A New Gene Coding for a Differentiation Antigen Recognized by Autologous Cytolytic T Lymphocytes on HLA-A2 Melanomas

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Summary

It has been reported previously that antitumor cytolytic T lymphocyte (CTL) clones can be isolated from blood lymphocytes of HLA-A2 melanoma patients, after stimulation in vitro with autologous tumor cells, and that some of these CTL clones lyse most HLA-A2 melanomas. A first antigen recognized by such CTL clones was previously shown to be encoded by the tyrosinase gene. We report here the identification of another gene that also directs the expression of an antigen recognized on most melanomas by CTL clones that are restricted by HLA-A2. The gene, designated Melan-A, is unrelated to any known gene. It is 18 kb long and comprises five exons. Like the tyrosinase gene, it is expressed in most melanoma tumor samples and, among normal cells, only in melanocytes.

When blood lymphocytes of melanoma patients are stimulated in vitro with autologous tumor cells in the presence of IL-2, one often observes proliferation of responder cells that exert a cytolytic activity on the tumor cells. From these responder cells it has been possible to obtain clones of cytolytic T lymphocytes (CTL)1, that lyse the autologous melanoma cells but do not recognize autologous fibroblasts, EBV-transformed autologous B cells, or targets cells that are lysed by NK-like effectors (1-5). Several groups have derived from HLA-A2 melanoma patients CTL that lyse not only the autologous tumor cells but also a large proportion of the melanoma cell lines derived from other HLA-A2 patients (6-9). Such CTL have been derived from both blood cells and from tumor-infiltrating lymphocytes.

By immunoselection with CTL clones derived from blood lymphocytes of patient SK29(AV), two distinct antigens were described that were shared by a high proportion of HLA-A2 melanomas. The antigens were named SK29-Aa and SK29-Ab (9). The latter is encoded by the tyrosinase gene, which is expressed only in melanocytes and melanomas (10).

We report here the identification of a second gene that codes for antigen SK29-Aa. The cloning approach involved the transfection of a cDNA library in COS cells, which are monkey kidney cells transfected with the gene coding for the SV40 large T antigen (11, 12). When these cells are transfected with plasmids containing the SV40 origin of replication, these plasmids replicate so as to produce $10^4$–$10^8$ copies per cell (13), resulting in a high level of expression of the cloned cDNA. Our data suggest that transfecting cDNA libraries into COS cells will be widely applicable for the identification of antigens recognized by T lymphocytes.

Materials and Methods

Cell Lines. Tumor cell lines LB39-MEL and LB33-MEL were derived from the metastatic melanomas of patients LB39 and LB33, respectively. The melanoma cells were cultured in Isco's medium supplemented with 10% FCS (both from GIBCO BRL, Gaithersburg, MD), l-arginine (116 mg/liter), l-asparagine (36 mg/liter), and l-glutamine (216 mg/liter). Melanoma cell lines SK29-MEL and SK23-MEL, and melanocyte samples, were gifts from Dr. L. Old (Memorial Sloan-Kettering Cancer Center, New York). SK29-MEL was cloned by limiting dilution and clone SK29-MEL.1 was selected for further experiments (9, 14). The antigen-loss variant of SK29-MEL has been described (9, 15). Melanoma cell line NA8-MEL was a gift from Dr. F. Jotereau (INSERM Unité 211, Nantes, France). SK29-MEL, SK23-MEL and NA8-MEL cells were cultured in DMEM (GIBCO BRL) containing 10% FCS,
10 mM HEPES and 4.5 g/liter glucose. COS-7 cells (American Type Culture Collection CRL 1651; Rockville, MD) were cultured in DME containing 10% FCS. WEHI-164 clone 13 (W13), a gift of Dr. T. Espevik (University of Trondheim, Trondheim, Norway; 16), was cultured in RPMI-1640 (GIBCO BRL) supplemented with 5% FCS.

Derivation and Culture of CTL Clones. Mixed lymphocyte-tumor cultures of PBL and tumor cells isolated from patient LB39 were performed as previously described (4) except that the medium was supplemented with 5 U/ml of human recombinant IL-2 (4 g/ml from R. Devos, Roche Research, Gent, Belgium). Derivation, long-term culture, and specificity analysis of CTL clones from PBL of patient SK29(AV) were previously described (9, 15).

