

# Tumor Antigens Discovery: Perspectives for Cancer Therapy

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## ABSTRACT

The adoptive transfer of cytotoxic T lymphocytes (CTLs) derived from tumor-infiltrating lymphocytes (TIL) along with interleukin 2 (IL-2) into autologous patients with cancer resulted in the objective regression of tumor, indicating that these CTLs recognized cancer rejection antigens on tumor cells. To understand the molecular basis of T cell-mediated antitumor immunity, several groups started to search for such tumor antigens in melanoma as well as in other types of cancers. This led to the subject I will review in this article. A number of tumor antigens were isolated by the use of cDNA expression systems and biochemical approaches. These tumor anti-

gens could be classified into several categories: tissue-specific differentiation antigens, tumor-specific shared antigens, and tumor-specific unique antigens. However, the majority of tumor antigens identified to date are nonmutated, self proteins. This raises important questions regarding the mechanism of antitumor activity and autoimmune disease. The identification of human tumor rejection antigens provides new opportunities for the development of therapeutic strategies against cancer. This review will summarize the current status and progress toward identifying human tumor antigens and their potential applications to cancer treatment.

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## INTRODUCTION

The immune system plays a critical role in immunosurveillance against cancer and in tumor regression. The importance of T cells in the regression of tumors has been demonstrated in experimental animal tumor models (1-3). Adoptive transfer of tumor-infiltrating lymphocytes (TIL) along with interleukin-2 (IL-2) into the autologous patients resulted in the objective regression of tumor (4,5). To further understand how T cells mediate antitumor responses and what molecules are recognized by T cells on tumor cells, a great deal of effort has been made to identify tumor antigens recognized by cytotoxic T lymphocytes (CTLs) with antitumor reactivity *in vivo* and *in vitro*. This has resulted in the molecular identification of a number of tumor

antigens in the past few years. These studies have provided the basis for understanding T cell-mediated antitumor activity, and a rationale for the development of anticancer vaccines. Clinical trials using peptides derived from the identified tumor antigens have shown promising results in the treatment of patients with melanoma. Recombinant viruses expressing tumor antigens combined with cytokines and costimulatory molecules are being evaluated for the potential therapeutic effect.

## APPROACHES TO IDENTIFICATION OF HUMAN TUMOR ANTIGENS

### Genetic Approach

The first approach is to transfect recombinant genomic DNA or cDNA libraries into cells expressing the appropriate MHC molecule. This

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system was recently improved by the use of cDNA libraries that are transfected transiently into COS or 293 cells along with a cDNA encoding the appropriate MHC molecule (6,7). Positive clones are identified on the basis of the ability to stimulate cytokine release from CTL. This simple and powerful approach has led to the identification of many genes encoding tumor antigens (7–16). Major drawbacks of this approach include the possibility of identifying cDNA clones that encode cross-reacting peptides recognized by T cells due to a high expression level in COS system, and the need to identify the antigenic peptides from the amino acid sequence of the antigenic proteins.

### Peptide Isolation

T cells recognize a peptide bound to the MHC class I molecule. Tumor-specific peptides can be eluted with acid from either the tumor cell surface or purified peptide–MHC complexes, and they can be separated subsequently by high-pressure liquid chromatography (HPLC). The eluted peptide fractions are then tested for their ability to stimulate cytokine secretion from CTL when pulsed onto MHC-matched antigen-presenting cells (APC). If the amount of peptide in the positive fraction is sufficient to allow sequence determination by Edman degradation, a naturally processed, tumor-specific peptide recognized by CTL can be directly identified (17). The peptide sequence can then be used to search databases to find the gene encoding the antigenic peptide. Drawbacks to this approach include the technical complexities associated with fractionation of the active peptides and the use of instruments such as tandem mass spectrometry.

It is worth noting that synthetic peptide libraries based on the peptide binding motif of a particular MHC molecule have been used for identification of an antigenic peptide recognized by CTL (18). However, the identified peptide may be a cross-reactive peptide, rather than an authentic peptide derived from a tumor antigen.

### “Reverse Immunology” Approach

In some cases, no tumor-reactive CTL exists. For example, it is relatively difficult to generate breast tumor-reactive CTL from a patient, but there is a putative tumor antigen whose expression is known to be associated with tumor (19,20). To prove that this is a true tumor antigen, it is necessary to generate CTLs in vitro

using peptides derived from a putative tumor antigen (19–23). However, in many cases, these CTLs do not recognize the tumor cells because of the T cell receptors (TCR) with low affinity to the peptides. Alternatively, the peptide recognized by CTLs in in vitro systems may not be naturally processed and presented on the tumor cell surface. Although significant progress has been made in understanding the rules of peptide-binding motifs for different MHC molecules (24), the predicted peptides that contain the MHC-binding motif may not, however, be naturally processed and presented on tumor cells at sufficient levels to be recognized by T cells. Use of antigen-presenting cells pulsed with peptide or protein, or transfected with a cDNA encoding the putative tumor antigen as stimulators, should be subjected to further investigation.

