Immunological Studies in Malignant Melanoma:

Importance of TNF and the Thioredoxin System

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Caminante, son tus huellas
el camino y nada más;
Caminante, no hay camino,
se hace camino al andar.
Al andar se hace el camino,
y al volver la vista atrás
se ve la senda que nunca
se ha de volver a pisar.
Caminante no hay camino
sino estelas en la mar.
A. Machado

We shall not cease from exploration and the end of all our exploring will be to
arrive where we started... and know the place for the first time.
T.S. Eliot

To all of you, who made this exploration possible.

ABSTRACT

Malignant melanoma is a tumor whose incidence is dramatically increasing in persons with light-coloured skin in all parts of the world. Due to its resistance against traditional chemo- and radiotherapy, melanoma has been a favourite target of alternative therapies, in particular those involving immunological mechanisms. Cytokines and particularly tumor necrosis factor (TNF) have been studied as possible antitumoral agents, but also as endogenous growth or differentiation factors. Previous studies showed that melanomas could express TNF *in situ* and that this expression correlated to decreased lymphocyte infiltration. On the other hand, redox reagents can modulate expression of cytokines, and the thioredoxin (Trx) system is particularly known to influence expression and secretion of TNF *in vitro*.

The overall aim of this research was to explore immunological aspects of melanoma, particularly the role of TNF both *in vitro* and *in vivo*, as well as its possible modulation by Trx.

In the *in vitro* studies first we developed a novel method for obtention of monoclonal antibodies against melanoma antigens, and generated and characterized specific monoclonal antibodies against both full-length and truncated Trx. We studied the cytokine expression of a panel of normal and transformed melanocytic cells by immunofluorescence, all of which presented TNF and Trx at levels comparable to monocytic cells, and TNF-receptors (TNFR) at low but detectable levels. Melanoma cells did not secrete TNF upon stimulation in spite of its presence in the Golgi apparatus. However, melanoma cells expressed the TNF-processing enzyme TACE and were capable of cleaving transfected GFP-tagged TNF. Imaging studies point to a possible cell-cell tranfer of endogenous TNF in melanoma cells.

On the other hand, TNF and Trx expression in melanoma cell lines correlated to resistance against exogenous TNF. We studied then the *in situ* expression of TNF and Trx by immunohistochemistry in a group of 44 cutaneous melanoma patients. Trx expression did not correlated to survival or other clinical-pathological parameters. TNF expression significantly correlated to better survival in tumors thicker than 0,8 mm, and constituted an independent prognostic factor.

These results point to a biological role of endogenous TNF in malignant melanoma, either by constituting an autocrine/paracrine differentiation factor or by modulating communication with other cell types, particularly of the host's immune system.

LIST OF PUBLICATIONS

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

Paper I. Barral, A. M., A. Fernández, M. E. Faxas, X. Pérez, C. A. García, and A. Rosén. 1997. Cell-cell adherence as a selection method for the generation of anti-melanoma monoclonal antibodies. *J Immunol Methods 203:103*.

Paper II. Sahaf, B., A. Söderberg, G. Spyrou, A. M. Barral, K. Pekkari A. Holmgren, and A. Rosén. 1997. Thioredoxin expression and localization in human cell lines: detection of full-length and truncated species. *Exp Cell Res 236:181*.

Paper III. Barral, A.M., R. Källström, B. Sander, and A. Rosén. 2000. Thioredoxin, thioredoxin reductase and TNFα expression in melanoma cells: correlation to resistance against cytotoxic attack. *Melanoma Res 10:331*

Paper IV. Barral, A.M., B. Sander, M. Söderström and A. Rosén. 2001. The intracellular dynamics of TNFα in malignant melanoma. *Manuscript.*

Paper V. Barral, A.M., H. Sjödin, A. Rosén and B. Sander. 2001. TNFα expression predicts a better outcome in thick malignant melanoma. *Submitted.*

Permissions to reprint the papers were granted by the publishers of the journals concerned.

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ABBREVIATIONS

ALM	acral lentiginous melanoma
ADAM	proteins containing *a d*isintegrin *a*nd *m*etalloprotease domain
AP	activator protein
ARE	AU-rich elements
ASK	apoptosis signal-regulating kinase
ATF	activating transcription factor
ATP	adenosine triphosphate
B-CLL	B-type chronic lymphocytic leukaemia
CHO	Chinese hamster ovary
CRD	cysteine-rich domain
DAB	diaminobenzidine
DD	death domain
DMXAA	5,6-dimethylxanthenone-4-acetic acid
ELISA	enzyme-linked immunosorbent assay
ER	endoplasmic reticulum
Ets	E26-transformation specific
FACS	fluorescence-activated cell sorter
GM-CSF	granulocyte-monocyte colony-stimulating factor
GMDP	glucosaminylmuramyl dipeptide
GSH	reduced glutathione
GSSG	oxidized glutathione
HLA	human leukocyte antigen (histocompatibility antigen)
IC_{50}	inhibitory concentration at 50%
IL	interleukin
IFN	interferon
mAb	monoclonal antibody
LMM	lentigo maligna melanoma
LPS	lipopolisaccharyde
LT	lymphotoxin
MAPK	mitogen activated protein kinase
MHC	major histocompatibility complex
MLE	maximum likelihood estimation
NADPH	nicotine adenine dinucleotide phosphate
NF-κB	nuclear factor-κB
NFAT	nuclear factor of activated T cells
NK	natural killer
NO	nitric oxide

NM	nodular melanoma
PDI	protein-disulfide isomerase
PKC	protein kinase C
PLAD	pre-ligand assembly domain
PMA	phorbol-myristate acetate
PSF	point spread function
ROS	reactive oxygen species
RT-PCR	reverse transcriptase polymerase chain reaction
SH	sulfhydryl group
SOD	superoxide dismutase
SSM	superficial spreading melanoma
TACE	TNF-alpha converting enzyme
TGF-α	transforming growth factor-α
Th1	T helper 1
Th2	T helper 2
TNF	tumor necrosis factor
TNFR	tumor necrosis factor receptor
TNFSF	tumor necrosis factor superfamily
TRAF	TNF-associated factor
Trx	thioredoxin
TrxR	thioredoxin reductase
UV	ultraviolet
WGA	wheat germ agglutinin

ABBREVIATIONS

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INTRODUCTION

Cutaneous malignant melanoma (MM) is a tumor whose incidence is increasing dramatically in persons with light-colored skin in all parts of the world. In most studies, the incidence is doubling every 6 to 10 years. In years of potential life loss, melanoma is second to adult leukaemia, as it affects younger individuals, causing a major public health problem.

While early melanoma can be effectively removed and results in >99 % disease-free survival for *in situ* and >90 % overall survival for thin melanomas (1), the prognosis of intermediate and thick melanomas is more sombre, due to the resistance of this tumor to traditional chemo- and radiotherapy.

As with other cancers, the causes of melanoma are several, involving environmental (particularly intermittent UVB exposure), genetic and immunological factors. From the normal melanocyte a series of tumor progression stages leads to the development of MM. The identification and studies of markers that can distinguish between these stages have been of great interest from the diagnostic/prognostic point of view, but also in order to elucidate the molecular mechanisms behind tumor progression.

These mechanisms include cytokines and growth factors, as well as their receptors, which play an important role in the inter-cellular communication. Up- or down regulation of their expression can alter vital signalling pathways. In particular, the role of TNF has evolved from a single cytotoxic molecule to member of a superfamily (TNF/TNFR superfamily) implied in complex processes such as organogenesis, inflammation and tumorigenesis.

All cellular processes can be influenced by the redox state in the microenvironment. Redox regulation comprehends both powerful buffering systems like glutathione and fine-tuning molecules like Trx/Trx-reductase (TrxR).

The studies presented in this thesis focus on different immunological aspects of malignant melanoma. Particularly we studied the role of TNF in MM and explored its regulation by redox mechanisms, both *in vitro* and *in vivo*.

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BACKGROUND

CUTANEOUS MALIGNANT MELANOMA

In 1995, an estimated 32,100 developed melanoma and 7,200 died of the disease in United States (2). In Sweden, the incidence of melanoma increased from 2.5 per 100,000 in 1958 to 11.6 per 100,000 in 1980 (3). Under 1992, 1,332 cases were registered. The average increase per year is between 5-6% (Source: Swedish Cancer Registry).

These alarming statistics together with the feasibility of melanoma as a tumor progression model explains its "attraction" in biomedical and clinical studies. Present molecular and clinical research of melanoma is now focused on (a) exploring the molecular defects that characterise the malignant process at each stage and (b) defining the biochemical and biological impact of those alterations on the mechanisms governing cellular processes like proliferation, differentiation and intercellular relationships.

Below, I will briefly outline (a) the general characteristics of normal melanocytes, (b) the currently accepted molecular progression model of melanoma, (c) the most important prognostic factors as well as (d) the immunological response observed for this tumor.

