Master's Thesis in Biomedicine

Identification of Immunodominant Regions of Melanoma

Antigen Tyrosinase by Anti-Tyrosinase Monoclonal Antibodies

Nele Jaanson

Faculty of Medicine

Tartu University

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Supervisor: prof. Mart Ustav

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Abbreviations

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AP	adapter protein
APC	antigen-presenting cells
BLAST	basic local alignment search tool
CDK4	cyclin-dependent kinase-4
CDKN2A	cyclin-dependent kinase inhibitor 2A
cDNA	complementary DNA
CMV IE promoter	cytomegalovirus immediate early promoter
CTL	cytotoxic T cells
DCT	dopachrome tautomerase
DHI	dihydroxyindole
DHICA	dihydroxyindole carboxylic acid
DM	deletion mutant
DNA	deoxyribonucleic acid
DOPA	dihydroxyphenylalanine
DTT	dithiothreitol
EDTA	diamine tetra-acetic acid
ELISA	enzyme-linked immunosorbent assay
ER	endoplasmic reticulum
FITC	fluorescein isothiocyanate
HLA	human leucocyte antigen
IFN	interferon
IMDM	Iscove's modified Dulbecco's medium
IgG	immunoglobulin G
Mab	monoclonal antibody
MC1R	melanocortin receptor 1
MTS	melanosomal transport signal
MW	molecular weight
OCA	oculocutaneous albinism
PAGE	polyacryl amide gel electrophoresis
PBMC	peripheral blood mononuclear cells
PBS	phosphate buffered saline
PCR	polymerase chain reaction
RASEF	RAS and EF hand domain containing
RGP	radial growth phase
RNA	ribonucleic acid
rVV	recombinant vaccinia virus
SV40	simian virus 40
TCR	T-cell receptor
Th	T helper
TIL	tumor infiltrating lymphocytes
TNF	tumor necrosis factor
TYRP	tyrosinase-related protein
UV	ultraviolet
VGP	vertical growth phase
WB	western blot

INTRODUCTION

Tyrosinase is the critical enzyme in melanin synthesis. As a melanocyte differentiation antigen it is also found to be expressed in most of the malignant melanomas including those of amelanotic lesions. Because of a series of demonstrations that tyrosinase gene products can be recognized by cytotoxic T cells (16, 100) and helper T cells (115) in humans, there is considerable interest in using tyrosinase as one of the potential antigenic targets for melanoma vaccines against tyrosinase positive melanomas. In order to develop serological reagents for immunodetection of human tyrosinase and to find the most immunogenic region of the protein for further DNA immunization experiments, we have raised a panel of monoclonal antibodies (mAb) against the recombinant tyrosinase expressed and purified from bacteria. Epitope mapping revealed the 79 amino acid long strech between 163 and 241 residues as the most immunodominant region of the tyrosinase. This region could be further divided into three parts by binding different mAbs. These mAbs were very useful tools for detection of tyrosinase expression from different constructs in tissue culture cells by immunocytochemistry and in melanocytes by immunohistochemistry. Some of the mAbs, which recognized epitopes between 163 and 204 amino acids, also recognized a distinct protein of about 70 kDa in Western-blot analysis of transfected and non-transfected COS-7 cells. One of them, 4B1 mAb was used in immunohistochemistry and cross-reaction with basement membrane of the human tissue was observed. The analysis of the 4B1 mAb epitope showed that the C-terminal part of that region almost entirely overlaps with the sequence of recently reported basement membrane protein β-netrin.

BACKGROUND

Tyrosinase

Tyrosinase (monophenol monooxygenase EC 1.14.18.1) is a copper-containing enzyme that is responsible for the formation of melanins and other polyphenolic compounds (99). This is a single-chain glycoprotein essential to initiate the cascade of reactions necessary to convert the amino acid tyrosine to melanin biopolymer (64), (Scheme 1). The principal subcellular site of tyrosinase activity has been determined to be the melanosome; however, tyrosinase is demonstrable in the soluble, ribosomal endoplasmic reticulum (ER), and Golgi apparatus fractions (41). Tyrosinase is a type I membrane glycoprotein whose cDNA predicts a protein of about 58 kDa, a 28-amino acid cytosolic tail, and five putative N-glycosylation sites (34). Tyrosinase occurs in four distinct microheterogenous forms as identified by PAGE; the high molecular weight membrane-bound form of tyrosinase found in melanosomes where the physiologic melanin production occurs is termed T_4 tyrosinase. The other three forms (T_1, T_2, and T_3) appear to be precursors of the enzyme, differing with respect of their
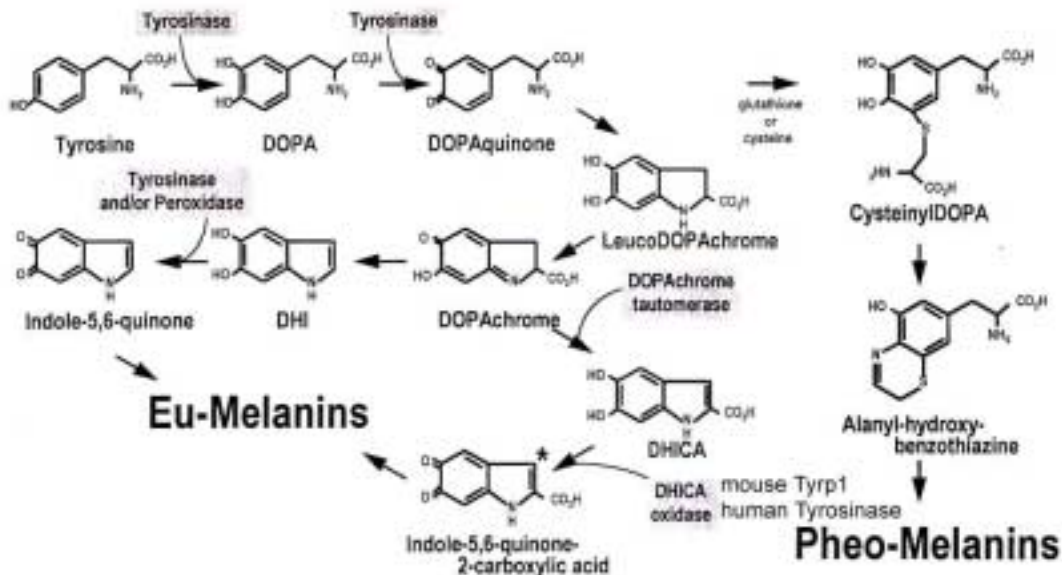
Scheme 1.
The functions for melanogenic enzymes, most of them demonstrated in murine melanocytes. Recently there has been found differences between functions of mouse and human couterparts, DHICA oxidase activity endowed to mouse Tyrp1 seems to be lost in human Tyrp1. This function is performed by tyrosinase.

posttranslational modifications (29). After solubilization of melanosomes with ionic detergents or trypsin, T_4 tyrosinase is dissociated into monomeric units and shows the same electrophoretic mobility as T_1 tyrosinase, with a molecular mass about 70 kDa. The molecular mass of tyrosinase has been reported to range from 60 to 75 kDa. After processing and glycosylation of the primary translation product leads to the mature tyrosinase molecules in the melanosomes, with a microheterogenous mass of 70-75 kDa. Variation in the size of the mature tyrosinase product has been attributed to the presence of isozymic forms (41) and/or alternate splicing of tyrosinase mRNA (92, 102, 108).

The tyrosinase gene on chromosome 11q14-21 comprises five exons spanning more than 50-65 kb (31, 91). The predicted amino acid sequence of the gene also contains a leader signal peptide and a transmembrane domain (69, 91), consistent with it being a protein anchored to the membrane of the melanosomes. It also codes for a short C-terminal cytosolic tail, which is needed for correct localization of protein to the melanosomal compartment (Figure 1.a). The active sites of tyrosinase are composed of two Cu-binding sites, designated Cu(A) and Cu(B)(93, 97). Both sites are located far apart in the primary structure of the protein (Figure 1.b), but come close enough to bind oxygen through the two copper atoms, as a result of the tridimensional folding of the molecule (Figure 8).
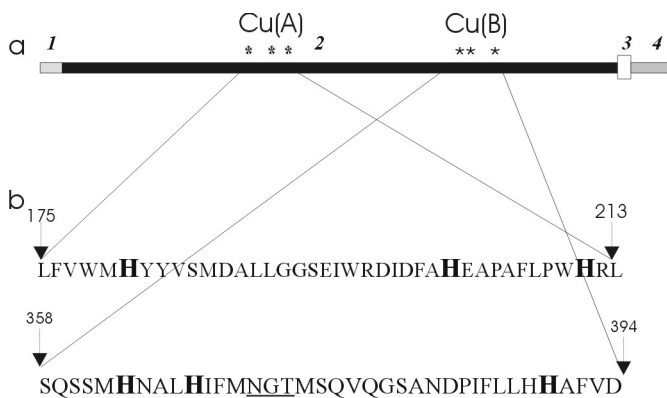
Figure 1.

a. Schematic representation of the structure of tyrosinase
1 – signal peptide, 2 – lumenal part, 3 – trans-membrane region,
4 – cytoplasmatic region.

b. Amino acid sequence of copper binding sites from first to third essential histidines which are shown in bold. A putative and conserved N-glycosylation site in the Cu(B) site is underlined.

Mutations of the tyrosinase gene have been documented in various forms of albinism (79, 111, 112). The disease is called oculocutaneous albinism (OCA) 1 and since tyrosinase is absolutely required for melanin biosynthesis, this is the most severe phenotype of all four currently known OCAs, resulting in virtually complete knockout of its function. Often those mutations themselves do not disrupt catalytic function, but the quality control system of the

ER is very specific and highly sensitive and can target mutant tyrosinase (even with minor point mutation) for proteosomal degradation (117, 118).

Tyrosinase-related protein (TYRP) family

Tyrosinase is a key enzyme catalyzing the first two reactions in melanin synthesis, the hydroxylation of L-tyrosine to 3,4-dihydroxyphenylalanine (DOPA) and the oxidation of DOPA to DOPAquinone (26, 43) (Scheme 1). A further cascade of reactions leads to the production of melanins, which are heterogenous biopolymers of quinone and hydroquinone monomer units. In addition to above mentioned two reactions, tyrosinase has proposed to have third function of oxidation of 5,6-dihydroxyindole (DHI) to indole-quinone (119). Tyrosinase is able to utilize tyrosine, DOPA and 5,6-dihydroxyindole (DHI) as substrates but in reality it is the hydroxylation of tyrosine that is the key, rate-limiting step (98). An example of the effect of a simple mutation in tyrosinase is seen in white (albino) mouse (Figure 2). However, the presence of functional tyrosinase is no guarantee of pigment production and there are many instances where significant amounts of tyrosinase are produced, yet little or no melanin is produced. This occurs in various forms of hypopigmentation disorders, in many instances of amelanotic melanoma, and probably also in lighter types of human skin, where activation of tyrosinase plays a role in determining skin phenotype (46).

