its cognate ligand TL1A has been scarcely studied in relation to apoptosis. After showing the expression of DR3 protein in melanoma cell lines using Western blotting, the activity of TL1A was analysed to shed light on the physiological activity caused by interaction between receptor and ligand in melanoma cells. The cytotoxicity of the ligand was measured by LDH release in growth medium after treatment of melanoma cells with the ligand. The results of the LDH release assays showed that TL1A induced early cytotoxicity in all 5 melanoma cell lines under investigation.

Several recent studies of TL1A and DR3 interactions bring TL1A in line with cytotoxicity in cells, and/or inflammatory conditions. Recombinant chicken TL1A protein showed cytotoxic activity in the murine fibrosarcoma cell line L929 and cultured chicken fibroblast cells, suggesting that chicken TL1A possibly functions as a substitute for TNF-α, since a homologous sequence of mammalian TNF-α has not been identified in the chicken genome database so far (Takimoto et al, 2005).
TL1A production was localized to the intestinal lamina propria in macrophages and CD4(+) and CD8(+) lymphocytes from Crohn's disease (CD) patients as well as in plasma cells from ulcerative colitis patients. The amount of TL1A protein and the number of TL1A-positive cells correlated with the severity of inflammation, most significantly in CD (Bamias et al, 2003).

The results of another study suggested the possibility that interaction between TL1A expressed on antigen-presenting cells and DR3 may be of particular importance for the pathogenesis of chronic inflammatory conditions, including inflammatory bowel disease. Blockade of the TL1A/DR3 pathway could offer therapeutic opportunities in Crohn's disease (Bamias et al, 2006). In fact, TL1A has been proven a mediator of gut inflammation (Young et al, 2006).

To investigate the role of TL1A and DR3 in the functioning of macrophage cells in relation to atherogenesis, the cellular events mediated by TL1A and DR3 in a human macrophage-like cell line THP-1 were analyzed. Treatment of THP-1 cells with recombinant TL1A in combination with IFN-gamma caused induction of pro-inflammatory cytokines MMP-9 and IL-8, suggesting that TL1A and DR3 interaction is involved in atherosclerosis (Kang et al, 2005).
There is also evidence to suggest that TL1A and DR3 interaction is involved in the aetiology of rheumatoid arthritis (Bossen et al, 2006). Severe rheumatoid arthritis (RA) stage was associated with highly elevated TL1A and DcR3 serum levels. TL1A concentrations were elevated in patients with RA by 5-fold ($P<0.00001$) (Bamias et al, 2008).
Gout et al (2006) also demonstrated in experiments with recombinant human TL1A in the DR3 expressing colon carcinoma cells HT29 that receptor activation through the ligand increases LDH release, and leads to activation of caspase 3 and nuclear fragmentation.

Thus, the cytotoxic activity of TL1A (LDH release) has been shown in different cell systems and appears to be of physiological consequence, especially in pathological situations related to inflammation. In this study, the cytotoxic effect of TL1A was described for the first time in melanoma cell lines. Since melanoma cells showed no DR3 expression, the cytotoxic activity of TL1A in melanoma might be unspecific.

4.3. TL1A had no apoptotic activity in melanoma cells, except in SK-Mel-13

Apoptotic activity has also been described for TL1A, although it received only marginal attention in released publications. In describing the identification and characterization of the TL1A ligand, Migone et al demonstrated that TL1A interacts specifically with membrane associated DR3 and that TL1A was able to induce caspase activation (initiator of the apoptotic pathway) in the human erythroleukemic cell line TF-1, which expresses DR3 endogenously.

However, TL1A activated caspases mainly in the presence of cycloheximide (CHX), which blocks protein synthesis (Migone et al, 2002).
Experiments similar to those of Migone et al (2002) were carried out with TL1A in the presence of CHX in melanoma cell lines. The results showed that CHX alone was such a potent apoptosis inducer in melanoma cells that the additive effects of TL1A were not significant (data not shown). Thus, TL1A showed no apoptotic activity in melanoma cells, excepting late, moderate apoptosis in SK-Mel-13.
These results mark the first description of TL1A effects in melanoma cells regarding its apoptotic activity.