## TUMOR ANTIGENS RECOGNIZED BY T CELLS

### Tissue-Specific Shared Differentiation Antigens

Five tissue-specific shared tumor antigens including tyrosinase, MART-1/Melan-A, gp100, TRP-1, and TRP-2 have been identified by TIL which induced tumor regression when administered to autologous patients along with IL-2 (Table 1). Thus, they may represent tumor rejection antigens. These antigens were expressed in melanoma, normal melanocytes, and retina, but not in other normal human tissues.

*TYROSINASE.* Tyrosinase is the first member of differentiation antigens identified and is an important enzyme involved in the synthesis of melanin (6,25). Two distinct epitopes, MLLAVLYLL and YMNGTMSQV, were identified from the tyrosinase molecule and recognized by CTLs in the context of HLA-A2 (26). The tyrosinase antigen was also shown to be recognized by an HLA-A24-restricted TIL888 that had been previously shown to result in the regression of multiple metastatic lesions when adoptively transferred into the autologous patient along with IL-2 (12). In addition, the peptide SEIWRDIDF derived from the tyrosinase protein was recognized by HLA-B44-restricted T cells (27). Interestingly, a naturally processed HLA-A2-associated peptide YMDGTMSQV was identified by peptide elution and mass spectrometry. This peptide results from the post-translational conversion of asparagine

TABLE 1. Tissue-specific differentiation antigens

Antigens	HLA restrictions	Peptides	Comments
Tyrosinase	HLA-A2	MLLAVLYCL	
	HLA-A2	YMNGTMSQV	
	HLA-B44	SEIWRDIDF	
	HLA-A24	AFLPWHRLF	
	HLA-DR4	QNILLSNAPLGPQFP	
	HLA-DR4	SYLQDSDPDSFQD	
MART-1/Melan-A gp100	HLA-A2	AAGIGILTV	
	HLA-A2	KTWGQYWQV	
	HLA-A2	ITDQVPFSV	
	HLA-A2	YLEPGPVTA	
	HLA-A2	LLDGTATLRL	
	HLA-A2	VLYRYGSFSV	
	HLA-A3	ALLAVGATK	
	HLA-A2	VYFFLPDHL	Epitope generated from an intron of an incomplete splicing RNA
gp75/TRP-1	HLA-A31	MSLQRQFLR	Epitope was derived from an alternative ORF
TRP-2	HLA-A31	LLGPRPYR	Mouse TRP-2 was identified as a tumor antigen for B16 melanoma

to aspartic acid (28). These results indicate that multiple epitopes and modified peptides can be generated from the same molecule and be presented to T cells by different MHC class I-restricted molecules.

While the role of CD8<sup>+</sup> T cells in tumor rejection has been well documented, CD4<sup>+</sup> T cells may also play an important role in antitumor immune responses. First, many melanoma cell lines express MHC class II molecules and are capable of presenting the antigenic peptides to CD4<sup>+</sup> T cells. Second, CD4<sup>+</sup> TIL derived from both melanoma and breast cancers have been shown to respond to the autologous tumor cells (29,30). For example, it was shown that the CD4<sup>+</sup> melanoma-reactive TIL1088 recognized antigenic peptides derived from the tyrosinase protein in an HLA-DR4-restricted fashion (31,32). Two distinct peptides, QNILLSNAPLGPQFP and DYSYLQSDPDSFQD, were identified to be recognized by the CD4<sup>+</sup> TIL1088 (33). Therefore, the tyrosinase gene product contains multiple CD8<sup>+</sup> T cell epitopes as well as CD4<sup>+</sup> T cell epitopes. The existence of other MHC class II-restricted tumor antigens from melanoma and breast cancers was recently reported (29,30,34). Studies directed toward identifying MHC class II-restricted tumor antigens are currently in progress.