Assay for Cytolytic Activity. The protocol was previously described (4). Target cells were treated for 48 h with 50 U/ml of human recombinant IFN-γ (Boehringer Mannheim, Mannheim, Germany). CTL and 1,000 ³Cr-labeled targets were incubated at various ratios in V-bottom microwells in a final volume of 200 µl. Chromium release in the supernatant was measured after 4 h of incubation.

Construction of the cDNA Library. Total RNA was isolated from clone SK29-MEL.1 and poly(A)⁺ RNA was prepared by oligo-dT binding (mRNA purification kit; Pharmacia Fine Chemicals, Piscataway, NJ). mRNA was converted to cDNA, ligated to EcoRI adaptors, and inserted into the EcoRI site of expression vector pcDNAI/Amp (Invitrogen Corporation, Oxon, UK) as described in the SuperScript plasmid system kit (GIBCO BRL). Recombinant plasmids were electroporated into JM101 Escherichia coli bacteria with a Gene pulser (Bio-rad Laboratories, Richmond, CA) at 1 pulse at 25 µF and 2,500 V, that were selected with ampicillin (50 µg/ml). Plasmid DNA from pools of bacteria was prepared as follows. Titrated suspensions of bacteria were seeded at 50, 100, or 150 bacteria per well in U-bottom microwells in 0.3 ml of tryptose, yeast extract, glycerol, phosphate, nitrate (TYGPN) medium (17). 10 microplates of each dilution were prepared. Bacteria were cultured for 48 h at 37°C. Control aliquots were titrated on agar, and only the set of microcultures containing close to 100 colonies per well were kept for the DNA extraction. Plasmid DNA was prepared in the microplates by the alkaline lysis method as described (17). The isopropanol-precipitated DNA were resuspended in 50 µl of Tris 10 mM, EDTA 1 mM, pH 7.4, containing 20 ng/ml of RNase. Southern blots were prepared with DNA isolated from 22 groups of 700,000 independent cosmids was constructed in cosmid c2RB (United States Biochemical Corp., Cleveland, OH). Complete sequences were obtained for introns 1, 2, and 4, and for the 3' and 5' ends of intron 3. The size of intron 3 was estimated as follows. Southern blots were prepared with cosmid DNA digested with EcoRI and Bgl II. A 7-kb EcoRI fragment hybridized with ³²P-labeled oligonucleotides deduced from the sequences of both ends of intron 3. The computer search for sequence homology was done with program FASTA on GenBank database release 79.0.

Transfection of a Melanoma Cell Line. Melanoma line NA8-MEL was transfected by the calcium phosphate precipitation method, as described (18). Briefly, 7.5 x 10⁶ cells were seeded in 4.5 ml of medium. 24 h later, they were transfected with 2 µg of plasmid pSVtkNeoB and 20 µg of plasmid pcDNAI/Amp containing cDNA AaG1. After 48 h, the cells were seeded in microcultures at 2,000 cells/well in 200 µl of medium containing 2 mg/ml of neomycin analog G418 (GIBCO BRL). NeoR colonies were selected for further experiments.

PCR Assays for Melan-A Expression. Isolation of total RNA from tumor samples was performed as described (20). RNA from melanocyte culture was a gift from Dr. Old. Reverse transcription was performed on 2 µg of total RNA with an oligo(dT) primer. cDNA corresponding to 100 ng of total RNA (10³ cell equivalents) was amplified for 33 cycles by PCR with the following primers: sense 5’-ACTGCTATCGGCTTG-3’, antisense 5’-TCAGCCATGTCAGGGT-3’. These primers are located in exons 2 and 5 to exclude the amplification of genomic DNA. An aliquot of the PCR reaction was run on a 1% agarose gel stained with ethidium bromide. To ensure that the RNA were not degraded, the cDNA products were tested for the presence of human β-actin.

Results

Blood lymphocytes of HLA-A2 melanoma patient LB39 were stimulated in vitro with irradiated cells of autologous tumor cell line LB39-MEL. From the responder T cell population, we isolated cytolytic T cell clone LB39-CTL-1/95, which lysed the autologous melanoma cell line (Fig. 1). This CTL clone also lysed melanoma cells from several other HLA-A2 melanoma patients, suggesting that its target antigen was presented by HLA-A2 (Fig. 1). This was confirmed by the observation that a previously described cell variant of melanoma line SK29-MEL that had lost HLA-A2 was not lysed by CTL 1/95 (Fig. 1).

