THE IMMUNOGENIC PROPERTIES OF HUMAN MELANOMAS AND MELANOMA-ASSOCIATED ANTIGENS RECOGNIZED BY CYTOTOXIC T LYMPHOCYTES

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IMUNOGÉNNE VLASTNOSTI ĽUDSKÝCH MELANÓMOV A S MELANÓMOM SÚVISIACICH ANTIGÉNOV ROZPOZNÁVANÝCH CYTOTOXICKÝMI T-LYMFOCYTMI

During the last years significant progress has been achieved in the identification of melanoma-associated antigens (MAA) recognized by cytotoxic T lymphocytes (CTL). These antigens belong to three main groups: tumor-associated testis-specific antigens (MAGE, BAGE, GAGE and PRAME), melanocyte differentiation antigens (tyrosinase, Melan-A/MART-1, gp100, TRP-1 and TRP-2) and mutated or aberrantly expressed antigens (MUM-1, CDK4, β-catenin, gp100-in4, p15 and N-acetylglucosaminyltransferase V). For the identification of these antigens, CTL cultures from mainly only 4 different melanoma patients have been used. These patients developed a strong anti-melanoma response resulting in long-lasting disease-free periods, pointing to the importance of the identification of highly immunogenic melanomas. In each of these patients, the immune response was observed against a unique set of 4 to 6 individual antigenic epitopes, on one hand suggesting the low immunogenicity of the individual antigens, and on the other pointing to the importance of the identification of additional highly immunogenic melanomas for the discovery of new MAA. The analysis of the available data on the immunogenic and protective properties of individual MAA confirms their low immunogenicity. In our study, we focused on the identification of especially highly immunogenic melanomas among a panel of 40 newly established melanoma cell lines. So far, only two such melanoma cell lines, FM3 and FM57 have been identified in this panel. The immunogenic properties of uncloned FM3 cells and several FM3 clones have been further investigated. It was found that the immunogenic properties of melanoma cells are mainly determined by the expression of progression-associated antigens as well as by ecto-ATPase, a molecule which is able to modulate cell adhesion. Cloning the cultures of PBL, stimulated with uncloned FM3 or with the highly immunogenic FM3 clone, FM3.29, has permitted us to identify the immune

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Počas posledných rokov sa dosiahol značný pokrok v identifikácii antigénov súvisiacich s melanómom (melanoma-associated antigens) (MAA) rozpoznávaných cytotoxickými T lymfocytmi (CTL). Tieto antigény sa delia do troch hlavných skupín: semenníkový špecifický antigén produkovaný tumorom (MAGE, BAGE, GAGE a PRAME), melanocytový diferenciačný antigén (tyrozináza, Melan-A/MART-1, gp 100, TRP-1 a TRP-2) a mutované gény alebo aberantná expresia antigénov (MUM-1, CDK4, β-katenín, gp100-in4, p15 a N-acetylglukózaminyltransferáza V). Na identifikáciu týchto antigénov sa použili iba CTL kultúry od prevažne štyroch rôznych pacientov s melanómom. U týchto pacientov sa vyvinula silná antimelanómová reakcia vyúsťujúca do dlhotrvajúceho obdobia bez príznakov ochorenia, čo poukazuje na dôležitosť identifikáce vysokoimunogénnych melanómov. V každom z týchto pacientov sa imunitná odpoveď pozorovala oproti jedinečnému súboru 4—6 jednotlivých antigénovýh epitopov, čo na jednej strane naznačuje nízku imunogénnosť jednotlivých antigénov a na druhej strane poukazuje na dôležitosť identifikácie prídavných vysokoimunogénnych melanómov pri objavovaní novej MAA. Analýza dostupných dát o imunogénnych a obranných vlastnostiach individuálnych MAA protvrdzuje ich nízku imunogénnosť. V našej stúdii sme sa zamerali najmä na identifikáciu vysokoimunogénnych melanómov v rámci panelu so 40 novozaloženými melanómovými bunkovými líniami. Dosiaľ boli iba dve takéto melanómové línie FM3 a FM57 v rámci tohto panelu identifikované. Imunogénne vlastnosti neklonovaných FM3 buniek a niekoľko FM3 klonov sa ďalej vyšetrovali. Zistilo sa, že imunogénne vlastnosti melanómových buniek sú prevažne určené expresiou antigénov súvisiacich s progresom, ako aj ekto-ATPázou, molekulou, ktorá je schopná modulovať bunkovú adnéziu. Klonovanie kultúr PBL stimulovaných neklonovaným FM3 alebo s vysoko imunogénnym FM3 klonom, FM3.29 nám umožnilo identifikovať imunitnú odpoveď proti 8 rôznym MAA, 5 z nich pravdepodobne predstavuje predtým neopísané antigény. *(Tab. 2, obr. 2, lit. 68.)*

Kľúčové slová: imumogénne vlastnosti, ľudské melanómy, s melanómom súvisiace antigény, cytotoxické T-lymfocyty, ekto-ATPáza.

response against eight different MAA, five of these probably representing not previously described antigens. ***(Tab. 2, Fig. 2, Ref. 68.)***

Key words: immunogenic properties, human melanomas, melanoma-associated antigens, cytotoxic T lymphocytes, ecto-ATPase.