4.4. TL1A induced late, moderate apoptosis, but not cytotoxicity in NHM

The physiological activity of TL1A was analysed in NHM, which are the natural precursors of malignant melanoma. In comparison to melanoma cells, significant DNA fragmentation in NHM was measured after 48 hours with factor 1.7 in treated cells. Interestingly, TL1A did not cause early cytotoxicity in NHM as compared to melanoma cell lines. Instead, LDH release was very low in treated NHM, and hence non-significant, whilst a higher release was seen in treated melanoma cells with up to factor 4 in A-375.
The human erythroleukemic cell line, TF-1 and activated T cells both naturally express endogenous DR3 (Migone et al, 2002). Interestingly, TL1A treatment of these cells did not result in significant apoptosis. A monoclonal agonistic antibody, F05, against DR3 was generated and TF-1 cells were treated with either F05 or recombinant TL1A. No effect on apoptosis or caspase activation was seen when F05 or TL1A was used in the absence of CHX, suggesting that neither F05 nor TL1A alone can trigger apoptosis in TF-1 cells (Wen et al, 2003).
Here, the results of this analysis show for the first time that TL1A may have different effects regarding its functions in non-transformed cells (NHM) and malignant cells.

4.5. Melanoma cells and NHM lacked surface expression of DR3 protein

The moderate apoptosis induction by TL1A in NHM, and the absence of apoptotic activity of TL1A in melanoma cells raised questions about the functionality of the death receptor. Migone et al (2002) showed that TL1A would only bind to the functional transmembrane DR3. In this light, FACS analysis was carried out to address the question of the expression of transmembrane DR3 in melanoma cells, which showed endogenous DR3 expression.

The FACS analysis showed no surface DR3 expression in any of the 6 melanoma cell lines (A-375, Mel-2a, Mel-HO, MeWo, SK-Mel-13 and Bro) or in 3 NHM. Jurkat cells, however, displayed a clear surface expression of DR3. These results were rather curious since all cell lines had endogenous DR3, although differing in the pattern of DR3 isoforms in the sense that the melanoma cell lines and NHM expressed only the glycosylated 66 kDa DR3 protein band, whilst Jurkat expressed the glycosylated 66 kDa and the 47 kDa DR3 protein bands.
This difference in DR3 expression suggests that the 66 kDa protein band could be unspecific, while the 47 kDa band represents the actual DR3.

Since it had been reported that TL1A would only bind to transmembrane DR3, we reasoned that melanoma cell lines, just like TF-1 and T cells, might naturally express DR3; the lack of surface expression of the death receptor, however, might be the cause of apoptosis resistance in the cells after treatment with TL1A. Therefore, ways of inducing surface DR3 expression were tried out.

4.6. Jurkat supernatant induced DR3 protein expression in melanoma cells

In quest of the cause(s) of the lack of surface DR3 in melanoma cells, a thorough investigation was done to screen out possibilities of the differences in expression resulting from the cell culture conditions of melanoma cell lines as compared to those of Jurkat cells, which showed both intracellular and transmembrane DR3. After ruling out any connection between cell culture conditions and DR3 expression, it was reasoned that transmembrane DR3 expression could be a result of a specific response of the melanoma cells to modulations in the immune system. Jurkat cells represent a specific part of the immune system since they are an immortalized line of T lymphocytes used to study T cell signaling. Therefore, after growing Jurkat cells overnight, the supernatant was obtained by discarding the cells per centrifugation. The melanoma cell lines A-375 and SK-Mel-13 were then treated with the Jurkat supernatant. Western blotting was carried out to find out the pattern of DR3 isoform expression in the same cells. After 24-hour treatment with 25% Jurkat supernatant, the 47 kDa protein band could be detected in both A-375 and SK-Mel-13 per Western blotting. At higher concentrations of the supernatant (50% and 75%), more protein bands could be seen, which showed stronger signals at 75%. The expression of the glycosylated 66 kDa and sometimes 54 kDa bands remained unchanged.
The experiment was performed on the melanoma cell lines Mel-2a and MeWo with the same results.

