Molecular Analysis of the HLA-A2 Antigen Loss by Melanoma Cells SK-MEL-29.1.22 and SK-MEL-29.1.29

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ABSTRACT

Due to the potential clinical relevance of HLA class I antigen losses in melanoma cells and the scanty information about the molecular mechanisms underlying these defects, we have characterized the cause of the HLA-A2 antigen loss by autologous melanoma cell lines SK-MEL-29.1.22 and SK-MEL-29.1.29. Both cell lines have structural defects of the HLA-A2 gene, which cause lack of their transcription. In SK-MEL-29.1.22 cells the 5'-flanking region, exon 1, intron 1, and a region at the 5' end of exon 2 of the HLA-A2 gene are deleted. The breakpoint of the HLA-A2 gene, which is recombined with a DNA fragment of unknown origin, was localized between two GTTCG sequence repeats at position 101 of exon 2. These repeats may provide the sequence basis for misalignment in the process of DNA deletion. In SK-MEL-29.1.29 cells, loss of HLA-A2 antigens, as well as of HLA-B44 and HLA-Cw5 alleles, is caused by the loss of one copy of chromosome 6. Down-regulation of the expressed HLA class I alleles in the two HLA-A2 loss variants and in the parental cells was found to be associated with a low TAP1 expression and a reduced function of peptide transporters. Therefore, multiple defects result in loss of down-regulation of HLA class I alleles in SK-MEL-29.1.22 and SK-MEL-29.1.29 melanoma cells.

INTRODUCTION

Immunohistochemical staining of a large number of surgically removed lesions has shown that malignant transformation of melanocytes is frequently associated with abnormalities in HLA class I antigen expression (for review, see Ref. 1). The potential negative impact of these abnormalities on the clinical course of the disease (2) and on the outcome of T cell-based immunotherapy (3) has stimulated interest in the characterization of their molecular mechanisms. This information provides the background to develop strategies to screen melanoma lesions for defects in HLA class I antigen expression and to correct them.

Distinct molecular lesions have been identified in melanoma cells with HLA class I antigen down-regulation. Structural abnormalities of the B2m gene(s) that inhibit its transcription and/or translation have been found in melanoma cells without detectable HLA class I antigen expression (4–6). In addition, a marked reduction of HLA class I antigen expression can be caused by defects in the stable assembly and intracellular transport of the HLA class I heavy chain-B2-m peptide complex due to TAP abnormalities (6). Down-regulation of the gene products of the HLA-B locus has been reported to reflect reduced transcription associated with overexpression of c-myc oncogene (7). Loss of a HLA haplotype has been shown to result from deletion of a chromosomal unit in the MHC region on chromosome 6 (8). To the best of our knowledge, no information is available about the molecular mechanisms underlying the loss of a single HLA class I allospecificity by melanoma cells. Thus, we have characterized the molecular lesions that cause HLA-A2 allele loss in the melanoma cell lines SK-MEL-29.1.22 and SK-MEL-29.1.29. These two clones were derived from the melanoma cell line SK-MEL-29.1 by sequential mutagenesis with γ-irradiation and selection with HLA-A2 antigen-restricted, MAA-specific CTLs (9). The process of their generation resembles the series of events leading to the isolation of melanoma cells with loss of HLA class I antigens in patients with melanoma. Specifically, ionizing irradiation has been shown to induce mutations similar to the spontaneous ones, but with a different frequency (10, 11). Furthermore, immune selection by MAA-specific, HLA class I-restricted CTLs leads to the isolation of melanoma cells with HLA Class I antigen loss both in vitro and in vivo (9, 12, 13). We have selected the HLA-A2 allospecificity for our studies because it is often down-regulated in melanoma lesions (14, 15), it presents a large number of MAA-derived peptides to CTLs (16), and it is apparently immunodominant in recognition of melanoma cells by CTLs both in vitro and in vivo (9, 17). In the present study, we have also analyzed the expression and function of the antigen-processing machinery in SK-MEL-29.1.22 cells, in SK-MEL-29.1.29 cells, and in their parental cells, because their low HLA class I antigen expression which could be enhanced by IFN-γ (9) resembles the phenotype of renal carcinoma cell lines with a TAP down-regulation (18).