Normal melanocytes

Melanocytes migrate from the neural crest into the skin during the first six to eight weeks of embryogenic development, and the cells are homogeneously distributed at the junction between the epidermal and dermal layers. These cells generate a light-absorbing shield of melanin that protects the skin from damage produced by UV-radiation. Synthesis of melanin takes place in specialized organelles called melanosomes. Melanocytes are dendritic cells with a big nucleus and scarce cytoplasm. They differentiate from melanoblast to mature melanocytes. While melanoblasts have the capacity to proliferate, mature melanocytes seldom do it, only when stimulated by action of ultraviolet (UV)-light (4, 5) or growth factors like fibroblast growth factor, hepatocyte growth factor and endothelins (6).

In the skin, melanocytes and keratinocytes form the 'epidermal melanin unit' in the epidermis. It is in this unit where melanocytes and keratinocytes are aligned along the basement membrane zone at a ratio of 1:5-8. Each melanocyte reaches with its dendrites into the upper

layers of the epidermis, transporting the pigment-containing melanosomes to approximately 35 keratinocytes (7). Keratinocytes, which are cells of exodermal origin, are capable of producing and secreting a wide range of cytokines and molecular mediators such as nitric oxide (NO), thus controlling melanocytes as well as other cell types like Langerhans cells (8). Melanocytes are capable of presenting antigens (9) and also generate cytokines themselves (10).

The most striking feature of melanocytes is their production of melanin, the pigment that protects the skin from the harmful UV-irradiation (for review see ref. (11). Melanin synthesis occurs in the melanosomes, and involves a complex enzymatic system, whose key actor is the enzyme tyrosinase (12). Melanin is stored and transported in melanosomes by an active mechanism involving myosine V (13). The exact mechanism of transfer of melanin from melanocytes to keratinocytes is unknown. Several mechanisms postulated include secretion into the intercellular space, direct translocation at membrane level, vesicular transport etc.

Molecular aspects of tumor progression in malignant melanoma

In this section I will discuss only the characteristics of cutaneous malignant melanoma, as other melanomas (uveal, mucosal) present a series of particularities not shared with the cutaneous type.

According to growth pattern, malignant melanomas can be classified into four main types:

1. Superficial spreading melanoma (SSM)

2. Nodular melanoma (NM)

3. Lentigo maligna melanoma (LMM)

4. Acral lentiginous melanoma (ALM)

The first two types are the most common, 70 % of the cases are SSM and 15-30 % is NM (2).

Clark and cols. developed a progression model of malignant melanoma based on the study of the invasion level of the tumor (14, 15).

According to this model (**Fig. 1**), the normal melanocyte could first give rise to a nevus cell presenting random chromosomal abnormalities. Some authors define a first step as the common nevus, where the melanocytes escape from the keratinocyte control and establish contact with each other, but without genetic alterations (16). The dysplastic nevus (or pre-malignant melanoma)

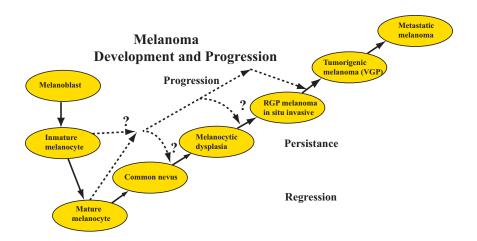presents atypical architectural and cytological features. In the primary melanoma stage, cells proliferate

Figure 1. Progression model of malignant melanoma. Adapted from (18). RGP: radial growth phase; VGP: vertical growth phase.

first within the epidermis (melanoma *in situ* or radial growth phase melanoma), then acquire the capacity to invade the dermis (invasive or vertical growth phase melanoma). In its most aggressive stage, metastatic melanoma cells are capable to migrate in to and grow at distant anatomic sites. The characteristics of cultured cells derived from the different progression stages are shown in **Fig. 2**, adapted from (17).

Parameter	Melanocytes	Nevus	Primary Melanoma		Metastatic Melanoma
			Early	Late	
Chromosomal abnormalities	None	None (few random)	(#1,6,7,9,) Nonrandom	(#1,6,7,9) Nonrandom	(#1,6,7,9,11) Nonrandom
Life span (doublings)	Finite (<60)	Finite (<50)	Infinite (>100)	Infinite (>100)	Infinite (>100)
Response to phorbol ester	Stimulation	Stimulation	Inhibition	Inhibition	Inhibition
Growth factor requirements	I, FGF, aMSH, TPA*	Same as melanocytes**	I only	None	None
Growth in soft agar (percent)	<0.001	0.001-3 (average 0.9)	5-10 (average 8)	5-20	5-70 (average 25)
Growth in nude mice (percent)	NOne	None	80	100	100

Figure 2. Characteristics of cultured melanocytic cells. * I= insulin; FGF= fibroblast growth factor; αMSH= a-melanocyte stimulating hormone. ** Cultures are often independent of FGF/TPA.

The molecular mechanisms underlying the different progression stages are summarized in **Fig. 3** adapted from (18):

1. Genetic instability manifested in chromosomal abnormalities (mostly 1, 6, 7, 9, 10 and 11), mainly affecting genes implicated in cell cycle regulation and DNA repair.

2. Deregulated proliferation due to alterations in genes related to cell cycle checkpoints (p53, p21, bcl-2).

3. Development of invasive potential, which comprises alterations in the normal tissue interactions, the ability of melanoma cells to evade inhibitory growth and motility signals, as well as production of cytokines and growth factors (see chapter Cytokines in melanoma).

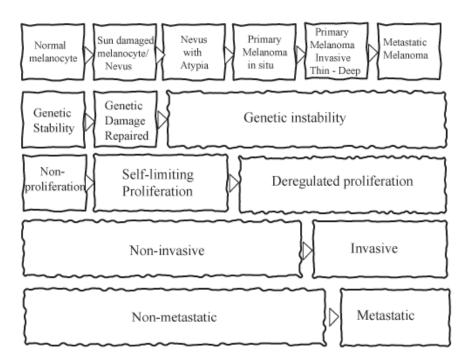4. Development of metastatic potential mainly related to the acquisition of neoangiogenic potential.

Figure 3. Mechanisms underlying the different progression stages of MM. Adapted from (18).

Diagnosis of and prognostic factors in MM

The typical melanoma lesion is relatively easy to identify, even macroscopically, as an irregularly growing, pigmented lesion that after a long time (even decades) of inactivity can debut with symptoms such as accelerated growth, itching and bleeding.

Pathologists have called malignant melanoma the "great pretender". When morphological characteristics of a lesion still cannot define if it is a melanoma or not, more specific cellular markers are needed. Several monoclonal antibodies recognizing melanoma-associated antigens are currently used in the pathological diagnosis, including HMB-45, which recognizes an early melanosomal antigen and S-100, a Ca-binding protein, usually in combination with diverse cell lineage-specific markers in order to differentiate from other neoplasms (reviewed in ref. 19). However, there is no unique 100% proof marker, and finding a melanoma-specific marker not present in benign lesions is still a dream for most pathologists.

Staging of melanoma is mainly based on the thickness of the tumor as total vertical height, described first time by Breslow in 1970 (20); and the level of invasion or Clark levels reflecting the penetration of the tumor into the dermal layers and the subcutaneous fat (14). The American Joint Committee on Cancer recently adopted a revised staging system for cutaneous melanoma based on tumor thickness and ulceration but not invasion level (21).

The total vertical height of melanoma (Breslow thickness) is the single most important prognostic factor in stage I and II (clinically localized) melanoma (2). Early melanoma (reviewed in ref. 22), including *in situ* melanoma and thin lesions (< 1 mm), has a survival greater than 99 and 90 %, respectively (1), and proposal for a new staging system with a cut-off at 1 mm has been suggested (23). Ulcerated melanomas appear to be more aggressive as they invade through the epidermis instead than pushing it upwards. Presence of ulceration is therefore indicative of worse survival, even after correction for tumor thickness (24, 25).

Other clinical variables such as age, gender, race and primary site; and pathologic factors as histologic type, mitotic index, tumor ploidy, S-phase fraction and DNA content have been studied. Particularly, lymphocyte infiltration of the tumor is considered a good prognostic indicator (26-28), although controversies exist about the exact nature of the infiltrate (29).

In metastatic disease, the number and the site of metastases are the most used prognostic indicators (2).

Immune response against melanoma

Tumor immunity has been a favourite subject of research during the twentieth century, balancing between extreme enthusiasm and deep scepticism, as new therapies based on experimental results were employed (mostly with partial or no success) on cancer patients (for reviews, see refs. 30, 31).

Malignant melanoma has been a preferred model for studies in cancer immunology and immunotherapy, because the availability of large panels of cultured melanoma cell lines corresponding to different progression stages, Besides, due to the known resistance of melanoma to traditional chemo- and radiotherapy, these patients are more likely to be included in new immunological protocols (32). The fact that melanoma is an immunogenic tumor has been supported by the following features (33):

1. Primary melanomas can spontaneously regress, either partially or completely

2. Primary melanomas often show large infiltrates of lymphocytes

3. Nevi can show "halos" (areas of depigmentation) around them

4. Primary melanomas can show areas of loss of pigmentation

5. Development of vitiligo in melanoma carries good prognosis.

However, other authors consider that it is the visibility and relative accessibility of melanoma that has helped to uncover the previous characteristics, which are not "special" for melanoma (32). Parallels have been drawn between the immune response in melanoma and the autoimmune reaction typical of vitiligo lesions, as two sides of the same coin (34).