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During the last years significant progress have been achieved in the identification of melanoma-associated antigens (MAA) recognized by cytotoxic T lymphocytes (CTL). These antigens belong to three main groups: tumor-associated testis-specific antigens (MAGE, BAGE, GAGE and PRAME), melanocyte differentiation antigens (tyrosinase, Melan-A/MART-1, gp100, TRP-1 and TRP-2) and mutated or aberrantly expressed antigens (MUM-1, CDK4, β-catenin, gp100-in4, p15 and N-Acetylglycosaminyltransferase V). A large number of antigenic epitopes have been characterized in these antigens, opening up new possibilities for the immunotherapy of malignant melanoma as well as of some other malignancies. The first clinical trials have already been started, but still the preliminary results obtained are not too promising (1). Despite the knowledge of the number of MAA, their real immunogenicity and protective activity are not known. Therefore, the fundamental question of anti-tumor immunotherapy — which antigens should be selected for vaccination and in which form they should be used — has not been solved until now. In the present paper we will summarize the available data on the immunogenic and protective properties of MAA defined by CTL clones and will also present our new data on the identification of highly immunogenic variants of FM3 melanoma cell line.

Testis-specific antigens

This group of antigens represents proteins expressed in many tumors and among normal tissues in testes and in some cases in the placenta. The group of testis-specific antigens consists of the MAGE, BAGE, GAGE and PRAME antigens.

The MAGE genes represent a family of 12 closely related genes located on the long arm of chromosome X (2). The ability to present peptide epitopes recognized by CTL has been shown only for MAGE-1 (3) and MAGE-3 (4). Two antigenic epitopes of MAGE-1, one restricted by HLA-A1 (EADPTGHSY) (5) and another restricted by the HLA-Cw16 (epitope SAYGEPRKL) (6) have been identified. For the MAGE-3 protein, four epitopes have been identified, one recognized by HLA-A1-restricted CTL clones of patient MZ2 (EVDPIGHLY) (4) and three other — on the ability of peptides, predicted by known HLA class I binding motifs, to stimulate the formation of CTL recognizing melanoma cells: the HLA-A2-restricted epitope FLWGPRALV (7), the HLA-B44-restricted epitope MEVDPIGHLY (8) and the HLA-A24-restricted epitope IMPKAGLLI (9).

The expression of MAGE antigens has been further investigated in a number of different studies. These antigens are generally highly expressed in cutaneous melanomas (up to 65 % for MAGE-3) (2), but not in ocular melanomas (10). To a lesser extent they are expressed in other types of tumors such as mammary carcinomas, head and neck tumors, lung carcinomas, sarcomas and bladder carcinomas (for review see (11)). A high expression of MAGE-1 (80 %) was found in hepatocarcinomas (12). In contrast, myelomonocytic leukemias have been consistently negative (13, 14), and colon or renal carcinomas are rarely positive. A correlation of the expression of MAGE antigens to tumor progression has been observed in a number of malignancies (15, 16, 17, 18). Peptides from MAGE-1 and MAGE-3 have been tested for their ability to induce anti-melanoma immune responses in vivo, and limited anti-tumor activity has been shown only for the MAGE-3 peptide EVDPIGHLY (1).

BAGE codes for a putative protein of 43 aminoacids and belongs to a family of several genes (19). Its pattern of expression in tumor samples is extremely similar to the pattern of the expression of MAGE antigens with an overall lower frequency of expression (22 % in melanomas, 15 % in bladder carcinomas, 10 % in mammary carcinomas and 8 % in head and neck squamous cell carcinomas). As for the MAGE antigens, the expression of BAGE correlates with the state of tumor progression. The antigenic peptide epitope was identified as AARAVFLAL (residues 2—10). However, experiments demonstrating the protective effect of this antigen are still absent.

An additional antigen present in melanoma cells from patient MZ2 was identified as the HLA-Cw6-restricted epitope (YRPRPRRY) encoded by the GAGE-1 gene (20). This gene belongs to a family of 6 genes. The GAGE-1 sequence contains an open reading frame coding for a protein of 138 amino acids. The two genes of the GAGE family that code for the peptide, namely GAGE-1 and GAGE-2, are expressed in a significant proportion of melanomas (24 %), non-small lung cancer (19 %), head and neck tumors (19 %), and bladder tumors (12 %).

During the investigation of the immunogenic properties of melanoma cells derived from the patient LB33, a variant of a melanoma cell line with the partial loss of HLA class I expression was found to induce the generation of a HLA-A24-restricted CTL clone recognizing a new antigen called PRAME (21). An interesting feature regarding this CTL clone was that it did not recognize the original melanoma cells, in spite of the fact that these cells were positive for the expression of PRAME antigen. The recognition was observed only when the NK inhibitory receptor expressed by the CTL clone was blocked. In contrast to the MAGE genes, PRAME is also expressed in some normal tissues other than the testis. Except for the endometrium (which expresses up to 30 % of the level found in the LB33 melanoma cells), the levels of expression in normal tissues corresponds to less than 3—5 % of that found in melanoma cells (21).