MATERIALS AND METHODS

Cell Lines. Cultured human cells were maintained in RPMI 1640 (Life Technologies, Inc., Grand Island, NY) supplemented with 10% serum plus (Hazelton Biologicals, Inc., Lenexa, KS) and 2 mm L-glutamine (Gemini Bio-Products, Inc., Calabasas, CA). Cells were cultured at 37°C in a 5% CO2 atmosphere and were harvested by incubation with PBS containing 1 mM EDTA.

mAbs and Conventional Antisera. The mAb W6/32 to a monomorphic determinant expressed on B2-m-associated HLA class I heavy chains, mAb TP25.99 to a determinant expressed on both B2-m-associated and B2-m-free HLA class I heavy chains, mAb HC-10 to a determinant expressed on B2-m-free HLA class I heavy chains, mAb LGII-220.6 to a determinant restricted to the gene products of the HLA-A locus, mAb H2-89-1 to a determinant restricted to the gene products of the HLA-B locus, anti-HLA-A2, A28 mAb CR11–351 and HO-1, anti-HLA-A2, B17 mAb HO-2 and MA2.1, anti-β2-m mAb BM0M.1, and anti-TAP1 mAb 148.3 were developed and characterized following the methodology described elsewhere (4, 19–26).

The rabbit antiserum R5996–4 reacting with denatured HLA class I heavy chains was developed as described (27). FITC-GAM antibodies, alkaline phosphatase-conjugated antiserum to IgG antibodies, and peroxidase-conjugated antiserum to IgG antibodies were purchased from Jackson Immunotech Research Inc. (Avondale, PA) and from Dako A/S, (Glostrup, Denmark), respectively.

Cytokine and Restriction Enzymes. Recombinant human IFN-γ was obtained from Hoffman-La Roche, Inc. (Nutley, NJ). Restriction enzymes HindIII, BglII, and Smal were purchased from Boehringer Mannheim (Indianapolis, IN). The restriction enzyme NalI was purchased from New England Biolabs Inc. (Beverly, MA).

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Molecular Mechanisms of HLA-A2 Loss by Melanoma Cells

Table 1 HLA class I heavy chain and β2-m-specific oligonucleotide primers

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<tr>
<th>Location</th>
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<th>Specificity</th>
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<tr>
<td>5pE2h</td>
<td>CCCGAAGGGCCGAGTTAAGAT</td>
<td>-</td>
<td>-237–217</td>
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<td>5pE1</td>
<td>CCTCTGCTCGCTACCTCCG</td>
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<tr>
<td>AP1</td>
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<td>5pE3</td>
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<td>5pB1E2</td>
<td>GACCCGGCCAGCACAGATCTTC</td>
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<td>HLA-B13, 15, 37, 40, 41, 44, 45, 47, 49, 50, 52</td>
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<td>27'</td>
<td>CCCGAAGGGCCGAGTTAAGAA</td>
<td>2</td>
<td>221–239</td>
<td>HLA-Cw5, 6, 15</td>
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<td>5pCE2</td>
<td>ACACAAGAACTTACACGCAGCCAGG</td>
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<td>P1</td>
<td>GTGGAGACATTCAAGACTGGTTC</td>
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<td>139–158</td>
<td>β2-m</td>
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<td>3'primers</td>
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<tr>
<td>AP2</td>
<td>TACCTTCTCGCTTCC</td>
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<td>3pE3W</td>
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<td>3pE3'</td>
<td>TGACGCTTCTTCTTCCCAGT</td>
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<td>3pB44E3</td>
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<tr>
<td>42'</td>
<td>CCGCCGGCTGAGCCGCTTT</td>
<td>3</td>
<td>258–275</td>
<td>HLA-Cw5</td>
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<tr>
<td>P2</td>
<td>GCAGCTGCTCATAATTCTGATC</td>
<td>1</td>
<td>166–185</td>
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* 5'-Flanking region of the class I gene.
* Described by Fernandez-Vina et al. (28).
* The first nine nucleotides at the 5'-end are homologous to the last nine nucleotides at the 3'-end of intron 1.
* Described by Oh et al. (29).
* Described by Bunce et al. (30).
* Described by Levine and Yang (31).
* Intron 2 of β2-m gene.

Synthetic Oligonucleotide Primers and Probes, cDNA Probes, HLA-A2 Gene, and Synthetic Peptides. The characteristics of the HLA class I and β2-m gene-specific synthetic oligonucleotide primers and sequence-specific oligonucleotide probes that were used in this study are listed in Tables 1 and 2, respectively. The oligonucleotides were synthesized on a DNA synthesizer (Milligen/Biosearch, Burlington, MA), with the exception of those kindly provided by Dr. S. Y. Yang (Memorial Sloan-Kettering Cancer Center, New York, NY). Oligonucleotide probes were end-labeled with [α-32P]ATP (5000 Ci/mmol) in the presence of T4 polynucleotide kinase (32). The HLA-A locus-specific cDNA probe pHLA-2a0.1 (33) was obtained from Dr. H. T. Orr (University of Minnesota School of Medicine, Minneapolis, MN). This probe was labeled with [α-35S]methionine, solubilization with NP-40, indirect immunoprecipitation, SDS-PAGE, one-dimensional IEF, and fluorography were performed as described (4, 38). Western blotting was performed using the Amersham ECL system (Amersham International plc, Buckinghamshire, United Kingdom) following the manufacturer's instructions.