*MART-1/MELAN-A.* The MART-1 gene was isolated by screening of a melanoma cDNA library with the HLA-A2-restricted melanoma-reactive TIL 1235 (35). MART-1 is identical to Melan-A independently cloned by another group (7). The MART-1 cDNA isolated from melanoma cells did not contain any mutation or other alterations in the coding region. Northern blot analysis indicated that MART-1 was expressed only in melanomas, melanocytes, and retina, but not in normal human tissues (35). The MART-1 gene product was found to be an immunodominant melanoma antigen recognized by the majority of HLA-A2-restricted melanoma-reactive CTL established from TIL in the Surgery Branch, NCI (35,36), as well as a large percentage of melanoma reactive clones derived from the peripheral blood lymphocytes (PBL) of HLA-A2<sup>+</sup> melanoma patients (37). One 9-mer peptide, AAGIGELTV (M9-27), was found to be recognized by HLA-A2-restricted melanoma-specific CTL (36). Although MART-1 is a dominant antigen recognized by many HLA-A2-restricted TIL, no correlation was found between T cell recognition of MART-1 and clinical response. In contrast, a strong correlation was found between the T cell recognition of gp100 and clinical response (38).

Some TILs recognize both MART-1 and gp100 (38).

*GP 100.* A cDNA clone encoding gp100 was isolated and shown to be almost identical to Pmel17, which had previously been cloned using anti-melanoma antibodies (39–41). Northern blot analysis demonstrated that gp100 was expressed in neonatal cultured melanocyte lines, most melanoma cell lines, and retina, but not in other normal tissues (40). The gp100 molecule was found to be recognized by 8 out of 21 HLA-A2-restricted melanoma-reactive TIL established from different patients with melanoma in the Surgery Branch, NCI, and appears to represent a highly immunogenic antigen. Some CTL lines can recognize multiple gp100 epitopes. Many gp100 epitopes have been identified by CTLs derived from different patients (38,40,42). Recently, a HLA-A3-restricted epitope was identified from the normal coding sequence of gp100 (43).

One of the gp100 epitopes, YLEPGPVTA, was independently isolated from an HPLC-purified fraction of peptides obtained from HLA-A2-positive melanoma (44). This observation validates both genetic and biochemical approaches to the identification of naturally processed peptides on tumor cells.

*TRP-1/GP75.* While all antigenic peptides discussed above were identified from the normal open reading frame, T cells are also capable of recognizing antigenic peptides from an alternative open reading frame or an intron of an mRNA transcript (Table 1). Several examples have recently been reported.

Previous studies showed that TIL586 derived from patient 586 recognized one or more antigens in the context of HLA-A31 (45). A tumor antigen encoded by TRP-1/gp75 was isolated following cDNA library screening. Interestingly, gp75 was previously reported to be recognized by IgG antibodies in the serum of a patient with melanoma (46). Northern blot and protein analysis indicated that the gp75 protein was one of the most abundant intracellular glycoproteins in melanocyte-lineage cells, but it was not detected in non-melanocytic cell types (45,47,48). The gp75 molecule has recently been shown to have DHI-2-carboxylic acid oxidase activity involved in the synthesis of melanin (49). To identify the epitope in gp75, DNA deletion constructs were made to localize an antigenic peptide to a small DNA fragment. A number of peptides were syn-

thesized on the basis of the predicted amino acid sequence of gp75 and the peptide binding motif for HLA-A31 (50). None of the peptides tested were found to stimulate cytokine release from TIL586 when pulsed onto HLA-A31-positive EBV-transformed B cells (51). However, two ATG start codons were present in different open reading frames relative to the normal gp75 ORF1 reading frame (Fig. 1). To explore this possibility, three peptides derived from ORF2 and two peptides from ORF3 were selected and synthesized on the basis of the HLA-A31 binding motif. Surprisingly, the peptide MSLQRQFLR, which was derived from ORF3, was capable of stimulating cytokine release from TIL586 when pulsed onto HLA-A31-positive EBV-transformed B cells (Fig. 2) (51). This represents one of the first examples of two overlapping open reading frames being used to translate two distinct polypeptides from a single eukaryotic cellular mRNA, although translation of overlapping reading frames has been reported in viral mRNAs (52–54). These results demonstrated a novel mechanism by which a human tumor antigen can be generated from an alternative open reading frame and presented to T cells by an MHC class I molecule.

Several related examples of the use of alternative open reading frames have also been reported recently. p16<sup>INK4a</sup> was demonstrated to encode an inhibitor of the cyclin D-dependent kinases CDK4 and CDK6 (55). p16 probably acts as a tumor suppressor since deletions and mutations of this gene were found in a variety of tumors (56). Interestingly, a second gene product was recently identified from an alternative open reading frame of the mouse p16<sup>INK4a</sup> gene to encode a 19 kDa (p19ARF) protein (57). The ectopic expression of p19ARF in fibroblasts was found to induce G1 and G2 phase arrest. Therefore, the p16 gene encodes two dissimilar proteins, both of which may be required for cell cycle regulation. In another study, a truncated cDNA encoding  $\alpha$ -tubulin was isolated from a mouse spleen cDNA library using lacZ-inducible T cells as a probe (58). Deletion and mini-gene constructs with an ATG start codon in frame with the  $\alpha$ -tubulin failed to identify the epitope recognized by T cells. However, a peptide derived from a non-ATG-defined alternative open reading frame was found to be recognized by T cells, suggesting that a cryptic translation product may also be presented to T cells by an MHC class I molecule. In another study, a gp100 epitope recognized by HLA-A2-restricted CTL clonoids was