In addition to tyrosinase, melanosomes contain two other proteins of TYRP family, TYRP-1 [dihydroxyindole carbolic acid (DHICA)-oxidase] and TYRP-2 (Dopachrome tautomerase, DCT), which are both implicated in eumelanin synthesis (Scheme 1). All these three proteins:

1)   are homologous (between 40% and 45 % of the amino acids) but distinct from one another in catalytic functions, modifying the types of melanins produced;

2)   have nearly identical molecular masses about 500 amino acids;

3)   have highly conserved cysteine residues that are implicated in tertiary structure;

4) have a secretory leader peptide, and an epidermal growth factor-like repeat present toward the C-terminus what is thought to mediate protein-protein interactions possibly in high molecular weight melanogenic complex

5) include two metal binding domains that are involved in enzymatic activities; for DCT Zn, for two others Cu-binding site

6) contain transmembrane domains at comparable positions (14)

7) are transported to melanosomes by a dileucine-based melanosomal transport signal (MTS), which locates in short cytoplasmic C-terminal tail of the protein (123).
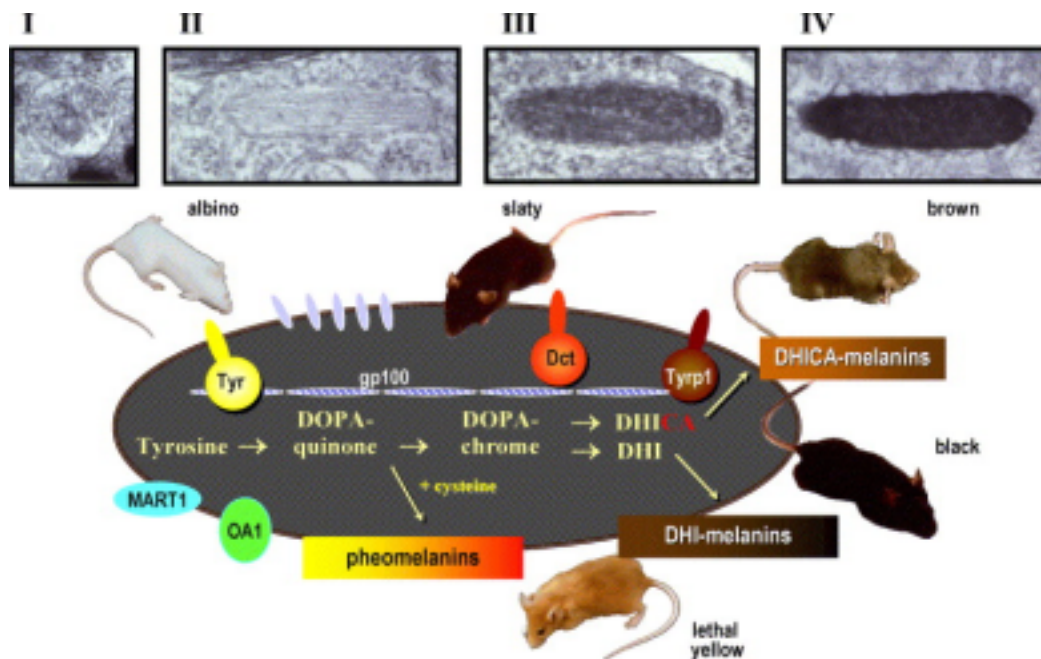
Figure 2. Schematic showing the maturation of melanosomes at the ultrastructural level top: from Stage I→II→III→IV. Melanosomal proteins that are involved in melanosome structure and/or function are shown aligned with the key steps in the production of melanin from tyrosine (bottom) and some instances of mutations of key genes in mice are depicted in the animal models (40). *Permitted by Elsevier*

In the presence of DCT, the carboxylic acid group of DOPAchrome, which would be spontaneously lost, is maintained and a carboxylated derivative (DHICA) is generated rather than DHI (120). This leads to a much slower further oxidation and polymerization, which results in a more soluble, lower molecular weight and lighter colored melanin known as DHICA-melanin. The presence of DCT, and formation of

DHICA rather than DHI, is also thought to have implications on the cytotoxicity of melanogenic intermediates to melanocytes. The disruption in pigmented phenotype resulting from a mutation in DCT is seen in slaty (gray) mouse (Figure 2).

TYRP1, at least in mice (64), is able to promote the oxidation of DHICA but in humans, this catalytic function of TYRP1 seems to be lost (Scheme 1) (13), and human tyrosinase seems able to perform that reaction (while mouse tyrosinase cannot) (81). However, in mice and in humans, TYRP1 also serves a more important function as a chaperone-like protein that assists tyrosinase processing, and dysfunction of TYRP1 elicits one form of albinism. In mice, mutations in Tyrp1 result in a brown coat color (40) (Figure 2).

Melanocytes, melanosomes, and melanin production

Melanin pigments are of no fixed molecular weight but are all derived by oxidation of the amino acid tyrosine and eventually produce two types of melanin in mammalian skin, black or brown eumelanin and red or yellow pheomelanin (93). The eumelanin precursors are derived from metabolites of dopacrome, whereas the pheomelanins derive from metabolites of 5-*S*-cysteinyldopa (Scheme 1). Melanins are produced by specialized dendritic cells, termed melanocytes, which are located as single cells primarily in the skin (within the basal layer of epidermis), but also in hair bulbs, eye, and inner ear, and are therefore responsible for skin, hair and eye colour. They are also situated in mucosa. Melanocytes synthesise melanin within discrete organelles termed melanosomes which are transferred to the surrounding keratinocytes. Developmentally, melanocytes arise from pluripotent cells of the neural crest, and their differentiation depends on spatial and temporal expression of variety of proteins including growth factors and adhesion receptors (105). Melanins are crucial in the absorption of free radicals generated within the cytoplasm, and in shielding the host from UV and visible radiation (110).

Melanosomes and their precursors can be classified into four stages of development based on morphology (Figure 2. upper panel). Early stages, or premelanosomes, lack

melanin and consist of vesicular structures with internal membranes (stage I), or characteristic, elongated structures with internal striations, or fibrillar structures, (stage II). Stage I of developing eumelanosome is spherical vacuole, derived from the endoplasmic reticulum, that elongates into an ellipsoidal organelle. As it is synthesized, polymerized melanins accumulate on striations, resulting in their thickening and blackening (stage III) until melanin fills entire melanosome (stage IV) (95).

To date only one melanosomal protein has been shown to be involved with the distinctive fibrillar structure of that organelle. Pmel17, also known as gp100 and silver, plays an important role in the formation of the characteristic fibrils that mark the transition of the melanosome from Stage I to Stage II (68). Such maturation is important to the subsequent sorting of melanogenic enzymes to melanosomes and to the eventual production of pigment (10). The known components of the endosomal sorting system, which employs a number of adapter protein complexes (AP1, AP2, AP3 and AP4 are known at this time) are functioning at various subcellular locations to transport proteins between different organelle components (94, 106). All known melanosomal proteins are glycosylated, at least through the early steps, and thus they all contain sorting signals at their amino termini that direct them to be taken into the ER immediately after their synthesis and to undergo early glycosylation events in the ER, and then to be sorted to the *cis*-Golgi for further processing. After that they are further glycosylated and processed through the *trans*-Golgi network (88). From that point they could potentially use the AP3 sorting system (which traffics proteins to early or late endosomes, and then to lysosomes/melanosomes). Tyrosinase is an example that uses that mechanism. At this time, the sorting system used by the other melanosomal proteins is not known, but is expected to involve other sorting pathways as well since disruptions in AP3 function alter tyrosinase sorting but does not affect Tyrp1 (50). Sorting signals are typically found at the carboxyl termini of proteins and several types are known, e.g. dileucine and tyrosine-based sorting signals (107).

Malignant melanoma

Etiology, risk factors

Melanoma is a disease in which malignant (cancer) cells arise from melanocytes. When melanoma starts in the skin, the disease is called cutaneous melanoma, which is most predominant place of arising this disease. But melanoma can occur everywhere in a body. If it occurs in eye it is called intraocular or ocular melanoma. Mucosal melanomas, far fewer in number than melanomas of the skin, manifest a far more aggressive and more rapid life-consuming biologic course. This behavior attends melanomas at any mucosal site, upper aerodigestive tracts, anorectum, and male and female genital tracts. Prognostic factors for both groups of melanoma – cutaneous and mucosal – are similar, but most mucosal melanomas have reached the dangerous limits, e.g., depth of invasion or thickness of melanoma at the time of diagnosis. In general, the mucosal melanomas are also more refractory to therapeutic modalities. In part, this may be due to anatomic restrictions of site and the large size of tumor when first discovered (4).

Although the aetiology is multifactorial, excessive ultraviolet exposure (UVB 290-320nm) appears to be the most important causative factor (2). UV light has been implicated in the genesis of several forms of cutaneous malignancies: squamous cell carcinoma, basal cell carcinoma, and melanoma, whereas the first two of them appear to be linked to total lifetime sun exposure, melanoma development is most closely associated with intense, intermittent exposure (7, 47). Melanomas occur relatively less frequently in areas that are continuously exposed to sunlight, like the face, hands, and arms, and more frequently in sun-protected areas receiving intermittent exposure, like the trunk in men and the backs of legs of women (83).

Experimental studies support the epidemiologic evidence implicating sun exposure in causing melanoma. Intense intermittent exposure apparently does not give melanocytes time to synthesize melanin to protect themselves from UV irradiation. This leads to DNA mutations. Although UVA (320-340 nm) light is more abundant in sunlight, than UVB light, the latter is responsible for several types of DNA lesions: it induces cyclobutane-pyrimidine dimers and pyrimidine-pyrimidone photoproducts. DNA

mutations result from incorrect repair of these lesions (30, 74). Prolonged sun exposure allows melanocytes to increase melanin production. UVB light can stimulate the transfer of melanin to form protective caps above the nuclei of suprabasal keratinocytes. During intense intermittent exposure, however, cells receive large doses of UV radiation without protection from increased melanin synthesis (87). In addition, melanocytes express several prosurvival factors, which may inhibit cell death following intense UV exposure (90). Therefore, melanocytes can receive intense mutagenic UV light and survive. If they are stimulated by growth factors, like basic fibroblast growth factor, endothelin-3, and stem cell factor, chances for transformation increase (9). In experimental models of melanoma UV radiation in the longer wavelength (UVA at 320-340 nm) has also been implicated.

Approximately 10% of melanomas arise due to a genetic predisposition. Germline mutations in the CDKN2A gene have been linked to melanoma incidence in many families with multiple cases of the disease. Studies of such families have indicated that the lifetime risk of melanoma in CDKN2A mutation carriers is very high, ranging from 58% in Europe to 91% in Australia by age 80 years (11). Although, there are some doubts of such high rate by some other authors who found that the risk of melanoma in CDKN2A mutation carriers was approximately 14% by age 50 years, 24% by age 70 years, and 28% by age 80 years. In addition, they suggested that the preponderance of familial clustering of melanoma occurs in families without identifiable mutations in CDKN2A (5). Major risk factors for melanoma include many nevi, especially dysplastic nevi, fair pigmentation, freckling, poor tanning ability, and germ line mutations in addition to the CDKN2A, also CDK4, or MC1R genes (33). These individuals are at risk of developing melanomas over their lifetime. A novel ocular and cutaneous malignant melanoma susceptibility locus maps to chromosome 9q21.32. Expression of RASEF, a known gene in this region, was examined in tumor tissue from 10 sporadic cutaneous malignant melanoma lesions and was found to be decreased in 70% of these tumors compared with RASEF expression in a human reference RNA pool from 10 different cell types and in 10 breast tumors (54).

The increase in melanoma incidence in U.S. Caucasian population is among the greatest for any cancer. From 1973 to 1994, melanoma incidence rates increased 120.5%

(37). While early, localized disease is effectively treated with wide excision, metastatic disease is almost universally fatal. Despite that white population is much more prone to melanoma, ratio between whites and blacks about 10:1, African Americans are more likely than whites to be initially seen by doctor with advanced disease and have a subsequent worse prognosis (18, 49). The median survival time for patients with disseminated melanoma is 8,1 months with only approximately 2% surviving for 5 years (70). Treatments for advanced disease are inadequate as most of disseminated cases are largely refractory to cytotoxic chemotherapy.

