Published in final edited form as:

J Immunother Emphasis Tumor Immunol. 1993 August ; 14(2): 88–93.

T-Cell Recognition of Human Melanoma Antigens

Yutaka Kawakami, Michael I. Nishimura, Nicholas P. Restifo, Suzanne L. Topalian, Bert H. O'Neil, Joel Shilyansky, John R. Yannelli, and Steven A. Rosenberg

Surgery Branch, National Cancer Institute, National Institutes of Health, Bethesda, Maryland, U.S.A

Summary

The adoptive transfer of tumor-infiltrating lymphocytes (TILs) with interleukin-2 (IL-2) has antitumor activity in some patients with metastatic melanoma. We have analyzed molecular mechanisms of TIL recognition of human melanoma. Some cultured TILs specifically lysed autologous and some allogeneic melanomas sharing a variety of class I major histocompatibility complex (MHC) molecules. HLA-A2-restricted melanoma-specific TILs lysed many HLA-A2⁺ melanoma cell lines from different patients but failed to lyse HLA-A2⁻ melanoma and HLA-A2⁺ nonmelanoma cell lines. However, these TILs were capable of lysing many naturally HLA-A2⁻ melanomas after introduction of the HLA-A2.1 gene by vaccinia virus. These results indicate that shared melanoma antigens (Ag) are expressed in melanomas regardless of their human leukocyte antigen types. In order to identify these shared melanoma Ags, we have tested some known proteins expressed in melanoma. Expression of tyrosinase or HMB45 Ag correlated with lysis of TILs. We are also attempting to isolate antigenic peptides by high performance liquid chromatography separation and genes encoding melanoma Ag by cDNA expression cloning. The T-cell component of the antimelanoma response was also analyzed by determining the genetic structure of the T-cell receptor (TCR) used by melanoma TILs. However, we did not observe common TCR variable region usage by different melanoma TILs. We could establish melanoma cell clones and lines resistant to TIL lysis due to the absence of or defects in the expression of Ag, MHC, or β_2 -microglobulin molecules. These data indicate multiple mechanisms for melanoma escape from T-cell immunosurveillance. These findings have important implications for the development of immunotherapies for melanoma.

Keywords

Tumor-infiltrating lymphocytes; Melanoma antigens; T-cell receptor; HLA-A2.1; Immunotherapy

The adoptive transfer of tumor-infiltrating lymphocytes (TILs) along with systemically administered interleukin-2 (IL2) can reduce the tumor burden in mice and humans. The response rate for patients with metastatic melanoma treated with IL2 + TIL therapy is ~40% (complete and partial remission), whether or not patients had been previously treated with IL2 (1). This is approximately twice the response rate observed with IL2 plus lymphokine-activated killer (LAK) cell therapy. Studies using ¹¹¹indium-labeled TILs showed accumulation of transferred TILs in melanoma lesions, and recent studies have shown that patients in whom radiolabeled TILs traffic to tumor sites have an increased response rate to TIL therapy (2). Immunohistochemical studies showed infiltrates of both CD4⁺ and CD8⁺ T-cells and macrophages, but not of B cells and natural killer (NK) cells, in biopsies from melanomas that regressed after IL2-related therapy. These results indicate an important role for T-cells in mediating melanoma regression in vivo.

MELANOMA-SPECIFIC T-CELLS DERIVED FROM TUMOR-INFILTRATING LYMPHOCYTES

We established TIL cultures that were primarily CD3⁺, CD8⁺ T-cells by dissociating cells from solid tumor specimens and culturing them in IL2-containing medium for > 1 month. These T-cell lines specifically lysed fresh and cultured autologous melanoma tumor cells in vitro in about one-third of cases (3,4). In many cases, they were also capable of releasing the lymphokines γ -interferon (γ -IFN), tumor necrosis factor- α (TNF α), and/or granulocyte-macrophage-colony-stimulating factor (GM-CSF) specifically following stimulation with autologous melanoma cells (5). Lysis and lymphokine release were blocked by anti-CD3 or by anti-class I major histocompatibility complex (MHC) antibody (Ab). Therefore, these TILs recognize autologous melanoma cells through an interaction of the T-cell receptor (TCR), the MHC, and tumor-associated antigenic peptide.