Immune surveillance, the concept that states that the immune system can recognize and destroy transformed cell clones, has been extensively debated and often rejected. However, the immune system does recognize and react against many tumors, and the lack of efficacy of this response is still a problem of outmost importance.

Tumor escape mechanism can be grouped in several (not exclusive) categories (35, 36):

1. Downregulation of immune responses by tumor cells or factors released by the tumor or tumor-infiltrating cells

2. Altered expression by the tumor cells of major histocompatibility complex (MHC) molecules and/or tumor antigens

3. Altered expression of adhesion or accessory molecules by tumor and/or dendritic cells

4. Utilization of the immune response to enhance tumor cell growth (immunostimulation).

A novel autocrine pathway denominated "antigen silencing" has recently been described in melanoma cells, which produce a soluble factor that diminishes the expression of the gene encoding the Melan-A/MART-1 antigen (37).

It is also important to define the differences between the antigen-specific tolerance, which is characteristic of early stages of tumor growth and the systemic immunodeficiency in later stages. Local mechanisms provoking "antigenic ignorance" in early cancer could later become systemic causing the general immune dysfunction (38).

In the case of melanoma, all these mechanism have been studied and in many cases correlated to prognosis, leading to new concepts for immunotherapeutic intervention (39). The identification of melanoma associated antigens such as the MAGE-family, MART/Melan, BAGE, tyrosinase and tyrosinase-related proteins prompted investigators to design cancer immunotherapy protocols (reviewed in ref. 31). Downregulation of MHC molecules and defects in the antigen-processing machinery are associated to a worse prognosis in melanoma (40-43).

The role of cytokines produced by both tumor and infiltrating lymphocytic cells in melanoma will be discussed in the chapter "TNF and other cytokines in MM".

CYTOKINES

According to the classical definition, cytokines are proteins secreted by the cells of the innate and adaptive immunity that mediate many of the functions of these cells (30). However, it has become increasingly evident that cytokines can be produced and/or secreted by a variety of cell types outside of the immune system, and regulate all type of biological functions. Today the term cytokine is used as a generic name for a diverse group of soluble proteins and peptides, which act as humoral regulators at nano- to picomolar concentrations and which, either under normal or pathological conditions, modulate the functional activities of individual cells and tissues. These proteins

also mediate interactions between cells directly and regulate processes taking place in the extracellular environment (44).

Cytokines share several properties, in spite of structural differences:

1. Cytokine secretion is a brief, self-limited event.
2. Cytokine action is often pleiotropic and redundant.
3. Cytokines often influence synthesis and actions of other cytokines.
4. Cytokines can act locally and/or systemically.
5. Cytokines act through binding to specific receptors.
6. External signals regulate cytokine receptor expression, and thus responsiveness.
7. Cellular responses to most cytokines consist of changes in gene expression of the target cells

Functionally, cytokines can be classified in three main categories (30):

1. Mediators and regulators of innate immunity
2. Mediators and regulators of adaptive immunity
3. Stimulators of hematopoiesis.

However, it should be noted that this classification responds more to the classical "immunological" functions of cytokines. A new vision of the role of cytokines in complex biological processes is now emerging, as in the case of the TNF/TNFR superfamily as multicellular "organizer" (45) (to be discussed in the chapter of TNF).

TUMOR NECROSIS FACTOR

TNF at a glance: a brief introduction to a fascinating molecule

The evolution of our knowledge about TNF follows a pattern similar to many scientific discoveries. A practical observation from the nineteenth century that cancer patients with infections sometimes presented tumor regression led to a quest for a link between infection, inflammation and antitumoral effect. In 1975 a factor that provoked tumor necrosis in LPS-primed mice (46) was discovered and hence fore named TNF. This factor later proved to be identical to the independently described cachectin (47), responsible for the wasting symptoms (cachexia) associated with chronic inflammatory diseases and cancers of certain type.

TNF was renamed TNFα to discriminate from lymphotoxin (LT), then called TNFβ. Later, after the discovery of two LT isoforms, by consensus they were designated TNF, LTα and LTβ.

In the following years a myriad of information was generated on the effects of TNF, mostly studied in relationship to those clinical conditions in which the factor was implicated: cancer and inflammation. Depending on the experimental model and the approach used, many and often-contradictory effects were described for this cytokine. Involvement of TNF in other pathological conditions such as diabetes, neurodegenerative diseases and obesity was discovered. Molecular studies and databases permitted the identification of a group of factors with structural similarities and diverse functions, and the term TNF-TNFR superfamily finally evolved (reviewed in refs. 45, 48-50; http://www.gene.UCL.ac.uk/users/hester/tnfinfo.html). The accumulation of experimental data eventually provided the basis for elucidating complex pathways at the cellular level, as the apoptotic cascade via the death receptors (50) or alternative gene activation pathways (51). With the use of knock-out mice, the function of these factors at a higher organizational level is being deciphered.

According to our present knowledge, TNF superfamily members play an important role in the organization of primary and secondary lymphoid organs, processes that require an effective and fine-tuned response. Differential expression of ligands and receptors in different cell types and tissues explain why the effect of these molecules can be restricted. Still, TNF remains one of the most ubiquitous and versatile members of the superfamily.

The TNF/TNFR superfamily is evolutionally highly conserved. It seems that most death domain (DD) containing genes come from a common ancestor, and a subsequent diversification took place with the appearance of the TNFR-associated factor (TRAF, adapter) domain. Thus, DD-containing receptors seem to play an important role in the evolution of the modern immune system, with the later addition of more specialized receptors (45).

Many functions and characteristics of TNF and TNFR have been derived from the study of their structure (49, 52). Much information was also gathered when looking at the different levels of control of TNF expression: transcriptional, post-transcriptional, intracellular dynamics, proteolytic processing and secretion.

Recent results point to similarities between the TNF and Notch processing pathways, opening new ways of integrating mammalian biology (53-56). Study of the mediators of the innate immune response (as Toll-like receptors) during

evolution is revealing relationships between inflammation and development (57). The circle completed, the link between inflammation and cancer (as two sides of the same coin) is being rediscovered, particularly by the study of proinflammatory cytokines, of which TNF is just a classical example (58).

How both TNF and the knowledge about it has evolved (its historicity), how associations to other seemingly unrelated molecules and processes have revealed new and decisive interconnections, how contradictions could be solved by integrating this knowledge at higher levels: this fascinating tale of scientific discoveries shows us the importance of avoiding the reductionistic approach in science. The whole cannot be explained by a simple addition of the dissected parts. To maintain an open-minded, dynamic and dialectical vision of the object of our research seems so evident and it is, however, so difficult to achieve (15, 59).

It is impossible to give an integral picture here of the TNF field (for a recent update see ref. 60), therefore after this brief general background I will focus on the role of TNF in cancer, and most specifically melanoma.

Gene organization, transcriptional and post-transcriptional control
The gene for human TNF lies on the short arm of chromosome 6, near the HLA locus flanking the centromere. **Fig. 4** shows the human MHC region and the TNF-related genes. In all vertebrate species, the genes encoding TNF and LT lie within the MHC, itself the most gene-dense and polymorphic region of the entire genome (61). TNF gene polymorphism have been extensively studied and several of them (shown in **Fig. 4**) associated to pathological conditions (61, 62).

Many of the polymorphisms are present in the promoter sequence of TNF. Binding sites for transcription factors such as NF-κB, NFATp, Ets, AP-1/ATF, AP-2, and some repressor sites are present in the promoter sequence, (63). Particularly, ATF2 has been shown to increase UVC-induced apoptosis in melanoma cells by downregulating TNF expression (64, 65). In NK cells, production of endogenous NO can inhibit NFAT, and thus downregulate TNF transcription, protecting the cells from activation-induced apoptosis (66). Interestingly, engagement of TNFR1 by TNF can also modulate TNF gene expression, by a mechanism involving ATF2 and p38 MAPK (67). Post-transcriptional control of TNF expression is achieved by regulating translational initiation, mRNA stability and polyadenylation. AU-rich elements (AREs) present in the 3' untranslated region of TNF mRNA can regulate protein expression. Knock-in mice expressing TNF transcripts that lack ARE overexpress TNF due to the increased stability of the transcripts

and the loss of translational silencing, resulting in a severe inflammatory phenotype (for review see ref. 68).
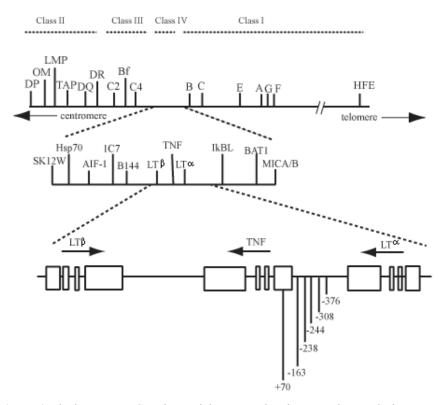

Figure 4. The human MHC region and the TNF-related genes. The MHC class IV region is detailed in the middle line. The bottom line shows the genomic organization of the *LT*β, *TNF* and *LT*α genes. Open boxes represent exons. Arrows indicate transcriptional direction. Polymorphisms involving G to A transitions at positions -376, -308, -244, -238 and -163, and a C insertion at position +70 of TNF are shown. Adapted from Ruuls and Sedgwick, 1999 (62).