Pathobiology of melanoma

Melanoma progression from normal melanocytes is divided into distinct steps characterized by histopathological and experimental features (Figure 3). Least is known about human melanocyte precursor cells, which can also be found in the adult.
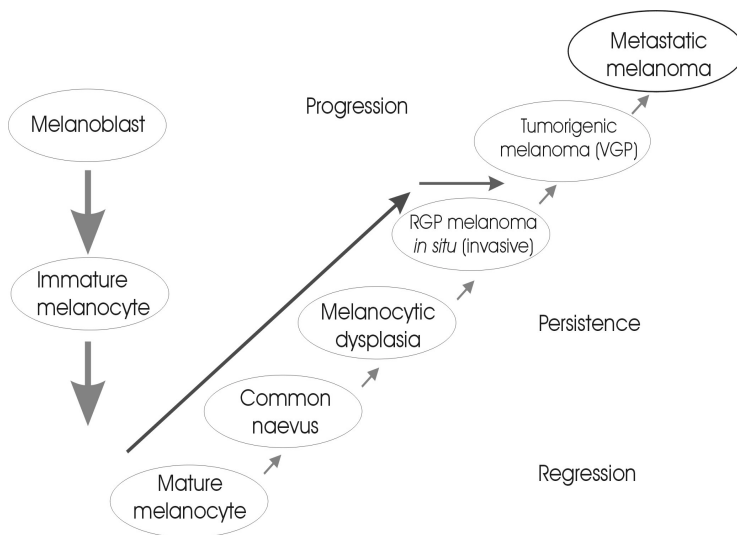
Figure 3 Melanoma development and progression. The model implies that melanoma commonly develops and progresses in sequence of steps from naevic lesions, which can be identified histologically approximately 35% of cases. Thus, melanoma may also develop directly from normal cells. The role of melanoblasts (immature melanocytes) remains poorly defined. Cells from naevic lesions show persistence, but the nontumorigenic lesions tend to disappear through apoptotic or differentiation pathways. RGP, radial growth phase; VGP, vertical growth phase. *Adapted from (105)*

Potentially, these cells are more likely to give rise to naevi and melanomas than mature, fully differentiated cells. Common naevi can progress to dysplastic naevi and melanomas, but more likely regress after four or five decades. Their development is potentially due to errors occurring in the growth regulation between melanocytes and keratinocytes. Both

cell types in normal skin exist in a fine balance (homeostasis) of growth and differentiation, and perturbance in adhesion and gap junction formation disrupts the control that keratinocytes exert over melanocytes. Dysplastic naevi are direct precursors of melanoma. However, their diagnosis and that of radial growth phase (RGP) primary melanomas is difficult and controversial. Surgical resection of RGP melanomas leads invariably to cure, suggesting that these lesions have not yet developed competence for tumorigenic growth and metastasis (104). Vertical growth phase (VGP) primary melanomas, on the other hand, contain multiple genetic abnormalities and have metastatic competence by growing invasively and independently of exogenous growth factors. They also produce tumours in immunodeficient mice (44).

Melanoma and tumor antigens

As melanoma is quite an immunogenic tumor it has been a valuable model for tumor immunology. Most of melanoma tumor specimens are infiltrated by reactive, so called tumor-infiltrating lymphocytes (TIL). Partial regression is common finding within primary cutaneous melanoma, and it occurs in 10% to 35 % of cases regardless of primary tumor thickness (12). Melanoma demonstrates regression 6 times more often than other malignant neoplasms, but complete regression of cutaneous malignant melanoma occurs more rarely (45). TILs are mostly cytotoxic T cells (CTL) that can be propagated in vitro by specific cytokines. Also, they can kill melanoma cells. This specific killing can be abrogated by monoclonal antibodies against CD3, CD8, T-cell receptors (TCRs) and against class I human leucocyte antigens (HLAs). This indicates that these CTLs can recognize the melanoma cells through the TCRs, in an HLA class I-restricted manner. Therefore, these cells and their products both are critical players in T cell-induced melanoma regression (52).

Recently, increasing importance is being given to the stimulation of a $CD4^+$ T helper cell (Th) response in cancer immunotherapy. Th cells are central to the development of an immune response by activating antigen-specific effector cells and recruiting cells of the innate immune system such as macrophages and mast cells (62). Th

cells can be divided in different types according to their cytokine profile. The Th1 is characterized by the production of IFNγ and IL-2 amongst others, whereas Th2 cells produce IL-4, IL-5, IL-10 and others. These cytokines can cross-regulate each other's function and development. The cytokines have a definitive influence on the outcome of the cellular and humoral immune response. IL-4 influences immunoglobulin class switching to IgG1 and IgE responses, whereas IFNγ influences the switching to IgG2a. Therefore these antibody isotypes are correlated with the Th type of the immune response. Furthermore, the cytokine IFNγ is well known for its effects on antigen processing and presentation. MHC class I and II and the expression of several other molecules involved in these processes, like TAP and proteasome components are under the control of IFNγ. Therefore, the Th1 type of T helper cell is correlated with an efficient cellular and CTL response (82). Th cells can also be induced to have a regulatory phenotype, which can significantly impede the function of Th cells in the tumor microenvironment and prevent tumor eradication. The resulting Th-like regulatory T cells produce IL-10 and other immunosuppressive cytokines (e.g. TGF-ß), which block the function of emerging antigen-specific Th cells (62).

Tumor antigen-specific Th cells can be activated by either antigen-presenting cells (APC) or directly by MHC class II-expressing tumors. The activation of the Th cell likely occurs when tumor antigens reach lymph nodes either through direct trafficking of tumor cells or during early immune activation, by APC delivery of antigens to lymph node T cells. The last is probably the predominant step since tumor cell migration (i.e. metastasis) is limited in the early stage of disease. Indeed, cancers can be infiltrated with dendritic cells (DC) early in the course of a tumor. For example, approximately 30% of node-negative breast cancers have significant DC infiltration. In addition, there is some evidence that tumor cells can act directly as APCs for activating Th cells, which of course, would be dependent on tumor expression of MHC class II. Melanomas typically have a high expression of MHC class II compared to other tumor types (62). MHC class II-negative tumor cells can be induced to express MHC class II molecule oftentimes unless there is some underlying defect in IFN responsiveness (78).

The initial trigger for a naïve CD4$^+$ T cell is the interaction of its T cell receptor (TCR) with the peptide ligand presented in MHC class II molecules. This leads within

several hours to cell surface expression of a member of the tumor necrosis factor (TNF) family called CD154 or CD40L (ligand). This molecule can interact with its receptor called CD40 (member of the TNF-receptor family) that is expressed on the APC. This interaction is responsible for activation and maturation of the APC that is characterized by the increased expression of several molecules involved in antigen presentation and co-stimulation, like MHC class II and I, ICAM-1, CD80 and CD86. This is a crucial step for efficient priming of specific CTL. The activation of APC by CD4$^+$ cells is essential for initiating a CTL. Th cells thus licence APC to induce killer cells (82). Analysis of the cellular interactions involved in CTL priming revealed that Th cells must recognize their antigenic peptide on the same APC that cross-presents the CTL epitope (6). Because cross-presentation of antigens is also required for MHC class I peptide loading, exogenous antigen has to enter the cytoplasm of the cell to be available for the proteasome-TAP dependent pathway. Antigen can be derived from different sources including apoptotic bodies. In particular immature DC posses the ability to ingest apoptotic cells via αvβ5 integrin and CD36 for cross-presentation to CTL. Normally the outcome of cross-presentation by immature DC is CTL tolerance. When the DC are activated by Th cells or inflammatory stimuli, this results in optimal CTL priming (82). In some murine models there has been shown that the prophylactic or post-challenge administration of both Th1-type and Th2-type CD4$^+$ clones specific for tumor leads to the induction of tumor-specific reactivity in vivo and concomitant tumor destruction, with quantitative rather than qualitative differences characterizing the anti-tumor activity of Th1 vs Th2 cells. Because the transferred CD4$^+$ cells lacked direct anti-tumor activity *in vitro* and required the *de novo* generation of tumor-specific CD8$^+$ T cells *in vivo*, these findings suggest that CD4$^+$ lymphocytes can enhance the ability of host APC to initiate an endogenous CD8$^+$ T cell response to authentic, poorly immunogenic tumor rejection Ag (28).

Other studies involving vaccination with irradiated tumor cells transduced to secrete GM-CSF show the dependence of CD4$^+$ T cells for tumor rejection. This lead to the simultaneous induction of Th1 and Th2 responses, both of which were required for maximal systemic antitumor immunity. It indicates a far broader role for CD4$^+$ T cells in orchestrating the host response to tumor. Cytokines produced by these CD4$^+$ T cells

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activate eosinophils as well as macrophages that produce both superoxide and nitric oxide. Both of these cell types then collaborate within the site of tumor challenge to cause its destruction (51).

It has became clear that antibodies play a role in antigen presentation in both the MHC class II route and the MHC class I proteasome-dependent route. Antigen-antibody immune complexes (IC) can be taken up by APC via Fc-receptors in a very efficient way. Antigen presentation by MHC class II and I is at least 1000-fold more efficient than the soluble protein in DC (96, 101). The different FcγR on DC might play a differential role. All three receptors CD64 (FcγRI), CD32 (FcγRII) and CD16 (FcγRIII) are expressed on DC. IC have been shown to be able to mature dendritic cells via CD64 and CD16 (96). The immunizing potential of IC in vivo in comparison to soluble protein has been shown by increased antibody responses against Ova (125).

All tumor antigens can be divided into 5 broad categories:

1)   *Mutations of genes or atypical gene products.*

Mutations can lead to increased immunogenicity of an epitope or changes in stability or trafficking. In the case of genes encoding p53, the p16/INK4a target CDK4, and β-katenin, the immune system can recognize the products of point mutations that are implicated in the pathogenesis of malignancy. Boon and colleagues were the first to show that unique antigens could be created by nucleotide point mutations resulting in individual amino acid changes (72). The immune system can also see products from alternative transcripts, including those from cryptic start sites and alternative reading frames, as well as pseudogenes (75) and antisense strands of DNA (121).

2)   *Cancer testis (CT) antigens.*

This class of antigens is not unique to cancer cells, but rather is shared with germ line cells that do not express MHC molecules. These normally silent antigens are sometimes expressed in cancer cells, perhaps due to changes in transcriptional regulation such as methylation of DNA. The MAGE-1 protein, which was the first human gene product recognized by $CD8^+$ T cells identified in a patient with cancer, is considered the prototype (122). The MAGE family, and related GAGE and BAGE families, as well as the NY-ESO-1 antigen (21) are the main CT antigens defined so far.

3)   *Differentiation antigens*

Differentiation antigens are antigens shared by cancer cells and their normal cell counterparts. It is thought that full T cell tolerance to these antigens during development does not always occur because these antigens are not expressed in the thymus. Therefore, reactive T cells are not completely deleted, although it is likely that T cells with high affinity are deleted. Melanoma and normal melanocytes share the melanosomal differentiation antigens that include tyrosinase, TRP-1/gp75, TRP-2/DCT, gp100/pmel17, and MelanA/MART-1. The melanosome membrane proteins are not only recognized by antibodies, but also by CD4$^+$ and CD8$^+$ T cells (86).

4)   *Over-expressed signal transduction molecule*

Forming another group of antigens, these molecules are present in normal tissue, but can also be over-expressed in cancers. The receptor tyrosine kinase HER2/neu is over-expressed in numerous types of cancers, including breast, ovarian, and lung. EphA3, another tyrosine kinase, was recognized by autologous T cells in patient with cancer (23).