Thus, for the first time, the induction of DR3 protein expression in melanoma cells through direct treatment with the supernatant of the T cell line Jurkat was demonstrated in this study.

4.7. Neither TNF-α, nor TL1A could induce DR3 protein expression in melanoma cells

Several reports state that the expression of death receptors can be boosted by cytokines such as interferon-γ and TNF-α. Fas expression, for example, can be effectively induced by TNF-α, as well as by the activation of lymphocytes (Golstein, 1997), (Medema et al, 1997).
In this study, the melanoma cell lines A-375 and SK-Mel-13 were treated with TNF-α and TL1A in a parallel experiment of the treatment with Jurkat supernatant. Neither TNF-α, nor TL1A was able to induce DR3 protein isoforms in melanoma cells. The expression of the glycosylated 66 kDa and sometimes 54 kDa bands remained unchanged.

4.8. Induction of DR3 protein in melanoma cells correlated with surface DR3 expression

The results of the Western blots after treatment of melanoma cell lines A-375, SK-Mel-13, Mel-2a and MeWo with Jurkat supernatant showed that Jurkat supernatant effectively induced DR3 as a 47 kDa protein band in melanoma cells. To address the question whether the induced DR3 protein was membrane bound, the melanoma cell lines A-375 and SK-Mel-13 were treated with Jurkat supernatant and then tested in FACS analysis for surface DR3 expression.
Surface DR3 was positive in A-375 and SK-Mel-13 cells after treatment with Jurkat supernatant in a dose and time dependency. The positive shifts in the FACS curves were comparable to those of Jurkat cells. These results demonstrated that, indeed, the induction of DR3 protein expression correlated with the expression of surface DR3 in melanoma cells.

Several independent studies of the full length transmembrane DR3, which is also known as the DR3 isoform 1, report that this protein runs as a 47 kDa protein band in Western blotting (Chinnaiyan et al, 1996; Kitson et al, 1996; Marsters et al, 1998).
In this study, it has been shown that the induction of DR3 protein of 47 kDa correlates with surface expression of DR3 in melanoma cells. Moreover, Jurkat cells, which differ in their basic DR3 expression only in the expression of the DR3 protein of 47 kDa, were always positive for surface DR3. It can therefore be deduced that the induced DR3 protein band of 47 kDa in the melanoma cells through Jurkat supernatant treatment could be the transmembrane DR3 protein, hence the positive surface DR3 in A-375 and SK-Mel-13 after treatment with Jurkat supernatant.

4.9. Jurkat supernatant induced apoptosis in melanoma cells

Jurkat supernatant alone effectively triggered apoptosis in melanoma cells and sensitized A-375 cells to TL1A activity, since significant apoptosis was detected in melanoma cells after treatment with Jurkat supernatant, especially in A-375, which was also sensitized to TL1A effects in the presence of Jurkat supernatant.
The cytotoxicity of melanoma cell lines A-375 and SK-Mel-13 after the treatment with Jurkat supernatant was not statistically significant and could be a result of necrosis due to high apoptotic activity.

Supposing that Jurkat supernatant's apoptotic activity in A-375 and SK-Mel-13 was mediated by the induced expression of surface DR3, the above results would fall in line with previous observations made by others: Overexpression of full length DR3 in cell lines triggered apoptosis (Chinnaiyan et al, 1996; Kitson et al, 1996; Bodmer et al, 1997; Marsters et al, 1998). Chinnaiyan et al reported apoptosis induction and NF-κB activation after ectopic expression of DR3 in MCF-7 cells. Kitson et al investigated apoptosis induction with constructs of full length DR3 and the truncated DR3 and reported significant apoptosis of 3T3 and 293T cells transfected with full length DR3 within 24 - 48 hours but not in cells transfected with the truncated form. After transiently overexpressing full length DR3 in 293T cells, Bodmer et al reported aggregation of the cytoplasmatic region, apoptosis and NF-κB activation. Finally, Marsters et al ectopically expressed full length DR3 in HEK293 and HeLa cells and reported a marked increase in DNA fragmentation and NF-κB activation.
It is important to note that all above-mentioned results were obtained in transient systems where DR3 and/or the signaling molecules were overexpressed. The biological signal transducers activated by DR3 through engaging its natural ligand TL1A have only been reported for TF-1 cells and activated T cells, which both endogenously express DR3. In these cells, TL1A binding induced apoptosis (measured by caspase activation) and NF-κB activation only in the presence of CHX. TL1A-induced cellular inhibitor of apoptosis protein c-IAP2 prevented DR3 mediated apoptosis in TF-1 cells (Wen et al, 2003).
Considering that TL1A could have differing activities depending on the cell system, the results in this study show that this ligand is redundant for DR3 mediated apoptosis in melanoma cells.

