Brief Definitive Report

Identification of a Gene Encoding a Melanoma Tumor Antigen Recognized by HLA-A31–restricted Tumor-infiltrating Lymphocytes

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Summary

The availability of antitumor cytotoxic T lymphocytes which can be generated from either peripheral blood lymphocytes after stimulation in vitro or tumor infiltrating lymphocytes (TIL) has made it possible to identify a number of melanoma antigens presented by major histocompatibility complex class I molecules. The present and previous studies indicated that TIL586 recognized an antigen expressed on most melanoma and normal melanocytes in the context of the HLA-A31 molecule. We report here the cloning of a cDNA that directs the expression of the shared melanoma antigen recognized by this TIL. The DNA sequence analysis revealed that the cDNA was almost identical to the gene encoding tyrosinase-related protein 1 or glycoprotein gp75 which was originally identified by serum antibodies in a patient with melanoma. The gene was found to be expressed only in melanoma, normal melanocyte cell lines, and retina, but not in other normal tissues tested. The gp75 antigen presented by HLA-A31 may therefore constitute a useful immune target for specific treatment of patients with melanoma, since both antibody- and T cell–mediated immune responses can be generated against this antigen.

The adoptive transfer of T cells can mediate tumor regression in patients with metastatic melanoma (1, 2). CTL which recognize melanoma cells in an MHC class I–restricted manner can be generated from either PBL or tumor-infiltrating lymphocytes (TIL) of patients with melanoma (3–7). The availability of these CTL has made it possible to isolate a number of tumor-associated antigens recognized by T cells (8). Identification of the antigens recognized by T cells on tumor cells is important for understanding the molecular basis of tumor recognition by T cells, and may lead to the development of new, more effective strategies for the treatment of patients with cancer.

Through the use of molecular cloning strategies, five melanoma antigens encoded by the MAGE-1 (9), MAGE-3 (10), tyrosinase (11, 12), gp100 (13, 14), and MART-1/Melan-A (15, 16) genes have recently been identified. The proteins encoded by MAGE-1 and MAGE-3 were shown to be recognized by CTL in association with HLA-A1 (9). Genes encoding tyrosinase, gp100, and MART-1/Melan-A were recently cloned and were recognized by HLA-A2–restricted CTL (11, 13–16) while the tyrosinase antigen was also recognized by HLA-A24–restricted CTL (12). Expression of three proteins, tyrosinase, gp100, and MART-1/Melan-A, has been shown to be restricted to melanoma, normal melanocytes, and retina whereas MAGE-1 and MAGE-3 expression has been found in normal testis as well as in a variety of tumors (9–11, 14, 15). Tyrosinase is a transmembrane glycoprotein expressed within melanosomes and has an enzymatic activity associated with the synthesis of melanin (17). Another enzyme associated with melanin synthesis is a tyrosinase-related protein (TRP-1), also called gp75. The gp75 protein was originally identified as an antigen recognized by serum IgG antibodies in a patient with melanoma (18, 19). Like tyrosine and gp100, gp75 is abundantly expressed in human pigmented melanocytic cells and melanoma (20).

The infusion of TIL586 plus IL-2 into the autologous patient with melanoma resulted in the objective regression of mediastinal metastases (21). This lead us to attempt to identify the tumor antigen recognized by TIL586. In the present study, we have used cDNA cloning strategy to isolate a gene encoding a shared human melanoma antigen recognized by TIL586 in association with HLA-A31. Analysis of the nucleotide sequence revealed that the cloned gene was almost identical to that previously reported for gp75.

Materials and Methods

Chemicals and Reagents. The following chemicals and reagents were purchased from the sources indicated: RPMI 1640, AIM-V media, Lipofectamine, G418 (GIBCO BRL, Gaithersburg, MD); the eukaryotic expression vector pcDNA3 (Invitrogen, San Diego, CA); anti–HLA-A31 monoclonal antibody (One lambda, Canoga Park, CA); anti–immunoglobulin M antibody conjugated with fluorescein isothiocyanate (Vector Laboratories, Inc., Burlingame, CA).

CTLs and Cell Lines. TIL586 were isolated from the tumor

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specimens of a patient with metastatic melanoma and grown in medium containing IL-2 (6,000 IU/ml) (Chiron Corp., Emeryville, CA) for 32–60 d as previously described (21). TIL586 were predominantly CD8$^+$ T cells. TIL888 were grown under the same conditions as described for TIL586.