Biochemistry of TNF

Human TNF (Accession number: X01394) is initially translated as a prohormone of 233 amino acids (26 kD), which is subsequently modified during postranscriptional processing (69-71). The latter includes acylation by myristic acid, via an irreversible amide bond on lysine residues (72), although other authors have detected reversible palmitoylation via a thioester bond on a cysteine residue (73). The human TNF sequence does not contain potential N-glycosylation sites, although O-glycosylation is possible (74). TNF sequence shows also a number of phosphorylation consensus sites (49). The propeptide leader sequence is of an unusual length, 76 amino acids, and functions as a

signal-anchor sequence. This transmembrane proTNF is proteolytically cleaved by TACE/ADAM17, a metalloproteinase (see below) to yield mature soluble TNF (17 kD, one disulfide bond, isoelectric point 5.8). The TNF monomer forms two antiparallel β-pleated sheets. Both TNF forms are active as self-assembling non-covalent trimers, whose individual chains fold as compact "jellyroll" β sandwiches and interact at hydrophobic interfaces (49, 52, 75). **Fig. 5** shows the three-dimensional structure of TNF. It is interesting to notice that this "jelly-roll" topology is strikingly homologous to several viral coat proteins (76). This and other similarities raise the possibility that TNF ligands represent descendants from the horizontal capture of a gene encoded by an ancient viral pathogen (45).

Processing of TNF

It is currently accepted that a metalloproteinase is the responsible for proteolytic cleavage of proTNF yielding the mature soluble TNF (77-79). TACE or ADAM17 is a member of the metalloproteinase-disintegrin family. ADAM proteases are "metzincins", Zn-dependent metalloproteases, containing a signal sequence, an inhibitory prodomain, a metalloprotease domain and a cysteine-rich region, a transmembrane domain and a cytoplasmic tail. All ADAMs described until now are membrane-bound. The prodomain inhibits the catalytic site via a cysteine-switch. ADAMs are implicated in a variety of processes, including sperm-egg binding, sperm migration, muscle cell fusion, ectodomain shedding and Notch-mediated signalling events (for review see ref. 80).

Besides proTNF, TACE is responsible for the shedding of other substrates like TNFR2, TGFα, L-selectin and β-amyloid precursor protein. Shedding of CD30 (another TNFSF member) from lymphoma cells is also mediated by TACE (81, 82). TACE knock-out mice present perinatal lethality, not the case of mice lacking either TNF, one or both TNFRs (83).

It seems that TACE must be membrane-bound to efficiently cleave the membrane-anchored pro-TNF, and that its action is cell autonomous (84, 85). Both recognition sequences and structural elements contribute to the shedding possibility of membrane proteins (86, 87). It seems also that an appropriate organization of the cytoskeleton is needed for the correct positioning of the shedding machinery and the substrates on the cell membrane (88).

In THP-1 monocytic cells, TACE is present on the cell membrane and internalized after phorbol ester stimulation (89). In another study, TACE was present mostly in a perinuclear compartment of COS cells, and removal of the

inhibitory prodomain took place in the trans-Golgi network by a furin-type proprotein convertase (90). This result raises the possibility that processing of proTNF can already take place in an intracellular compartment. It has been postulated that synthesis and insertion of TNF in the ER membrane occurs co-translationa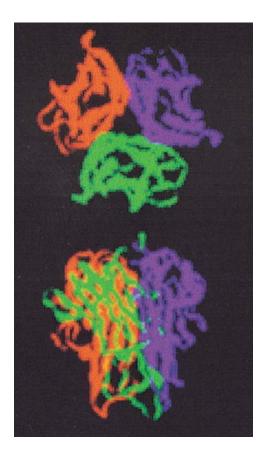lly. Then, probably via Golgi-mediated transfer, TNF appears on the plasma membrane as a trimeric, type II transmembrane protein that can be then cleaved (91). Other results point to the Golgi compartment as the main reservoir of TNF in monocytes (92), from where TNF localizes directly to the membrane and, if not processed, is recycled by endocytosis (93). Solomon et al. (94) described the presence of mature TNF at a postendoplasmic reticulum site.

Figure 5. Trimeric structure of TNF. Structure of the TNF trimer shown from the top (upper) and side (lower) views with each monomer differentially shadowed. The β-pleated sheets assume a "jellyroll" orientation in each monomer. Adapted from (76).

TACE prodomain acts through the inhibition of the Zn atom present in the catalytic site by a thiol group. The redox environment can regulate this interaction, and increased ectodomain shedding by PMA-induced reactive oxygen species (95) and NO (96) has been observed.

Recently a dominant negative form of TACE has been described, that inhibited both TNF and TNFRII shedding. Thus, the regulation of ectodomain, and particularly TNF shedding could be far more complicated than presently thought (97). Moreover, alternative cleaving sites have been described for TNF (74, 98), and it is the length of the linking domain, permitting the protease access, which determines the cleavage process (91). Other proteases, such as the serine protease PR3 (99), can also process proTNF. Moreover, a cell line with reconstituted TACE activity

but no proTNF processing has been described, suggesting other mechanisms of control for ectodomain shedding (54).

TNF-receptors

Two types of TNFRs have been described: TNF receptor I (p55 in mouse, p60 in humans, CD120a, TNFRSF1A, accession number: M75866) and TNFRII (p75/p80, CD120b, TNFRSF1B, accession number: M32315). Founder members of the TNFR superfamily, these molecules also represent its two main categories: receptors containing a DD (TNFRI) and those containing a TRAF domain (TNFRII).

The extracellular ligand-binding region of all TNFSF receptors is characterized by variable numbers of cysteine-rich domains (CRD) (**Fig. 6**), permitting the formation of trimer structures.

The elongated receptor chains fit into the "grooves" between protomers within the ligand trimers (for review see refs. 45, 49, 50, 100). Receptor dimerization is supposed to occur by ligand-induced oligomerization, but recent results point to the presence of pre-ligand assembly domain (PLAD). Thus, receptor chains self-assemble in the absence of ligand and signalling involve rearrangement of the preassembled chains (101, 102). The clustering and activation of TNFRs by UV-light and osmotic shock, in absence of ligands has also been observed (103, 104).

TNFRs can be also cleaved proteolytically and exist as soluble receptors. Shedding of TNFRs can be regulated by SH-reagents (105).

The "classical" localization of TNFRs is on the cell membrane. However, recent studies point to an intracellular, mainly Golgi localization of TNFRs in endothelial cells (106-108). Myosin II is involved in the translocation of TNFRI from the *trans*-Golgi network to the membrane (109). Also, the presence of a TNF-binding protein has been described in mitochondria (110, 111).

Biological roles of TNF

The differential roles of transmembrane and soluble TNF are still unclear. It is postulated *a grosso modo* that soluble TNF mediates the systemic effects of the cytokine while transmembrane acts on the autocrine/paracrine level. The latter can mediate cytotoxic effects (112). The possibility of reverse signalling for transmembrane TNF has also been suggested, as the intracellular domain of TNF is phosphorylated (113) on a casein kinase I motif present in TNF and other TNF ligand family members (114). Outside-to-inside signaling through membrane TNF induces E-selectin expression in activated CD4+ T-cells (115). TNF prosequence possesses a putative nuclear signal sequence, and it was shown to translocate to the nucleus after processing, similar to the
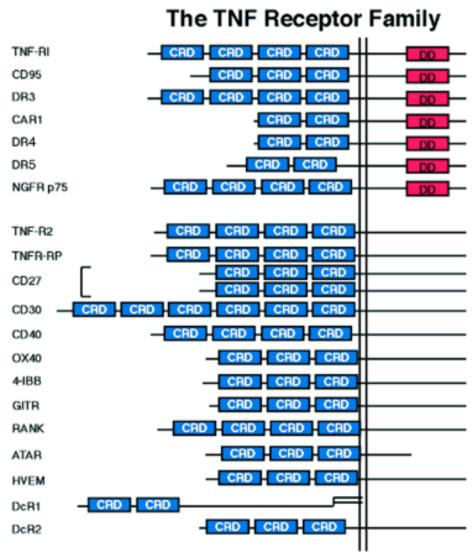
The TNF Receptor Family

Figure 6. Structural comparison of the members of the TNF receptor family. The extracellular ligand-biding regions of the receptors are characterized by variable numbers of cysteine-rich domains (CRD). Death receptor contain a death domain (DD) in their intracellular region, which is essential for apoptosis signaling. Adapted from (100).