4.10. Conclusions and future perspectives

In this study, DR3 ligand TL1A was unable to induce apoptosis in melanoma cells. Instead, melanoma cells reacted with cytotoxicity after treatment with TL1A. While the basic expression of functional transmembrane DR3 in melanoma cells was negative, the expression of a 66 kDa protein band seemed self-evident. This finding suggests that the 66 kDa protein band could be unspecific, especially since its expression remained unaltered after induction of the surface receptor in melanoma cells. On the other hand, DR3 protein bands of high MW have been described as glycosylated/truncated DR3 proteins. The 66 kDa protein, therefore, may represent a DR3 protein with other functions in melanoma cells.
In a recent study, Gout et al (2006) discovered DR3 of high MW (glycosylated DR3) as a high affinity functional and signaling counter-receptor for E-Selectin. The study showed that interaction of glycosylated DR3 with E-Selectin triggered the activation of the prosurvival extracellular signal regulated kinase (ERK) and promigratory p38 mitogen-activated protein kinase (MAPK) pathways in primary colon carcinoma cells. However, this interaction did not induce apoptosis in the colon carcinoma cells. Instead, the cells showed high attachment to purified E-Selectin and E-Selectin expressing endothelial cells, suggesting that glycosylated DR3 regulated metastasis and apoptosis escape in colon carcinoma cells.
It would be very interesting to confirm these findings in melanoma cells, which show very early metastasis and express DR3 of high MW.

In conclusion, transmembrane DR3, designated DR3 isoform 1 of 47 kDa, can be induced by treating melanoma cells with Jurkat supernatant. This induced expression of DR3 in melanoma cells could be mediated through activity of the T cell line Jurkat probably via cytokine release. Moreover, Jurkat supernatant alone induced apoptosis in melanoma cells. Up-regulation of DR3 caused by Jurkat supernatant might have induced apoptosis in melanoma cells. At the same time, apoptosis of melanoma cells caused by Jurkat supernatant might be responsible for up-regulation of DR3 in melanoma cells, probably also via cytokine release. The correlation between induction of DR3 expression and apoptosis induction in melanoma cells opens questions about regulation of DR3 expression and function. It would be interesting to find out which cytokine is responsible for the activity of the T cell line Jurkat in mediating DR3 in melanoma cells. Further studies are therefore needed in order to target this complex death receptor.

5. Summary

Melanoma develops through malignant transformation of melanocytes and accounts for only 4% of all skin cancers; however, it is the most aggressive form of skin cancer, causes the greatest number of skin cancer related deaths and shows increasing incidence rates worldwide. The hallmark of melanoma aggressiveness is the early metastasis. Current systemic therapeutic approaches, either as mono or poly chemotherapy, and combination of chemo immunotherapy have produced low response rates while maintaining toxic side effects and high expenses. Recent studies, however, have identified defects at multiple levels of the apoptosis program in melanoma, which provide new clues to drug resistance of this highly aggressive neoplasm.

Apoptosis defines a genetically preserved, specific physiological form of cell death with a distinct set of morphological and biochemical changes involving the cytoplasm, nucleus and plasma membrane. Two major apoptotic pathways have been described, namely the intrinsic pathway and the extrinsic pathway. The intrinsic pathway, also known as the mitochondrial pathway, is activated by a variety of extra and intracellular stresses, including oxidative stress and treatment with cytotoxic drugs. The receptor-ligand mediated extrinsic pathway is activated through binding of death receptors (DR) and their cognate death ligands. The two apoptosis signaling pathways converge at the level of specific proteases, the caspases. Decoy receptors (DcR) are members of the tumour necrosis factor receptor (TNFR) superfamily that bind death ligands without activating the apoptosis pathway.