From an immunological point of view, the MAGE antigens represent very good targets for immunotherapy. They are widely distributed in a number of tumors and not present in normal tissues only in testis which is not accessible to the cells of the immune system due to the lack of the direct contact of testis cells with the immune cells (22) and the lack of HLA class expression on the surface of germ cells (23) which are the only cells in the testis expressing MAGE antigens (24). The MAGE proteins are relatively large proteins with a number of potential HLA class I binding epitopes. But so far, only one patient has actually shown an immune response to these antigens (patient MZ2), suggesting an extremely low immunogenicity of MAGE antigens. A possible reason for their low immunogenicity could be their high content of regular secondary structures (25) retarding their unfolding and processing in proteasomes.

Melanocyte differentiation antigens

The recognition of normal melanocyte gene products has been demonstrated for a number of CTL lines and clones and indicates the absence of strong tolerance to this group of „self" proteins (26). Several such melanocyte differentiation antigens have been identified so far, including tyrosinase, Melan-A/MART-1, gp100, TRP-1 and TRP-2.

The first melanocyte differentiation antigen found to be recognized by tumor specific HLA-A2.1-restricted CTL was tyrosinase (27). The HLA-A24-restricted melanoma specific TIL 888 line was also shown to recognize tyrosinase (2). Tyrosinase is a 529-amino-acid melanosomal membrane protein previously shown to be required for the synthesis of melanin (29). For HLA-A2-restricted CTL, two nonapeptide epitopes were later identified (30), namely the signal sequence 1—9 (MLLAVLYCL) and the peptide 369—377 (YMNGTMSQV). The HLA-A24-restricted peptide recognized by TIL 888 could not be determined despite great efforts to achieve this (Dr. Yutaka Kawakami, personal communication), but for another HLA-A24-restricted TIL line from patient 1413, it was determined as AFLPWHRLF (31). Recently another peptide epitope, 192—200 (SEIWRDIDF) have been shown to be recognized by a HLA-B44-restricted CTL clone, established from PBL of patient MZ2 (32).

The epitope 369-377, YMNGTMSQV, predicted from the amino acid sequence of the protein, was found to be slightly different from the tyrosinase epitope YMDGTMSQV identified by mass spectroscopy of peptides eluted from the HLA-A2.1 melanoma cell line (33). This particular peptide results from posttranslational conversion of asparagine to aspartic acid. The modified peptide was recognized by the CTL clone much more efficiently than the unmodified peptide, indicating that the posttranslationally modified peptide in fact is the natural epitope.

In general, the generation of a tyrosinase-specific response in melanoma patients is a relatively infrequent event and is usually seen only in patients showing a high response against several melanoma-associated antigens (as exemplified by patients MZ2, 888 and SK29 (AV)). The only data indicating its possible involvement in tumor rejection is the complete rejection of multiple melanoma metastases in patient 888 upon injection of TIL established from resected melanoma (28), but the same TIL recognize several other antigens. Therefore, antigens other than tyrosinase could constitute major rejection antigens which could mediate tumor rejection caused by TIL 888.

Another melanocyte differentiation antigen, Melan-A/MART-1, was identified independently by two different groups (34, 35). This is a small transmembrane protein consisting of 118 amino acid residues widely distributed in melanomas, but absent in other tumors. Only HLA-A2-restricted CTLs have been demonstrated so far and the 9-mer immunodominant peptide 27—35 with the sequence AAGIGILTV was identified and was shown to be recognized by all TIL cultures reacting against Melan-A/MART-1 (36). Another epitope, 32—40 (ILTVILGVL), was identified by sequencing of natural processed peptide from melanoma cells (37). The peptide 27—35 probably represents the immunodominant peptide of Melan-A/MART-1 because only this peptide was able to induce the generation of melanoma-specific CTL upon immunization in vitro of PBL from melanoma patients (38). It is of interest to note that other Melan-A/MART-1 peptides having higher affinity to HLA-A2.1 antigen did not induce the generation of melanoma-specific CTL (39), pointing to the existence of predominantly intermediate and low affinity T cell receptors which recognize „self" antigens. The relative immunogenicity of Melan-A/MART-1 is thought to be the highest among the melanocyte-specific differentiation antigens, and CTL recognizing this protein are easily induced after stimulation of PBLs with peptides or allogeneic melanoma cells (38, 40) or expansion of TILs isolated from melanoma tumors (36, 41). On the other hand, the role of this antigen in the generation of protective immunity is probably not highly significant, because the ability of different TILs to induce tumor rejection did not correlate with the recognition of Melan-A/MART-1 (42) and the immunization of the melanoma patients with immunodominant peptide 27—35, increasing the frequency of Melan-A/MART-1-specific CTL, did not induce tumor regression (43).

The gene encoding gp100 was originally identified as a melanocyte lineage-specific antigen recognized by the antibodies NKI-beteb, HMB-50 and HMB-45, which are used as diagnostic markers for human melanoma (44). Using TIL 1200, which induced complete regression of multiple melanoma metastases, the antigen recognized by these TILs was identified and found to be identical to the melanocyte differentiation antigen gp100 (45. 46) and the 661-aminoacid gp100 glycoprotein appears to contain a signal peptide as well as a single transmembrane domain.