Melanoma cell lines 397mel, 526mel, 586mel, 624mel, 888mel, and 928mel and EBV transformed B cell line 586EBVB were established in this laboratory and cultured in RPMI 1640 medium containing 10% FCS. Normal cultured melanocytes derived from infant foreskin (NHEM530 purchased from Clonetics Corp., San Diego, CA; FM902 and FM906 provided by Dr. M. Herlyn, The Wistar Institute, Philadelphia, PA) were cultured in melanocyte growth medium (MGM; Clonetics Corp.). The COS-7 cell line was provided by Dr. W. Leonard (National Institutes of Health, Bethesda, MD).

GM-CSF Secretion Assay. Responder cells (1×10^5) and stimulator cells (5×10^4–10^5) were mixed in 200 μl of AIM-V medium containing IL-2 (120 IU/ml) in a 96-well flat bottomed microplate. After incubation for 24 h, 100 μl of supernatant was collected and the amount of GM-CSF was measured in a standard ELISA assay (R+D System, Minneapolis, MN). Optical density was measured at 405 nm and the concentration of GM-CSF was calculated by comparison to GM-CSF standards in the same assay.

Isolation of the HLA-A31 Gene. Total mRNA was prepared from 586mel cells using an RNA isolation kit (Stratagene, La Jolla, CA). After the first-stranded cDNA was made using reverse transcriptase, a PCR was carried out using a pair of primers (5UTA and 3UTA) which was specific for the HLA-A allele and flanked the coding region (22). The PCR products were separated on 1.0% agarose gel and a 1.3-kb DNA fragment was isolated. Following BamHI and HindIII digestion, the DNA fragment was cloned into the pBK vector (Stratagene). The resultant plasmid was called pWBK101. The HLA-A31 gene was then sequenced using the dideoxynucleotide chain termination method (sequenase 2.0; United States Biomedical Corp., Cleveland, OH) to confirm that the sequence corresponded to the published DNA sequence.

cDNA Expression Cloning and Screening. A cDNA library prepared from 888mel was constructed as previously described (12) and used for screening. Plasmid DNAs were isolated from pools of bacteria containing 50–100 colonies. DNA was transfected into COS-7 cells using Lipofectamine (GIBCO BRL). Briefly, 5×10^4 cells were plated in 100 μl of DMEM (Biofluids, Inc., Rockville, MD) without serum in a flat bottomed 96-well microplate. 200 ng of DNA from each of the pools and 50 ng plasmid pWBK101 containing the HLA-A31 gene were mixed with 2 μl of lipofectamine in 100 μl of DMEM for 15–45 min. The DNA/lipofectamine mixture was then added to the COS-7 cells and incubated overnight. The following day, the transfection medium was removed, cells were washed twice with DMEM medium. TIL586 was added at a concentration of 10^5 cells/well in AIM-V medium containing 120 IU/ml of IL-2. After 18–24-h incubation, 100 μl of supernatant was evaluated for the release of GM-CSF.

Northern Blot Analysis. Total RNA was isolated by the guanidine isothiocyanate/cesium chloride centrifugation method. Total RNA from human normal tissue was purchased from Clontech (Palo Alto, CA). 20 μg of total RNA was subjected to electrophoresis in a 1.2% agarose formaldehyde gel and transferred to a nylon membrane. A 2.2-kb ApaI DNA fragment of gp75 gene was labeled by the random priming method. Prehybridization and hybridization were performed according to the QuickHyb protocol (Stratagene). Membranes were washed twice with 2× SSC/0.1% SDS at room temperature for 15 min and twice with 0.1× SSC/0.1% SDS at 60°C for 30 min. The autoradiography was performed at −70°C.

PCR Detection for gp75 Expression. Reverse transcription was performed using 5 μg of total RNA with an oligo(dT) primer. cDNA corresponding to 100–500 ng of total RNA was amplified for 30 cycles by PCR using primers gp1 and gp11B. 5 μl of PCR products were run on a 4% nusieve agarose gel stained with ethidium bromide.

Results

Identification of HLA-A31 as a Restriction Element for TIL586. Previous studies had shown that TIL586 lysed the autologous melanoma cell line and several allogeneic melanoma targets derived from different patients which shared HLA-A31, -A29, -Cw7, or -B44 (23). However, TIL586 failed to lyse autologous nonmelanoma targets including EBV-transformed B cells. Results in Table 1 indicated that an allogenic melanoma matched at the HLA-A31 locus stimulated GM-CSF release from TIL586 while four of five melanoma cell lines not expressing HLA-A31 or an EBV-transformed B cell line expressing HLA-A31 failed to stimulate significant GM-CSF release. A recent study showed that the HLA-A29 allele was deleted in the 586mel line (24) and that TIL586 recognized an antigen on the normal melanocyte cell line NHEM680 which shared HLA-A29, A31 and B44 (15). Therefore, these results suggested that TIL586 recognized a melanocyte/melanoma-specific antigen in the context of the HLA-A31. Interestingly, one melanoma cell line, 888mel (non-HLA-A31), did not express HLA-A31, but was found in this (Table 1) and other experiments to stimulate GM-CSF release from TIL586. TIL888, previously shown to recognize tyrosinase as

