Human tyrosinase is a type I transmembrane glycoprotein with tyrosine hydroxylase and DOPA oxidase activities, being the key regulatory enzyme in melanin production. Antigenic peptides generated from processing of human tyrosinase and different mutants of tyrosinase were considered very promising for the development of vaccines that could improve the immune response in melanoma patients. In this review we consider some aspects of human tyrosinase processing and different strategies adopted to develop an effective anti-melanoma immunotherapy.

Key words: tyrosinase, melanoma, antigenic peptide, vaccine.

INTRODUCTION

Melanocytes are specialized pigment-producing cells that are responsible for the coloration of the skin, eyes and hair. Transformation of melanocytes in cancerous cells gives birth to malignant melanoma, the most aggressive skin cancer. At present, surgery for early melanoma is curative in most patients, but management of patients with metastatic melanoma has been proved to be ineffective in most cases. Inefficiency of the standard therapy, applied to control or cure malignant melanoma, is due to resistance of the transformed melanocytes to conventional therapy and to aggressiveness of the dissemination process.

Melanoma is considered highly responsive to the attack of the immune system. Nowadays, one of the major goals of scientists is the identification of tumor-associated antigens and the induction of effective tumor-specific responses to eradicate melanoma. Diverse categories of melanoma-associated antigens have been identified and studied. Among them, tyrosinase is the most important melanoma-associated antigen that has been proved to be recognized by autologous T lymphocytes. Immunogenic peptides derived from tyrosinase and presented in the context of MHC class I or class II pathways have been identified, making tyrosinase an ideal target for the development of vaccination strategies against melanoma.
MALIGNANT MELANOMA

Currently, cutaneous melanoma accounts for approximately one percent of cancer death. The incidence of malignant melanoma has increased in the past 50 years for more than six times. The American Cancer Society estimated that incidence rates of malignant melanoma will surpass the frequency of primary tumors of the brain, Hodgkin's disease, carcinoma of the larynx or pharynx and thyroid carcinoma. Survival chances of patient with malignant melanoma in the next years are 90 percent for stage I melanoma patients, 70 percent for stage II, 45 percent for stage III and only 10 percent for stage IV (1, 2).

MELANOMA PROGRESSION

Multivariate studies have been conducted in an effort to identify clinical and histological parameters of prognostic significance in melanoma (3). Melanoma lesions are defined by multiple changes in architectural and cytological features: a large size of the lesion, asymmetry, intraepidermal migration of melanocytes, cellular enlargement and abnormal size and shape of the nucleus. There are several distinctive signs of melanoma, called the “ABCD of melanoma”, where every letter is used to designate a characteristic of the affected region (A=asymmetrical, B=border irregularity, C=color change and D=diameter enlarging).

Usually, a four stage system is used for characterization of melanoma lesions: stage I refers to localized disease with no evidence of regional lymph node involvement, stage II indicates either palpable regional lymph node or pathologic documentation of lymph node involvement by melanoma and stages III and IV of disease consist of disease spread beyond regional lymph nodes (4).

ANTI-MELANOMA IMMUNOTHERAPY

Immunotherapy uses the ability of the immune system to fight against cancer. Immunotherapy may be classified into several types, including: a) active immunotherapy, which consists in specific stimulation of patient’s immune system with vaccines and/or nonspecific stimulation using adjuvants, b) passive immunotherapy, consisting in the treatment with exogenously produced antibodies, c) adoptive immuno-therapy, which means the transfer of lymphocytes and/or cytokines, d) restorative therapy, designed to restore deficiencies in the patient’s immune response and e) cyto-modulatory therapy, that aims to enhance the expression of major histocompatibility complex (MHC) molecules on the surface of the tumor cells (5).

Development of anti-melanoma vaccines is the most important goal of the anti-melanoma immunotherapeutic approach. Tumor vaccines are tumor antigen preparations in the form of whole tumor cells, tumor cell lysates, purified or
synthetic tumor peptides, cDNA encoding tumor antigens, or antiidiotypic antibodies bearing an internal image of the antigen.

There are two principal types of tumor vaccines: 1st generation (whole cell vaccines, autologous vaccines, allogeneic vaccines, tumor cell lysate vaccines, defined antigen vaccines and antiidiotypic antibodies vaccines) and 2nd generation (genetically modified cellular and recombinant tumor vaccines, whole cell vaccines and autologous, allogeneic, mixed and recombinant vaccines) (6).