Five different peptide epitopes, all recognized by CTL in a HLA-A2-restricted manner, have been identified (42, 46). Some CTL lines were found to recognize multiple gp100 epitopes (42). The peptide epitope 280—288 (YLEPGPVTA), which was recognized by 6 of 8 different gp100-reactive TIL derived from different patients (42) was also isolated from the HLA-A2 molecule on melanoma cells and was reported to be recognized by 5/5 CTL raised from PBLs (47). Melanoma-reactive CTL could be induced by in vitro stimulation with these gp100 peptides, but not as efficiently as with the MART-1 peptide 27—35 (48). Recently an epitope of gp100 (amino acids 17—25 (ALLAVGATK)) recognized by a HLA-A3-restricted melanoma-specific CTL have been identified (49).

A significant correlation between T cell recognition in vitro and tumor regression in patients receiving TIL therapy has been demonstrated for gp100 (42), suggesting that this protein may be a potent tumor regression antigen. However, as for tyrosinase, the same TIL cultures recognized other antigens which could induce regression, either by themself or in combination with the CTL clones recognizing gp100. Nevertheless, this antigen until now probably represents the most promising HLA-A2-restricted differentiation antigen yet described for immunotherapeutic applications.

Two other proteins which are involved in the biosynthesis of melanin have been shown to contain epitopes recognized by CTL. The tyrosinase-related protein-1 (TRP-1), or gp75, is responsible for the formation of a CTL epitope recognized in HLA-A31-restricted manner (50). Attempts to identify a peptide epitope resulted in the identification of an epitope which was not in the protein sequence of TRP-1 itself but the product of an alternative reading frame (ORF3) of TRP-1 (51). The identified peptide has the sequence MSLQRQFLR. The same HLA-A31-restricted TIL culture was used for the identification of another antigen — TRP-2 (52). The peptide epitope of TRP-2 was identified as LLPGGRPYR.

The infusion of TIL 586, predominantly recognizing TRP-1 along with into the same melanoma patient resulted in the objective regression of the tumor (53), indicating that both of these two antigens serve as regression antigens in HLA-A31-positive patients.

It should be expected, that the immunogenicity of melanocyte differentiation antigens is low, which can be explained by immunological tolerance against these potential highly immunogenic epitopes of these „self" proteins. Indeed, the majority of peptide epitopes have low or intermediate affinity for HLA class I antigens and the corresponding peptide therefore can activate peptide-dependent lysis of CTL clones only at relatively high concentrations (30, 54).

Mutated or aberrantly expressed melanoma-specific antigens

The presence of mutated melanoma-associated antigens, leading to the generation of unique antigenic determinants, is probably not a rare event and the frequency of CTL clones recognizing unique antigens could be significant (55). It is interesting to note that for the majority of highly immunogenic melanomas, the recognition of such unique antigens have been demonstrated (Tab. 1). These antigens are usually present in primary tumors and could be significant for the generation and/or progression of tumors, which makes it important to characterize these antigens not only in connection with immunotherapy but also for understanding their involvement in the molecular mechanisms of malignant transformation. With respect to melanoma, a total of 6 characterized antigens belong to this group.

MUM-1 (standing for melanoma ubiquitous mutated) antigen was identified as an HLA-B44-restricted antigen present on melanoma cell line LB33-MEL (56). It is encoded by a gene that is present in many normal tissues. The sequence coding for the antigenic peptide is located across an exon-intron junction. A point mutation that generates the antigenic epitope is located in the intron region. The function of this protein is not yet known. It is interesting to note that more than one third of the CTL clones established against the melanoma cell line LB33-MEL. A were in fact directed against this antigen (57) indicating the high immunogenicity of this mutated epitope.

The first unique antigen identified having a well known biological function was mutated CDK4 (58), a key protein involved in the regulation of cell cycle progression as a part of the CDK-4-p16-Rb pathway, which has been shown to be often inactivated in human melanomas (59). This antigen was identified using three HLA-A2-restricted CTL clones obtained from the PBL of patient SK29. The mutation, an arginine-to-cystein exchange at residue 24, is part of the CDK4 peptide recognized by CTL. The exact naturally processed epitope recognized by these CTL clones was not identified, but it probably is the 10-mer ACDPHSGHFV, being recognized by a CTL clone 1000 times more efficiently than the parent epitope ARDPHSGHFV, while the 11-mers KACDPHSGHFV and KARDPHSGHFV were recognized with an equally high efficiency. The mutation was demonstrated to have the inactivating effect on the normal function of CDK4 protein (58, 60), thus playing an important role in the malignant transformation of melanoma line SK29(AV) (60).