Table 1. *Specificity of Antigen Recognized by TIL586*

Stimulators		GM-CSF release	
Cell lines	HLA-A31 expression	TIL586	TIL888*
		pg/ml	
397mel	−	31	13
526mel	−	38	23
586mel	+	>5,000	17
624mel	−	25	12
888mel	−	323	2,750
928mel	−	15	14
1353mel	+	926	15
586EBVB	+	12	16

TIL586 secreted GM-CSF when cultured with HLA-A31 melanoma cells. GM-CSF in the supernatant was measured after TIL586 and stimulator cells were coincubated for 24 h. GM-CSF secreted by TILs alone without stimulators (<50 pg/ml) was subtracted. 586EBVB was a EBV transformed B cell line. All others were melanoma cell lines.

* TIL888 is a HLA-A24–restricted CTL which recognizes the tyrosinase antigen in 888mel (12).

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a tumor antigen in an HLA-A24–restricted fashion and sharing the HLA-Cw7 class I allele with TIL586, failed to respond to 586mel.

To confirm that HLA-A31 was the restriction element for TIL586, we isolated the HLA-A31 gene by RT-PCR and cloned it into pBK-CMV vector. The resultant plasmid was called pWBK101. We transfected pWBK101 into COS-7 cells and the human embryonic kidney cell line 293. The expression of the HLA-A31 gene expressed on the cell surface was confirmed by FACS® analysis using anti–HLA-A31 antibody (data not shown). To test whether HLA-A31 was functional in presenting the melanoma antigen to TIL586, we transfected the HLA-A31 cDNA into the HLA-A31 negative melanoma cell lines 397mel, 526mel, 624mel, and 888 mel and selected stable transfectants in G418-containing media. The expression of the HLA-A31 gene in the stable transfectants was evaluated by FACS® analysis. Transfectants of 526mel, 624mel, and 888mel expressing HLA-A31 significantly enhanced the stimulation of GM-CSF release from TIL586 whereas the HLA-A31 transfectant of 397mel failed to stimulate increased GM-CSF release (Table 2). This failure of 397mel to stimulate cytokine release from TIL586 was attributed to the lack of expression of the antigen recognized by TIL586 (Table 3). FACS® analysis demonstrated that a high level of HLA-A31 expression was found on the surface of 397mel-A31 (data not shown). These results indicated that TIL586 recognized a shared antigen expressed on a number of melanomas presented by the HLA-A31 MHC class I molecule.

Table 2. *Specific Secretion of GM-CSF by TIL586 Is HLA-A31 Restricted*

	Stimulators		
Cell lines	Transfected gene	HLA-A31 expression	GM-CSF secretion
			pg/ml
None	None	–	<10
397mel	None	–	47
397mel	HLA-A31	+	99
526mel	None	–	36
526mel	HLA-A31	+	537
624mel	None	–	39
624mel	HLA-A31	+	1,120
888mel	None	–	327
888mel	HLA-A31	+	1,090
586mel	None	+	>5,000
586EBVB	None	+	29

GM-CSF in the supernatant was measured after TILs were coincubated for 24 h with melanoma cell lines with or without stably transfected HLA-A31. GM-CSF secreted by TILs alone without stimulator (<50 pg/ml) was subtracted.

Table 3. *Expression of gp75 in Different Cell Lines Tested*

Melanoma cell lines	Expression of gp75	Melanoma cell lines	Expression of gp75
397mel	–	1011mel	+
526mel	+	1088mel	+
537mel	+	1102mel	–
553Bmel	+	C32	–
586mel	+	RPMI7951	–
624mel	+	WM115	–
677mel	+	HS695T	–
679mel	–	A375	–
697mel	+	SKnel	–
729mel	–	586EBVB	–
883mel	+	Melanocytes	
836mel	–	FM906	+
888mel	+	FM902	+
928mel	+		
938mel	–	Other tumor lines	
952mel	–	Daudi	–
978mel	–	MDA231	–

Expression of gp75 was tested by Northern blot analysis with 10–20 μg of total RNA and probed with the 2.2-kb ApaI DNA fragment and by RT-PCR amplification on total RNA with primers gp1 and gp11B. Daudi is a Burkitt's B cell line and MDA231 is a breast cancer cell line.