MELANOSOMAL PROTEINS AS ANTIGENS

There are numerous ways to classify cancer antigens. Human melanoma antigens can be classified into three groups: antigens expressed in melanoma, normal melanocytes and retina, antigens expressed in several cancers and testis, and antigens specific for individual tumors (5, 6). Tissue-specific antigens are expressed in normal melanocytes, retina or melanoma cells, but not in other tissues. Melanosomal proteins that are immune targets in transformed melanocytes can be structural proteins (gp100) or enzymatic proteins (tyrosinase, tyrosinase-related proteins).

Tyrosinase, a copper containing enzyme, is a type I transmembrane glycoprotein with seven potential N-glycosylation sites that catalyzes the rate-limiting reactions in melanin synthesis. It converts tyrosine to DOPA-quinone and subsequently oxidizes 5, 6-dihydroxyindole (DHI) to indole-5,6-quinone. Tyrosinase is essential for melanin biosynthesis (7–10).

Tyrosinase-related proteins (TRP1 and TRP2) are expressed in normal melanocytes and melanoma cells. They are type 1 transmembrane glycoproteins with structural features similar to tyrosinase. TRP1 is the enzyme that oxidizes 5, 6-dihydroxyindole-2-carboxylic acid (DHICA) monomers into melanin. TRP2 is involved in the tautomeration reaction of DOPA chrome to DHICA (11, 12).

Tyrosinase, TRP1 and TRP2 are synthesized on ribosomes and transported through the rough endoplasmic reticulum (ER) and Golgi apparatus, where they are post-translationally modified. They are targeted to early melanosomes, where they will catalyse pigment biosynthesis (13).

Mutations in genes involved in melanin synthesis lead to oculocutaneous albinism (OCA), a disease associated with reduced pigmentation in the skin, hair, and eyes. Toyofuku et al. compared the intracellular processing, sorting and degradation of tyrosinase and TRP1 and their effects on the catalytic function and melanin synthesis, in wild-type and mutant melanocytes. The experiments showed that mutations in tyrosinase or TRP1 genes increased the time of association of these melanosomal proteins with molecular chaperones calnexin and BiP, resulting in retention of the mutant product in the ER. The obtained results proved that OCA1 and OCA3 are ER retention diseases (14).
The Pmel 17 (gp100) is a melanosomal resident glycoprotein containing 661 amino acids. It has five potential N-glycosylation sites. All five N-glycosylation sites are occupied. Pmel 17 is synthetized in the ER as a 70 kDa polypeptide, being rapidly exported to stage I melanosomes as a 100 kDa mature protein, which is Endo-H sensitive. Only a small fraction (5%) of Pmel 17 protein acquires complex N-glycans in the Golgi apparatus, becoming Endo-H resistant. In the melanosomes, Pmel 17 is proteolytically cleaved in three fragments (15, 16). Bakker and collab. have proved that antigenic peptides derived from processing of gp100 are recognized by melanoma-derived tumor-infiltrating lymphocytes (17).

The P protein is a melanosomal protein expressed in normal melanocytes and melanoma cells. It contains 838 amino acids and has a molecular weight of 110 kDa. It has four potential N-glycosylation sites, none of them being occupied (18). The experimental studies suggested that this protein could function like a proton pump, having an important role in maintaining a low pH value in melanosomes (19).

**HUMAN TYROSINASE**

Human tyrosinase is a type I glycoprotein that catalyzes the first two steps of melanin synthesis. It has 533 amino acids, 7 N-glycosylation sites, 17 cysteine residues distributed in two cysteine rich domains, two copper binding domains, a transmembrane domain and a cytoplasmatic domain (20) (Figure 1).

![Figure 1](image-url)  
*Fig. 1. – Schematic representation of human tyrosinase (SS=signal sequence, TM=transmembrane region, CYS=regions reach in conserved cysteine residues, Cu=copper binding domains).*

**MATURATION AND DEGRADATION OF HUMAN TYROSINASE**

The investigation of maturation and degradation of wilde-type tyrosinase has been done in different types of cell lines. Popescu and colleagues have proved that human tyrosinase is synthesized in B16F1 murine melanoma cells as a high mannose precursor that acquires complex N-glycans in the Golgi apparatus in approximately 30 min after synthesis and is degraded during 2 h of chase. The proper folding of tyrosinase is calnexin-dependent. The DTT sensitivity experiments showed a direct correlation between calnexin interaction and formation of critical disulfide bonds that lead to a stable three-dimensional conformation. Wilde-type tyrosinase also associates with calreticulin and BiP. Interaction of wilde-type tyrosinase with molecular chaperones started from the early stages of synthesis and decreased in time (21).
The polypeptidic chains that acquire the native conformation are exported to the Golgi apparatus, where are modified, and subsequently are targeted to melanosomes (in melanocytes and melanoma cells) or to lysosome-like vesicles (in non-melanocytic cells).