The main objective of this study was to investigate and describe new apoptosis regulatory molecules which have not yet been described in melanoma, but may play a role in apoptosis regulation. Established human melanoma cell lines and isolated normal human melanocytes (NHM) maintained under cell culture conditions were used as models for this investigation.

Firstly, the basic expression of various apoptotic molecules was confirmed at the mRNA and/or protein levels. FLICE (FAS-associated death-domain-like IL-1beta-converting enzyme)-inhibitory protein FLIP has been identified as an inhibitor of death receptor signaling. In this study, melanoma cell lines and NHM showed a basic expression of cellular FLIP mRNA.

Fas ligand (FasL/CD95L) is the natural ligand for the death receptor Fas/CD95; the binding of FasL to Fas triggers apoptosis in Fas expressing cells. FasL mRNA expression was very low and inconsistent in melanoma cells and NHM in this study, which is inline with the view that FasL expression plays a less significant role in the apoptosis resistance and immune escape of melanoma cells. DcR3 is reported to bind to FasL and TL1A (TNF like ligand 1A). In this

investigation, DcR3 mRNA and protein were detected in all melanoma cell lines and NHM analysed, thus DcR3 being described for the first time in melanoma.

Ectopic expression of DR6 has been shown to induce apoptosis in the human cervical carcinoma cell line HeLa. Since the expression of death receptor 6 (DR6) has not yet been described in malignant melanoma, DR6 mRNA levels were also investigated. All melanoma cell lines and NHM analysed showed a basic DR6 mRNA expression, confirming previous data that DR6 was expressed in most human tissues.

TL1A is the natural ligand for death receptor 3 (DR3). TL1A mRNA was detected in all melanoma cell lines and NHM analysed, with this death ligand being shown in melanoma for the first time here. TL1A appears to be of physiological consequence in different cell systems and has been described in pathological situations related to inflammation. In this study, TL1A induced early cytotoxicity in all melanoma cell lines under investigation. Interestingly, TL1A did not cause early cytotoxicity in NHM as compared to melanoma cell lines. Instead, LDH release was very low in treated NHM. TL1A showed no apoptotic activity in melanoma cells, except late, moderate apoptosis in SK-Mel-13. On the other hand, NHM showed late apoptosis after treatment with TL1A.

The precise role of death receptor 3 (DR3) in a physiopathologic context remains unclear at the moment. Its ectopic expression in mammalian cells has been shown to induce apoptosis. To date, 12 distinct isoforms of DR3, produced by alternative pre mRNA splicing, have been described. The major isoform, DR3-1, is a 47 kDa protein that extends to include the transmembrane and death domains. Moreover, due to glycosylation of DR3 proteins, protein bands of higher sizes between 60 kDa and 200 kDa may be expected. DR3 ligand, TL1A, interacts specifically with membrane associated DR3.

While the expression of DR3 mRNA remained inconsistent in melanoma cell lines, a protein band of 66 kDa was constantly shown in all melanoma cell lines and NHM under investigation. This protein band has been described by others as a glycosylated DR3 protein. On the other hand, Jurkat cells, an acute human leukaemia cell line, consistently displayed DR3-1 protein of 47 kDa and were also positive for functional, membrane bound DR3. The results of this analysis describe DR3 expression and functionality for the first time in melanoma.

In this study, it was shown for the first time that Jurkat supernatant induced DR3-1 protein of 47 kDa in melanoma cells. The induction correlated with the expression of functional, transmembrane DR3 in the melanoma cells. These results confirm previous findings that the 47 kDa DR3 protein is the functional, membrane bound death receptor. The DR3 ligand, TL1A, and TNF-α did not mimic this effect of Jurkat supernatant in melanoma cells, suggesting that DR3

induction could be caused by a cytokine release of the Jurkat cells. Moreover, Jurkat supernatant alone induced apoptosis in melanoma cells.