5)   *Viral antigens*

Viral infections are implicated in the pathogenesis of some types of cancers, such as hepatoma (hepatitis virus), lymphomas and nasopharyngeal cancer (Epstein-Barr virus), cervical cancer (human papillomavirus) and leukemias (human T cell leukemia virus). T cells can recognize the strong antigens expressed by viral infected cells, but these viruses have evolved mechanisms to escape the immune system (86).

Tyrosinase as melanoma differentiation antigen

As a melanocyte differentiation antigen, tyrosinase is commonly expressed in malignant melanoma (16), including those of amelanotic leasons. It has also been reported to have a more homogenous pattern of expression in melanomas than gp75 and gp100 (22). Peptides derived from tyrosinase are frequently presented on melanoma cells by major histocompatibility molecules (56, 115). The development of immunotherapies for patients with melanoma is based in part on employing cytotoxic T-cell recognizing tyrosinase peptides as the immunogen (25). The production of these peptides is contingent on the presence of tyrosinase and its proteolytic degradation products.

However, tyrosinase peptide presentation is an aberrant phenotype of melanoma cells, because tyrosinase in normal melanocytes is a stable enzyme that is localized to the melanosomes, the site of melanin synthesis (35). The production of antigenic peptides involves an accumulation of tyrosinase in the ER as a 70-kDa high mannose glycoform and its subsequent routing to the cytoplasm for degradation by the proteasome. Failure of tyrosinase in these melanoma cells to be processed in the medial Golgi as indicated by endoglycosidase H digestion and confocal microscopy is reminiscent of albino mutant forms of tyrosinase that contain loss-of-function mutations and are retained in the ER (34, 77).

Melanoma-reactive T lymphocytes that specifically recognize tyrosinase in context of both class I and class II MHC molecules have been isolated and expanded from TIL and peripheral blood mononuclear cells (PBMC), and T cell epitopes have been identified that are restricted by HLA-A1 (57, 60), HLA-A2.1 (127), HLA-A24 (56), HLA-B35 (76), HLA-B44 (17). Many epitopes from melanoma antigens generate and maintain immune responses when presented in the context of HLA-DR molecules. For tyrosinase it has been reported in context of HLA-DR1 (71), HLA-DR4 (114), and HLA-DR15 (63).

Vaccination strategies against tumor

The immune system has evolved to protect the host from infections by distinguishing "self" and "non self". This is required for preventing immune recognition of host's own tissues. Developing T and B-lymphocytes undergo an educative process where immature cells expressing receptors that are self-reactive are usually deleted from the immune repertoire. The process, by which immature T-lymphocytes expressing receptors reactive to self are deleted in the thymus, is called central tolerance. As lymphocytes develop in the thymus (T cells) or the bone marrow (B cells), they undergo positive or negative selection. Central tolerance comes from the deletion of lymphocytes specific for ubiquitous self-antigens presented by APCs in the thymus and bone marrow (86).

Central tolerance is unable to delete all self-reactive T-lymphocytes however. T cells recognizing extra-thymic self-antigens, such as tissue-restricted antigens, are found in secondary lymph organs (128). Unlike developing lymphocytes, mature naïve lymphocytes that recognize antigen in secondary lymph tissue undergo activation or tolerance. Extra-thymic self-antigens influence the pool of circulating T cells. Antigens expressed on low levels, with inherently low affinity, with qualitatively poor agonistic properties, or sequestered antigens may be ignored by immune system. In contrast, high expression of agonist antigens may lead to activation followed by deletion of these T cells (20). This process is dependent on the level of antigen expressed in the peripheral tissue, and on the ability of bone-marrow-derived cells to "cross-tolerize" by taking up the antigen in the periphery and delivering it to the naïve T lymphocytes present in secondary lymph tissue (86).

Lymphocyte tolerance to some melanosomal differentiation antigens is effectively maintained in the periphery. T and B-lymphocytes reactive to these self-antigens are present in the immune repertoire but remain inactive upon exposure to the self-antigen. Exposure to altered forms of the self-antigen can induce these self-reactive lymphocytes to become activated. This in turn leads to autoimmunity, as evidenced by the destruction of melanocytes resulting in vitiligo.

Two signals are required for activation of naïve T cells. The first, or stimulatory, signal is transmitted via the TCR-CD3 complex, and the second, or co-stimulatory, signal is delivered by co-stimulatory molecules on professional APCs. In the absence of the second signal, anergy can be induced. One way tumor cells may evade immune recognition is by providing signal 1 in the absence of signal 2. So, one of the major problems faced one by inducing immunity against self-antigens is to overcome tolerance to self-antigens. This can be achieved in number of different ways:

1) Immunization with altered forms of a self-protein exploiting the presence of low/intermediate affinity T cells

Selected missense mutations in self-peptides increase peptide binding to MHC-I or -II molecules by altering single amino acid residues that anchor the peptide to the MHC molecule. Enhanced binding of the mutant peptides to MHC molecules provides more favorable energy conditions at the APC/TCR synapse for T cell activation. These

mutations increase the cell surface density of self-peptides at this synapse on the surface of APCs through increased affinity for MHC molecules. These missense mutations are not really non-self, but rather have been termed altered self (48). Such mutations in self-peptides increase TCR signaling sufficiently to trigger activation of naive T cells. Once these T cells are primed, they may respond to the parental non-mutant peptide presented by cancer cells and normal cells. Such antigens have been called heteroclitic. Previous experiments have shown that vaccination with a mutant form of a non-immunogenic peptide, rationally designed to enhance binding to MHC-I molecules, leads to T cell–dependent rejection of tumors expressing the parental non-mutant peptide (32).

2)   Xenogeneic DNA immunization

The use of xenogeneic antigens has been investigated as means for overcoming tolerance and/or ignorance to otherwise poorly immunogenic differentiation antigens on tumor cells. There has been shown that tolerance is broken by priming mice with human TYRP1 for sub-optimal series of two immunizations followed by a third immunization with either human or mouse TYRP1. This resulted in tumor immunity by antibody response, specially strong when the priming was done by human TYRP1 and then followed by boosting with syngeneic mouse TYRP1. These auto-antibodies are responsible for tumor rejection through activating Fc receptors (24). Most of such immunizations are performed by xenogeneic antigens delivered in the form of plasmid DNA. Vaccines made from bacterial plasmid DNA are simple and relatively inexpensive to prepare and contain immunostimulatory CpG sequences which may serve an adjuvant effect by inducing local cytokine release (61). DNA vaccines, in contrast to peptide vaccines, contain numerous potential epitopes. Plasmid encoding the antigen of interest is introduced into the skin or muscle with an intra-dermal or intra-muscular injection where it is taken up by professional APCs, such as DCs.

3)   Use of recombinant viruses expressing tumor antigens

Recombinant vaccinia viruses (rVV) have been used in several studies to induce immunity to self-antigens. Vaccination with rVV encoding murine gp75^{TYRP1} resulted in tumor-protective immunity and autoimmunity, whereas plasmid DNA encoding the same antigen failed to do so (84).

4)   Whole cell vaccines (typically transfected with cytokines or co-stimulatory molecules) and exogenous use of cytokines.

EXPERIMENTAL PART

Aims of the study

There were several aims of this study, which is a part of a bigger project of elaborating anti-melanoma DNA vaccination strategies:

1) Development of serological reagents for immunodetection of tyrosinase, as for checking the expression tyrosinase from DNA plasmid constructs, as well as for immunophenotyping of melanoma specimens by immunohistology. The last is definitely needed for proving that vaccination against tyrosinase is reasonable for that specific patient.

2) To find out the most immunogenic part of tyrosinase. It was done by the production and purification of recombinant tyrosinase, generation of mAbs that recognize tyrosinase, and by identification of the immunodominant region of the protein by epitope mapping.

Materials and methods

Expression constructs

Construction of prokaryotic expression plasmid pET 19b-Tyr for production and purification of truncated tyrosinase protein

Truncated human tyrosinase cDNA, encoding 452 of the 511 amino acids of the mature tyrosinase molecule in vector pQE9tyrA (Qiagen, Germany) was a gift by Pärt Peterson (Institute of Medical Technology, University of Tampere). This sequence did not contain a leader signal peptide and transmembrane domain of tyrosinase protein

coding sequence. The tyrosinase cDNA was cleaved off at first digesting with *Bam*HI, the end was filled in with Klenow's enzyme, and further digested with *Bpu*1102I. The expected 1389-bp tyrosinase cDNA fragment was purified by Qiagen gel extraction kit and cloned into pET19b vector (Novagen, Wisconsin, USA). *E. coli* DH 5 cells (Invitrogen) were transformed by the resulting plasmid pET19btrTyr. Restriction analysis and DNA sequencing selected the clone with the correct structure.

Construction of eukaryotic expression plasmids

a)   **Truncated tyrosinase** expression plasmid

Tyrosinase cDNA from pQE9tyrA was cleaved off with *Bam*HI and *Hin*dIII, and cloned into the same restriction sites of pCG3F12 expression vector, which contains BPV-1 protein E2 epitope 3F12 (Quattromed, Tartu, Estonia), CMV IE promoter, SV40 replication ori, ampicillin resistance gene and multicloning site (55). The resulting plasmid was named pCG3F12tyr.

b)   T**he entire coding sequence of human tyrosinase** was constructed designing two sets of oligonucleotide primers based on sequence in UniProtKB/Swiss-Prot entry, accession number P14679

For the signal peptide:

5` primer

5′-CC<u>TCTAGA</u>ACTAGAGGAAATGCTCCTG-3′, designated as XbaIfsign,

encoding after Xba I restriction site the signal peptide sequence,

3` primer

5′-AGTTGAATCCCATGAAGTTGC-3′, designated as BstXIrsign,

derived from exon 1 starting BstXI restriction site which naturally occurs in tyrosinase coding sequence.

For the transmembrane and cytoplasmic domain:

5` primer:

5`-GGACATAACCGGGAATCCTAC-3` designated BstXIftrans,

derived from exon 4 starting before another BstXI restriction site with different overhanging sequence than the one in exon 1.

3` primer:

5`-GGCTCGAGCCCTACTCTATTGCCTAAGC-3` designated XhoIrtrans, starting at Xho I restriction site and containing 3` sequence of exon 5 transmembrane region. Restriction sites are underlined.

Signal peptide was amplified by PCR of 40 cycles on genomic DNA of human melanoma cell line C-32 (103). For the transmembrane region cytoplasmic RNA of the same cell line was purified by RNeasy kit (Qiagen) and used as a template. Reverse transcriptase reaction was done using Avian Myeloblastosis Virus Reverse Transcriptase (Promega, USA), using appropriate primers and followed by PCR of 40 cycles. The PCR products were cleaved with respected restriction endonucleases resulting with 291 bp product for signal peptide and 290 bp product for transmembrane reagion. In addition, tyrosinase cDNA from pQEtyrA was cleaved with BstXI resulting with 1058 bp product. These three fragments with all different overhanging regions were purified and ligated into pCG expression vector digested previously with XbaI and XhoI. The resulting plasmid was named pCGtyrtp.

c) **For epitope mapping studies** the set of tyrosinase deletion mutants (DM) was generated with appropriate oligonucleotide primers containing an initiation methionine codon in the optimal Kozak context in their 5' part, and stop codon in 3' part.

The first range of the DMs was done dividing truncated tyrosinase into three regions (fig. 2. b), resulting with plasmids *pCG3F12*DM1, *pCG3F12*DM2, and *pCG3F12*DM3.