Cloning of a cDNA Encoding a Melanoma Antigen Recognized by TIL586. Since 888mel transfected with HLA-A31 increased the stimulation of GM-CSF release from TIL586 in comparison to non-transfected 888mel, 888mel seemed to express a shared antigen recognized by TIL586 and a cDNA library, which had previously been prepared with RNA from the 888mel cell line (12), was chosen for cloning this antigen.

Each cDNA pool containing 50–100 cDNA clones was cotransfected with the pWBK101 containing the HLA-A31 gene into COS-7 cells using lipofectamine and transfected COS-7 cells were tested for the ability to stimulate GM-CSF release by TIL586. After the screening of 300 cDNA pools, we identified four positive cDNA pools; three cDNA pools consistently conferred the ability to stimulate secretion of GM-CSF from TIL586 twofold higher than did the control plasmid, and one cDNA pool (pool 776) strongly stimulated GM-CSF release from TIL586. Individual cDNA clones were prepared from pool 776 and cotransfected into COS-7 cells with the HLA-A31 gene, and 14 out of 100 clones tested were found to confer recognition by TIL586. Restriction analysis demonstrated that the 14 positive clones appeared to contain a single 2.4-kd DNA insert which was called pcDNA776. A representative experiment is shown in Fig. 1. The level of GM-CSF release stimulated by COS-7 cells transfected with pcDNA776 was comparable to that stimulated by the 586mel

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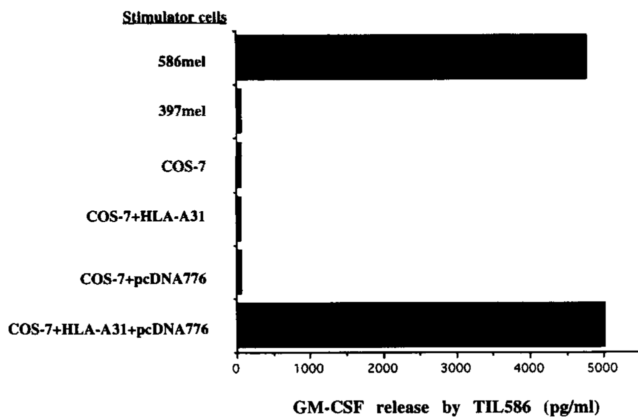

Figure 1. Northern blot analysis of the gp75 expression. 20 μg of total RNAs from different human tissues was loaded. After blotting, the membrane was probed with a 2.2-kb ApaI DNA fragment of the gp75 gene. The detected band for gp75 was marked. The 28S and 18S RNA were also marked and served as a indication of similar amount of RNA being used.

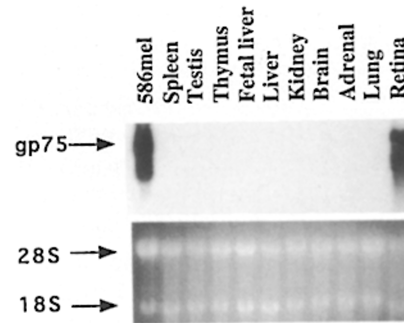

Figure 2. GM-CSF release by TIL586. GM-CSF secretion from TIL586 was significantly increased when co-incubated with COS-7 cells transfected with gp75 and HLA-A31 genes as described in Materials and Methods. Control stimulatory cells included HLA-A31 586mel, 397mel, COS cell alone, and COS cells transfected with either HLA-A31 or the gp75 cDNA. TIL586 was coincubated with stimulatory cells for 24 h and GM-CSF in the supernatant was measured.

cells, indicating that this may be a dominant antigen recognized by TIL586.

The pcDNA776 insert was sequenced and found to be almost identical to the cDNA sequence previously shown to encode melanocyte lineage specific protein, gp75 (19, 25). Sequence comparison between pcDNA776 and gp75 cDNA from GenBank (25) revealed that pcDNA776 represented a partial cDNA clone which lacked the first 375 bp and had a different 3′ end which appeared to be due to the usage of different polyadenylation sites. A full-length cDNA fragment (776FL) containing the entire coding region of gp75, isolated by PCR using primers located in the 5′ and 3′ untranslated regions of the gene, was cloned into the expression vector pcDNA3. Transfection of either plasmid into COS-7 along with the HLA-A31 gene conferred the ability to stimulate comparable level of GM-CSF secretion from TIL586 (data not shown).

The truncated cDNA776 clone lacked the authentic start codon and the leader peptide of the gp75 protein. The full length cDNA contained an open reading frame of 1,584 nucleotides coding for a polypeptide of 527 amino acids with a molecular weight of 60 kD. No difference was found between the amino acid sequence encoded by pcDNA776FL insert and gp75, indicating that TIL586 recognized a nonmutated peptide(s) derived from gp75. gp75, also called TRP-1 has approximately 40–45% amino acid sequence identity to tyrosinase (11, 12), gp100 (14, 26, 27) and TRP-2 (28).