Current published data describe the apoptotic activity of DR3 via its ectopic expression. It has also been demonstrated that alternative splicing of DR3 mRNA takes place during the activation of lymphocytes, which results in up-regulation of the transmembrane form of DR3. However, the signals leading to this up-regulation of transmembrane DR3 have not yet been elucidated. The correlation between up-regulation of transmembrane DR3 and apoptosis induction in melanoma cells, as shown in this study, may help answer questions about regulation of DR3 expression and function. The up-regulation of transmembrane DR3 in melanoma cells may be induced by cytokines such as interferon gamma (IFN-γ) and various interleukins in the Jurkat supernatant. Further studies are therefore needed in order to target this complex death receptor. The induction of DR3 in melanoma cells could lead to developing new therapeutic strategies in targeting melanoma in future.

6. Zusammenfassung

Das Melanom entsteht durch maligne Transformation von Melanozyten. Zwar sind nur vier Prozent aller Hauttumore Melanome, jedoch sind es hoch aggressive Hauttumore, die zudem eine weltweit stetig ansteigende Inzidenz und ungebrochen hohe Mortalität zeigen. Für die Mortalität des Melanoms ist die frühe Metastasierung bedeutend. Gegenwärtige therapeutische Maßnahmen, entweder als Einzel- oder Polychemotherapie und die Kombination von Chemo-Immuntherapie, zeigten nur wenig Erfolg, haben aber gleichzeitig toxische Nebenwirkungen und verursachen hohe Kosten. Neue Studien haben Defekte in der Signaltransduktion der Apoptose in Melanomzellen aufgezeigt, die Hinweise zum Verständnis und zur Entwicklung neuer Ansätze in der Therapie dieses hoch aggressiven Tumors geben.

Apoptose definiert eine genetisch bedingte, physiologische Form des Zelltodes mit spezifischen morphologischen und biochemischen Veränderungen, die das Zytoplasma, den Zellkern und die Plasmamembran betreffen. Zwei Signalwege der Apoptose wurden beschrieben, zum einen der durch intrazelluläre Signale wie DNA-Strangbrüche, oxidativer Stress und Chemotherapeutika induzierte intrinsiche Signalweg, zum anderen der extrinsische Signalweg, der durch die Bindung so genannter Todesliganden an spezifische Todesrezeptoren an der Oberfläche der Zellmembran aktiviert wird. Die Todesrezeptoren sind eine Untergruppe der TNF (Tumor-Nekrose-Faktor)-Rezeptorfamilie, zu denen auch *decoy* (Köder)-Rezeptoren (DcR) gehören. Letztere binden zwar Todesliganden, aber das apoptotische Signal wird nicht weiter geleitet.

Das Hauptziel dieser Arbeit war die Untersuchung und Beschreibung der Gen- und Proteinexpression apoptoserelevanter Moleküle, die in Melanomzellen noch nicht beschrieben wurden. Als Modelle für diese Untersuchung wurden verschiedene humane Melanom-Zelllinien sowie normale humane Melanozyten (NHM) verwendet.

Zunächst wurde die Genexpression von FLIP (FLICE *(FAS-associated death-domain-like IL-1beta-converting enzyme)-inhibitory protein)* untersucht. FLIP ist ein Inhibitor des extrinsischen Apoptosesignalweges. Alle untersuchten Melanom-Zelllinien und NHM zeigten eine konstitutive Genexpression von FLIP.

Fas ligand (FasL/CD95L) ist der natürliche Todesligand für den Rezeptor Fas/CD95; die Bindung von FasL an den zellmembranassoziierten Fas-Rezeptor induziert Apoptose. Die Genexpression von FasL in den untersuchten Melanom-Zelllinien und NHM war nur gering. Das steht in Einklang mit bisherigen Ergebnissen, dass FasL-Expression eine weniger bedeutende Rolle in der Progression von Melanomen spielt. DcR3 bindet FasL und TL1A (*TNF like ligand 1A*). Sowohl die DcR3-mRNA als auch das DcR3-Protein waren in allen untersuchten Melanom-

Zelllinien und NHM konstitutiv exprimiert. Hierdurch wird die Expression von DcR3 in Melanomzellen erstmalig dokumentiert.