The antigen recognized by CTL 1/95 appeared to be different from the previously described melanoma antigen SK29-Ab, which is encoded by tyrosinase, because an antigen-loss variant of SK29-MEL, which was resistant to antityros-
inase CTL clone SK29-CTL-IVSB, was still lysed by CTL 1/95 (Fig. 1). The target antigen of CTL 1/95 was named LB39-Aa. When CTL 1/95 was tested on other antigen-loss variants obtained from melanoma SK29-MEL, it showed the same lytic pattern as CTL clones obtained from patient SK29(AV) that recognize an antigen described as SK29-Aa (9). This suggested that antigen LB39-Aa was the same antigen as SK29-Aa.

Identification of a cDNA Coding for Antigen LB39-Aa. Because melanoma line SK29-MEL was lysed by CTL 1/95, we used a cDNA library that had been prepared with RNA from this cell line. This library had been prepared with expression vector pcDNAI/Amp. This vector carries the SV40 origin of replication which enables it to replicate autonomously in COS cells to large copy numbers (13). This library was divided into 800 pools of 100 bacteria. Each pool was expected to contain approximately 25 different cDNAs because only 50% of the plasmids contained an insert and the cloning was not directional. The HLA-A2.1 gene of patient SK29(AV) was also cloned into plasmid pcDNAI/Amp.

Each cDNA pool was cotransfected with the HLA-A2 construct into duplicate microcultures of COS-7 cells. After 48 h, the transfected COS cells were tested for expression of antigen LB39-Aa by their ability to stimulate the production of TNF by CTL 1/95. The CTL were added to the transfecants and after 24 h the concentration of TNF in the culture supernatant was measured by its cytotoxic effect on W13 cells. The amounts of TNF found in the supernatant showed a wide variation ranging from 2 to 7 pg/ml in most microcultures (Fig. 2). Six values stood out above 8 pg/ml. Among those we found two pairs of high duplicates. From each of the two pools of bacteria corresponding to these duplicate positives, we subcloned 800 bacteria. Plasmid DNA was extracted from each of the 1,600 bacteria and cotransfected into COS cells with the HLA-A2 construct. One clone, named AaG1, was found to confer recognition by CTL 1/95: COS cells transfected with this cDNA clone stimulated TNF release by the CTL (Fig. 3). They also stimulated CTL clone SK29-CTL-10/196, an HLA-A2-restricted CTL known to recognize on SK29-MEL the SK29-Aa antigen (9) (Fig. 3). The antityrosinase CTL IVSB was not stimulated by COS cells transfected with cDNA AaG1 (Fig. 3).

Stable transfectants were made in order to confirm the results obtained with COS cells. We used HLA-A2 melanoma cell line NA8-MEL which was not recognized by anti-Aa CTL 1/95. The cells were cotransfected with the pcDNAI/Amp plasmid containing cDNA AaG1 and with a plasmid conferring resistance to geneticin. Clones were isolated from the geneticin-resistant transfectants. They proved sensitive to lysis by CTL 1/95 (Fig. 4 A) and they also stimulated the production of TNF by this CTL (Fig. 4 B).

Structure of the Gene Coding for Antigen LB39-Aa. The sequence of cDNA clone AaG1 proved to be 675-bp long. When a Northern blot prepared with RNA of melanoma cell line SK29-MEL was hybridized with this cDNA, a band of ~0.75 kb was observed, suggesting that clone AaG1 was incomplete. It was used to screen again the cDNA library derived from SK29-MEL and a cDNA clone of 760 bp, named Aa84/3, was obtained. This cDNA clone was strictly identical to AaG1 except for the addition of 85 bp at the 5' end.

The sequence of cDNA Aa84/3 is shown on Fig. 5. No significant homology with any gene recorded in data banks was observed. The putative protein comprises 118 amino acids. It has no signal sequence. It is rich in proline residues (9%) and contains a hydrophobic region spanning residues 27-48 (Fig. 5).