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ATGAGTGCCTAAACTCCTCTCTCTGGGCTGTATCTTCTTCCCCTTGCTACTTTTTTCAG 60
ORF1 M S A P K L L S L G C I F F P L L L F Q
CAGGCCCGGGCTCAATTCCCAAGACAGTGTGCCACTGTTGAGGCTTTGAGAAGTGGTATG 120
ORF1 Q A R A Q F P R Q C A T V E A L R S G M
TGTGCCCCAGACCTGTCCCCTGTGTCTGGGCTGGGACAGACCGCTGTGGCTCATCATCA 180
ORF1 C C P D L S P V S G P G T D R C G S S S
GGGAGGGGCAGATGTGAGGCAGTGACTGCAGACTCCCGGCCCCACAGCCCTCAGTATCCC 240
ORF1 G R G R C E A V T A D S R P H S P Q Y P
CATGATGGCAGAGATGATCGGGAGGTCCTGGCCCTTGGCGTTCTTCAATAGGACATGTCAC 300
ORF1 H D G R D D R E V W P L R F F N R T C H
ORF2 M I G R S G P C A S S I G H V T
ORF3 M S L

TGCAACGGCAATTTTCTCAGGACACAACCTGTGGGACGTGCCGTCCTGGCTGGAGAGGAGCT 360
ORF1 C N G N F S G H N C G T C R P G W R G A
ORF2 A T A I S Q D T T V G R A V L A G E E L
ORF3 Q R O F L R T Q L W D V P S W L E R S C

GCCTGTGACCAGAGGGTTTCTCATAGTCAGGAGAAATCTTCTGGACTTAAGTAAAGAAGAA 420
ORF1 A C D Q R V L I V R R N L L D L S K E E
ORF2 P V T R G F S STOP
ORF3 L STOP

                ApaI
AAGAACCCTTTGTCCGGGCCCTGGATATGGCAAAGCGCACAACTCACCCCT...ATATGA 1584
ORF1 K N H F V R A L D M A K R T T H P

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**FIG. 1. The nucleotide, amino acid sequence, and open reading frames of the gp75 gene**

The partial nucleotide and amino acid sequences of the first 157 amino acids are shown from the start codon for translation of ORF1 (gp75). The DNA fragment that conferred the ability to stimulate GM-CSF release from TIL586 is underlined. Two putative start codons, ATG (254–256) and ATG (294–296), are in bold and may result in the translation of ORF2 and ORF3, respectively. The peptide sequence recognized by TIL586 from ORF3 is in bold and underlined.

identified from an intron of an incomplete splicing form of the gp100 RNA (59).

*TRP-2.* Since TIL586 has antitumor activity *in vivo*, it is possible that TIL586 consists of oligoclonal T cell populations that recognize multiple tumor antigens. To test whether TRP-1/gp75 was the only antigen recognized by TIL586, a number of CTL clones were established from TIL586. The recognition pattern of CTL clones from TIL586 could be classified into several categories: the first class of CTL clones recognized TRP-1/gp75, 586mel, and HLA-A31 positive melanocytes; the second class of CTL clones did not recognize TRP-1/gp75, but strongly recognized autologous tumor and HLA-A31 positive normal melanocytes, suggesting that these CTL clones recognized a new antigen; the third class of CTL clones recognized autologous tumor, but neither TRP-1/gp75

nor HLA-A31 positive normal melanocytes. This led to identification of TRP-2 as a tumor antigen recognized by CTL clones from TIL586 (Table 1) (16). TRP-2 is a member of the tyrosinase-related protein family and has approximately 40–45% of amino acid sequence identity to tyrosinase or TRP-1/gp75. An antigenic peptide was then identified from the normal open reading frame of TRP-2. Interestingly, a mouse TRP-2 was identified as a tumor antigen recognized by CTL reactive with B16 melanoma (60). This represents the first example of which both human and mouse TRP-2 act as tumor antigens. Therefore, the mouse TRP-2 can be tested as an ideal antigen in studies important for human cancer vaccines.