RNA and Genomic DNA Isolation. Total RNA was isolated from cells using the method described by Davis et al. (39). Genomic DNA used for Southern blot analysis and for PCR amplification was extracted from cells using the method described by Verbal (40) and the cell lysis method (41) followed by proteinase K digestion, respectively.

Transfection of Cells with a Wild-type HLA-A2 Gene. Plasmid pHLA-A2 was transfected into cells using the electroporation method as described elsewhere (4). Three days after transfection, G418 was added to the medium to a final concentration of 0.8 mg/ml and maintained for 2 weeks. Cell colonies were picked and cultured for 1 month in medium supplemented with G418 (0.4 mg/ml).

PCR and cDNA and Genomic DNA Amplification. cDNA reverse-transcribed from total RNA as described (42) was used as a template for PCR amplification. PCR amplification of cDNA and of genomic DNA was performed as described (30, 37).

Southern Hybridizaion. PCR products and restriction enzyme-digested genomic DNA were size-fractionated, transferred to nitrocellulose filters or nylon membranes, and hybridized with probes, as described elsewhere (4, 5, 33).

Table 2 HLA class I allele-specific oligonucleotide probes

<table>
<thead>
<tr>
<th>Location</th>
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<th>Exon</th>
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<tr>
<td>62G</td>
<td>GAGGAGGAGGACACGG</td>
<td>2</td>
<td>176–190</td>
<td>HLA-A2</td>
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<tr>
<td>142TK</td>
<td>ACCACCAAGCACAAG</td>
<td>3</td>
<td>180–194</td>
<td>HLA-A2, 28</td>
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<td>95V</td>
<td>CACACCGCTCAGAGG</td>
<td>3</td>
<td>7–21</td>
<td>HLA-A2, 69</td>
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<td>62RN</td>
<td>GAGGAGGAGGACACGG</td>
<td>2</td>
<td>176–192</td>
<td>HLA-A19, 25, 26, 28, 33, 34, 66</td>
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<tr>
<td>236CE2</td>
<td>GAGGAGGAGGACACGG</td>
<td>2</td>
<td>232–246</td>
<td>HLA-B13, 44</td>
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<td>76TAE3</td>
<td>ACCACAGTGCTCAGC</td>
<td>3</td>
<td>70–84</td>
<td>HLA-B13, 45, 49, 50, 54, 55, 56</td>
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<tr>
<td>229CE</td>
<td>CGGAGGAGCACTCCA</td>
<td>3</td>
<td>223–235</td>
<td>HLA-B44, 45</td>
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<td>97W</td>
<td>ACATCACTGGAG</td>
<td>3</td>
<td>12–24</td>
<td>HLA-Cw1, 6, 14</td>
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<td>138K</td>
<td>GGCCTCTGTCCG</td>
<td>3</td>
<td>136–148</td>
<td>HLA-Cw5, 8</td>
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</tbody>
</table>

* Described by Oh et al. (29).
* Provided by Dr. S. Y. Yang.
* Described by Levine and Yang (31).

Immunochemical Methods. Radiolabeling of cells with [35S]methionine, solubilization with NP-40, indirect immunoprecipitation, SDS-PAGE, one-dimensional IEF, and fluorography were performed as described (4, 38).

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Inverted PCR and DNA Sequencing. Genomic DNA (5 µg) was digested with NdeIII. After phenol-chloroform extraction and ethanol precipitation, DNA (2 µg/ml) was self-ligated in the presence of T4 DNA ligase under conditions favoring circularization. Following linearization by digestion with SmaI, DNA (200 ng) was amplified by PCR using primers 5'pE3 and 3'pE2. PCR products were cloned into the TA cloning vector (Invitrogen, San Diego, CA) following the manufacturer’s instructions. Plasmid DNA was isolated from white colonies and digested with EcoRI. The insert was analyzed by 1.5% agarose gel electrophoresis and by dot blot with HLA-A2-specific probe 62G. DNA sequencing of selected inserts was performed as described elsewhere (43), using the Sequenase Version 2.0 kit (United States Biochemical Corp., Cleveland, OH).

Peptide Translocation Assay. This assay was performed as described (44).