To isolate the gene corresponding to cDNA AaG1, DNA was prepared from 22 groups of 70,000 cosmids of a human genomic library. The cDNA hybridized to nine cosmid groups. We subcloned the group that produced the strongest hybridization band and we identified one cosmid that hybridizes with cDNA clone AaG1. Genomic sequences corresponding to exons and adjacent intron regions were obtained by sequencing the cosmids with primers deduced from the cDNA...
sequence. The comparison of the cosmid and cDNA sequences indicated that gene Melan-A comprises five exons (Figs. 5 and 6). Further intron sequences were obtained using primers deduced from the first sequences. This led to the complete sequence of introns 1 (1,512 bp), 2 (5 kb), 4 (1,462 bp), and a partial sequence of intron 3 (Fig. 6). By hybridizing restriction fragments of the cosmid with oligonucleotides cor-

Figure 2. Stimulation of CTL 1/95 by COS cells cotransfected with HLA-A2 and pools of approximately 25 different cDNAs obtained from melanoma cell SK29-MEL. Each symbol represents the TNF content of one individual microculture, and the duplicate microplates are named A and A', B and B', etc. Duplicates of seemingly positive microcultures are connected with a line. Each pool of cDNAs was transfected into duplicate microcultures. Both the HLA-A2 gene and the cDNAs were cloned into expression vector pcDNAI/Amp. They were cotransfected with DEAE-Dextran into subconfluent COS cells. 48 h after transfection, CTL 1/95 (2,000 cells per well) was added. The culture supernatants were harvested 1 d later and tested on W13 cells for their TNF content.

Figure 3. Stimulation of CTL 1/95 by COS cells transfected with pcDNAI/Amp constructs containing HLA-A2 and cDNA AaG1 as described in Materials and Methods. Control stimulator cells included HLA-A2 melanoma cell line SK29-MEL and COS cells transfected with HLA-A2 alone. The transfectants also stimulated HLA-A2-restricted CTL clone 10/196 of patient SK29, which has been previously found to be directed against antigen LB39-Aa, and did not stimulate antityrosinase CTL clone SK29-CTL-IVSB.

Figure 4. Recognition of antigen LB39-Aa expressed in a transfectant obtained by cotransfecting HLA-A2 melanoma cell line NA8-MEL with the pcDNAI/Amp-cDNA AaG1 construct and pSVtkneoB. (A) Lysis of genicin-resistant transfectant clone NA8-MEL.c.l.1 by anti-LB39-Aa CTL 1/95. (B) Production of TNF by CTL 1/95 stimulated with clone NA8-MEL.c.1.1.
Figure 5. Sequence of the five exons of gene Melan-A and sequence of the protein encoded by the longest open reading frame. The nucleotide indicated as the first in exon 1 corresponds to the 5' end of cDNA clone Aa84/3. Box shows a polyadenylation site. A hydrophobic stretch of the protein is underlined. The cDNA sequence data are available from EMBL/GenBank/DDBJ under accession number HSU06654.

Table 1. Expression of the Melan-A gene

| Normal tissues          | Proportion of positive samples |
|-------------------------|--------------------------------|
| Melanocytes             | 2/2                            |
| Skin                    | 2/3                            |
| Liver                   | 0/1                            |
| Kidney                  | 0/1                            |
| Heart                   | 0/1                            |
| Prostate                | 0/1                            |
| Breast                  | 0/4                            |
| Ovary                   | 0/1                            |
| Testis                  | 0/2                            |
| Adrenals                | 0/3                            |
| Lung                    | 0/2                            |
| Fetal brain             | 0/1                            |
| Cerebellum              | 0/1                            |
| Substantia nigra        | 0/1                            |

Tumors

| Melanoma samples        | 26/26                          |
| Melanoma cell lines     | 12/21                          |
| Breast tumor samples    | 0/5                            |
| Sarcoma samples         | 0/5                            |
| Non small cell lung tumor samples | 0/5 |
| Renal carcinoma samples | 0/4                            |
| Colon carcinoma samples | 0/4                            |

The expression of gene Melan-A was tested by reverse transcription and specific PCR amplification. The melanoma samples and cell lines were derived from a random sampling of HLA-A2 and non HLA-A2 patients. The amount of PCR product did not vary significantly among the positive samples. All RNAs presented here showed similar amplification of the β-actin cDNA.

Discussion

Both the tyrosinase gene (10) and the Melan-A gene (this report) have been identified as genes coding for antigens recognized by autologous CTL on melanoma cells, using a procedure that involves high level transient expression of HLA genes and cDNAs in COS cells. This method differs from other procedures in that it is performed on primary cells such as melanoma cells and on autologous cells, which is likely why these genes were not recognized by CTL on other cell systems.

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