To broad clinical utilities of tumor antigens such as TRP-1 and TRP-2 that are restricted by HLA-A31, it is important to identify T cell

TABLE 2. Tumor-specific shared antigens

Antigens	HLA restrictions	Expression patterns <sup>a</sup>				
		Normal tissues	Melanoma (%)	Breast (%)	Prostate (%)	Bladder (%)
MAGE-1	HLA-A1, -Cw16	Testis	30	5	15	19
MAGE-3	HLA-A1, -A2	Testis	65	5	15	34
BAGE	HLA-Cw16	Testis	17	10	0	15
GAGE-1	HLA-Cw6	Testis	20	0	10	12
CAG-3/NY-ESO-1	HLA-A31	Testis	35	30	25	80

<sup>a</sup>Gene expression was assayed by RT-PCR of RNA extracted from tumor samples.

epitopes restricted by other MHC class I alleles. On the basis of structural similarities of a group of HLA alleles and peptide binding motifs, several supertypes were proposed (61). The HLA-A31 molecule belongs to a member of the HLA-A-3-like supertype that covers 45–50% of all ethnic populations. In a recent study, it was demonstrated that both TRP-1 and TRP-2 peptides were capable of binding to HLA-A3, -A11, -A31, -A33 and -A68 (62). Furthermore, the TRP-2 peptide was recognized by T cells in the context of HLA-A31 and -A33 (62). These studies raised a possibility that the TRP-1 and TRP-2 peptides could be used in the peptide-based vaccines to treat patients expressing one of the HLA-A3 superfamily members.

### Tumor-Specific Shared Antigens

**MAGE-1 AND -3.** MAGE-1 was the first tumor antigen identified on a human melanoma using a genetic approach. A genomic library derived from the tumor cell line was transfected into an MHC-matched, antigen-loss variant (8). Stable transfectants were screened by CTLs derived from the melanoma patient who had been repeatedly immunized with mutagenized autologous tumor. MAGE-1 was isolated from the transfectants on the basis of its ability to stimulate cytokine release from the CTLs (8). The peptide epitope EADPTGHSY, was subsequently identified and recognized by CTL in the context of HLA-A1 (63). A second antigenic peptide, SAYGEPKRL, was identified from the MAGE-1 protein and recognized by HLA-Cw16-restricted CTLs (64). The MAGE-1 gene is expressed in

approximately 30% of melanomas as well as in other types of tumors (Table 2), but it is not detected in normal human tissues with the exception of testis (8).

DNA hybridization analysis indicated that MAGE-1 is a member of a multiple-gene family. A similar peptide, EVDPIGHLY, derived from MAGE-3, was shown to be recognized by HLA-A1-restricted CTLs (9). The second epitope peptide was identified using HLA-A2-restricted CTLs generated in vitro with a synthetic peptide derived from MAGE-3 (65). Like MAGE-1, MAGE-3 was not expressed in human normal tissues except testis. Because MAGE-3 expression was detected in approximately 60% of melanomas, it may represent a good candidate for the development of vaccine strategies.

**BAGE AND GAGE.** Two additional antigens were identified from a cDNA library made from the same MZ2 melanoma cell line (Table 2). A cDNA clone encoding BAGE was isolated using a class I HLA-Cw16-restricted T cell clone derived from the MZ2 patient (11). This gene encodes a polypeptide of 43 amino acids and appears to be expressed in a pattern similar to that of members of the MAGE family. A peptide of nine amino acids was recognized by CTLs in the context of HLA-Cw16. Using the same approach, a gene encoding an antigen recognized by a class I HLA-Cw6-restricted T cell clone was isolated. This led to the identification of a new family of genes named GAGE (10). Among them, GAGE-1 and GAGE-2 encoded a peptide that was recognized by CTLs in association with HLA-Cw\*0601. GAGE genes were found to have an expression

pattern similar to that of the MAGE and BAGE genes (10).

*CAG-3/NY-ESO-1.* It was demonstrated that several CTL clones established from TIL586 did not recognize TRP-1 or TRP-2, or normal HLA-A31-positive melanocytes, but they still strongly recognized the autologous tumor 586mel (16). After screening a cDNA library, several positive cDNA clones were identified (R. Wang and S. Rosenberg, unpublished data). DNA sequence analysis revealed that this gene is identical to NY-ESO-1, which was recently reported to be recognized by the autologous serum from a patient with esophageal cancer (66). Like members of the MAGE gene family, this gene is not expressed in normal human tissues except testis, but it is highly expressed in melanoma, breast, prostate, bladder, and other types of cancers (Table 2) (R. Wang and S. Rosenberg, unpublished data, 66).