DM 1 primers:

5' primer 5´-GC GGA TCC GAT GAC GAT GAC AAA GCC-3´, named *DM1F*

3' primer 5´-GGG AAG CTT TCA AAA GCC AAA CTT GCA GTT -3´, named *DM1R*

DM 2 primers:

5' primer 5´-CC GGA TCC TGG GGA CCA AAC TGC ACA -3´, named *DM2F*

3' primer 5´-GGG AAG CTT TCA TGC ATC CCG CCA GTC CCA -3´, named *DM2R*

DM 3 primers:

5' primer 5´-CC GGA TCC GAA AAG TGT GAC ATT TGC -3´, named *DM3F*

3' primer 5′-GGG AAG CTT TCA GAT CCG ACT CGC TTG TTC -3′, named *DM3R*

The second set of the DMs was cloned by dividing DM2 into 3 parts (fig. 2. c), resulting with plasmids *pCG 3F12*DM2$_1$, *pCG 3F12*DM2$_2$, and *pCG 3F12*DM2$_3$

DM 2$_1$ primers:

5' primer 5′-GC GGA TCC GAT GAC CGG GAG -3′, designated as *75F*

3' primer 5′-GGG AAG CTT TCA ATC TCC TGT CAG CTT -3′, designated as *228R*

DM 2$_2$ primers:

5′primer *75F*, the same as for DM 2$_1$

3′ primer 5′-GGG AAG CTT TCA TGC TTC ATG GGC AAA -3′, designated as *204R*

DM 2$_3$ primers:

5′primer *DM1F*

3′ primer 5′-GGG AAG CTT TCA TCC ATT TTT CAT TTG GCC ATA GGT -3′ designated as *162R*.

All 5′ primers contained also *Bam*HI restriction site in 5′ end and 3′ primers contained *Hin*dIII restriction site in 5′ end for cloning. Restriction sites are underlined.

The tyrosinase deletion mutants were generated by PCR of 25 cycles at annealing temperature of 55°C with appropriate oligonucleotide primers. All PCRs for DMs were performed using Taq polymerase (Fermentas, Lithuania). The resulted products were cleaved with *Bam*HI and *Hin*dIII, purified, and cloned to pCG3F12 expression vector. All enzymes used for restriction were also from Fermentas.

All the cloned plasmid constructs used in this study were sequenced using BigDye® v3.1 cycle sequencing kit (Applied Biosystems) on Megabase^{TM} 1000 (AmershamPharmacia) sequencer apparatus. Sequence analysis was performed using BioEdit sequence alignment editor version 5.0.9 and BLAST (Basic Local Alignment Tool), located at National Center for Biotechnology Information web site.

Production and purification of truncated tyrosinase protein

E. coli BL 21-CodonPlus(DE3)-RIL and BL 21-CodonPlus(DE3)RP cells (Stratagene, La Jolla, USA) were transformed by pET19btrTyr. The transformed cells

were grown in LB broth containing ampicillin 100μg/ml. After the cell culture reached an OD of 0.5, the synthesis of truncated tyrosinase was induced by adding 1mM of isopropyl-β-D thiogalacto-pyranoside (IPTG). The culturing was continued for additional 3.5 hours. The bacteria were harvested by centrifugation at 4000 x g for 20 min. The expression and solubility of the protein was analysed by gel electrophoresis of the fractions of the bacterial lysate in the 10% SDS-PAGE gel.

The purification of recombinant protein from inclusion bodies was done by the following protocol. Bacterial pellet was washed with buffer: 10 mM Tris-HCl (pH 8.0 4°C), 1 mM EDTA, and 150 mM NaCl. After harvesting the bacteria were resuspended in lysis buffer: 50 mM Tris-HCl (pH 8.0 4°C), 1 mM EDTA, and 0.1 M NaCl. Then the solution of phenylmethylsulphonyl fluoride (PMSF) in 2-propanol was added to final concentration of 0.2 mM and lysozyme to 0.2 mg/mL. The mixture was incubated on ice during 20 minutes, shaking occasionally. The cells were lysed by adding of Na deoxycholate to a final concentration of 0.2 % (w/v). Lysate was incubated on ice 20 minutes and sonicated for shearing the DNA and lowering of the viscosity. The lysate was cleared by centrifugation at 10000 g 10 minutes at 4°C. Precipitate was suspended in extraction buffer: 50 mM Tris-HCl (pH 8.0 4°C), 10 mM EDTA, 0.1 M NaCl, and 0.5 % (v/v) Triton X-100 and incubated then at room temperature for 5 minutes and the insoluble inclusion bodies were precipitated by centrifugation at 10000 g for 10 minutes at 4°C. The resuspension and precipitation steps were repeated 4-5 times to get sufficiently clean suspension of inclusion bodies. The purified inclusion bodies were solubilised in 8 M urea and the protein solution was dialysed against decreasing concentrations of urea in 1 x PBS, finally only against 1 x PBS. The concentration of the soluble protein was determined by Bradford method (15) by using bovine serum albumin as a standard.

Purification of DNA

Ultra Clean kit (Eppendorf, Germany) was used for the purification of PCR products from TBE and TAE agarose gels. Modified boiling lysis (103) was used for plasmid DNA isolation and purification in the case of DMs. For purification of plasmid DNA for transfection into eukaryotic cells Eppendorf's commercial plasmid purification kit was used.

Cell lines, electroporation, tissue samples

For eukaryotic protein expression were used COS-7 cells ECACC (European Collection of Animal Cell Cultures No: 87021302). Cells were grown in IMDM (Gibco, UK), supplemented with 10% fetal calf serum (Sebak, Germany), penicillin 1 U/ml and streptomycin 0.1 mg/ml at 37ºC in a 5% CO2 atmosphere. Electroporation into COS-7 cells was done at 975 µF and 180V setting using Gene pulser system (BioRad) and electroporation cuvettes with a 0,4 cm gap width (BioRad). The amount of DNA used for one half confluent 9 cm diameter plate (about 10^6 of cells) was 2 µg. Cells were harvested 48 h after electroporation and denatured by Laemmli sample buffer (2% SDS, 50 mM TrisHCl pH 6,8, 0,1% bromophenol blue, 100 mM DTT, 20% glycerol).

Tissue samples from human pigment naevi were obtained from the Department of Pathology and Neuropathology of Tartu University Clinics.

Electrophoresis and Western blotting

The expression of the proteins was analysed in the 10% SDS-polyacrylamide gel according to the method of Laemmli. Coomassie Blue R-250 was used for staining the protein on acrylamid gel for prokaryotic expression. The molecular-mass marker used was SDS Molecular Weigh Marker (Amersham, UK). Western blotting was performed according to the method of Towbin (116). The tagged truncated proteins were detected

using 3F12 mAb as primary antibody (67) raised against the E2-tag, continued by Goat Anti-Mouse Alkaline Phosphatase Conjugated antibody (LabAS, Estonia). Specific signals were detected using the substrate 5-bromo-4-chloro-3-indolyl phosphate in combination with nitro blue tetrazolium. For the epitope mapping and for detection of full length tyrosinase hybridoma supernatants were used containing mAbs against tyrosinase. 1E2 mAb directed against BPV-1 E2 protein was used as a negative control. For secondary antibody Goat Anti-Mouse Peroxydase Conjugate (LabAS, Estonia) was used. Specific reaction was detected with Enhanced Chemiluminescence (ECL^{TM}, Amersham Biosciences).

Immunization of mice, generation of hybridomas, screening for mAb production

Female Balb/c mice were immunized with 50 µg of purified bacterially produced recombinant tyrosinase 3 times at 3-4 weeks intervals. The injections were intraperitoneal, initially in Freund's complete adjuvant and subsequently in PBS. Following the final injection, mice were allowed to rest for 5 weeks and then injected with 100 µg of antigen. One week later, final boosts with 100 and 200 µg of protein in PBS at 4 and 3 days before fusion, were performed. The spleen cells of immunized mice were fused with mouse myeloma cell line SP 2/0. Hybridomas producing anti-tyrosinase antibodies were cloned, and screened by solid-phase ELISA using the immunizing purified protein as the target antigen. 19 clones were isolated. Each of them was further subcloned and controlled by Western blot analysis using purified protein from inclusion bodies as antigen.

The subtypes of antibodies were analysed by subtype specific peroxidase conjugated antibodies (LabAs, Estonia).

Immunocytochemical and -histochemical procedures

Immunofluorescence assay on transfected COS-7 cells was done by (39). Supernatants of positive hybridomas were used as primary antibodies. FITC conjugated goat anti-mouse IgG antibodies (LabAS, Tartu, Estonia) were used as secondary antibodies.

Immunohistochemistry was performed on frozen tissue sections of human pigment naevus. The primary antibodies used were 5E1, 4B1, 1B8 and 1D7 hybridoma supernatants. For secondary antibodies peroxidase conjugated goat anti-mouse IgG antibodies were used. Diaminobenzidine tetrahydrochloride was used as chromogen.

Results

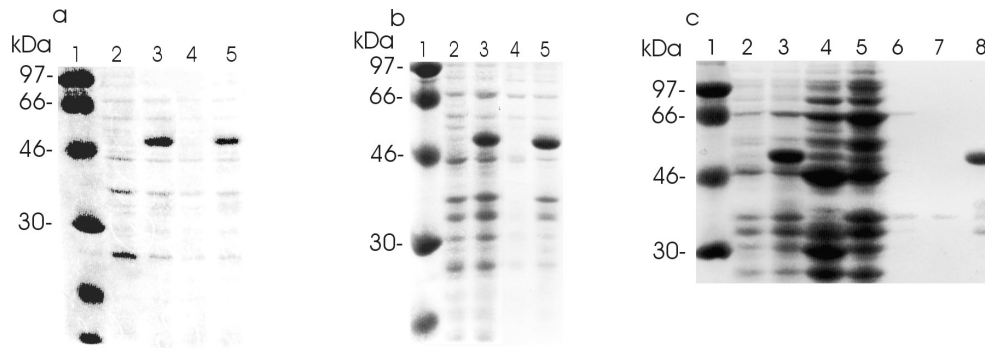## Production of recombinant truncated tyrosinase protein

Figure 4. Bacterial expression of tyrosinase, analysis of solubility and purification from inclusion bodies.
a: lane 1, mass standard; lane 2, uninduced E. coli BL 21-CodonPlus(DE3)-RIL cells transformed with pET19b trTyr; lane 3, induced E. coli BL 21-CodonPlus (DE3)-RIL cells transformed with pET19b trTyr; lane 4, uninduced E. coli BL 21-CodonPlus(DE3)-RP cells transformed with pET19b trTyr; lane 5, induced E. coli BL 21-CodonPlus(DE3)-RP cells transformed with pET19b trTyr;
b: lane 1, mass standard; lane 2, uninduced E. coli BL 21-CodonPlus(DE3)-RIL cells transformed with pET19b trTyr; lane 3, induced pET19b trTyr; lane 4, soluble fraction of induced pET19b trTyr; lane 5, insoluble fraction of induced pET19b trTyr;
c: lane 1, mass standard; lane 2, uninduced E. coli BL 21-CodonPlus(DE3)-RIL cells transformed with pET19b trTyr; lane 3, induced pET19b trTyr; lane 4, supernatant of lysis buffer; lane 5, supernatant of extraction buffer I (Na deoxycholate), lane 6, supernatant of extraction buffer II; lane 7, wash fraction with 1xPBS; lane 8, suspension of inclusion bodies (2 μl).

E. coli BL 21-CodonPlus(DE3)-RIL and BL 21-CodonPlus(DE3)-RP cells were transformed by pET19b trTyr. There was a problem in using regular *E. coli* DE3 strain possibly because of the different codon usage from mammalian cells. We found best expression in *E. coli* BL 21-CodonPlus(DE3)-RIL strain (Figure 4.a) with expected distinct band of 52 kDa. Despite good over-expression of the recombinant tyrosinase, unfortunately all of it was in the insoluble fraction (Figure 4.b) and did not bind to the Ni-affinity column, even under the denaturating conditions (8 M urea). Most of the protein was present in the flow through and first wash fractions (data not shown).