A product of the mutated β-catenin gene was shown to be recognized by TIL 1290 which was derived from a recurrent tumor in the melanoma patient 888 (61). As for tyrosinase, previously identified with TIL 888, this recognition was restricted by HLA-A24. The β-catenin peptide SYLDSGIHF was found to induce half-maximal lysis of target cells at a concentration of 1 pM, whereas a 1-μM concentration of the normal peptide (SYLDSGIHS) was required to obtain an equivalent level of sensitization. The original 888 melanoma cell line as well as the 1290 melanoma cell line derived from the recurrent tumor both appeared to express the mutated and the normal β-catenin gene product.

In addition to β-catenin, TIL 1290 was previously found to recognize p15, a protein with unknown function, which is responsible for a CTL epitope only in melanoma cells but not in normal cells (62). The peptide epitope was identified as AYGLDFYIL. Northern blot analysis indicated that this gene was expressed in all of the normal tissues examined. The mechanism responsible for the selective expression of this protein in tumor cells has not been identified, but it has been suggested that posttranslational regulation of gene expression may be involved. TIL 1290 recognized other A24-positive melanomas but not normal HLA-A24-positive melanocytes. This suggests that p15 could be a shared tumor-specific antigen present on several HLA-A24-positive melanomas, but this suggestion should be made with caution because of the possibility that TIL 1290 may also recognize other antigens.

In addition to the MUM-1 epitope, which spans an intron-exon boundary, a novel product of the N-acetylglucosaminyltransferase V (GnT-V) gene, transcribed from the 3' end of one of the GnT-V introns, was shown to encode a T cell epitope recognized by HLA-A2-restricted melanoma-reactive T cells (63). The antigenic epitope was identified as the decapeptide VLPDVFIRCV or the nonapeptide VLPDVFIRC. The results of anchored PCR amplification suggest that messengers coding for the antigenic peptide are generated by the activation in melanoma cells of a promoter located in this intron. In contrast to the fully spliced GnT-V mRNA, which was found in a wide range of normal and tumor tissues, the mRNA containing the intron region, was found at insignificant levels in normal tissues including normal melanocytes. The corresponding mRNA was observed to be present in about 50 % of melanomas.

The HLA-A24-restricted TIL 1290 was recently found to recognize one additional antigen, a variant of the gp100 gene that had retained the entire fourth intron of this gene, termed gp100-in4 (64). The gp100-in4 transcript could be detected by reverse transcriptase-PCR but could not be detected in Northern blots conducted with melanoma RNA, indicating that it represents a relatively rare transcript. Read-through of this transcript into the region corresponding to the fourth intron gave rise to an additional 35 amino acids not found in the normal gp100 protein, and a peptide within this region (VYFFLPDHL) was shown to be recognized by a T cell subline isolated from TIL 1290. HLA-A24-matched allogeneic melanoma cell lines and melanocytes were found to be recognized by the T cell line, demonstrating that this represents a nonmutated epitope.

The selection of highly immunogenic melanoma variants

In our own experiments we concentrated on the selection of highly immunogenic melanomas from the collection of more than 40 melanoma cell line established in our laboratory from several

Tab. 1. Antigenic epitopes of melanoma-associated antigens recognized by CTL.
Tab. 1. Antigénne epitopy antigénov súvisiacich s melanómom rozpoznávané cytotoxickými T-lymfocytmi.

Antigen Antigén	HLA class I HLA trieda I	Epitope Epitop	Sequence Sekvencia
Testis-specific antigens *Semenníkové špecifické antigény*			
MAGE-1	A1	161-169	EADPTGHSY
	Cw16	230-238	SAYGEPRKL
MAGE-3	A1	168-176	EVDPIGHLY
	A2	271-279[a)]	FLWGPRALV
	B44	167-176[a)]	MEVDPIGHLY
	A24	195-203[a)]	IMPKAGLLI
BAGE	Cw16	2-10	AARAVFLAL
GAGE-1	Cw6	9-16	YRPRPRRY
PRAME	A24	301-309	LYVDSLFFL
Melanocyte differentiation antigens *Melanocytové diferenciačné antigény*			
gp100	A2	154-162	KTWGQYWQV
	A2	209-217	ITDQVPFSV
	A2	280-288	YLEPGPVTA
	A2	457-466	LLDGTATLRL
	A2	476-485	VLYRYGSFSV
	A3	17-25	ALLAVGATK
MART-1	A2	27-35	AAGIGILTV
	A2	32-40	ILTVILGVL
Tyrosinase	A2	1-9	MLLAVLYCL
	A2	369-377	YMNGTMSQV
			YMDGTMSQV[b)]
	A24	206-214	AFLPWHRLF
	B44	192-200	SEIWRDIDF
TRP-1/gp75	A31	1-9[c)]	MSLQRQFLR[c)]
TRP-2	A31	197-205	LLPGGRPYR
Mutated and aberrantly expressed antigens *Mutované a aberantne exprimované antigény*			
MUM-1[d)]	B44	nt782-808	EEKLIVVLF
β-Catenin	A24	29-37	SYLDSGIHF
P15	A24		AYGLDFYIL
CDK4	A2		ACDPHSGHFV
GnT-V[e)]	A2		VLPDVFIRCV
gp100-in4	A24	170-178	VYFFLPDHL