Notch signaling pathway (56). Mice expressing non-cleavable transmembrane TNF were protected from endotoxin induced lethal shock, and interestingly, presented significantly higher IL12 levels than wild type mice after LPS stimulation (116).

BACKGROUND 27

Both TNFRs can bind soluble TNF, but transmembrane TNF binds preferentially to TNFRII (117, 118), and the possibility of an autotropic signalling pathway had been described in an experimental model (119)

Most studied are the effects of TNFRI triggering and the resulting apoptotic cascade. Recruitment occurs via DD containing mediators, leading to the activation of caspases and cell death (for reviews, see refs. 50, 100, 120, 121). Recent results showed that apoptotic stimuli often can result in necrosis, depending on factors such as absence of caspase activation and the intracellular balance of ATP and Bcl-2 family members. An active necrotic signaling pathway through DD receptors can also exist (122). TNF generates ROS (123) and those in turn can modulate expression of other cytokines like IL8 (124)

Less known are the effects mediated by TNFRII. It was originally thought that this receptor mediated the survival signals, mainly through activation of NF-κB, but many results point to cross-talk between both signalling pathways (51, 125, 126). In the TF-1 eryhtroleukemic cell line, TNFRII was responsible for both apoptotic and proliferative signaling, depending of its mitotic activity (127). Cell surface and secreted forms of both TNFRs has been correlated to cell cycle phases, S-phase presenting the highest and M-phase the lowest receptor levels (128). Recently the induction of cell death by TNFRII was reported, through production of endogenous transmembrane TNF and consequent activation of TNFRI (129).

It is generally accepted that cells expressing endogenous TNF are resistant to exogenous TNF (130), due to the downregulation of TNFRs (131). This effect takes place independently of intracellular signalling pathways (132), and requires TNF membrane retention (133, 134).

TNF AND OTHER CYTOKINES IN MALIGNANT MELANOMA

Originally, cytokines were considered mediators of the immune response, produced mainly by lymphocytes and monocytes. However, since the mid-'80s it has been increasingly evident that multiple cell types, both normal and transformed, can express cytokine genes and produce cytokine proteins. An explosion of studies during the '90s described production of diverse cytokines by tumor cells and/or the effect of cytokines upon them.

In the case of melanoma research, panels of melanoma cell lines were scrutinized for presence of cytokine mRNAs (135-137), and/or protein, mainly secreted (135, 138). It was found that the majority of melanoma cell lines, and often normal cultured melanocytes, presented transcripts for a large number of cytokines, particularly IL1, IL6, IL8, GM-CSF and TNF.

Presence of TNF mRNA and protein has been detected in cultured normal melanocytes and malignant melanoma cell lines. Interestingly, expression of TNF, IL1α and IL6 mRNA correlated to that of mutated *N-ras* oncogene (139). Lugassy and Escande first described the presence of TNF protein in melanoma cells in 1991 (140). Co-culture of melanoma cells with angioma fibroblasts increased TNF expression in the former. However, TNF protein expression in normal skin melanocytes could not be detected by electron microscopy (141). Constitutive secretion of TNF was observed in two out of 19 melanoma cell lines tested by Colombo (135) but in none of the cell panel studied by Bennicelli and Guerry (138). Recently, secretion of TNF in the malignant melanoma cell line BRO by stimulation with the immunomodulator glucosaminylmuramyl dipeptide was reported (142).

Interestingly, TNF inhibits melanogenesis both in melanocytes (143) and B16 mouse melanoma cells (144, 145).

Presence of cytokines has also been investigated in biopsy material or tissue sections, either using molecular biology techniques as RT-PCR (146-148), *in situ* hybridization (149) or immunohistochemistry (150-154). *In vivo* not all primary melanomas are TNF-positive, and this expression correlates to infiltration by CD3-positive lymphocytes (152). In another study, TNF appeared to be a marker of more benign lesions (151).

Cytokine receptors in melanoma have been studied at a lesser extent, but the presence of TGFαR, IL1R, IL6R, GM-CSFR and TNFR has been detected *in vitro* and *in vivo* (137, 151). Expression of TNF receptors in melanoma cell lines could be regulated by IFNγ and dbc-AMP (155). *In vivo*, TNFR expression was more pronounced in advanced primary and metastatic melanomas (151).

Several hypotheses have been postulated about the role of cytokines in melanoma, which are supposed to influence the growth of the tumor (autocrine role), its interaction with the tumor microenvironment (stroma, infiltrating immune cells) and its metastatic spread (angiogenic role) (156-158).

In the case of IL6 it has been shown that it is growth inhibitory for early stage melanomas but stimulates the growth of advanced melanoma cells (159-161). High serum levels of IL6 were found in melanoma patients (162, 163).

Expression of IL8 has mainly been associated with the metastatic potential of the tumor, both *in vitro* (164, 165) and *in vivo* (166, 167). Multiple levels of regulation by other cytokines and the organ environment have been described for this cytokine (168-171).

GM-CSF expression in vivo correlates inversely with tumor depth,

suggesting a tumor inhibitory effect (172).

IL15 expression has also been detected in malignant melanoma (173, 174), and it seems to regulate melanoma cell progression through a juxtacrine mechanism (175, 176).

In non-small cell lung carcinoma, the expression of intratumoral TNF by immunohistochemistry was associated with a better survival of patients (177). In the same type of tumor, TNF expression quantified by RT-PCR (178) correlated positively with Bcl-2 expression and better survival, and negatively to microvessel production.

Both tumoral and infiltrating cells can produce cytokines in a tumor tissue, a fact that has contributed to contradictory results. Studies based only on detection of mRNA transcripts in tumor homogenates, or only on detection of the presence of the intracellular cytokine protein should be analyzed carefully (see Methodological considerations).

As lymphocyte infiltration is considered a sign of anti-tumoral response, even if not efficient, many studies have been undertaken to characterize these cells, including their cytokine profile, and possible correlation to clinical outcome. Regressing melanomas, representing a succesful immune response, tend to present a Th1 phenotype (179), with preferential expression of IL2, GM-CSF and IL15, although tumoral cells also expressed the latter cytokine also (180). Another study showed higher IL10 levels in metastatic lesions that responded to an IL2-vaccine treatment, and an increase in time of IFNγ expression (181).

The role of tumor-infiltrating macrophages (as a typical TNF-producing cell type) is still much debated. Macrophage infiltration is considered to promote neoangiogenesis, mainly by releasing factors like TNF and IL1α (182, 183). Hydrogen peroxide secreted by these cells inhibits tumor-specific T-cell and NK cell-mediated cytotoxicity (184). On the other hand, recent report sustain the role of macrophages in tumor killing (185) and as orchestrators of the immune response to tumor cell death (186).

High were the hopes about administration of TNF as an antitumoral agent, but the elevated systemic toxicity of this factor was disappointing. However, it has become increasingly evident that the antitumoral effect of TNF is not mediated by direct action on the tumoral cells, but through disruption of the tumor-associated vasculature. The use of isolated limb perfusion with TNF for melanomas and sarcomas localized to the limbs resulted in an improved response (187-189).

In a recent report, DMXAA, a new antitumor drug was shown to exert its effect on a mouse colon adenocarcinoma level by induction of intratumoral TNF and subsequent hemorrhagic necrosis (190). Melphalan, a cytostatic agent often employed in isolated limb perfusion protocols, was shown to upregulate TNF expression in tumor-bearing mice through a mechanism involving IFNβ (191).

On the other hand, a certain tumor promoting effect of TNF has been suggested due to the fact that TNF-deficient mice are refractory to skin carcinogenesis (58, 192).

REDOX REGULATION BY THIOREDOXIN

Reactive oxygen species and oxidative stress

In aerobic cells, ROS are generated when oxygen is partially reduced as electrons leak out of the electron transport chain during respiration in mitochondria. In addition to mitochondria, other sources of ROS generation include endogenous enzyme systems, e.g., plasma membrane NADPH-oxidase and cytoplasmic xanthine oxidase, as well as organellar sources, e.g. peroxisomal cytochrome P-450 oxidase (193). Phagocytic cells have a unique O_2^- production system named respiratory burst (194) and there is a constant generation of ROS in melanocytic cells as a byproduct of melanin synthesis (195). ROS are also generated as a response to external stress, e.g. UV-irradiation, ionizing radiation, NO, and certain chemical compounds (196).

The redox state of the cell is determined by the balance between the oxidizing (ROS) and antioxidant, reducing equivalents. Elevation of ROS exceeding the buffering capacity and enzymatic activities designed to modulate ROS levels result in the harmful "oxidative stress". Highly reactive radicals can damage DNA, RNA, as well as protein and lipid components, which may lead to cell death (193). This can occur by necrosis if ROS levels are extremely high; on the contrary, moderate ROS levels can act as second messengers and activate apoptotic cell death (197). ROS can also act as intracellular signaling molecules, modulating gene expression and enzymatic activities (for reviews see refs. 193, 198, 199).