### Tumor-Specific Unique Antigens

Many mutations have been identified from tumor suppressor genes such as *ras*, *p53*, and *p16* tumor samples. Because the mutated proteins or peptides could be more immunogenic and be seen as foreign by the host immune system, it has long been assumed that many tumor antigens would be mutated antigens recognized by T cells. Surprisingly, the majority of tumor antigens identified are nonmutated self-proteins. To test whether mutated *ras* and *p53* are tumor-specific antigens, several groups have raised CTLs against normal or mutated peptides from the *ras* proto-oncogene (67) and *p53* tumor suppressor gene (19,68–71). However, in most cases these CTLs failed to recognize tumor cells. One possibility is that peptides selected on the basis of peptide-binding motif are not naturally processed or that the density of MHC-peptide complexes is too low on tumor cells to allow recognition by these CTLs. It should be noted, however, that despite the numerous mutations identified in *ras* and *p53*, only a very small subset of mutations enhance, if they are processed, the MHC-binding affinity or T cell recognition. Several mutated gene products have been recently identified as tumor-specific antigens recognized by CTL derived from patients by screening cDNA libraries using reactive CTLs (Table 3).

MUM-1 (melanoma-ubiquitous mutated) was isolated following the transient transfection of COS cells with HLA-B44 and pools of cDNAs

TABLE 3. Tumor-specific unique antigens

Antigens	HLA presentation	Peptides
MUM-1	HLA-B44	EEKLIVVLF
CDK4	HLA-A2	ACDPHSGHFV
$\beta$ -catenin	HLA-A24	SYLDSGIHF
HLA-A2	HLA-A2	Altered HLA-A2

derived from the LB33 melanoma cell line. The peptide epitope EEKLIVVLF was found to be recognized by CTL (72). DNA sequence analysis revealed that a point mutation in the sequence of the cDNA isolated from the tumor led to a change of one amino acid (Ser to Ile) at position 5 of the peptide. Since both the normal and mutated peptides bound efficiently to the class I HLA-B44 molecule, but only the mutated form could be recognized by T cells, this indicated that the mutation appeared to have an effect on T cell recognition. Further analysis indicated that the antigenic peptide spanned the intron-exon boundary of an incompletely spliced transcript.

A second mutated gene was isolated from a cDNA library of melanoma patient SK29 (14). This gene encodes cyclin-dependent kinase 4 (CDK4), an enzyme involved in cell cycle control. DNA sequence analysis indicated that a point mutation (a C-to-T transition) led to a substitution of a cysteine for an arginine residue at codon 24. It was found that the mutated peptide was capable of sensitizing the target cells at 100- to 1000-fold lower peptide concentrations than the normal peptide. The CDK4 protein usually forms a complex with cyclin D1 and phosphorylates the pRB protein, and therefore promotes the cell cycle progression from G1 to S phase (56). However, assembly of CDK4 with cyclin D1 as well as its kinase activity was found to be inhibited by p16<sup>INK4a</sup>. Interestingly, p16<sup>INK4a</sup> cannot bind to the mutated CDK4 and fails to inhibit the kinase activity of CDK4/cyclin D, implying that the mutation in the CDK4 gene leads to a loss of cell cycle control.

The mutated  $\beta$ -catenin gene product was also recently identified and shown to be recognized by TIL1290 derived from a melanoma patient 888 (15). Partial cDNA sequence analysis indicated that a point mutation was found to be responsible for a change of serine to phenylalanine in the coding region. The mutated  $\beta$ -catenin

peptide SYLDSGIHF was found to actively sensitize target cells for lysis at a concentration of 1 pM, whereas the normal peptide (SYLDSGIHS) required a much higher peptide concentration to sensitize lysis of target cells. A competitive peptide binding assay indicated that this substitution resulted in an increase in binding affinity of the mutated peptide to the HLA-A24 molecule. The  $\beta$ -catenin protein has been shown to be a cytoplasmic protein that interacts with the cellular adhesion molecule E-cadherin (73). A number of mutations have been found in the  $\beta$ -catenin gene product from different tumors (74,75). Loss of cell adhesion molecules may play a role in the metastatic process (76). Recently, three groups reported that the upregulation or stabilization of  $\beta$ -catenin may contribute to tumorigenesis and cancer progression because of mutations in the adenomatous polyposis coli tumor suppressor protein (APC) or  $\beta$ -catenin (77–79).

An HLA-A2 mutation was found to be recognized in renal carcinoma cells by CTL. Interestingly, T cells recognized the mutated whole HLA-A2 molecule, instead of recognizing the mutated peptide epitope (80).

### Tumor Antigens Ubiquitously Expressed

The majority of human melanoma antigens identified thus far are tissue-specific. However, one melanoma-specific T cell line was found to recognize an antigen termed p15, whose expression is ubiquitous in normal tissues (81). A non-mutated peptide, AYGLDFYIL, was identified as a T cell epitope derived from the p15 protein. It is not clear whether this peptide represents a true T cell epitope or is a cross-reactive one with the product derived from an unknown gene but is still recognized by TIL1290 on tumor cells. The low level of expression of this antigen in normal tissues may not generate a sufficient amount of MHC-peptide complex on the cell surface to elicit a T cell response.