[a)] Predicted epitopes, for which induced CTL can kill tumor cells.
Predpovedané epitopy, proti ktorým indukované CTL môžu usmrtiť nádorové bunky.
[b)] Natural peptide identified by elution from HLA-A2.
Prirodzené peptidy identifikované vyluhovaním z HLA-A2.
[c)] Antigenic peptide resulted from translation of an alternative open reading frame (ORF3) of thr gp75 gene.
Antigénny peptid vzniknutý transláciou alternatívneho otvoreného čítacieho rámca (ORF3) génu thr gp75.
[d)] Mutation occurs in an intron sequence followed by stop codon.
Mutácia sa vyskytuje v intrónovej sekvencii nasledovanej stop kodónom.
[e)] N-acetylglucosaminyltransferase V. Peptide is encoded by an intron sequence.
N-acetylglukózaminyltransferáza V. Peptid je kódovaný intrónovou sekvenciou.

melanoma patients. Comparison of their immunogenic properties in mixed lymphocyte tumor cultures have shown that only few melanoma cell lines are able to induce a strong CTL response in syngeneic mixed lymphocyte-tumor cell cultures (MLTC) (65). The most immunogenic cell line, FM3 have been used for the production of melanoma-specific CTL clones which recognized four groups of probably new melanoma-associated antigens. Preliminary data indicate that FM3 is still the most immunogenic cell line in our collection, but some other cell lines could also induce a strong and specific immune response, for example, FM57.

According to the expression on normal melanocytes and changes in the expression during tumor progression, the antigens identified with the FM3 melanoma cell line were subdivided into two groups — differentiation and progression-associated antigens. An important observation concerning these two groups of antigens was the discovery of different types of regulation of their expression by the interferon-γ (IFN-γ) — down-regulation of differentiation-associated antigens and up-regulation of progression-associated antigens (66). This observation suggests the necessity of the induction of the immune response against both these groups of melanoma-specific antigens in order to avoid the induction of the immunological escape due to the selective loss of certain tumor-associated antigens. The immunogenic capacities of the differentiation-associated and progression-associated antigens have also been compared on different melanoma clones with varying levels of the expression of these antigens. The most immunogenic melanoma cell clone (the clone FM3.29) was found to express the largest amounts of progression-associated antigens indicating their pivotal role in the overall immunogenicity of melanoma cells. The cloning of CTL lines established by immunization of syngeneic PBL with this melanoma cell clone resulted in the generation of significantly higher proportion of the cytotoxic clones than by the immunization with the unselected melanoma cell line (Fig. 1). The primary screening of the specificity of the resulting CTL clones indicates the recognition of at least six different antigens (with uncloned FM3 it was only four), four of them were not identified upon previous screening of CTL clones against uncloned FM3 and was found to be three different epitopes of gp100 (epitopes 154—162, 280—288 and an unidentified, probably new epitope) and epitope 27—35 of MART-1. It is interesting, that immune response against epitope 154—162 of gp100 has been previously observed only in TIL cultures, but or in PBL, stimulated with tumor cells. Totally, immune response against 8 different antigens has been observed in stimulated cultures of PBL from patient MAVN, indicating the significance of the immune response to several melanoma epitopes in creating strong protective immunity. This patient is free of disease more than five years after operation.

The role of ecto-ATPase in the control of the interaction of CTL with tumor cells

The fact that the recognition of FM3 melanoma cells by some melanoma-specific CTL clones increased with tumor progression (67) could indicate a higher efficiency of escape at the early stages of tumor progression. In search of such mechanisms, it was found that ecto-ATPase activity being rather low in normal melanocytes is significantly elevated in a highly differentiated melanoma subline and is decreased with tumor progression. Thus, ecto-ATPase activi-

Tab. 2. Melanoma-associated antigens identified using highly immunogenic melanoma.
Tab. 2. Antigény súvisiace s melanómom identifikované pomocou vysokoimunogénnych melanómových buniek.

Patient	Source of CTL	Number of antigens identified by CTL	Identified antigens
Pacient	Zdroj CTL	Počet antigénov identifikovaných pomocou CTL	Identifikované antigény
MZ2	PBL, stimulated with tumor cells PBL, stimulované nádorovými bunkami	6	MAGE-1, MAGE-3, BAGE GAGE, Tyrosinase
SK29(AV)	PBL, stimulated with tumor cells PBL, stimulované nádorovými bunkami	6	Tyrosinase, Melan-A, CDK4
LB33	PBL, stimulated with tumor cells PBL, stimulované nádorovými bunkami	6	MUM-1, PRAME
888	TIL	4	Tyrosinase, p15, β-catenin, gp100-in4
MAVN	PBL, stimulated with tumor cells PBL, stimulované nádorovými bunkami	8	gp100, MART-1