Still, a significant population of wilde-type tyrosinase goes to unproductive folding cycles, remains Endo-H sensitive, is retained in the ER and is further subjected to proteasomal degradation by the ERAD pathway (21).

Popescu and colleagues have also proved that the transmembrane domain is critical for interaction of wilde-type tyrosinase with calnexin. A truncated form of tyrosinase that is lacking the cytosolic and transmembrane domains proved to be inefficiently processed. The interaction with calnexin was compromised by lack of the transmembrane domain. Mutant protein was retained completely in the ER by calreticulin and BiP and was subjected to proteasomal degradation. Analysis of tyrosinase mutants that lacked the cytosolic domain and of mutants in which the transmembrane domain was replaced with the transmembrane domain from RPTPase Mu or with a GPI anchor proved that are efficiently processed in the ER, being exported to the Golgi apparatus (22).

Ujvari and collab. showed that the translation rate of human tyrosinase determines its N-linked glycosylation level, by using normal melanocytes, melanoma cells, an in vitro cell-free system and semi-permeabilized cells. Glycosylation at Asn290 (Asn-Gly-Thr-Pro) was suppressed, particularly when translation proceeded rapidly, producing a protein doublet with six or seven N-linked core glycans. The inefficient glycosylation of Asn290, due to the presence of a proximal Pro, was enhanced in melanoma cells compared with normal melanocytes. Slowing the translation rate with the protein synthesis inhibitor cycloheximide increased the glycosylation efficiency in live cells and in the cell-free system (23).

Halaban and collab. have demonstrated that the substrates DOPA and tyrosine induce in melanoma cells a transition of the misfolded wild type tyrosinase to the native form, which is resistant to proteolysis, competent to exit the ER, and able to produce melanin (24).

Degradation of proteins that are retained in the ER due to their improper or suboptimal processing involves retrotranslocation, to reach the cytosolic ubiquitin-proteasome machinery. The ubiquitin-proteasomal pathway is involved in the degradation of substrates originated in the ER (25). Halaban and collab. followed the kinetics of synthesis, degradation, processing, chaperone binding and inhibitor sensitivity, and also the subcellular localization of tyrosinase in normal and malignant melanocytes. In amelanotic melanoma cell lines, tyrosinase failed to reach the melanosome, which is the organelle for melanin synthesis, because it was retained in the ER and then degraded. Tyrosinase appeared mostly as a 70 kDa core-glycosylated, endoglycosidase H-sensitive, immature form bound to the ER chaperone calnexin and had a life-span of only 25% of normal. Maturation and
transit from the ER to the Golgi compartment was facilitated by lowering the temperature of incubation to 31°C (26).

TYROSINASE – A PROMISING ANTIGEN IN MELANOMA TREATMENT

The immune system of a melanoma patient maintains the stability and controls the progression of melanoma. Efficiency of immune defense depends on several factors such as the state of the immune system, the level of expression of the melanoma-associated antigens, the processing and presentation of the melanoma-associated antigens, etc. We shall present several studies that made from human tyrosinase a promising tissue-specific melanoma antigen considered for the development of an efficient anti-melanoma vaccine.

The antigenic peptides production requires accumulation of tyrosinase in the ER and degradation by the ERAD pathway. Melanosomal proteins seem to be especially antigenic. Epitope immunization can induce production of reactive T cells in melanoma patients and sometimes can determine the regression of the tumor. Numerous studies were pointed to the identification of the antigenic epitopes derived from tyrosinase. We show in Table 1 the sequences of the antigenic epitopes derived from human tyrosinase.

Table 1

<table>
<thead>
<tr>
<th>Amino acid sequence</th>
<th>Epitope</th>
<th>MHC-I specific class</th>
<th>Tissue</th>
</tr>
</thead>
<tbody>
<tr>
<td>368–376</td>
<td>YMDGTMSQV</td>
<td>HLA-A2</td>
<td>Normal melanocytes</td>
</tr>
<tr>
<td>1–9</td>
<td>–</td>
<td>HLA-A*0201</td>
<td>Retina</td>
</tr>
<tr>
<td>146–156</td>
<td>SSDYVIPGTY</td>
<td>HLA-A1</td>
<td>Melanoma</td>
</tr>
<tr>
<td>243–251</td>
<td>KCDICTDEY</td>
<td>HLA-A1</td>
<td></td>
</tr>
<tr>
<td></td>
<td>MLLAVCZLL</td>
<td>HLA-A*0201</td>
<td></td>
</tr>
<tr>
<td></td>
<td>AFLPWHLRF</td>
<td>HLA-A*2402</td>
<td></td>
</tr>
<tr>
<td></td>
<td>SEIWRDIDF</td>
<td>HLA-B44</td>
<td></td>
</tr>
<tr>
<td>56–70</td>
<td>QNILLSNAPLGPQFP</td>
<td>HLA-DR4</td>
<td></td>
</tr>
<tr>
<td>460–462</td>
<td>SYLQDSDPDSFQD</td>
<td>–</td>
<td></td>
</tr>
</tbody>
</table>