HER-2/neu was recently identified as a shared tumor antigen recognized by T cells in breast and ovarian cancers. The HER-2/neu proto-oncogene encodes a tyrosine kinase protein whose expression has been shown to be increased in 30% of breast and ovarian cancers. In breast cancer, HER-2/neu overexpression was reported to be associated with aggressive disease. Cytotoxic T lymphocytes isolated from tumor-associated lymphocytes can specifically recognize a synthetic peptide corresponding to amino acids 971–980 of the HER-2/neu protein (82). This is

the first demonstration that CTLs isolated from human tumors recognize HER-2/neu as an ovarian tumor antigen. Recently, four ovarian tumor-reactive CTLs were established from different HLA-A2<sup>+</sup> patients and were capable of recognizing both freshly isolated HER-2/neu<sup>+</sup> tumor cells and non-HLA-A2 ovarian tumor lines transfected with HLA-A2 cDNA (20). A common epitope, KIFGSLAFL, was found to be recognized by four of four CTL lines (20). Another epitope peptide (971–980) was also found to be recognized by two of four CTL lines. These results were supported by findings of other groups (83,84). Recognition and lysis of ovarian cancer cells by CTLs were also shown to correlate with the expression level of HER-2/neu in the tumor cells (83). Most importantly, the breast and ovarian cancer-specific CTLs recognized the same epitope peptide (GP2; amino acids 654–662) derived from the HER-2/neu protein in the context of HLA-A2 (85,86). It appears that the GP2 peptide represents a common epitope shared by different epithelial tumors because it was recognized by CTL lines derived from breast, ovarian, non-small-cell lung and pancreatic cancers (86).

Another tumor antigen in this class is the mucin-associated epitope from the muc-1 gene product, whose expression has been shown to be associated with breast and pancreatic adenocarcinomas (87). The *muc-1* gene is expressed on epithelial cells, fibroblasts, and B cells, and it can serve as a target for T cell recognition. However, T cell recognition of the muc-1 gene product appeared to be non-MHC restricted (88). The epitopes for T cell recognition were found in the tandem repeat of the muc-1 protein (89).

## PERSPECTIVES FOR CANCER THERAPIES

The identification of tumor antigens recognized by T cells has important implications in the understanding of T cell-mediated antitumor activity and provides opportunities for the development of new strategies for cancer vaccines. Because the majority of tumor antigens identified thus far are self-proteins, a potential consequence of active immunization with these cancer peptides is the development of autoimmune disease (90,91). Development of vitiligo has been found to be correlated with good prognosis or clinical responses to immunochemotherapy in mela-



noma patients (92–94). The adoptive transfer of autologous TIL along with IL-2 into patients with melanoma resulted in the objective regression of tumor, but only occasionally resulted in the depigmentation of skin in treated patients, suggesting that the treatment of patients with anti-self T cells does not necessarily cause tissue destruction in normal organs (40,45). Patients developed neither depigmentation nor any retinal tissue destruction after treatment with tyrosinase-specific TILs (95). Recently, Houghton and co-workers have demonstrated that passive immunization with a mouse monoclonal antibody (TA99) against gp75 induced protection against and rejection of the gp75<sup>+</sup> B16F10 melanoma in syngeneic mice (96). There was no evidence of decrease in pigmentation, inflammation, or changes in cellular morphology or tissue architecture in the eyes of mice treated with antibody (96), suggesting that the threshold required for tumor regression is lower than that for normal tissue destruction.

Identification of these new cancer peptides has led to several clinical trials conducted in the Surgery Branch, National Cancer Institute. Active immunotherapy involves the direct immunization of cancer patients with cancer antigens in an attempt to boost immune responses against the tumor. The immunodominant peptides derived from tumor antigens could readily be synthesized *in vitro* and used for immunization either alone or in a form intended to improve their immunogenicity, such as in combination with adjuvant, linkage to lipids/liposomes or helper peptides, or pulsed onto antigen-presenting cells. It has recently been reported that peptide-pulsed dendritic cells (DC) induce antigen-specific anti-tumor immune responses in mice (97). Human DC derived from PBMC have been transduced with retroviral vectors encoding tumor antigens and have induced tumor-specific CTL *in vitro* (98,99). Modification of the immunodominant peptides to improve binding efficiency to MHC antigens can potentially increase immunogenicity and induce stronger antitumor activity. Preliminary studies have indicated that peptide vaccines using gp100 G9-280 or G9-280M peptides combined with IFA result in 40–50% clinical responses in HLA-A2<sup>+</sup> melanoma patients (S. Rosenberg, unpublished data). These results are very encouraging; since multiple tumor antigens and multiple epitopes are identified from patients with cancer, it is likely that the use of multiple epitope peptides will enhance antitumor activity.