SHARED MELANOMA ANTIGENS RECOGNIZED BY TILS

Further studies of TILs reactive with autologous melanoma cells showed cross-reactivity of some TILs against allogeneic melanoma cells sharing at least one class I MHC determinant. This observation suggests that some tumor-associated antigen (Ag) peptides are shared by melanoma derived from different patients. We have so far identified HLA-A1, -A2, -A24, -A31, -B8, and -Cw7 as human leukocyte antigen (HLA) molecules capable of presenting these shared melanoma antigens (6,7).

We focused on HLA-A2-restricted melanoma-specific TILs for further analysis of shared Ag, since HLA-A2 is the most frequently expressed class I HLA molecule (45% in Caucasians) and may be a dominant restriction element for the induction of melanoma-specific T-cells (8). The structure, function, and peptide binding motifs of the HLA-A2.1 molecule, the major HLA-A2 subtype, have been characterized and have been useful in identifying antigenic peptides recognized by HLA-A2-restricted T-cells (9,10). We established several TIL lines and clones from HLA-A2⁺ patients and analyzed three important components in the T-cell recognition of melanoma: MHC, tumor-associated Ag, and TCR.

Depending on the TIL lines or clones tested, these TILs lysed eight to 13 of 15 (53–87%) HLA-A2⁺ melanoma cell lines derived from 20 patients but did not lyse nonmelanoma cell lines, including Epstein-Barr virus (EBV)-transformed B cells, fibroblasts, and other types of cancer, whether or not these cells expressed HLA-A2 (Table 1). We could also detect this cross-reactivity by measuring lymphokine secretion by TILs (7). These results suggest the existence of shared melanoma-specific Ag peptides that are recognized by T-cells in the context of HLA-A2 molecules.

We also examined whether melanoma-reactive cytolytic T lymphocytes (CTLs) recognized melanocytes, the presumed cell of origin of melanomas. Using HLA-A2⁺ allogeneic cultured melanocytes from the foreskin of infants as targets, we occasionally detected low levels of lysis by melanoma TILs (10% lysis at Effector/Target ratio = 40–80:1). However, these melanoma TILs released high levels of γ -IFN when incubated with almost all HLA-A2⁺ cultured melanocytes tested. It has recently been reported that HLA-A2-restricted melanoma-specific CTLs can lyse cultured HLA-A2⁺ allogeneic melanocytes (11). Although cultured melanocytes differ from melanocytes in situ in terms of expression of many proteins, these data, together with the data that melanoma antigens are shared among patients, suggest that TILs might recognize tissue (melanocyte lineage)-specific normal self peptides.

We then investigated whether these shared melanoma antigens were expressed on HLA-A2⁺ melanomas by introducing the genomic HLA-A2.1 gene into HLA-A2⁺ melanoma cell lines

and testing the lysability of the transfectants. After Cotransfection with pSV2neo and selection in G418-containing medium, we isolated tumor cell clones and analyzed their HLA-A2 expression by flow cytometry. All clones that expressed HLA-A2 on their surfaces were lysed by HLA-A2–restricted melanoma-specific CTLs, but none of the HLA-A2[−] clones were lysed, whereas all clones lysed by the autologous TILs, which were restricted by MHCs other than HLA-A2 (Table 2) (12).

In order to screen many HLA-A2[−] cell lines for the presence of shared melanoma Ag, we have used vaccinia virus as a vector that permits transient expression of HLA-A2.1. By this method, virtually 100% of the melanoma cell lines expressed HLA-A24 h after infection. After HLA-A2 transduction, 11 of 17 (65%) naturally HLA-A2[−] human melanoma cell lines were lysed by several HLA-A2–restricted, melanoma-specific TILs, but none of 16 nonhuman melanoma cell lines, including murine melanoma, and other types of human cancer lines were lysed. Thus, these shared tumor antigens seemed to be expressed specifically on human melanomas or melanocyte-lineage cells (13).