RESULTS

Lack of HLA-A2 Antigen Cell Surface Expression by Melanoma Cell Lines SK-MEL-29.1.22 and SK-MEL-29.1.29. The clones SK-MEL-29.1.22 and SK-MEL-29.1.29 derived from the HLA-A2, A28, B44, Bw45, Cw5, Cw6 melanoma cell line SK-MEL-29.1 were not stained in IIF by anti-HLA-A2, B17 mAb HO-2, and MA2.1, although the latter two mAb stained the parental melanoma cell line SK-MEL-29.1. In contrast, SK-MEL-29.1.22 and SK-MEL-29.1.29 cells, like the parental cell line, were stained by mAb TP25.99 and W6/32 to monomorphic determinants of HLA-A, B, and C antigens, by anti-HLA-A mAb LGIII-220.6, by anti-HLA-B mAb H2-89.1, and by anti-HLA-A2, A28 mAb CR11-351. Incubation for 72 h with IFN-γ (100 units/ml) did not induce reactivity of SK-MEL-29.1.22 and SK-MEL-29.1.29 cells with anti-HLA-A2, B17 mAb HO-2, and MA2.1 but increased the percentage of stained cells and the intensity of staining by all of the other anti-HLA class I mAb tested. Representative staining profiles are shown in Fig. 1. These results indicate a selective lack of HLA-A2 antigen cell surface expression by SK-MEL-29.1.22 and SK-MEL-29.1.29 cells. It is noteworthy that the intensity of staining of SK-MEL-29.1.22 and SK-MEL-29.1.29 cells by anti-HLA class I, anti-HLA-A, and anti-HLA-A2 A28 mAb is not lower than that of the parental cells. These results are compatible with the possibility that SK-MEL-29.1.22 and SK-MEL-29.1.29 cells compensate the lack of HLA-A2 antigen expression by increasing that of the other allospecificities.

Lack of HLA-A2 Heavy Chain Synthesis by SK-MEL-29.1.22 Cells and of HLA-A2, B44, and Cw5 Heavy Chain Synthesis by SK-MEL-29.1.29 Cells. To determine whether HLA-A2 heavy chains are synthesized by SK-MEL-29.1.22 and SK-MEL-29.1.29 cells, a NP-40 extract of the two [35S]methionine-labeled cell lines was immunoprecipitated with anti-HLA-A2, B17 mAb HO-2, anti-β2-m-associated HLA-A, -B, and -C heavy chain mAb W6/32, and rabbit anti-HLA class I heavy chain serum R5996-4. The isolated antigens were analyzed by SDS-PAGE and by one-dimensional IEF. mAb HO-2 immunoprecipitated no components from SK-MEL-29.1.22 and SK-MEL-29.1.29 cells, but immunoprecipitated the characteristic 44- and 12-kDa subunits of HLA class I antigens from the parental cell line (data not shown). The latter two components were also immunoprecipitated from the three melanoma cell lines by the remaining anti-HLA class I mAb. Analysis by one-dimensional IEF did not detect the HLA-A2 heavy chain among the components immunoprecipitated from IFN-γ-treated SK-MEL-29.1.22 and SK-MEL-29.1.29 cells by mAb W6/32 and by rabbit anti-HLA class I heavy chain serum R5996-4. In contrast, HLA-A2, B44, Bw45, Cw5, and Cw6 heavy chains were detected in the immunoprecipitates from IFN-γ-treated SK-MEL-29.1.22 cells and only HLA-A2, Bw45 and Cw6 heavy chains in the immunoprecipitates from IFN-γ-treated SK-MEL-29.1.29 cells. Representative results are shown in Fig. 2. All of the above-mentioned HLA class I heavy chains, as well as HLA-A2 heavy chains, were detected in the immunoprecipitates from parental cells by the anti-HLA class I mAb used. The intensity of the components corresponding to HLA-A2 and Bw45 alloantigens isolated from IFN-γ-treated SK-MEL-29.1.22 and SK-MEL-29.1.29 cells was higher than that of the components isolated from IFN-γ-treated parental cells. In contrast, the intensity of the component corresponding to HLA-B44 alloantigen isolated from SK-MEL-29.1.22 cells is lower than that of the component isolated from parental cells. Besides corroborating the serological findings, these results indicate that SK-MEL-29.1.22 and SK-MEL-29.1.29 cells do not synthesize HLA-A2 heavy chains and that, in addition, SK-MEL-29.1.22 cells do not synthesize B44 and Cw5 heavy chains.