To prove that the tumor antigens identified thus far are true tumor rejection antigens, it is necessary to generate a large number of tumor-reactive, peptide-specific CTL *ex vivo*. Adoptive transfer of these CTL into the autologous patient will be evaluated.

The most effective cancer vaccines involve the incorporation of genes encoding tumor antigens into recombinant plasmid or viruses such as vaccinia, fowlpox, or adenovirus. Because tumor cells transduced with genes encoding cytokines or costimulatory molecules have been shown to elicit *in vivo* antitumor activity, the combination of cancer antigen genes with other genes encoding cytokines such as IL-2 and costimulatory molecules such as B7.1 may enhance the immune response following viral infection. Alternatively, cancer vaccines using recombinant virus encoding tumor antigens can be enhanced by the exogenous administration of immunostimulatory cytokines (100). The major problem associated with recombinant viruses encoding tumor antigens is that patients develop strong antibody responses against the recombinant viruses, resulting in inefficient or low infection activity. "Naked" DNA vaccines are an alternative to the use of recombinant viruses. It has been shown that the injection of plasmid DNA encoding model tumor antigens directly into muscle or into the skin resulted in both cellular and humoral immune reactions (101).

## FUTURE DIRECTIONS

The current cDNA cloning approach requires the determination of a restriction element for T cells. In some cases, it is difficult to determine which restriction molecule is used because of the point mutation of tumor antigens or unique tumor antigens. To overcome this problem, a retroviral-based cDNA expression system is being developed for the cloning of tumor antigens without information on restriction presentation. This will facilitate the identification of tumor antigens restricted by different MHC class I molecules.

Since successful vaccination may require both CD4<sup>+</sup> and CD8<sup>+</sup> T cells, it is important to develop systems for cloning MHC class II-restricted tumor antigens. Utilization of MHC class I- and II-restricted cancer peptides combined with cytokines and costimulatory molecules may mount an improved antitumor response against human cancer.

The majority of tumor antigens identified to

date are melanoma antigens. It is important to extend our studies in melanoma to other tumors such as breast and prostate cancers. However, there are technical problems that need to be solved, including difficulties in generating CTL against breast and prostate tumor cells and a lack of knowledge of the putative tumor antigens expressed by these tumors. One approach is to clone tumor antigens shared by many types of tumors, including breast and prostate cancers, by the use of CTL clones derived from melanoma-reactive TIL. Several examples, including MAGE-1, MAGE-3, BAGE, GAGE, and CAG-3/NY-ESO-1, are well documented. Of particular interest, CAG-3/NY-ESO-1 is expressed in 30% of breast, 25% of prostate, and 80% of bladder cancers.

The second strategy is the "reverse immunology" approach. In general, tumor antigens are overexpressed in the tumor compared with the normal counterpart, making them potential targets for immunotherapy. Recently developed DNA microchip technology will provide new opportunities to test and evaluate the gene expression pattern on a large scale. DNA microchip technology allows one to load thousands of cDNAs or to synthesize potentially hundreds of thousands of oligonucleotide probes on a chip (102,103). With this technology, one is able to quickly monitor the expression of thousands of genes or to detect mutations in a gene of interest in a single chip. The availability of a large amount of information from the Human Genomic Project and many cDNA databases has made this approach feasible. A recent study using a cDNA microarray showed that TRP-1/gp75, which was previously shown to be recognized by CTL, was among the genes detected with a high level of expression in melanoma cells (104). This is in agreement with our Northern blot results showing that TRP-1/gp75 is expressed 10-fold higher in melanoma than in normal melanocytes (16). Evaluation of the changes in gene expression in cancer cells versus normal cells may allow us to define genes associated with malignancy and to identify potential tumor antigens. Once we have a few candidate genes in hand, the next step will be to generate antigen-specific CTL and test for their ability to recognize tumor cells. Improvement of quick and efficient methods for generating antigen-specific CTL will enhance our ability to identify tumor antigens.

In conclusion, the identification of human tumor antigens has provided a great opportunity for the development of effective cancer vaccines

against cancer. Improvement of methods for generating CTL in vitro for breast, prostate, and other type of cancers and development of strategies for cloning MHC class II tumor antigens are important challenges. Cancer vaccines based on the identification of genes encoding tumor antigens combined with different adjuvants, cytokines, and costimulatory molecules need to be evaluated.

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