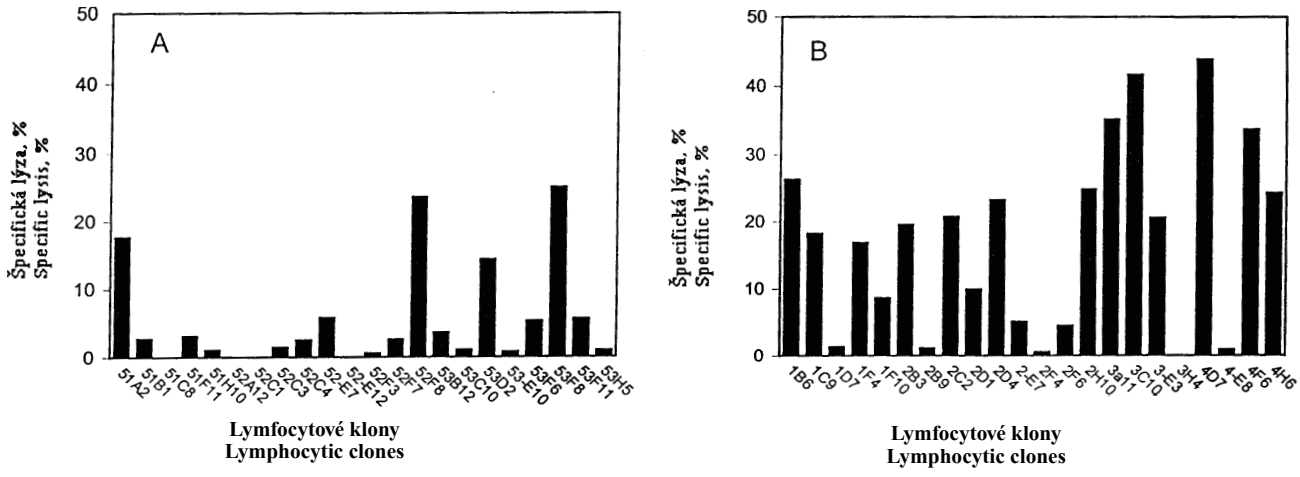

Fig. 1. Primary screening of the cytolytic activity of growing lymphocyte clones in cloned cultures of CTL lines raised against the uncloned FM3 line (A) or the preselected clone FM3.29 (B).
Obr. 1. Primárny skríning cytolytickej aktivity narastajúcich lympfocytových klonov v klonovaných kutúrach CTL línií proti neklonovaným FM3 líniám (A) alebo preselektovaným klonom FM3.29 (B).

ty appeared to be inversely related to the recognition by CTL. CD39, a marker described originally in activated lymphoid cells and recently shown to be one of ATP- and ADP-hydrolases, showed the same pattern of expression as ecto-ATPase, indicating that CD39 is the major ecto-ATPase in melanomas (Fig. 2). Involvement of CD39 in cell adhesive interaction has been proposed before (68). Using the EBV-transformed B cell line JY, which usually grows as a mixture of aggregates and single cells, it was found that single cells have a higher ATPase activity and that extracellular ATP, but not AMP+P_i stimulates de-adhesion. Since the same protein, CD39, is responsible for ATP hydrolysis in both EBV-B cells and melanomas, these results suggest that the elevated levels of ecto-ATPase in

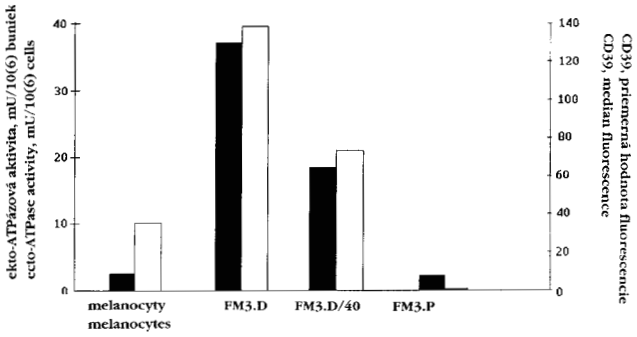

Fig. 2. Ecto-ATPase activity and CD39 expression of melanocytes and FM3 sublines differing in the state of progression. FM3.D — differentiated variant of FM3 isolated at early passages and showing high expression of differentiation markers; FM3.D/40 — the same cell line after passages40; FM3.P — FM3 cells after prolonged cultivation in vitro and having a low expression of differentiation markers. The properties of the FM3.D and FM3.P cell lines have been described earlier [66].
Obr. 2. Ekto-ATPázová aktivita a CD39 expresia melanocytov a FM3 sublínií odlišujúcich sa stavom progresie. FM3.D — diferencovaný variant FM3 izolovaný v skorých pasážach a preukazujúcich vysokú expresiu diferenciačných markerov, FM3.D/40 — tá istá línia po 40 pasážach, FM3.P — FM3 bunky po dlhotrvajúcej kultivácii in vitro a majúce nízku expresiu diferenciačných markerov. Vlastnosti FM3.D a FMR.P bunkových línií boli už opísané (66).

highly differentiated melanomas may represent an escape mechanism enabling target tumor cells by inducing de-adhesion to escape recognition by T cells at the early steps of tumor formation.