Lack of HLA-A2 mRNA in SK-MEL-29.1.22 Cells and of HLA-A2, B44, and Cw5 mRNA in SK-MEL-29.1.29 Cells. To characterize the defects in HLA class I antigen expression at the mRNA level in SK-MEL-29.1.22 and SK-MEL-29.1.29 cells, cDNA was synthesized from RNA isolated from the two cell lines using the HLA class I-specific 3'-primer 3pE3. DNA was amplified by PCR using the primers indicated in Fig. 3. RT-PCR products for HLA-A, -B, and -C alloantigens with the expected sizes of 434, 352, and 340 bp, respect-
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Fig. 2. One-dimensional IEF analysis of HLA class I allospecificities isolated from SK-MEL-29.1.22 and SK-MEL-29.1.29 cells. Following a 72-h incubation at 37°C with IFN-γ (100 units/ml) SK-MEL-29.1.22 and SK-MEL-29.1.29 cells were metabolically labeled with [35S]methionine and solubilized with 1% NP-40. Antigens immunoprecipitated by anti-β2-m-associated HLA-A, -B, and -C heavy chain mAb W6/32, by anti-HLA-A2, B17 mAb HO-2 and by anti-HLA-A2, A28 mAb HO-1 were treated with neuraminidase and analyzed by one-dimensional IEF using a vertical slab gel apparatus. Gels were then processed for fluorography. HLA class I allospecificities isolated from IFN-γ-treated parental SK-MEL-29.1 cells were used for comparison.

Fig. 3. Southern blot analysis of HLA class I heavy chain cDNA amplified by RT-PCR from RNA isolated from SK-MEL-29.1.22 and SK-MEL-29.1.29 cells. HLA class I heavy chain cDNA was synthesized from total cellular RNA isolated from IFN-γ-treated (100 units/ml for 72 h at 37°C) SK-MEL-29.1.22 and SK-MEL-29.1.29 cells by RT reaction using HLA class I-specific 5′-primer 3pE3, HLA-A, -B, and -C allele cDNAs were then amplified by PCR using HLA-A locus-specific 5′-primer 5pE2A (A), HLA-B13, -15, -37, -40, -41, -44, -45, -47, -48, -49, -50, and -52-specific 5′-primer 5pB1E2 (B), and HLA-C locus-specific 5′-primer 5pC2E (C), respectively, paired with primer 3pE3. RT-PCR products were fractionated by 1.5% agarose gel electrophoresis and transferred to nylon membranes. Blots were hybridized with the indicated 32P-labeled oligonucleotide probes. RT-PCR products from parental SK-MEL-29.1 cells were used as positive controls. RT-PCR products from (HLA-A11, A24) Colo 38 melanoma cells and from (HLA-A24, B60) DKB and (HLA-A29, B12, Cw3) MANN lymphoid cells were used as negative controls.

Table 1. Southern blot analysis of HLA class I heavy chain cDNA amplified by RT-PCR from RNA isolated from SK-MEL-29.1.22 and SK-MEL-29.1.29 cells. Following a 72-h incubation at 37°C with IFN-γ (100 units/ml) SK-MEL-29.1.22 and SK-MEL-29.1.29 cells were metabolically labeled with [35S]methionine and solubilized with 1% NP-40. Antigens immunoprecipitated by anti-β2-m-associated HLA-A, -B, and -C heavy chain mAb W6/32, by anti-HLA-A2, B17 mAb HO-2 and by anti-HLA-A2, A28 mAb HO-1 were treated with neuraminidase and analyzed by one-dimensional IEF using a vertical slab gel apparatus. Gels were then processed for fluorography. HLA class I allospecificities isolated from IFN-γ-treated parental SK-MEL-29.1 cells were used for comparison.

MOLECULAR MECHANISMS OF HLA-A2 LOSS BY MELANOMA CELLS

Restoration of HLA-A2 Antigen Expression by SK-MEL-29.1.22 and SK-MEL-29.1.29 Cells Following Transfection of a Wild-type HLA-A2 Gene. To determine whether HLA-A2 antigen expression by SK-MEL-29.1.22 and SK-MEL-29.1.29 cells could be restored by transfection of a wild-type HLA-A2 gene, the two cell lines were transfected with a plasmid containing a wild-type HLA-A2 gene and neomycin resistance gene. Following selection in medium supplemented with G418, two G418-resistant SK-MEL-29.1.22 clones and one G418-resistant SK-MEL-29.1.29 clone were stained in IIF by anti-HLA-A2, B17 mAb HO-2 (Fig. 4). Furthermore, the HLA-A2 heavy chains were identified by one-dimensional IEF analysis in the immunoprecipitates by mAb W6/32 and TP25.99 from two SK-MEL-29.1.22 transfected clones pretreated with IFN-γ. The intensity of HLA-A2 heavy chains was higher than that of those immunoprecipitated from parental cells SK-MEL-29.1 (Fig. 5). In contrast, the intensity of HLA-B44 and Cw6 heavy chains immunoprecipitated by mAb W6/32 from the two transfectant clones was lower than that of the heavy chains immunoprecipitated by mAb TP25.99. These findings may reflect the low efficiency of association of HLA-B44 and Cw6 heavy chains with β2-m. The reconstitution of HLA-A2 antigen expression by SK-MEL-29.1.22 and SK-MEL-29.1.29 cells following transfection with a wild-type HLA-A2 gene indicates that lack of HLA-A2 antigen expression is caused by structural abnormalities in the HLA-A2 genes in these two cell lines.