To counteract the deleterious effects of ROS, the cell possesses different antioxidant systems. Thiol containing moieties (as the SH residues) have reducing power. The most powerful thiol antioxidant buffer in the cell is reduced glutathione (GSH) present in millimolar concentrations in the cytoplasm (200). Other enzymatic systems include catalase, SOD, glutathione peroxidase,

methallothionein, quinone reductase and hem oxygenase. The Trx system, consisting of Trx, TrxR and NADPH operate as a powerful NADPH-dependent protein disulfide reductase system (201, 202).

Thioredoxin

Trx is an evolutionarily highly conserved 12 kD molecule, first described in 1964 as a hydrogen donor for the enzyme ribonucleotide reductase. Nowadays, Trx is considered not only one of many intracellular redox proteins, but it has been implicated in signalling pathways as cell proliferation, differentiation and the apoptotic cascade and a modulator of gene expression (for reviews see refs. 194, 203-205).

Overexpression of Trx has been associated to resistance to cytotoxic agents like adriamycin and cisplatin, as well as exogenous TNF, which are ROS-generating agents (206-208). There are contradictory results about its role in apoptosis. Trx in some studies protects against apoptosis (209-211), and it has been shown to bind to ASK-1, an important mediator of TNF-induced apoptosis (209, 212). However, Trx was also shown to participate in the apoptosis induced by IFN and retinoic acid (213, 214) and IFNβ plus tamoxifen (215). Moreover, Trx and other reducing agents are necessary for activation of caspases (216).

Trx can enhance the induced expression of several cytokines, including TNF in the MonoMac monocytic cell line (217), and in synovial fibroblasts Trx exerted a costimulatory role in TNF-induced IL6 and IL8 production (218). B-CLL cells exposed to Trx release TNF in a dose-dependent manner (210).

The Trx system is considered the major antioxidant catalyst in the human epidermis, and TrxR activity seems to be associated to tumor thickness in malignant melanoma (219). TrxR is highly expressed in human melanoma cell lines, and it can also be secreted (220). UV-B irradiated keratinocytes release Trx that can act as a survival factor for both keratinocytes and melanocytes (221). An immunohistochemical study (222) indicated a possible association between Trx and p53 expression in breast cancer, and in gastric cancer Trx was predominantly expressed in undifferentiated rather than differentiated tumors and cell lines (223).

MAJOR AIMS OF THIS STUDY

The primary aim of the studies included in this thesis was to study some immunological aspects, particularly the role of TNF and the thioredoxin system in malignant melanoma, from the basic to the applied level. The specific objectives were as follows:

1. To develop a novel, more biological selection method for generation of anti-melanoma monoclonal antibodies
1. To study the expression and localization of the full-length and truncated Trx in different cells, particularly melanoma, using novel monoclonal antibodies.
2. To study the expression of cytokines, particularly TNF and its receptors in cultured melanocytic cells.
3. To explore the effect of Trx on TNF expression and release in melanocytic cells.
4. To assess the possible correlation between Trx/TrxR/TNF expression and resistance to exogenous TNF in melanoma cell lines.
5. To study the processing of TNF in malignant melanoma cell lines.
6. To explore a possible correlation between *in situ* TNF and Trx expression and disease-specific survival in primary malignant melanoma patients.

Barral 2001

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METHODOLOGICAL CONSIDERATIONS

The methods employed in the different studies are described in detail in the corresponding Materials and Methods sections of each article. However, I will discuss below some aspects related to the selection of a particular method and its quality control.

Cytokine detection

The biochemical characteristics of a particular cytokine render the molecule sometimes difficult to detect on a reliable basis (224). When detecting mRNA expression for cytokines in tissues, many have recurred to the use of e.g. RT-PCR from whole cell lysates. Since tissues can contain multiple cell types, and in the case of tumors, both tumoral and infiltrating immune cells, which may produce cytokines, this approach should be interpreted carefully. A method that permits assignment of cytokine expression to cell type, such as immunohistochemistry or *in situ* hybridization is recommended to employ in parallel.

Detection of mRNA for a given cytokine does not imply the automatic expression of the protein. Although in this thesis we do not report methods for detection of cytokine mRNA (particularly TNF), previous studies showed by *in situ* hybridisation that TNF was indeed produced in melanoma specimens (149). We also performed *in situ* hybridisation in several melanoma cell lines using digoxigenin-labeled TNF and Trx riboprobes (results not shown).

Detection of a cytokine protein can be achieved by immunochemical methods such as ELISA or Western blot (both in supernatants and cell lysates), ELISPOT (secreted), immunofluorescence, immunohistochemistry or by biological assays. We used ELISA in **Paper III** for detection of TNF in culture supernatants. We employed the matched antibody pair from R&D systems, but we also tested the mAb used for the other assays (CY-014) instead of the coating antibody, yielding almost identical results (unpublished observations). Although the ELISA method offers high sensitivity, it is not optimal for detection of cytokines that are not secreted into the culture medium but taken up by the same or neighbouring cell. This is the case of IL15 produced by melanoma cells (175) or in our study TNF. To detect release of a cytokine at the single-cell level it is recommended to use the ELISPOT assay (205, 225, 226). This method is very suitable for cells that grow in suspension, but in the case of

adherent cells such as melanoma modifications for culturing have to be taken into account.

Immunofluorescence

One of the most versatile and powerful methods for detection of proteins within a cell is immunofluorescence (227). Several variants of this technique were used in our papers. In flow cytometry, the combination of size, granularity and fluorescence intensity gives a quantitative and high-output information. In **Paper I**, flow cytometry on an acetone-fixed melanoma cell line determined the selection of the final antibody-producing hybridoma clone. Acetone is a coagulant fixative that acts through extraction of phospholipides from the cell membrane, thus causing also permeabilization of the cells. However, milder coagulant fixatives such as paraformaldehyde or glutaraldehyde had been shown to preserve better the cellular integrity with minimal antigen loss (228). Besides, acetone fixation provokes leakage of Trx from the cells (J. Nilsson, personal communication). Therefore in the following papers we recurred to fixation by paraformaldehyde and permeabilization with saponin, a method specially suited to cytokine detection (224). In **Paper II**, **III** and **IV** this method was used for flow cytometric detection of intracellular antigens (Trx, TrxR, cytokines, and intracellular markers). Alternatively, for detection of surface-bound Trx and TNFR in **Paper II** and **III**, respectively, cells were stained without permeabilization. To avoid internalization or shedding of the mAb, cells were metabolically inhibited using 0.1 % sodium azide and low temperatures (0 ºC). Quantitation of fluorescence intensity still present many practical difficulties in spite of calibration standards and methods (229). The specific aim in **Papers II** and **III**, when measuring the expression of Trx/TrxR and TNF/TNFR in the cells was not to determine absolute levels of expression, but to compare expression between melanocytic and control monocytic cells. In order to achieve this, cells were analyzed in the same experiment, including isotype controls, for comparison and/or substraction.

In **Paper III**, the initial screening for cytokine expression in the cell line panel was done using immunofluorescence on cells immobilised on adhesion slides. This variant is effective, requiring few cells and little amount of reagents, and very appropriate for discrimination of positive and negative cells. However, in the case of adherent cells like melanoma, if the intracellular localization of a molecule is to be determined it is necessary to stain cells growing on a surface and then analyze them by high-resolution microscopy. In **Paper III** the intracellular localization of TNF was studied using confocal microscopy and double staining for TNF using a specific mAb and the Golgi-marker WGA. In

Paper IV we used mostly wide-field microscopy and deconvolution for the colocalization studies and live-cell imaging.

Image restoration

Microscopic immunofluorescence volume images are usually affected or distorted due to several factors. Details can be hidden by noise or masked by other features, artefacts may confuse the viewer, and the system can even add structures to the image. Image restoration aims at reversing the degradation process in order to recover a true image. Images from microscopes with non-intrinsic 3D imaging capabilities (like wide-field microscopes) suffer from severe degradation due to blurred out-of-focus areas contributing to in-focus areas. However, when the object is not too dense or complex, deconvolution (see below) can remedy the situation. In the case of microscopes with intrinsic 3D capabilities (like confocals), a pinhole is used for exclusion of information outside the focal plane, resulting in a tremendous increase of resolution. However, the intensity of the image diminishes, and the very high intensities used for excitation can cause photodamaging and photobleaching. Images can also suffer from anisotropy and degradation due to noise (230).

Deconvolution

Deconvolution is a mathematical process that uses a theoretical or measured point spread function (PSF) to make an inverse filtration or deblurring of the image. A record of a 3D set of data (in form of a stack of 2D images taken at constant Z-distances) of a specimen will be the basis for the mathematical calculation back to the original dimensions. The PSF can be calculated theoretically on the basis of the electromagnetic diffraction theory using the information from the microscope properties. A measured PSF, calculated from a 3D image stack from an object with known dimensions (like latex beads) has the advantage of considering the aberrations present in the optics. Several mathematical algorithms have been used, such as maximum likelihood estimation (MLE) (231).