These results suggest that Ag proteins for shared melanoma antigenic peptides exist in HLA-A2[−] melanoma cell lines derived from patients expressing a variety of class I HLA types. It is possible that different peptides derived from the same Ag protein bind to different MHC molecules and present to T-cells that are restricted by MHC loci other than HLA-A2 (Fig. 1). The isolation of such Ag proteins may allow for the development of vaccines to induce T-cell responses in patients expressing a variety of HLA types, particularly combined with effective new immunization methods, including the use of vaccinia virus, bacillus Calmette–Guerim, liposome, lipoprotein conjugates, and genetically engineered cell lines expressing high levels of Ag, B7, and cytokines that stimulate the immune system.

IDENTIFICATION OF SHARED MELANOMA ANTIGENS

Three approaches have been used to attempt to isolate and identify common melanoma antigens. The first approach is to evaluate known candidate molecules expressed preferentially on melanomas. These include proteins recognized by melanoma-reactive Ab (p97, HMWMAA, HMB45 Ag) or T-cells (MAGE-1), molecules specifically expressed on melanomas or melanocytes (tyrosinase, tyrosinase-related proteins), or oncogene products (p53, N-ras).

We examined the correlation between expression of some of these candidate molecules and lysis by TILs. We first determined that most melanoma cell lines contained the mechanisms necessary for T-cell interactions, including Ag processing, expression of adhesion molecules, and the capacity to be lysed, since most melanoma cell lines were lysed by HLA-A2–restricted influenza M1-specific CTLs after incubation with exogenously added influenza M1_{58–66} peptide or infection with vaccinia virus containing M1 protein gene. Therefore, melanoma cell lines expressing Ag recognizable by TILs should be lysed by HLA-A2–restricted TILs. Among candidate molecules tested, expression of tyrosinase and the melanosomal glycoprotein recognized by the monoclonal antibody HMB45 correlated with cytolysis by TILs, but p97, HMWMAA, and tyrosinase-related protein did not correlate. We are currently investigating the importance of these proteins by screening HMB45 Ag and tyrosinase transfectants for sensitivity to lysis by TILs.

MAGE-1 Ag was the first reported human melanoma Ag to be cloned and is recognized by HLA-A1–restricted melanoma-specific CTLs. MAGE-1 is also expressed by melanoma cell lines from patients who do not express HLA-A1 (14). However, our HLA-A2–restricted TILs do not appear to recognize the MAGE-1 Ag in the context of HLA-A2, since there was no correlation between cytolysis by TILs and MAGE-1 mRNA expression in melanoma target cells (15). A peptide, ILESLFRV, containing HLA-A2 binding motif in a MAGE-1 protein

was synthesized and the possibility that it may be recognized by HLA-A2–restricted TILs was tested. The HLA-A2–restricted TILs tested did not recognize this peptide. In addition, HLA-A2⁺ fibroblast cell lines transfected with MAGE-1 gene were not lysed by our HLA-A2–restricted melanoma-specific TILs.

A second approach to the identification of melanoma Ag is the isolation of tumor-related peptides bound to class I MHC molecules. Peptides were eluted from HLA-A2 molecules and fractionated by HPLC, and the lysability of HLA-A2⁺ EBV B cells or T2 cell lines pulsed with each fraction was tested using melanoma-specific TILs. In preliminary studies, we have identified multiple fractions that rendered B-cell lines susceptible to lysis by TILs. Since those fractions presumably contain the melanoma antigenic peptides, recent technological advances that allow sequencing of femtomolar amounts of peptide with tandem mass spectrometry may permit direct identification of the melanoma antigenic peptides in these fractions (10). Multiple HPLC fractions containing melanoma antigenic peptides shared among melanoma cell lines from different patients have recently been reported, using similar methods (16).

The third approach to the identification of melanoma antigens is the cloning of genes coding for them. We made a cDNA expression library from HLA-A2⁺ melanoma cells and transfected it into HLA-A2⁺ cell lines that are resistant to lysis by melanoma-specific TILs, but nevertheless have intact Ag processing and presenting capacity. We are now screening transfectants using cytotoxic or lymphokine release assays to identify transfectants that can be recognized by TILs.