Purification of the protein

As all of the protein was in insoluble fraction and His tag was inaccessible, it was better to purify the protein from inclusion bodies by fractionation using Na Deoxycholate (Figure 4.c). Purified and concentrated suspension of inclusion bodies containing truncated tyrosinase in 1xPBS solution was obtained. The inclusion bodies were solubilised in 8 M urea and protein solution was dialysed against decreasing concentrations of urea in 1 x PBS, finally only against 1 x PBS. The amount of protein obtained was about 3-4 mg/L of cultured bacteria.

Generation of the hybridoma cell lines and screening their anti-tyrosinase mAb production

Mouse mAbs were generated against purified recombinant truncated tyrosinase protein and screened by ELISA. We chose a long immunization schedule in order to get high affinity IgG1 subtype antibodies. After five months immunization of BALB/c mice, the spleen cells were fused with mouse myeloma cell line SP2/0. 19 clones secreting mAbs showing reactivity with recombinant tyrosinase were harvested and subcloned. Each of them was controlled by immunoblot using truncated tyrosinase purified from bacteria, lysates of COS7 cells transfected with *pCG*tyrtp, or *pCG3F12*tyr or *pCG3F12*DM2 as antigen. 17 out of the 19 antibodies gave positive results in immunoblot, showing linearity of these epitopes (Figure 6.a). There is shown on this figure only DM2 expression and mAb binding as purified bacterial protein and full-length tyrosinase gave the same results. Two of the remaining – 4G9 and 1D3 – gave positive results only in ELISA and were figured as recognizing conformational epitopes. Most of mAbs were, as expected, IgG1 subtype, with exceptions of two – 2C9 and 3F6 – which were of IgM subtype (Table 1). All this work was done in cooperation with Jüri Parik and Erkki Juronen who did immunizations, fusion, subcloning, and subtype identification.

Expression of truncated and full-length tyrosinase in the tissue culture cells

To analyse eukaryotic expression of truncated and full-length tyrosinase the plasmids pCG3F12tyr and pCGtyrtp DNA were electroporated into COS-7 cells (Fig. 5.a). pCGE2 DNA expressing BPV-1 E2 protein was used as negative control. The expression of these
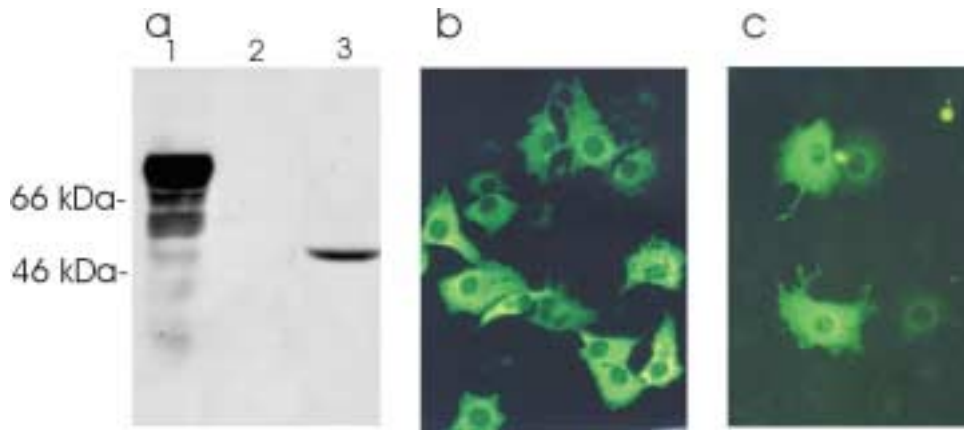

Figure 5.
Comparison of the expression of full-lenth and truncated tyrosinase in COS-7 cells
a: Western blot analysis: lane 1, COS-7 cells electroporated with pCGtyrtp (wt tyrosinase); lane 2, COS-7 cells electroporated with carrier for negative control; lane 3, COS-7 cells electroporated with pCG3F12tyr (truncated tyrosinase).
Immunocytochemical analysis in COS7 cells
b: wt tyrosinase (pCGtyrtp) was detected predominantely in coarse granular structures associated with the juxtanuclear region and extended into the cytoplasm.
c: the expression of truncated tyrosinase (pCG3F12tyr) was more homogenous, detected all over the cytoplasm and less condensated to the perinuclear region.

different constructs was analyzed in Western blot with mixture of 1B8, 1C1, and 1D7 anti-tyrosinase antibodies. Truncated tyrosinase was expressed as distinct ~52 kDa band. Full-length tyrosinase cDNA expression was observed as 55-75 kDa microheterogenous protein, most of this migrated at about 70-75 kDa.

Immunocytochemical reactivity

Immunocytochemical reactivity was tested on COS-7 cells, transfected with truncated and full-length tyrosinase cDNA. The full-length tyrosinase was detected predominantly in coarse granular structures associated with the juxtanuclear region (Figure 5.a) and extended into the cytoplasm. The expression of truncated tyrosinase construct was more homogenous, not containing coarse granularity (Figure 5.b)

Table 1. Summary of the features of anti-tyrosinase antibodies described in this study (-), reaction not observed; (+/-) extremely weak expression; (+,++,+++) mild, moderate, and strong reactions. I, II, III: first, second, and third immunodominant regions found by epitope mapping. Conf: conformational epitope

antibody		1D3	2C9	5E 1	4B 1	1B 8	4F 2	5H 12	5F 9	1D 8	5F 2
Expression of Δtyr in COS-7 cells on Wb		-	+/-	+	+	+++	+/-	+++	+/-	+/-	++
Nonspecific reaction in COS-7 cells		-	-	-	++	-	-	+	++	+++	+
Immuno-	Δtyr	+/-	+	++	+	+++	+	++	+	+	+/-
fluorescence.	wt.tyr	+	+++	+++	++	+++	+++	+++	+++	+++	+
Antibody binding region		conf	I	III	I	III	I	I	I	I	I
mAb subtype		IgG1	IgM	IgG1	IgG1	IgG1	IgG1	IgG1	IgG1	IgG1	IgG1

antibody		3F 6	4G 9	5B 10	5B 5	4C 7	1C 1	1D 7	1E 7	5A 2
Expression of Δtyr in COS-7 cells on Wb		+/-	-	+/-	+/-	+++	+++	+++	+	+/-
Nonspecific reaction in COS-7 cells		-	-	++	-	-	-	-	-	-
Immuno-	Δtyr	+/-	-	+/-	+/-	++	+++	++	+	+/-
fluorescence	wt.tyr	+	-	+	+	+++	+++	+++	+	+/-
Antibody binding region		I	conf	I	II	III	III	III	I	III
mAb subtype		IgM	IgG1	IgG1	IgG1	IgG1	IgG1	IgG1	IgG1	IgG1

found with full-length tyrosinase. The expression was detected all over the cytoplasm, with less condensation to the perinuclear region. Such a pattern of expression was observed by almost all mAbs with different strength. With 5A2 mAb the signal was very weak and with mAb 4G9 not expressed at all (Table 1).

Epitope mapping of the mAbs

In order to identify the most immunodominant regions of tyrosinase we carried out the epitope mapping (done by Kaidi Möll) of generated antibodies using deletion mutants generated by PCR described in the Materials and Methods. Three eukaryotic expression plasmids were generated: *pCG3F12*DM1 (amino acids 23 – 107), *pCG3F12*DM2 (amino acids 108 – 241), and *pCG3F12*DM3 (amino acids 242 – 474) (Scheme 2.b).
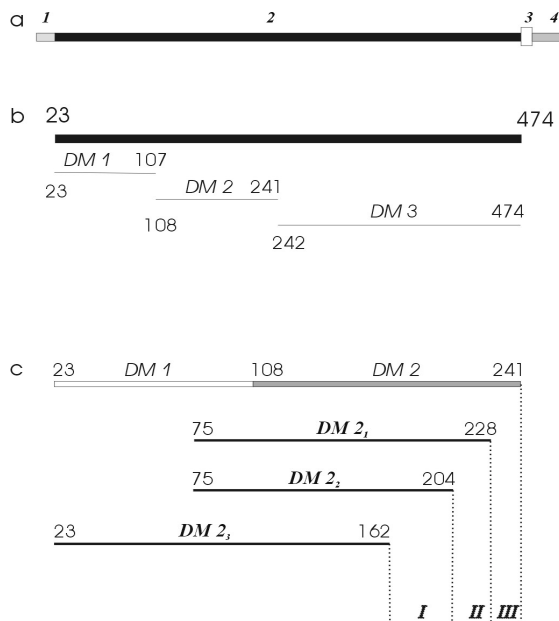
Scheme 2.
The structure of the full size and truncated forms of the tyrosinase used in this study

a. Full-length tyrosinase, 1 – signal peptide, 2 – lumenal part, 3 – trans-membrane region, 4 – cytoplasmatic region.
b. Scheme of making first range of deletion mutants.
c. Scheme of making second range of deletion mutants.

I, II, III – first, second, and third immunodominant regions found by epitope mapping.

All proteins expressed from these plasmids contained 3F12 epitope of the BPV 1 E2 protein at the N terminus as a positive tag for identification of protein expression. The DM plasmids were electroporated into COS-7 cells. After 48h the lysis of the transfected cells was done and analysis of protein expression in immunoblot was followed. All DMs had expected molecular weight as detected by 3F12 mAb: DM 1 at 10 kDa, DM 2 at 16.4

kDa, and DM 3 at 26.9 kDa. None of the hybridoma supernatants did react with DM1 and DM3 (data not shown). All the supernatants, except 1D3 and 4G9 reacted with DM2 (Figure 6. a) in the western blot analysis. Anti-BPV-1 mAb 1E2 did not react with any of DMs, which was used as a negative control of the assay.

The second set of DMs was generated by shortening DM 2 from C-terminus (Scheme 2.c) using the primers descibed in the Materials and Methods. Three eukaryotic expression plasmids were generated, *pCG 3F12*DM2$_1$ (expressing aa 75 – 228), *pCG 3F12*DM2$_2$ (aa 75 – 204), and *pCG 3F12*DM2$_3$ (aa 23 – 162), all encoding 3F12 epitope of BPV E2 protein at the N-terminus of DM for positive control. Their expression was analyzed as previously. As seen (Figure 6. b), the mAbs 2C9, 4B1, 4F2, 5H12, 5F9, 1D8, 5F2, 3F6, 5B10, 5B5 and 1E7 had the epitope on DM 2$_1$(amino acids 75 – 228), also there were mAbs, which did not bind to DM 2$_1$, but bound efficiently to DM2. These data indicate that the epitope for mAbs 5E1, 1B8, 4C7, 1C1, 1D7, and 5A2 is within 13 amino acid stretch between residues 229 to 241. There is also seen in this figure the additional band about 15 kDa recognized by antibodies 5H12 and 5F2, faintly also by 4F2 and 5F9. As this band was seen with no other expression constructs in WB it might be the degradative product of this special DM 2$_1$ or just the nonspecific signal, expressed stronger with in higher concentrations of antibodies as 5H12 and 5F2.

DM 2$_2$ (amino acids 75 – 204)(Figure 6. c) was recognized by mAbs 2C9, 4B1, 4F2, 5H12, 5F9, 1D8, 5F2, 3F6, 5B10 and 1E7, however, the mAb 5B5, which recognized DM 2$_1$ was not binding to DM 2$_2$, indicating that the epitope for this mAb is within residues 205 to 228. None of the mAbs did bind to the DM 2$_3$ (Figure 6. d), which suggests that epitopes for the antibodies 2C9, 4B1, 4F2, 5H12, 5F9, 1D8, 5F2, 3F6, 5B10 and 1E7 are mapped within the region of 163 – 204 residues. These data suggest that the 79 amino acid long sequence between amino acids 163 to 241 is the immunodominant region of the tyrosinase. This region can be divided into three parts (Scheme 2.c):

The first region is 42 amino acids long, located between 163 and 204 amino acids, which carried the epitopes recognized by mAbs 2C9, 4B1, 4F2, 5H12, 5F9, 1D8, 5F2, 3F6, 5B10 and 1E7. Six of them - 4B1, 5H12, 5F9, 1D8, 5F2, 5B10 - gave the additional band at ~70 kDa (Figure 6.c).