Change of Restriction Pattern of HLA-A Gene(s) in SK-MEL-29.1.22 Cells. To define the abnormalities of HLA-A2 gene in SK-MEL-29.1.22 and SK-MEL-29.1.29 cells, genomic DNA isolated from these two cell lines and from the parental cell line was digested with HindIII and BglII. Hybridization with the HLA-A locus-specific cDNA probe pHLA-2a.1 identified a 5.1- and a 15.1-kb fragment in DNA from the three cell lines digested with HindIII and BglII, respectively (Fig. 6). Furthermore, the pHLA-2a.1 probe hybridized to a 7.1-kb fragment in HindIII-digested DNA from SK-MEL-29.1.22 cells (Fig. 6). These results suggest the presence of a lesion in the 5′ region of HLA-A2 gene involving the HindIII restriction site in SK-
MOLECULAR MECHANISMS OF HLA-A2 LOSS BY MELANOMA CELLS

SK-MEL-29.1.22 HLA-A2 gene SK-MEL-29.1.29 HLA-A2 gene mAb

Fig. 4. Reconstitution of HLA-A2 antigen expression by SK-MEL-29.1.22 and SK-MEL-29.1.29 cells following transfection with a wild-type HLA-A2 gene. Following transfection with a wild-type HLA-A2 gene and subsequent selection with G418, G418-resistant SK-MEL-29.1.22 cell clones (a and b) and SK-MEL-29.1.29 cell clone (c) were expanded. Cells were then harvested and incubated with anti-HLA-A2, B7 mAb HO-2. Following washings with PBS, cells were stained with FITC-GAM. Fluorescence intensity was determined on a FACS analyzer. Background was determined by incubation of cells with FITC-GAM alone.

Fig. 5. One-dimensional IEF analysis of HLA class I allospecificities isolated from SK-MEL-29.1.22 cells transfected with a wild-type HLA-A2 gene. Following a 72-h incubation at 37°C with IFN-γ (100 units/ml), two HLA-A2-transfected SK-MEL-29.1.22 clones were metabolically labeled with [35S]methionine and solubilized with 1% NP-40. Antigens immunoprecipitated by anti-β2-m-associated HLA-A, -B, and -C heavy chain mAb W6/32 and by anti-β2-m-associated and β2-m free HLA class I heavy chain mAb TP25.99 were treated with neuraminidase and analyzed by one-dimensional IEF using a vertical slab gel apparatus. Gels were then processed for fluorography. HLA class I allospecificities isolated from IFN-γ-treated parental SK-MEL-29.1 cells were used for comparison.