Bereits belegt war, dass die ektopische Expression von DR6 (death receptor/Todesrezeptor 6) Apoptose in der humanen Gebärmutterkrebs-Zelllinie HeLa induzieren kann. Für das Melanom gab es hierzu noch keine Daten. Es wurde daher die Genexpression von DR6 in dieser Arbeit näher untersucht. In allen analysierten Melanom-Zelllinien und in NHM konnte DR6 mRNA nachgewiesen werden. Dies steht in Einklang mit bisherigen Berichten, dass DR6 in den meisten humanen Geweben exprimiert wird.

TL1A ist der natürliche Ligand für den Todesrezeptor 3, DR3. In dieser Arbeit konnte erstmalig dokumentiert werden, dass die mRNA von TL1A generell in Melanom-Zelllinien und in NHM exprimiert ist. TL1A steht im physiologischen Kontext in Zusammenhang mit Entzündungen in verschiedenen Zellsystemen. In dieser Arbeit wurde nachgewiesen, dass TL1A in allen untersuchten Melanom-Zelllinien frühe Zytotoxizität induziert, aber vergleichsweise geringe Zytotoxizität in NHM. TL1A induzierte jedoch keine Apoptose in Melanom-Zellen, außer geringe, späte Apoptose in einer Zelllinie. Demgegenüber zeigten NHM späte Apoptose nach Behandlung mit TL1A.

Welche Rolle DR3 in pathophysiologischen Prozessen spielt, ist momentan nicht geklärt. Bisher konnte gezeigt werden, dass die ektopische Expression von DR3 Apoptose induzieren kann. Aktuell sind 12 verschiedene Isoformen von DR3 bekannt. Die Hauptisoform DR3-1 ist ein 47 kDa Protein, das sowohl die Transmembran- als auch die Todesdomäne (DD, *death domain*) besitzt. Zudem werden DR3-Proteine glykosyliert, so dass auch DR3-Proteine mit höherem Molekulargewicht zwischen 60 kDa und 200 kDa erwartet werden können. Ein weiteres erstmalig dokumentiertes Ergebnis dieser Arbeit ist die Expression und Funktionalität von DR3 in Melanom-Zelllinien und NHM. Während die Genexpression von DR3 in den Melanom-Zelllinien nicht reproduzierbar war, konnte mittels eines DR3-Antikörpers ein 66 kDa großes Protein in allen untersuchten Melanom-Zelllinien und NHM detektiert werden. Ein Protein mit diesem Molekulargewicht wurde bereits als glykosylierte Modifikation des DR3 beschrieben. Hingegen konnte in Jurkat-Zellen, eine humane akute Leukämie-T-Zelllinie, sowohl das DR3-1 Protein von 47 kDa, als auch das funktionelle, membranassoziierte DR3-Protein registriert werden.

In dieser Arbeit konnte dokumentiert werden, dass der Zellkulturmedium-Überstand von Jurkat-Zellen die Proteinexpression von DR3-1 in vier Melanom-Zelllinien induzieren kann. Zudem wurde auch dadurch die Expression von funktionellem, membrangebundenen DR3 in den Melanom-Zellen induziert. Die Induzierbarkeit von DR3-Expression in zellulären Systemen

wurde hier erstmalig gezeigt. Es wurden hier auch vorherige Ergebnisse bestätigt, dass das DR3-Protein mit dem Molekulargewicht von 47 kDa der funktionelle, membrangebundene Rezeptor ist. Der DR3-Ligand TL1A und TNF-α allein konnten keine Induktion von DR3 in den Melanom-Zelllinien hervorrufen. Daraus folgt, dass die DR3-Induktion wahrscheinlich über bestimmte sezernierte Zytokine von T-Zellen vermittelt wurde. Übereinstimmend induzierte der Zellkulturmedium-Überstand von Jurkat-Zellen Apoptose in den Melanom-Zellen.