Expression of Gene gp75. Northern blot analyses were performed using cDNA776 gene as a probe to evaluate the expression pattern of pg75 in different tissues. As shown in Fig. 2, only retina was shown to be positive among the ten normal human tissues tested. This result was confirmed by RT-PCR analysis (data not shown). The expression pattern of gp75 in melanoma cell lines and other cell lines is listed in the Table 3. 12 of 26 melanoma cell lines were positive. The level of expression in the two melanocyte cell lines tested was about 10-fold lower than that found in the positive melanoma cell lines. Two tumor lines tested, the Burkitt's B cell line Daudi and the breast cancer cell line MDA231, were negative. Thus, like tyrosinase, gp100 and MART-1, the expression pattern of this gene appeared to be restricted to melanoma, normal melanocyte cell lines, and retina.

Discussion

The gp75 gene is the sixth gene that has been identified as an antigen recognized by autologous CTL in patients with melanoma. MAGE-1 is expressed on a variety of tumor cells (melanoma, lung cancer, breast cancer) and testis, but not in other normal human tissues (9). Tyrosinase (11, 12), MART-1/Melan-A (15, 16), and gp100 (13, 14) have been identified as melanoma antigens and they were expressed only in normal melanocytes, melanomas, and retina, but not in other normal tissues.

Though sera from some melanoma patients contain antibodies against gp75, gp75 has not previously been identified as an antigen recognized by T cells. By the use of cDNA cloning, we have now identified the nonmutated gp75 as an antigen recognized by HLA-A31–restricted TIL586. This is further supported by the fact that TIL586 appeared to recognize the antigen on HLA-A31$^+$ allogeneic melanocyte and melanoma cell lines.

The gp75 protein is the most abundant intracellular glycoprotein expressed in human pigmented melanocytic cells and melanoma cells (20) and is the homologue of the mouse b (brown) locus gene product (19, 29). The gene coding for gp75 has been mapped to chromosome 4 in the mouse (29) and to chromosome 9, 9q23 region in the human by in situ hybridization (30). Genetic studies have shown that the murine b locus influences the biogenesis of melanosomes and controls the type of melanin produced (17). There was a direct correlation between melanin content and the expression of the four melanogenic proteins, tyrosinase, gp100, gp75 (TRP-1), and TRP-2 (17). The gp75 protein has recently been demonstrated to have DHI-2-carboxylic acid oxidase activity, which is important in melanin synthesis (31).

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The presence of CTL and IgG antibodies against gp75 in some patients with metastatic melanoma indicates that autoreactive T cells and B cells existed and may be activated in vivo. Since it is thought that high affinity lymphocytes are deleted in the induction of tolerance, only CTL with low or intermediate affinity are likely to exist against such an antigen (32). The presence of an immune response to an antigen shared by tumor and normal cells raises important questions about the nature and mechanism of immune responses to self antigens on growing tumor cells. The increased expression of gp75 in melanoma cells relative to that in melanocytes, as demonstrated by Northern blot analysis, might be one important factor in the induction of a T cell response to the self antigen gp75.

Shared differentiation antigens like those encoded by tyrosinase, MART-1/Melan-A, gp100, and gp75 may serve as useful targets for immunotherapy. Local depigmentation in melanoma patients has been reported to correlate with prolonged survival (33, 34) and clinical response to chemoimmunotherapy (35). The depigmentation may result from the destruction of melanocytes as a consequence of an immune response directed against these differentiation antigens. After receiving the infusion of 2.3×10^{11} TIL586 cells plus IL-2, patient 586 experienced partial regression (<50% reduction) of all measurable lesions, including two cervical cutaneous metastases, anterior mediastinal mass and a mass in the right lung. There were no adverse effects related to melanocyte destruction. These results suggest that CTLs directed against shared differentiation antigens may be useful for the immunotherapy of patients with melanoma. Nevertheless, it will be of importance to determine any adverse side effects on normal tissue upon immunization with these melanocyte lineage specific antigens including gp75, gp100, MART-1, and tyrosinase.

We would like to thank Drs. S. Topalian and J. R. Yannelli for providing cell lines and A. Mixon and E. B. Fitzgerald for FACS® analysis. We also would like to thank M. El-Gamil for her help in preparing the cDNA library, and Y. Li and S. Eliyahu for technical assistance.

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Received for publication 24 October 1994 and in revised form 8 November 1994.

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