Theoretically, melanoma antigens may be foreign proteins (such as viral proteins), mutated peptides (such as mutated oncogene products), or normal self peptides expressed only in early developmental stages or even expressed in normal adult tissues (Table 3). For example, MAGE-1 Ag seems to be a normal self protein. Our immunological analysis of melanoma Ag suggests that at least some antigens are probably nonmutated self peptides specifically expressed in melanocyte-lineage cells, although truly melanoma-specific antigens might exist. It has been reported that depigmentation that resulted from destruction of melanocytes was associated with clinical response after chemoimmunotherapy of melanoma (17). Questions remain as to whether these self proteins can be used as the basis for cancer immunotherapies. The number of self peptides expressed on the surface of melanocytes in situ in the context of MHC needs to be addressed. In active immunization, we have to consider two extreme possibilities: strong immunization may induce an autoimmune disorder destroying normal tissues in the body, or it may be difficult to immunize patients because of existing tolerance mechanisms for self Ag. It is reassuring, however, that we have not observed massive destruction of melanocytes after the adoptive transfer of TILs capable of causing the regression of established melanomas in humans.

T-CELL RECEPTORS USED BY MELANOMA-SPECIFIC TILS

The T-cell component of antimelanoma responses was also analyzed by determining TCR V region use in melanoma-specific TILs, using the polymerase chain reaction (PCR) and cDNA cloning. We analyzed V α and V β use of TCR on melanoma-specific polyclonal TIL lines from 15 patients. Genes were identified with PCR, using a variety of known 5'-V α or -V β region primers. We could detect V α 1, V α 2, V α 7, V α 21, and V α 22 more frequently than other V regions in melanoma-specific TILs. However, it is difficult to correlate V segment use and melanoma recognition in heterogeneous TIL lines because the sensitivity of PCR allows for amplification of TCR genes used by small percentages of T-cells that may not be active in specific melanoma lysis. In fact, genomic Southern blot analysis with a C β probe and Northern blot analysis with a V α 1, 2, or 7 probe suggested the existence of several major T-cell clones, the TCRs of which were likely to recognize melanoma.

We also analyzed TCRs of five TIL clones from different patients. Three HLA-A2–restricted TIL clones, two from the same patient, used different V α segments. These results suggest the existence of multiple T-cell clones capable of recognizing multiple melanoma antigens in the same patient, as previously described (16,18). It will be interesting to analyze TCR genes on T-cell clones for which both the MHC restriction element and the relevant melanoma peptide are known. We are now attempting to transduce T-cells with TCR α and β chain genes cloned from HLA-A2–restricted, shared melanoma Ag-specific TILs. Such genetically modified melanoma-specific T-cells may be useful for research and treatment of patients.

MECHANISMS BY WHICH MELANOMAS MAY EVADE T-CELL RECOGNITION

Loss or dysfunction of any of three important components of specific T-cell recognition (TCR, MHC, or Ag) may allow tumor cells to escape T-cell recognition. We were able to establish melanoma cell lines resistant to TIL lysis in vitro by repeated coculture with TILs. Adhesion molecules and lytic machinery for CTL lysis of these resistant cell lines were intact, since they could be lysed by other CTLs. It is likely that these melanoma cell lines either lacked the Ag or had a defect or absence in the MHC molecules that presented the antigenic peptide to T-cells (19). We have also identified five melanoma cell lines that do not express class I MHC molecules on their surface, from >50 melanoma cell lines tested. All of these MHC nonexpressing melanoma cell lines had defects in β_2 -microglobulin expression, since they expressed class I MHC on their surfaces after transduction of the normal β_2 -microglobulin gene. This mechanism for lack of surface MHC expression differed from that in small-cell lung cancer cell lines, in which lack of class I MHC expression appeared to result from different defects of the peptide transporter and proteasome genes (20). Thus, multiple mechanisms may exist for melanomas to escape T-cell immuno-surveillance.

CONCLUSION

The mechanisms by which T-cells interact with human melanomas were analyzed at a molecular level. This information may improve our understanding of the immune response against melanoma and may lead to improvements in immunotherapy against this malignancy.