The second region is 24 amino acids long, located between 205 and 228 amino acids where the epitope recognized by mAb 5B5 is located.

The third region was 12 amino acids long, located between 229 and 241 amino acids where the epitopes recognized by mAbs 5E1, 1B8, 4C7, 1C1, 1D7 and 5A2 are located.
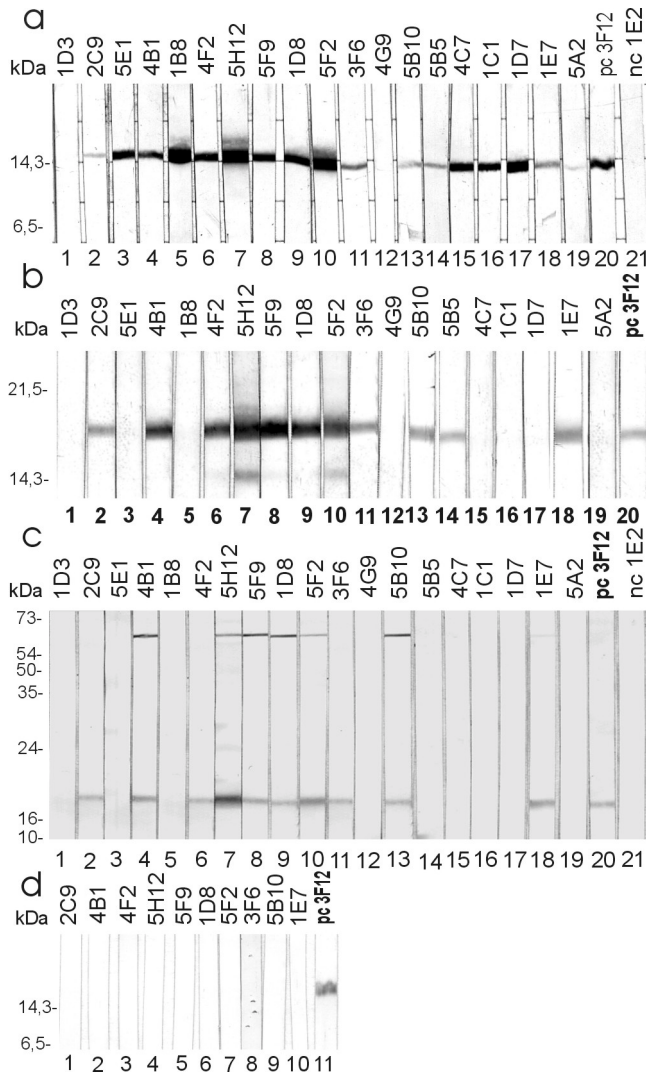

Figure 6

The results of the immunoblot analysis of the tyrosinase deletion mutants (DM).

a) All the supernatants, except 1D3 and 4G9 reacted with DM2.

b) The mAbs 2C9, 4B1, 4F2, 5H12, 5F9, 1D8, 5F2, 3F6, 5B10, 5B5 and 1E7 recognized the epitopes on DM 2_1.

c) DM 2_2 was recognized by mAbs 2C9, 4B1, 5H12, 5F9, 1D8, 5F2, 3F6, 5B10 and 1E7. Six of them gave the additional band (4B1, 5H12, 5F9, 1D8, 5F2, 5B10).

d) None of the mAbs did bind to the DM 2_3.

Nc: 1E2 is a negative control using mAb 1E2 directed against BPV E2 protein epitope that is not encoded by pCG3F12 vector.
Pc: 3F12 is a positive control using mAb 3F12 directed against BPV E2 protein epitope that is encoded by pCG3F12 vector.

Two mAbs – 1D3 and 4G9 – did not give results in immunoblot and were considered as recognizing the conformational epitopes as they gave signals only on ELISA where the antigen used was bacterially purified protein in it's more or less renatured state. There might be possibility that one of them, namely 4G9, is directed against bacterially purified protein and does not recognize the glycosylated variant in eukaryotic expression, indicated by absence of signal in immunoflorescence. But as we

had plenty of working, good signal-giving mAbs, we did not start to check it out as this was not our goal in this study.

Immunohistochemical reactivity

Immunohistochemical reactivity was tested on the frozen tissue sections of *naevus pigmentosus.* 5E1, 1B8, 1D7 and 4B1 mAbs were used for testing. Melanocytes and naevus cells showed intense cytoplasmic staining with 5E1, 1B8 and 1D7, whereas keratinocytes were negative (Figure 7. a). 4B1 mAb gave the same expression pattern, but stained also intensively the basement membrane zone (Figure 7.b).

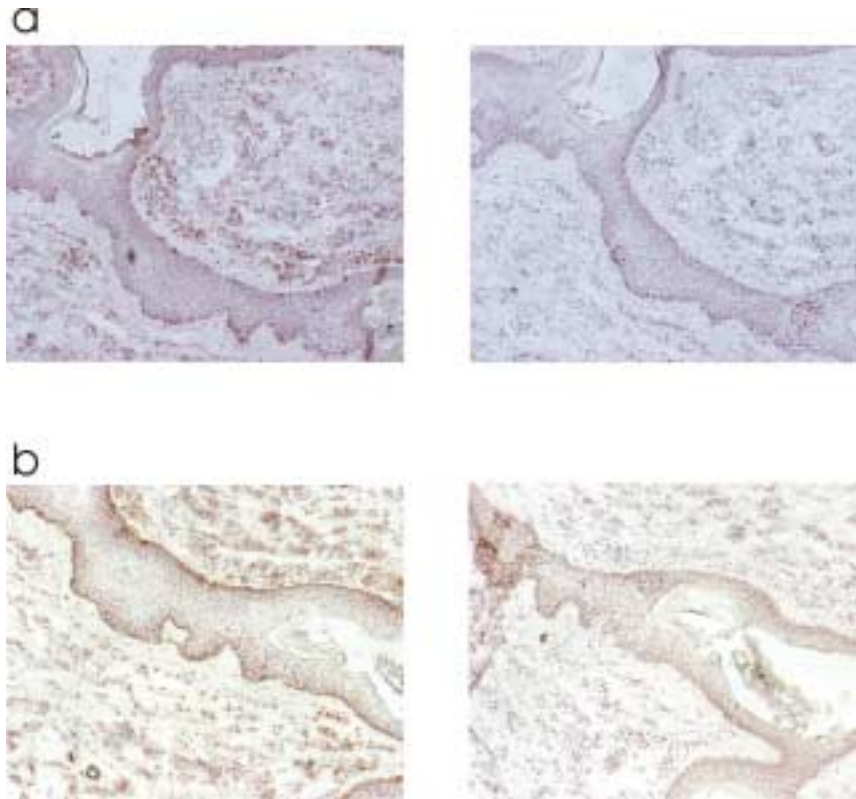Figure 7. Immunohistochemistry of frozen sections of naevus pigmentosus
a: left, with anti-tyrosinase antibody 1B8, right, negative control.
b: left, with anti-tyrosinase antibody 4B1, right, negative control.
Melanocytes and naevus cells show intense cytoplasmic staining, with mAb 4B1 the basement membrane is also stained.
Chromogenic substrate diaminobenzidine, background staining with hematoxylin-eosin

Considering the cross-reaction observed with mAb 4B1, we did BLAST analysis of the region where mapped the antibodies that gave the additional band. It revealed that basement membrane protein β-netrin almost entirely mapped with tyrosinase in that region having 5 identical amino acids, one which share biochemical properties (F vs W) and only one which does not map (A vs C):

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197 – DIDFAHE – 203 tyrosinase
468 – DIDWCHE – 474 β-netrin
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Discussion

Tyrosinase is a key enzyme in the synthesis of pigments known as melanins. It is also one of the first tumor antigens characterized for melanoma. As a melanocyte differentiation antigen, it is commonly expressed in malignant melanoma, including those of amelanotic lesions. Active immunotherapy in the form of DNA vaccination represents one potential strategy for that otherwise fatal disease when discovered too late or relapsed. Although DNA vaccines have induced immune responses to viral proteins, vaccinating against tissue specific self-proteins on cancer cells is clearly a more difficult problem. One way to induce immunity against a tissue specific differentiation antigen on cancer cells is to vaccinate with xenogeneic antigen or DNA that encodes orthologous DNA from another species (124). Immunization with xenogeneic human DNA encoding tyrosinase family proteins induced antibodies and cytotoxic T-cells against syngeneic B16 melanoma cells in C57BL/6 mice, but immunization with mouse tyrosinase-related DNA did not induce detectable immunity (124). Development of xenogeneic DNA vaccination in canine malignant melanoma has demonstrated that vaccination of dogs with human tyrosinase cDNA develops specific anti-tyrosinase humoral immune responses, is safe, almost not having side effects, and prolongs substantially the survival time of advanced disease (8). Tyrosinase has also been reported to have more homogenous expression within individual tumor specimen, distinctly different of gp100

expression (22), which should give better effect in vaccination killing most of the cancer cells in case of good immune response.

With these issues in mind, we chose tyrosinase as our target in mice melanoma model for future experiments. Tyrosinase is well expressed in B16 mouse melanoma cells, and syngeneic for C57BL/6 mice. This model is currently the most used all over the world for studying melanoma, its development and treatment modalities. Using tyrosinase cDNA in vaccination studies should give, at least in challenge studies, some protection against melanoma. The truncated variants should also work well but this must be established by experiments comparing two different DNA vaccinations. For that purpose the cDNA of human tyrosinase was cloned into eukaryotic expression vector and the expression of truncated and full-length tyrosinase was demonstrated in WB analysis.

The molecular mass of glycosylated tyrosinase protein has been reported to range from 60 kDa (73) to 75 kDa (41, 113). The primary unglycosylated translation product of tyrosinase gene is about 58 kDa (108), which, after processing and glycosylation, leads to the mature tyrosinase molecule in melanosomes, with a microheterogenous mass of 70-75 kDa (85). Variations in size of the mature tyrosinase product have been attributed to the presence of isozymic forms (41) and alternate splicing of tyrosinase mRNA (108). We found in COS-7 cells electroporated with full-length tyrosinase cDNA expression of a set of proteins with molecular mass from 55 to 75 kDa with predominant band at 70-75 kDa. Truncated tyrosinase was expressed as about 52 kDa distinct band, as expected. Our series of mAbs were generated against the unglycosylated peptide synthesized in *E.coli*, and the recognized antigenic epitopes would therefore be present in mature tyrosinase as well as in precursors and intermediate forms. This would explain the weaker bands from 55 to 70-kDa present in COS-7 transfectants. There was an essential difference also between localization and expression pattern of full-length compared to truncated tyrosinase in immunofluorescence analysis in COS-7 cells. As the latter do not contain melanosomes, the full-length tyrosinase was detected predominantely in coarse granular structures associated with the juxtanuclear region and extending into the cytoplasm. These structures were consistent with a lysosomal and late endosomal localization of tyrosinase reported previously in non-melanocytic cell types (19), (89), (117). The expression of truncated tyrosinase construct was more homogenous showing fine

granularity, different from full-length tyrosinase, all over the cytoplasm with less condensation to the perinuclear region. As tyrosinase cytosolic tail is found to mediate sorting to its targeting to endosomes and lysosomes in non-pigmented cells (109), (27), this fine granular, more homogenous pattern of truncated tyrosinase is the expected result.