Concluding remarks

Tumor cells express a number of potentially immunogenic tumor-associated antigens, but the overall immunogenicity of tumor cells is nevertheless generally low, probably because of the lack of some other necessary attributes which are characteristic only for professional antigen-presenting cells such as dendritic cells. Therefore the success of the immunotherapy depends on the identification of several potentially immunogenic tumor-associated antigens, the selection of the appropriate combination for the inclusion in vaccine and the development of the effective immunization protocols. The analysis of the immunogenic properties of the so far identified melanoma-associated antigens indicates that only a few such antigens really are highly immunogenic, emphasizing the need of the identification of new tumor-associated antigens. It is important to note that in the majority of cases where the molecular characterization of melanoma-associated antigens has been achieved the melanoma cell lines were capable to induce the immune response against unique set of several antigens (Tab. 2). For this reason there is the hope, that the identification of new highly immunogenic melanomas will permit the characterization of new melanoma-associated antigens. Therefore we have concentrated on such identification of highly immunogenic melanomas and further selection of highly immunogenic clones from such melanomas. We have already identified 2 highly immunogenic melanoma cells lines, and for one of them, FM3, immunogenic clone have been isolated. The PBLs primed with this immunogenic clone were cloned and it was found that the majority of proliferating clones specifically recognized melanoma cells. These CTL clones, in addition to the previously described four new antigens (65), also recognized several epitopes of gp100 (including new, not previously characterized epitopes), a dominant immunogenic differentiation antigen, capable to induce protective immune response (42), as well as MART-1.

Our very recent results have pointed out that the ecto-ATPase might be an important factor in modulating the adhesion between tumor target cells and the corresponding immune effector cells. The investigation of ecto-ATPase activity as a factor in the immunological escape developed on the melanoma cell surface and the ways to overcome this will represent another complementary approach for the optimization of immunotherapy.

The identification of immunogenic protective tumor antigens, development of the effective methods of immunization and inactivation of the escape mechanisms might be the basis for development of future more effective methods of immunotherapy.*

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PREDSTAVUJEME NOVÉ KNIHY

Sedláčková E., Tichý M.: Generál MUDr. Ján Paškan (1907—1989). Zakladateľ vojenského zdravotníctva na Slovensku. Bratislava, JUGA 1997.

Knihu vďaky s úvodným príhovorom syna—lekára chirurga doc. MUDr. Jána Paškana, CSc., prečítate na jeden dúšok. Dozviete sa v nej o detstve a štúdiách, o vojnových rokoch krutosti, o organizácii zdravotnej služby v SNP, o hlade, biede, neopísateľnej únave po čistočnom potlačení SNP za krutej zimy v horách. Na druhej strane sa dozviete o odhodlanosti, ochote pomôcť, čo nepoznala hraníc.

Kto bol generál Paškan?

Človek, ktorý sa tešil vzácnej obľube každého, kto ho bližšie poznal a stretol sa s ním. Sám mám naň spomienky najvzácnejšie, pretože mi zachránil život v pohnutých časoch SNP, keď stál na čele zdravotnej služby.

Hneď v prvých dňoch povstania presťahoval ružomberskú vojenskú nemocnicu, v ktorej bol veliteľom, do Korytnice. Tu pracovala nemocnica v bojových podmienkach ako poľná vojenská nemocnica pod vedením MUDr. Bohuša Štekláča.

V Korytnici bola kúpeľná prevádzka len v troch letných mesiacoch a pracoval som tam v lete už piaty rok ako pridelený kúpeľný lekár. Opis ďalších udalostí súvisiacich so záchranou môjho života MUDr. Paškanom preberám z mojej knihy *Spomienky a vyznania lekára:* "Asi týždeň po oficiálnom vyhlásení SNP sme išli sanitkou do nemocnice v Banskej Bystrici pre chirurgický materiál. Šofér, traja lekári a vtedajší šéf vojenského zdravotíctva MUDr. Paškan, domnievam sa, že mal vtedy hodnosť podplukovníka. Po ceste už blízko Banskej Bystrice nás pristavili dvaja sovietski partizáni so samopalmi a legitimovali nás. Mali sme riadny vysielací rozkaz, všetko bolo v poriadnu, len mne kázali vystúpiť, ostatní môžu pokračovať v ceste. Bol som v civile, ostatní vo vojenských uniformách, príčinou, prečo ma chceli zadržať, bolo miesto môjho narodenia — Budapešť. Jeden z partizánov, ktorý vedel čítať latinku, sa zúčastnil na bojoch o Budapešť a mal na ne zlé spomienky. Tvrdil, že som špión z Maďarska. Bol som mu podozrivý najmä preto, že som bol v civile. Zastali sa ma solidárne všetci spolucestujúci a náčelník zdravotnej služby povstaleckej armády Paškan vyhlásil, že bezo mňa nepôjdu ďalej, vrátia sa, ale následky si ponesú obaja partizáni. Nakoniec nás pustili ďalej všetkých, bol som im vďačný za záchranu." Vtedy sa totiž neuvážňovalo, nesúdilo, ale strieľalo.

Kniha o generálovi Paškanovi podáva obraz o človeku, ktorého dominantou života bola činorodá práca, humánnosť, čestnosť, priateľský vzťah ku kolegom, čím sa zapísal nielen do histórie zdravotníctva, ale aj národa.

Týmto príspevkom chcem ešte raz poďakovať generálovi Paškanovi za záchranu života.

I. Sečanský