Mosse and collab. showed that treatment of melanoma cells with proteasomal inhibitors determined accumulation of two forms of tyrosinase in the cytosol: a glycosylated form that is proteolytically cleaved at the C-terminus by an inhibitory resistant protease and a deglycosylated form. This result leads to the conclusion that there are at least two alternative pathways of degradation for human tyrosinase into the cytosol (27).

Skipper and collab. have identified a natural peptide as the target of a clone of cytotoxic T lymphocytes (CD8+). This peptide is present in normal melanocytes...
and in melanoma cells as an antigen, in a MHC I restricted fashion. The recognized epitope is YMDGTMSQV and it is identical with the sequence 368–376 in human tyrosinase, excepting the aspartic acid residue (Asp or D) that replaces the asparagine residue (Asn or N) in the third position. This peptide was proved to be the result of post-translational conversion of Asn to Asp. This modification is highly important for antigen recognition by specific T cells but has no importance for MHC-I loading with the antigenic peptide. The enzymatic deamination of Asn to Asp most probably occurs under the control of PNGase enzyme that catalyzes the hydrolysis reaction between complex or oligomannosidic glycans and Asn during the degradation process. The proteasomal degradation of the tyrosinase is essential for processing and presentation of its antigenic peptides in a MHC-I restricted fashion. In the sequence 145–156 there are other two antigenic peptides: SSDYVIPGTY (TYR146–156) and ISSDYVIPGTY (TYR145–156), which are recognized by the MHC-I restrictive T cells (28).

Kittlesen and collab. developed several studies on cytotoxic T lymphocytes taken from melanoma patients. They found that the sequence KCDICTDEY (TYR243–251) is efficiently recognized by T cells. The same clone of T cells also recognizes the sequence DAEKCDICTDEY (29).

Hosseau and collab. showed that certain carbohydrate moieties are required for processing of tyrosinase peptides recognized by helper T lymphocytes (CD4+). Hosseau proved by site directed mutagenesis that four sites were required to generate forms of tyrosinase that could be recognized by individual T cell clones. These findings proved once again that the posttranslational modifications of human tumor-associated proteins such as tyrosinase could be a critical factor for the development of anti-tumor immune responses (30).

Meyer and collab. analyzed the immunological response after vaccination in an adjuvant setting with recombinant modified vaccinia virus Ankara carrying the cDNA for human tyrosinase (MVA-hTyr). The delivery of MVA-hTyr was safe and did not cause any side effects. A strong response to the viral vector was achieved, indicated by an increase in the frequency of MVA-specific CD4+ and CD8+ T cells and an increase in the virus-specific antibody titers. No tyrosinase specific T-cell or antibody response was observed with MVA-hTyr in any of the vaccinated patients. Thus, although MVA-hTyr provided a safe and effective antigen-delivery system, it does not elicit a measurable immune response to its transgene product in patients with stage II melanoma (31).

Reynolds and collab. developed a polyvalent vaccine containing multiple antigens, including MAGE-3, Melan-A/MART-1, gp100, tyrosinase, melanocortin receptor (MC1R) and dopachrome tautomerase (TRP-2). During his experiments he identified multiple melanoma-associated peptides that could stimulate CD81 T cell responses in vivo in humans, and proved the existence of a marked MHC independent heterogeneity in the ability of these peptides to induce CD81 T cell responses in different individuals (32).
CONCLUSIONS

Nowadays, much effort is done in order to discover new tumor biomarkers that could be used for early detection of melanoma lesions, and new strategies are considered very promising for the development of a universal anti-tumor vaccine. But, so far, scientists failed to design an efficient vaccine that could be used to cure melanoma. In this picture, experimental studies put human tyrosinase in a central place. New mutants of tyrosinase are designed and tested in order to obtain an elevated immune response that could provide the basis for an efficient anti-tumoral therapy.

REFERENCES