Tyrosinase has proved to be more complicated to isolate than many other proteins. The difficulties are in part due to the strong hydrophobic binding of tyrosinase to other integral membrane proteins (126). The latter is used to purify tyrosinase from melanoma cells by using hydrophobic interaction chromatography. Some of the investigators have used prokaryotic tyrosinase cDNA over-expression combined with poly-his tag (22), (66) to purify protein on the Ni-affinity column. We tried to use the same method without much of success. As most of the protein occurred in insoluble fraction and in flow through by Ni-column purification, we decided to go different way. The purification from inclusion bodies, which has not been used before for tyrosinase, proved to be useful for getting sufficient quantity of the recombinant protein at satisfactory purity for immunizing purposes and for later ELISA analyses.

Antibodies against mammalian tyrosinase have been produced previously. Rabbit anti-hamster tyrosinase polyclonal antibodies (36), mouse tyrosinase (53), human tyrosinase (14), also against mouse T_4 tyrosinase (53) and human tyrosinase (73). The latter 5C12 mAb was reported to recognize an antigenic epitope residing in the carbohydrate moiety of tyrosinase. The series of antibodies against the tyrosinase family proteins have been generated by Hearing and coworkers (42) and antibody of human recombinant tyrosinase by Chen and coworkers (22). Our purpose was to generate the set of specific anti-tyrosinase mAbs for immunophenotyping melanomas by tyrosinase expression, and also for detecting antigen expression of gene therapy constructs, which is the requirement for going on to mice studies. Another goal was to locate the most immunodominant regions on tyrosinase by epitope mapping for using these parts later on in DNA vaccine therapy against malignant melanoma. Although much of the current effort in tumor immunology is directed to the generation of effective cytolytic T-cell responses, the cytotoxic response mediated by antibodies is just one possible effector mechanism contributing to the anti-tumor response. Macrophage-mediated antibody-

dependent cellular cytotoxicity is found to be a critical point in this (24). The anti-tyrosinase antibodies have been found to be involved in the regression of the tumor in some patients (3).
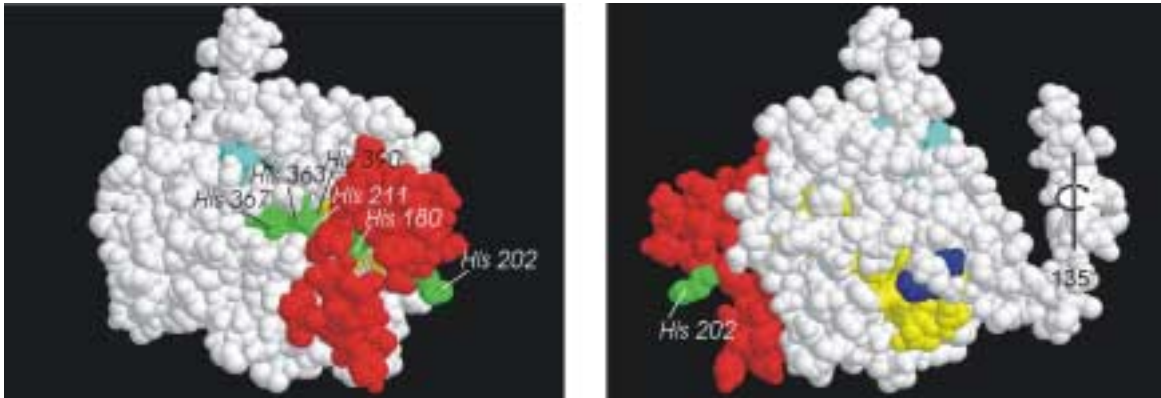

Figure 8. Three-dimensional structure of human tyrosinase based on RasMol program version 2.6. The right image is -135º turn over y-axis of the left image
The first region is marked by red including amino acids 163 to 204 and three histidine molecules 180, 202, 211, recognized by 10 mAbs: 2C9, 4B1, 4F2, 5H12,5F9, 1D8, 5F2, 3F6, 5B10, and 1E7, Histidine molecules are colored green despite of belonging to immunodominant region or not
The second region from 205 to 228 amino acids is colored in yellow, recognized by only one 5B5 mAb
Third region colored in blue is recognized by mAbs 5E1, 1B8, 4C7, 1C1, 1D7, and 5A2

The identification of B cell epitopes on tyrosinase has been described using vitiligo patients sera containing tyrosinase auto-antibodies (58). Our findings are different from those of experiments carried out under totally different conditions. We found the distinct immunodominant region between 163 and 241 amino acid of tyrosinase (Figure 8), where all the antibodies mapped. Several cytotoxic T cell epitopes have been also found to locate into this region between 163 and 241 amino acids (59), (57), (56). This is also the region where is located one of the metal binding sites, Cu(A) site (Figure 1 and 8). The metal binging sites are the active sites of the enzyme. Cu(B) site has proposed to be involved in binding and recognition of substrate (at least in DHICA), the Cu(A) site is suggested to be involved in the different catalytic potentials (81). It needs, of course, further studies, but probably some of those mAbs could be, theoretically, also useful tools for tyrosinase functional research. An interesting parallel has been found by group who

performed proteomic scan for tyrosinase peptide antigenic pattern in vitiligo and melanoma (71). They found that the immunoreactive peptide differentiating humoral response of vitiligo/melanoma patients from that of healthy subjects is only represented by a tyrosinase sequence crucial for tyrosinase activity, both for the presence of copper binding His[180] and OCA I-A variant position F176 (80). That is the same area of our immunodominant region.

Part of the mAbs, which had epitopes in first region between 163 and 204 amino acids (4B1, 5H12, 5F9, 1D8, 5F2, 5B10), gave an additional distinct band about 70 kDa in WB analysis of COS-7 transfected cells and also with negative control. 4B1 mAb was used for immunohistochemistry and cross-reaction with basement membrane was observed. BLAST analysis of this short amino acid sequence showed almost perfect match of its C-terminal part with recently reported basement membrane protein β-netrin (65). The amino acid sequence of tyrosinase from aa 197 to 203 is DIDFAHE and β-netrin sequence between 468 and 474 is DIDWCHE. The F (in tyrosinase) and W (in β-netrin) share the same biochemical properties, the only different amino acid is A (in tyrosinase) versus C (in β-netrin). Also the MW of the additional band on WB correlates well with this of β-netrin 69,9 kDa. β-netrin is reported to be expressed in basement membranes of kidneys and also in cells of renal tubular epithelium and mesenchymal cells. As COS-7 cells have kidney origin, it might be the reason for cross-reaction observed.

One way for tumor therapy is of using antibodies against tumor antigens triggering antibody dependent cell cytotoxicity, or to deliver by mAbs the cytotoxic enzyme or radioactive ligand to tumor cells. MAbs against TYRP-1 has been used for passive immunization against melanoma in mouse model (38). Passive transfer of mAb against gp75 was able to lead to rejection even of established B16F10 tumors in the lung. The similar effects have been observed with mAb treatment against other antigens expressed by B16F0 cells. For further development into clinic the possible hurdles connected to mAbs' murine origin could be overcome by the generation of chimeric and humanized mAbs that contain human Fc domains and retain targeting specificity by incorporating portions of murine variable regions. This can be accomplished by grafting either the entire murine variable regions (chimeric antibodies) or the murine

complementary-determining regions (humanization) into the human IgG framework (1). It is temping to speculate that the powerful tools as antibodies are, in our case some of them could be of use in tumor passive treatment, too. This needs, of course, to be proven in further experiments.

Summary

The following summarizes briefly the essence of the presented work:

✓ We have cloned human full-length tyrosinase cDNA and its truncated form into eukaryotic expression vector for gene expression and further immunization studies against malignant melanoma in mouse model

✓ The cloning, expression and purification of truncated human tyrosinase protein was done for the immunization of mice and for gaining specific mAbs against tyrosinase

✓ These mAbs were used for immunodetection of tyrosinase in tissue sections

✓ The identification of the most immunodominat region of the protein was done by epitope mapping, and revealed distinct 79 amino acid long stretch between 163 and 241 residues, where all the linear epitopes mapped

✓ An interesting finding was that some of the mAbs which were directed against the region from 163 to 204 amino acids reacted very specifically also with basement membrane and BLAST analysis revealed very close match with basement membrane protein ß-netrin

✓ Generated antibodies can be very useful tools for immunodetection of human tyrosinase expression, also for tyrosinase functional studies, and possibly in immunotherapy of malignant melanoma

Kokkuvõte

Melanoomi antigeeni türosinaasi immuundominantsete regioonide kindlakstegemine kasutades türosinaasi-vastaseid monoklonaalseid antikehi

- ✓ Täispikk türosinaasi cDNA ja selle trankeeritud vorm sai kloneeritud eukarüootsesse ekspressioonivektorisse nende järgnevaks geeniekspressiooni kontrollimiseks ja edasisteks immuunkatseteks hiire melanoomi mudelil

- ✓ Spetsiifiliste monoklonaalsete antikehade saamiseks türosinaasi vastu sai trankeeritud türosinaasi kodeeriv DNA järjestus kloneeritud prokarüootsesse ekspressioonivektorisse, ekspresseeritud bakteris ja sellest kultuurist puhastatud valku kasutati hiirte immuniseerimiseks

- ✓ Saadud antikehi kasutati koelõikudel türosinaasi immuun-detekteerimiseks

- ✓ Antikehade epitoop-mappimist kasutades selgitati välja kõige immundominantsem türosinaasi piirkond, mis asus 79 aminohappe pikkusel lõigul, 163-st amonohappest 241-ni, kuhu kõik lineaarsed epitoobid kaardistusid

- ✓ Üks huvitav leid seejuures oli, et osa antikehi, mis tundis ära lõiku 163 ja 204 aminohappe vahel reageeris ka väga spetsiifiliselt basaalmembraaniga ning BLAST analüüsiga leidsime me väga sarnase piirkonna olevat ühes basaalmembraani valgus ß-netriinis

- ✓ Antud antikehad väga kasulikud vahendid türosinaasi ekspressiooni kontrollimisel, samuti selle valgu funktsionaalsetes katsetes ja võimalik, et ka maliigse melanoomi immuunteraapias

Tänuavaldused

Tänan oma juhendajat Mart Ustavit, et ta selle teema mulle kunagi usaldas ja maksimaalselt selleks vabadust on andnud. Samuti tänan Urvet, kes juhendas minu esimesi iseseisvaid ponnistusi sellel okkalisel rajal ja luges kriitilise pilguga läbi käsikirja.

Suured tänuavaldused Kaidile epitoop-mäppimise katsete tegemise ja vormistamise eest. Sama Erkki Juronenile hübridoomiliinide tegemise eest. Jüri Parikule immuniseerimise läbiviimise eest, samuti ka tema rohkete kommentaaride ja õpetuste eest, mida ta jagas valgupuhastamise ja antikehadega töötamise kohta, lisaks lõbusatele anekdootidele, mis leevendasid ebaõnnestumiste masendust. Aitäh Kadrile heade kommentaaride eest käsikirja valmimisel. Tänud Aarele, Ivarile ja Andresele, kes alati igale mu kõige tüütumale küsimusele on olnud valmis vastama ja mind aitama. Samuti Annele ja Merikesele, et nad alati vajalikul hetkel olemas on olnud. Ning Daimale ja Tõnisele aitäh nende meeldiva naabruse ja alatise abivalmiduse eest. Ja kõigile teistele inimestele viroloogia õppetoolist kõigi toredate hetkede eest.

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