Acknowledgements

We thank C. H. Delgado, S. Eliyahu, M. Custer, A. Mixon, E. B. Fitzgerald, Dr. S. S. Hom, Dr. L. Rivoltini, Dr. P. Shamamian, Dr. F. Marincola, Surgery Branch, NCI; Dr. M. Dibrino, Dr. J. Coligan, Biological Resources Branch, NIAID; Dr. J. W. Yewdell, Dr. J. R. Bennink, Laboratory of Viral Diseases, NIAID; Dr. M. Herlyn, Wistar Institute of Anatomy and Biology; Dr. P. Charmley, Virginia Mason Research Center; and Dr. L. E. Hood, Department of Molecular Biotechnology, University of Washington, who took part in various aspects of this study.

References

1. Rosenberg SA, Packard BS, Aebersold PM, et al. Use of tumor-infiltrating lymphocytes and interleukin-2 in the immunotherapy of patients with metastatic melanoma: a preliminary report. *N Engl J Med* 1988;319:1676–80. [PubMed: 3264384]
2. Pockaj BA, Sherry R, Wei J, et al. Localization of 111 indium-labeled tumor infiltrating lymphocytes to tumor in patients receiving adoptive immunotherapy: augmentation with cyclophosphamide and association with response. (submitted)
3. Topalian SL, Solomon D, Rosenberg SA. Tumor specific cytolysis by lymphocytes infiltrating human melanomas. *J Immunol* 1989;142:3714–25. [PubMed: 2785562]
4. Kawakami Y, Rosenberg SA, Lotze MT. Interleukin 4 promotes the growth of tumor-infiltrating lymphocytes cytotoxic for human autologous melanoma. *J Exp Med* 1988;168:2183–91. [PubMed: 3264324]

5. Schwartzentruber DJ, Topalian SL, Mancini M, Rosenberg SA. Specific release of granulocyte-macrophage colony-stimulating factor, tumor necrosis factor- α , and IFN- γ by human tumor-infiltrating lymphocytes after autologous tumor stimulation. *J Immunol* 1991;146:3674–81. [PubMed: 1902860]
6. Hom SS, Topalian SL, Simonis T, Mancini M, Rosenberg SA. Common expression of melanoma tumor-associated antigens recognized by human tumor infiltrating lymphocytes: analysis by human lymphocyte antigen restriction. *J Immunother* 1991;10:153–64. [PubMed: 1868040]
7. Hom SS, Schwartzentruber DJ, Rosenberg SA, Topalian SL. Specific release of cytokines by lymphocytes infiltrating human melanomas in response to shared melanoma antigens. *J Immunother* 1993;13:18–30.
8. Crowley NJ, Darrow TL, Quinn-Allen MA, Seigler HF. MHC-restricted recognition of autologous melanoma by tumor-specific cytotoxic T-cells: evidence for restriction by a dominant HLA-A allele. *J Immunol* 1991;146:1692–9. [PubMed: 1671580]
9. Falk K, Rotzeschke O, Stevanovic S, Jung G, Rammensee HG. Allele-specific motifs revealed by sequencing of selfpeptides eluted from MHC molecules. *Nature* 1991;351:290–6. [PubMed: 1709722]
10. Hunt DF, Henderson RA, Shabanowitz J, et al. Characterization of peptides bound to the class I MHC molecule HLA-A2.1 by mass spectrometry. *Science* 1992;255:1261–6. [PubMed: 1546328]
11. Anichini A, Maccalli C, Mortarini R, et al. Melanoma cells and normal melanocytes share antigens recognized by HLA-A2–restricted cytotoxic T-cell clones from melanoma patients. *J Exp Med* 1993;177:989–98. [PubMed: 8459226]
12. Kawakami Y, Zakut R, Topalian SL, Stotter H, Rosenberg SA. Shared human melanoma antigens: recognition by tumor infiltrating lymphocytes in HLA-A2.1 transfected melanomas. *J Immunol* 1992;148:638–43. [PubMed: 1729379]
13. O’Neil BH, Kawakami Y, Restifo NP, Bennink JR, Yewdell JW, Rosenberg SA. Detection of shared MHC restricted human melanoma antigens following vaccinia virus mediated transduction of genes coding for HLA. *J Immunol* 1993;151:1410–8. [PubMed: 8335937]
14. van der Bruggen P, Traversari C, Chomez P, et al. A gene encoding an antigen recognized by cytolytic T lymphocytes on a human melanoma. *Science* 1991;25:1643–7. [PubMed: 1840703]
15. Zakut R, Topalian SL, Kawakami Y, Mancini M, Eliyahu SE, Rosenberg SA. Differential expression of MAGE-1, -2, and -3 messenger RNA in transformed and normal human cell lines. *Cancer Res* 1993;53:5–8. [PubMed: 8416750]
16. Slingluff CL, Cox AL, Henderson RA, Hunt DF, Engelhard VH. Recognition of human melanoma cells by HLA-A2.1–restricted cytotoxic T lymphocytes is mediated by at least six shared peptide epitopes. *J Immunol* 1993;150:2955–63. [PubMed: 7681084]
17. Richards JM, Mehta N, Ramming K, Skosey P. Sequential chemotherapy in the treatment of metastatic melanoma. *J Clin Oncol* 1992;10:1338–43. [PubMed: 1634924]
18. Wolfel T, Klehmann E, Muller C, Schutt K-H, Buschenfelde K-HM, Knuth A. Lysis of human melanoma cells by autologous cytolytic T-cell clones: identification of human histocompatibility leukocyte antigen A2 as a restriction element for three different antigens. *J Exp Med* 1989;170:797–810. [PubMed: 2788708]
19. Topalian SL, Kasid A, Rosenberg SA. Immunoselection of a human melanoma resistant to specific lysis by autologous tumor-infiltrating lymphocytes: possible mechanisms for immunotherapeutic failures. *J Immunol* 1990;144:4487–95. [PubMed: 2160503]
20. Restifo NP, Esquivel F, Kawakami Y, et al. Identification of human cancers deficient in antigen processing. *J Exp Med* 1993;177:265–72. [PubMed: 8426105]