Fig. 6. Southern blot analysis of HLA-A locus genes isolated from SK-MEL-29.1.22 and SK-MEL-29.1.29 cells. Genomic DNA (20 μg) isolated from SK-MEL-29.1.22 and SK-MEL-29.1.29 cells was digested overnight at 37°C with restriction enzymes BglII and HindIII. Digested DNA was fractionated on a 0.8% agarose gel and transferred to a nylon membrane. Blots were hybridized with 32P-labeled HLA-A locus-specific cDNA probe pHLA-2a.1. The region to which this probe is homologous and the restriction sites of BglII (B) and HindIII (H) in the HLA-A2 gene are shown at the bottom of the figure. DNA from parental SK-MEL-29.1 cells was used as a control.
Identification of the Breakpoint in the HLA-A2 Gene in SK-MEL-29.1.22 Cells. To identify the breakpoint in the HLA-A2 gene in SK-MEL-29.1.22 cells, a DNA fragment spanning from a region upstream of position 114 in exon 2 to position 139 in exon 3 of the HLA-A2 gene was generated by digesting genomic DNA with the restriction enzyme NsiI (Fig. 8A). Self-ligation of this fragment resulted in the insertion of the upstream region between the 5′ end of the remaining exon 2 and the 3′ end of exon 3. This DNA segment was amplified by PCR using the primers 5pE3 and 3pE2 (Fig. 8A). Genomic DNA from the parental cells SK-MEL-29.1 and DNA from the pHLA-A2 plasmid carrying a HLA-A2 gene were used as controls. A 277-bp and a 250-bp fragment were obtained from DNA from SK-MEL-29.1.22 cells, whereas only a 277-bp fragment was obtained from DNA from SK-MEL-29.1 cells and from the HLA-A2 gene (Fig. 8B). The HLA-A2-specific probe 62G hybridized only to the 250-bp fragment from SK-MEL-29.1.22 cells and to the 277-bp fragment from SK-MEL-29.1 cells and from the HLA-A2 gene (Fig. 8B). These results suggest that the 250-bp fragment obtained from SK-MEL-29.1.22 cells results from the recombination of exon 2 of the HLA-A2 gene with a DNA fragment of unknown origin. To prove this possibility, PCR products from SK-MEL-29.1.22 cells were cloned into the TA cloning vector. Plasmids carrying the 250-bp fragment were identified by digestion with EcoRI, agarose gel electrophoresis, and dot blot with the probe 62G. Plasmids containing inserts with the appropriate size, which hybridize to the 62G probe, were used for DNA sequencing. At position 102 of exon 2, the HLA-A2 gene is recombined with a DNA fragment, which shows no homology with HLA class I genes (Fig. 8C). This DNA fragment shows an 80% homology to the nucleotide sequence from position 12878 to 12944 of intron 7 of the gene for cytokeratin 20.

Loss of HLA-B44 and Cw5 Genes in SK-MEL-29.1.29 Cells. The lack of HLA-A2 gene detection and of HLA-B44 and Cw5 gene expression in SK-MEL-29.1.29 cells reflects loss of the corresponding genes, because no fragment was amplified by PCR from genomic DNA, using the HLA-B44-specific primers 5pBI2E and 3pB44E3 and the HLA-Cw5-specific primers 27 and 42 (Fig. 9). These results do not reflect low efficiency of PCR amplification, because the amount of a 342-bp Bm gene fragment amplified from the DNA of SK-MEL-29.1.29 cells was similar to that from control cell lines. These findings indicate that HLA-B44 and Cw5 genes, as well as the HLA-A2 gene, are deleted in SK-MEL-29.1.29 cells.

Abnormalities of the HLA class I Antigen-processing Machinery in SK-MEL-29 Cells. The low HLA class I antigen expression by SK-MEL-29.1.22 and SK-MEL-29.1.29 cells, as well as by the parental SK-MEL-29.1 cells, as compared to that by the autologous B lymphoid cells SK-MEL-29.1 EBV (Table 3) reflects an impaired antigen processing, because the stability of HLA class I antigens is reduced in the three melanoma cell lines. As shown in Table 3, incubation at 26°C for 16 h induced an approximately 2-fold increase of the level of HLA class I antigen expression by the three melanoma cell lines but did not affect that by the autologous lymphoid cells. Incubation with IFN-γ (400 units/ml) for 24 h enhanced the level of HLA class I antigen expression on melanoma and autologous lymphoid cells almost 2-fold. The existence of deficiencies in the antigen-processing machinery in the three melanoma cell lines is also indicated by the reduced level of TAP1 as compared to that in the autologous B lymphoid cells (Table 3). The level of TAP1 was increased by about 50% in all melanoma cell lines following incubation with IFN-γ (400 units/ml) for 24 h. Furthermore, the peptide translocation assay showed that the activity of the peptide transporters is about 50% lower in the three melanoma cell lines than in the autologous B lymphoid cells (Table 4), whereas no difference was found among the three melanoma cell lines.

DISCUSSION

The present study has shown that distinct structural defects underlie the HLA-A2 allele loss by the autologous melanoma cell lines SK-MEL-29.1.22 and SK-MEL-29.1.29. HLA-A2 allele loss is associated with loss of the HLA-B44 and Cw5 alleles in SK-MEL-29.1.29 cells. As detected by karyotyping analysis, this phenotype reflects the loss of one copy of chromosome 6 where the MHC region maps (45). Loss of one copy of chromosome 6 in melanoma cells has also been described by Ochi et al. (46). If the MHC region within the deleted
chromosome 6 is not translocated, one HLA haplotype is lost. An alternative mechanism for haplotype loss is deletion of a genomic fragment within chromosome 6 that is undetectable by karyotyping (8).