Immunohistochemistry

Immunohistochemistry combines the advantages of immunological detection (using specific antibodies) and morphological analysis. Thus, it can be correctly established in which cell type of a given tissue is the antigen of interest expressed. However, as this method is inherently subjective, a rigorous quality control is necessary to ensure reliable and repetitive results (232). Here we will focus only on immunohistochemistry on formalin-fixed, paraffin-embedded material, used in **Paper V** and in part in **Paper III**.

Factors to consider include: quality of the tissue sample; quality of the antibody ; reliability of the procedure and evaluation.

Quality of the tissue sample includes the physical quality of the tissue specimen and the representative quality of the particular sample (tissue section for the whole specimen). The first depends on factors such as fixation and embedding, which are relatively standardized in routine pathology laboratories but should be considered in the case of very old samples; and the quality of sectioning. Paraffin-embedded specimens are considered very stable, but there are reports of loss of antigenicity of sectioned tissue. The representative quality of the samples is very important to consider. In our case, melanoma specimens were carefully selected in order to ensure sections from the central area of the tumor. Those samples that contained no tumoral or invasive component were excluded. This can cause certain under-representation of very small tumors (there were 15 cases that lacked enough material to be processed), something to taken into account when analyzing the population studied.

A particular problem when doing immunohistochemistry in melanoma is the presence of the melanin pigment. Melanin grains are similar in colour to the most employed substrate, diaminobenzidine (DAB), although experienced observers can distinguish melanin on the basis of its different structural appearance. More difficult to evaluate is the unspecific binding of antibodies or secondary reagents to the pigment (unpublished observations). Therefore in **Paper V,** we excluded those melanomas that were heavily pigmented and therefore difficult to evaluate.

Quality of the antibody. Any antibody to be used in immunohistochemistry should be previously evaluated and optimized for staining. In the particular case of paraffin sections, the fixation and embedding procedure can alter the antigenicity of proteins, making not all antibodies suitable for this technique. Several quality controls for the antibodies include specificity control, by blocking with the antigen (or epitope). Also it is recommendable to test if the expression of the protein is associated in the same cell to the expression of the corresponding mRNA by *in situ* hybridization, to discriminate if the cell produces or takes up the studied antigen. In the case of the anti-TNF mAb CY-014, both controls were made in previous studies (149, 152). In the case of the anti-Trx mAb, we checked for specificity blocking the staining with recombinant Trx (results not shown). The use of control antibodies of the same isotype is mandatory to control possible unspecificities due to the

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secondary reganets. Several pretreatments were tested for antigen demasking. In the case of the anti-TNF mAb, only microwave treatment resulted in staining. For the anti-Trx mAb, several pretreatments were successful, including trypsin digestion (**Paper III**), addition of boiling citrate buffer (**Paper V**) and antigen retrieval solution (results not shown).

Reliability of the procedure. Prior to the study described in **Paper V**, the staining procedure was optimized and assayed in different tissues. For TNF, different lymphoid tissues (lymph nodes, spleen) and inflammatory tisses (such as appendicitis); for Trx also lymph nodes and placenta (known for high Trx content) were evaluated (results not shown). Also normal skin was subjected to staining.

In order to achieve as homogenous staining as possible, after optimization of the procedure, all samples were stained with each antibody the same day.

Evaluation. In a study with clinical samples (like **Paper V**) it is of outmost importance to make the evaluation blindly. Slides were evaluated by two observers at least twice separately and then two more times simultaneously. Agreement between observers was high although we have not quantitated it. Only when a final scoring was established did we retrieve the clinical data.

Cytotoxicity

In **Paper III** we used the neutral red uptake assay for measuring the cytotoxic effect of TNF on melanoma cells. Neutral red assay has been shown to be a reliable and simple assay for measuring cell viability (233, 234). This method is based on the lysosomotropic properties of neutral red, and therefore its applicability is much dependent of the lysosomic activity of the cell to be studied, not all cell types taking up the dye at the same level (our unpublished observations). However in the case of cultured melanoma cell lines there was correspondence between cell number and intensity of the dye, as well as good reproducibility of the experiments (unpublished observations). Experiments were carried out in 6 replicates, and the outer wells of the plates were not used to avoid differences due to evaporation.

RESULTS AND DISCUSSION

As described in detail in the Background section, MM constitutes an important and practical model for immunological studies. In this thesis I focused on different immunological aspects of this tumor.

Paper I. Cell-cell adherence as a selection method for specific antibodies recognizing malignant melanoma. *J Immunol Methods*, 203: 103-109 (1997).

Mabs recognizing melanoma-associated antigens generated by immunization with complex antigenic mixtures and selected by traditional immunochemical methods (as ELISA) were not always useful for the study of biological samples. This prompted us to search for alternative methodologies. We focused on a selection method that would favor the recognition of native antigens expressed on the cell surface by the mAbs. This cell-cell adherence method proved to be simple and reliable, resulting in hybridomas producing specific mAbs. Posterior immunohistochemical studies revealed a high degree of specificity of Mel-3 mAb for malignant melanoma (unpublished results).

Paper II. Thioredoxin expression and localization in human cell lines: Detection of full-length and truncated species. *Exp Cell Res*, 236: 181-192 (1997).

Trx is a redox molecule whose expression is augmented during malignant transformation (201, 204). In this paper hybridomas producing mAbs against full-length and truncated Trx were generated by immunizing with the recombinant molecules. Specific mAbs were selected by ELISA against the immunogens. The mAbs were employed to study the expression of the two types of Trx in different cell types. All melanoma cell lines studied by intracellular immunofluorescence were positive with the exception of the FM28.4 cell line. The latter proved to be overgrown by mouse fibroblasts used as feeder cells. Another clone derived from the same tumor was later tested positive for Trx (**Paper III**). None of the cell lines was positive for membrane Trx. The truncated form of Trx was also present in melanoma cells but at lower percent and intensity compared to full-length Trx.

Paper III. Thioredoxin and thioredoxin reductase and TNFα expression in melanoma cells: correlation to resistance against cytotoxic attack. *Melanoma Res*, 10: 331-343 (2000)

This study was based on the following rationale: (a) the high Trx/TrxR expression observed for melanoma cells in **Paper II**; (b) reports that Trx could enhance *in vitro* the expression and release of cytokines, particularly TNF, IL1 and IL6 (217) and (c) immunohistochemical data showing a variable

expression of TNF in primary malignant melanoma, which correlated negatively to lymphocytic infiltration (152).

First, we wanted to test whether there was any association between Trx and cytokine expression in cultured normal and transformed melanocytic cells. As shown by immunofluorescence, all cells presented high levels of Trx, TrxR and the cytokines TNF, IL1α and β.

It is interesting to speculate why all melanoma cell lines studied by us were TNF-positive. We know from previous studies (152) and our own (**Paper V**), that not all primary melanomas are TNF-positive. Particularly, the TNF-positive FM28.7 melanoma cell line originated from a TNF-negative metastasis. Normal melanocytes of the skin were reported to be TNF-protein negative (141), but cultured melanocytes in our study were TNF-positive. Either upregulation of TNF expression is a phenomenon associated to *in vitro* culture, or the melanoma cell lines used in our study have some common characteristic that conveys TNF-positivity. In this case, we have used cell lines selected by their ability to provoke cytotoxic T-lymphocyte responses (235).

In the following studies we focused on TNF, because of the previous *in situ* results and the possibility to detect TNF by flow cytometry, thus permitting a more quantitative approach.

We detected the presence of both TNFR on the surface of melanoma cells, although at much lower levels than in U937 monocytic cells. Later studies showed the presence of intracellular receptors in the FM3 cell line, both TNFR1 (**Paper IV**) and TNFR2 (unpublished results).

Cells expressing endogenous TNF are reported to be resistant to the action of exogenous TNF (130). This effect has been studied in detail by the group of Fiers et al. using TNF-sensitive and resistant variants of the murine fibrosarcoma line L929 and different TNF constructs. Resistance to TNF required TNF membrane retention and consequent downregulation of TNFR (131-133, 236). Sensitivity to TNF has also been related to expression of receptor subtype, cell cycle and metabolic activity (127, 134, 237, 238). As the intracellular signaling pathways for TNF began to be elucidated, it was obvious that the metabolic inhibitors often used in classic cytotoxic assays prevented the synthesis of survival proteins also induced by TNF.