Aktuelle, veröffentlichte Daten beschreiben die Apoptose-Aktivität von DR3 in Zusammenhang mit seiner ektopischen Expression. Die Korrelation zwischen Hochregulierung von funktionellem DR3 und Apoptoseinduktion in Melanom-Zellen, wie in dieser Arbeit gezeigt, könnte helfen, Fragen zur Regulierung der DR3-Expression und deren Funktion zu beantworten. Es ist auch dokumentiert worden, dass das alternative Spleißen von DR3-mRNA während der Aktivierung von Lymphozyten verstärkt stattfindet, welches zur Hochregulierung des funktionellen DR3 führt. Die Signale, die zu dieser Hochregulierung führen, sind noch nicht beschrieben. Die Regulierung von DR3 in Melanom-Zellen könnte möglicherweise durch Zytokine wie Interferon gamma (IFN-γ) und Interleukine, die sich im Jurkat- Zellkulturmedium-Überstand befinden, vermittelt werden.

Weitere Studien sind erforderlich, um die Regulation und Funktionalität dieses hochinteressanten Todesrezeptors und seines Liganden zu entschlüsseln. Die Apoptoseinduktion in Melanomzellen durch Hochregulierung von DR3 könnte sich zu Hoffnungsträger für neue therapeutische Verfahren gegen das maligne Melanom entwickeln.

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8. Abbreviations

ALM	Acral lentiginous melanoma
APP	ß-amyloid precursor protein
APS	Ammonium persulphate
BCA	Bicinchoninic acid
Bcl	B-cell lymphoma
BFGF	Basic fibroblast growth factor
BPE	Bovine pituitary extract
BSA	Bovine serum albumin
CHX	Cycloheximide
CM	Cutaneous melanoma
CT	Computed tomography
Da	Dalton
DcR3	Decoy receptor 3
DD	Death domain
DED	Death effector domain
DISC	Death inducing signaling complex
DMEM	Dulbecco's modified Eagle' medium
DMSO	Dimethyl sulfoxide
DNA	Desoxyribonucleic acid
DR3	Death receptor 3
DR6	Death receptor 6
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme-linked immuno sorbent assay
et al	and others
FACS	Flourescence activated cell sorting
FADD	Fas-associated death domain
FasL	Fas ligand
FCS	Fetal calf serum
FLICE	FAS-associated death-domain-like IL-1beta-converting enzyme
FLIP	FLICE-inhibitory proteins
Gy	Gray
hr	hour
IgG	Immune globulin G
IL	Interleukin
LDH	Lactate dehydrogenase
LMM	Lentigo maligna melanoma
MRT	Magnetic resonance tomography
NHM	Normal humane melanocytes
NM	Nodular melanoma
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction

PET	Positron emmision tomography
PMSF	Phenylmethansulfonyl fluoride
RNA	Ribonucleic acid
RT-PCR	Reverse transcription-polymerase chain reaction
SDS	Sodiumdodecylsulfat
SSM	Superficial spreading melanoma
TAE	Tris-Acetat
TBE	Tris-Buffered Electrophoresis
TBS	Tris buffered saline
TEMED	N,N,N',N'-tetramethylethylenediamine
TL1A	TNF-like ligand 1A
TNFR	Tumor necrosis factor receptor
TNF-α	Tumor necrosis factor alpha
TRADD	TNF receptor associated death domain
TRAIL	TNF related apoptosis inducing ligand
VEGI	Vascular endothelial growth inhibitor

Selbständigkeitserklärung

Ich, Amma Yeboah, erkläre, dass ich die vorgelegte Dissertation mit dem Thema: „Expression and functionality of death receptors and death ligands in cultured melanoma cells“ selbst verfasst, keine anderen als die angegebenen Quellen und Hilfsmittel benutzt, ohne die (unzulässige) Hilfe Dritter verfasst und auch in Teilen keine Kopien anderer Arbeiten dargestellt habe.

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Amma Yeboah

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Mein Lebenslauf wird aus Datenschutzgründen in der elektronischen Version meiner Arbeit nicht mit veröffentlicht.