FIG. 1.

T-cell recognition of melanoma antigenic peptides in the context of different class I MHCs. Common (Pr1) or different (Pr2, 3) melanoma Ag proteins may provide Ag peptides (Ag1, Ag2) to different MHCs, and these Ag peptides may be recognized by T-cells in the context of different MHCs. If such common Ag proteins exist, we may be able to use them to immunize patients expressing a variety of HLA types.

TABLE 1
TIL 660, 620, and 501 lyse HLA-A2⁺ melanomas but not HLA-A2⁻ melanomas nor nonmelanoma cell lines^a

Target	TIL			Target	HLA-A2	TIL		
	HLA-A2	660	620 (% lysis)	501		660	620 (% lysis)	501
501mel	+	43	54	52	510mel	42	38	57
526mel	+	22	27	16	501 EBV B cell	0	3	0
624mel	+	36	43	36	501 fibroblast	ND	ND	2
677mel	+	38	39	18	501 ConA blast	ND	ND	0
697mel	+	32	32	23	660 EBV B cell	-5	-6	ND
397mel	-	3	5	-2	660 PHA blast	6	3	ND
883mel	-	-1	-3	-1	Colon tumor	2	-2	ND
978mel	-	2	-3	1	B-cell lymphoma	-2	-4	ND
SKmel2	-	0	-3	8	Daudi	0	8	-2
SKmel28	-	-2	-5	2	K562	ND	ND	5

ND, not determined; PHA, phytohemagglutinin.

^a ⁵¹Cr release assay (E/T = 40:1).

TABLE 2HLA-A2–restricted TIL 620 lyses HLA-A2–transfected 397mel tumor clones^a

Targets	HLA-A2	TIL620 ^b	TIL397 ^b
501mel	+	91	7
397mel	–	–1	43
397cl-1	–	–2	35
397cl-6	–	–2	46
397cl-5	+	37	53
397cl-9	+	47	24

^a 5-h ⁵¹Cr release assay (E/T= 40:1).^b Results given as percentage specific lysis.

TABLE 3
Possible melanoma antigens recognized by T-cells

Foreign peptides (viral proteins)

Self peptides

 Mutated peptides (oncogene products)

 Normal peptides

 Oncofetal

 Normally expressed