In our system, melanoma cell lines proved relatively resistant to the action of exogenous TNF, when compared to the WEHI-164 cell line. There was no significant effect on cell viability after 24 h, a reason why we prolonged the assay to 72 h. The IC_{50} for TNF was obtained using a dose –response curve

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from 0.5 to 50 ng/ml (7 points) that was analyzed using a sigmoidal fit. Only TNF concentrations higher than 5 ng/ml resulted in >50% cell death in most cells. In some cases, at low (up to 1 ng/ml) TNF concentrations a slight increase in viable cell number could be observed (unpublished observations). In our study, melanoma cell lines did express endogenous TNF without downregulating TNFRs (although both were expressed at low levels) while still being sensitive to TNF at doses and times similar to those reported for sensitive (and TNF-negative) L929 cells (236). "TNF-sensitivity" as a concept is very flexible depending of the experimental conditions (time, addition of metabolic inhibitors, TNF and target cell species etc). *In toto*, our results contradict the paradigm that TNF-positive cells are wholly resistant to exogenous TNF.

We observed a correlation between Trx/TrxR content and resistance to TNF in our study. It could explain the slower death process in our melanoma cells because of the presence of antioxidant buffering molecules to counteract the generation of ROS produced by TNF (123). However, considering recent results about TNF signalling and the role of Trx in apoptosis (as an activator of caspase-3), a slow apoptotic process could take place in these cells as a product of activation of multiple signalling pathways, both cytotoxic and survival. Dissection of the precise mechanism of TNF-mediated cell death in melanoma cells will require both powerful molecular modeling due to the complexity of these pathways and at the same time a careful interpretation of the data.

Stimulation of melanoma cells with the phorbolester PMA, alone or in combination with Ca-ionophore or Trx did not provoke release of TNF as in the case of the U937 cell line. In other studies we have tried several other stimulators such as UV-light, NO, IFNα and LPS, but no significative TNF release was observed. Valyakina et al (142) reported release of TNF by the BRO melanoma cell line after stimulation with the muramylpeptide GMDP. This substance was tested in the FM3 cell line, where we observed a maximal release of 25 pg/ml after 24-hr stimulation at the highest dose of GMDP employed (50 µg/ml). In comparison, the usual amount of TNF measured by us in the supernatants of stimulated U-937 cells is above 150 pg/ml. Therefore we can conclude that the lack of secretion of TNF in the melanoma cells tested by us is not restricted to stimulation by phorbol esters.

As a first step to explore the lack of release of TNF in melanoma cells we double stained for TNF and the Golgi-marker wheat germ agglutinin (WGA). Co-localization was present, indicating that TNF in melanoma also followed the Golgi pathway described for macrophages (92, 93, 239).

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Paper IV. Intracellular dynamics of TNF in malignant melanoma cell lines. (2001) *Manuscript.*

This paper is a logical continuation of the previous study on TNF processing. We wanted to clarify whether the lack of release of TNF from melanoma cells was due to insufficiency in the processing machinery and to explore the intracellular dynamics of TNF in these cells.

First, we studied if TACE, the enzyme necessary for TNF processing was present in melanoma cells. Immunofluorescent studies utilizing flow cytometry and microscopy, showed that FM3 and FM55M2 cell lines presented TACE both on the surface and intracellularly, at levels comparable to the classic TACE-expressing monocytic THP-1 line. The striking similarity of TACE staining to that of actin prompted us to double-stain with TACE and phalloidin-TRITC. We obtained partial colocalization after deconvolution image restoration (unpublished observations), and it would be interesting to study a possible intracellular association of TACE to the cytoskeleton.

Next, we generated a vector coding for GFP-tagged TNF for intracellular imaging. To check if this GFP-TNF construct could be secreted, we transfected CHO cells, which do not produce TNF (74), and tested for the presence of the cytokine in the supernatants by ELISA. GFP-transfected CHO cells released TNF spontaneously and increasingly after PMA stimulation (results not shown). To ensure that GFP-TNF would localize to the same areas as native TNF, we double-stained the transfected FM3 cell line with mAb CY-014, and could demostrate that native and transfected TNF had a similar intracellular localization.

GFP-TNF transfected FM3 and FM55M2 cell lines were used to study the intracellular localization of TNF. Double-stainings with the specific marker for Golgi apparatus calnexin and the ER-marker PDI showed co-localization. The transport of melanin in the melanosomes is a process characteristic of melanocytes. We double-stained transfected melanoma cells with the melanosome marker HMB-45 but could not detect any co-localization with TNF, concluding that TNF does not follow the melanin pathway.

Stimulation of cells with PMA resulted in a concentration of intracellular TNF to the apex of the dendrites. We also observed co-localization with TNFRI. AS TNFRI in FM3 cells has been observed mainly intracellularly, a ligand-receptor interaction would be spatially possible after stimulation, although at present we do not know if it is involving any downstream signaling.

RESULTS

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We could detect the presence of cleaved TNF in stimulated FM3 cells by Western blot, although at very low levels compared to the uncleaved protein. The processing of TNF was further studied using a construct containing TNF tagged with the epitope FLAG in its N-terminus and GFP in its C-terminus. CHO cells were also transfected with this construct and the secretion of TNF as well as the intracellular localization was corroborated (results not shown). With this construct we could discriminate between uncleaved and cleaved TNF, observing processing of TNF already after 15 minutes. While the cleaved prosequence was preferentially bound to the membrane, cleaved TNF seems to localize to the cytoplasm. Further studies with cocultured cells should clarify if the cleaved TNF recirculates inside the cell or if it is transferred to neighbouring cells.

Paper V. Endogenous TNFα predicts a better survival in thick melanoma. (2001) *Submitted.*

In this paper we wanted to test *in vivo* the hypothesis arising from the *in vitro* studies of **Papers II** and **III**: that the presence of TNF and Trx would confer resistance to melanoma cells, thus resulting in a worse survival of the patients. We studied a group of 44 primary melanoma patients by immunohistochemistry for TNF and Trx. In tumors with thickness > 0,8 mm TNF expression was significantly correlated to better disease-free survival. Moreover, by multivariate analysis TNF proved to be an independent prognostic factor. Trx, on the other hand, was not related to survival or the other evaluated parameters.

This result only shows how complicated "the real life" is compared to the experimental models. As in a previous immunohistochemical study TNF expression in melanomas was negatively correlated to lymphocyte infiltration, which has been considered a favourable prognostic factor, we expected TNF to be a marker of worse prognosis. However, the presence of lymphocytic infiltrate in a solid tumor does not mean that those cells are functionally active (35, 36, 240-242). On the other hand, our previous results and the fact that TNF expression did not correlate to mitotic index suggests that TNF is not an autocrine growth factor for melanoma cells. On the contrary, it could be a differentiation factor, as other authors have reported the preferential expression of TNF in more benign lesions (151). Thus, loss of this differentiation factor would result in a more aggressive behaviour.

Finally, a positive effect of TNF expression on the host's immune response could be another reason to the favourable prognosis. TNF is known to

CONCLUSIONS

upregulate MHC class I antigens in melanoma cells (243-246), thus possibly increasing the antigenic presentation of melanoma antigens.

RESULTS

CONCLUSIONS

1. Cell-cell adherence constitutes a reliable and simple method for generation of mAbs against native tumor antigens.

2. Trx and TrxR were highly expressed intracellularly in cultured melanoma cells, but not on the surface. Truncated Trx was present at lower levels.

3. In a panel of cultured normal and transformed melanocytic cells all cells expressed intracellular TNF at levels comparable to monocytic cells.

4. Melanoma cells also expressed TNF receptors, at low levels on the surface and at higher levels intracellularly.

5. Stimulation with phorbol esters and other agents, particularly Trx did not provoke significant TNF release in the panel of melanocytic cells studied.

6. This secretory block was not due to defects in the processing machinery, as cleaved TNF was detected in melanoma cells.

7. After stimulation of the melanoma cells there was accumulation of TNF at the apex of the dendrites, which co-localized with TNFR1.

8. TNF, Trx and TrxR expression in melanoma cells correlated to resistance against exogenous TNF *in vitro*.

9. The *in situ* expression of TNF in melanoma tissue correlated to a significantly better survival in patients with tumors thicker than 0,8 mm.

10. In melanomas > 0,8 mm thick TNF expression constituted an independent prognostic factor.

FUTURE PERSPECTIVES

1. The intracellular processing mechanisms of TNF in melanoma cells have been studied transfecting TNF cloned from immune cells. Isolation of TNF as well as its purification and biochemical characterization from melanoma cells could reveal alterations in the sequence or posttranslational variations that could explain e.g. the lack of secretion of this cytokine.

2. Is TNF in melanocytic cells transferred to other cells by direct cell-cell contact and if so, by which mechanism? Cocultivation of melanocytes and keratinocytes and tracking of TNF by advanced imaging methods, alternatively ultrastructural studies could elucidate these questions.

3. Is TACE associated to the cytoskeleton? A deeper exploration of the association of TNF and its mediators with the cytoskeleton could explain the spatial organization and the dynamics of the signalling pathways of this and other cytokines.

4. What is the reason of the better prognosis associated with TNF expression in MM patients? The study of differential immune responses against TNF-positive and negative (either "natural" or knock-out) melanoma cells could clarify if this effect is due to a more effective immune response of the host against the tumor.

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