In SK-MEL-29.1.22 cells, the HLA-A2 gene is not transcribed because of the loss of its transcriptional regulatory region, exon 1, intron 1, and the first 101 bases of exon 2. The size of the deleted region is likely to be more than 830 bp, based on the structure of the HLA-A2 gene (47). The fragment of HLA-A2 gene is joined to a DNA fragment with no sequence homology to HLA class I allospecificities ranging from the loss of a single allele to that of a full haplotype depending on the position of DNA breakage and wild-type HLA-A2 allele has been selectively eliminated from the genome of the cell line SK-MEL-29.1.22.

The region from the middle of exon 2 to all of the downstream exons and introns of the HLA-A2 gene is present in SK-MEL-29.1.22 cells, because part of exon 2 and the whole exon 3 were amplified by genomic PCR. Furthermore, Southern blot analysis detected no change in the BglIII restriction site in intron 3. Lastly, exon 8 of the HLA-A2 gene was detected in SK-MEL-29.1.22 cells by hybridization to an HLA-A locus-specific probe. The HLA-A2 gene fragment does not appear to be transcribed to form an abnormal unstable and rapidly degraded mRNA, because HLA-A2 cDNA could not be amplified using the primers corresponding to the regions downstream the position 101 of exon 2 of the HLA-A2 gene.

It is interesting that the DNA breakpoint in exon 2 of the HLA-A2 gene in SK-MEL-29.1.22 cells is located between two GTTCG repeats. One copy of the repeats and the sequence between them are deleted from the HLA-A2 gene. This type of change resembles the spontaneous deletion following misalignment of one of the sequence repeats on the other one and excision of DNA strand by endonuclease (48). Such a mutational event and γ-irradiation-induced DNA damage might be instrumental in causing the molecular lesion in exon 2 of the HLA-A2 gene in SK-MEL-29.1.22 cells.

The partial deletion and rearrangement of HLA-A2 gene found in SK-MEL-29.1.22 cells are similar to those described in two lymphoid cell clones with spontaneous HLA-A2 antigen loss (49). Changes of HindIII restriction pattern of the HLA-A2 gene was the only abnormality described in the latter two cell clones. In contrast, the structural lesions that we have found in SK-MEL-29.1-22 and SK-MEL-29.1.29 cells are different from those found in other melanoma cell lines (8) and other types of malignant cells with selective loss of HLA class I allospecificities (50, 51). Among the lesions identified is the deletion of genomic DNA fragments, which may result in the loss of HLA class I allospecificities ranging from the loss of a single allele to that of a full haplotype depending on the position of DNA breakage and
the extent of the deleted region. These abnormalities have been recently described in the melanoma cell lines 553-MEL and 1195-MEL, which have lost multiple HLA class I specificities, including HLA-A2, as well as in the melanoma cell line 586-MEL, which has lost a full haplotype. It is noteworthy that rearrangement of chromosome 6, where the MHC region maps (45), frequently occurs in malignant melanoma with progression of the disease (52).

In agreement with our previous findings (15), the present study has shown a selective increase of HLA-A28 and B45 allele expression by the two HLA-A2 loss mutant melanoma cell lines as compared with their HLA-A2 positive parental cell line. These results are likely to reflect the availability of the class I heavy chains resulting from the lack of competition by HLA-A2 heavy chains, because the levels of B27-associated HLA-A28 and B45 heavy chains immunoprecipitated from the two HLA-A2 loss mutant melanoma cell lines was increased. In contrast, the level of B35 free HLA-A28 and B45 heavy chains was not changed. This interpretation, which is supported by the selective decrease of the level of B27-associated HLA-B44 and Cw6 heavy chains immunoprecipitated from the HLA-A2-loss mutant cell lines following transfection with a wild-type HLA-A2 gene, is consistent with the information in the literature. Measure of the binding of ß2-m to HLA class I heavy chains (53) and analysis of the in vivo assembly and processing of HLA class I molecular complexes in lymphoid cell lines (54) have shown a differential ability of the gene products of HLA-A, -B, and -C loci to associate with B27. An alternative, although not exclusive, possibility is that the reduced level of B27-associated HLA-B44 and Cw6 heavy chains immunoprecipitated from HLA-A2 transfectants by mAb W6/32 reflects the decreased loading of peptides on HLA class I allospecificities due to TAP downregulation. HLA class I alleles have been shown to differ in their ability to bind to TAP (55), as well as to use TAP-independent pathways to bind peptides (56).

HLA class I antigen down-regulation in SK-MEL-29.1.22 and SK-MEL-29.1.29 cells, as well as in parental cells, was partially corrected by incubation of cells at low temperature, indicating a defect in antigen processing. This defect was also shown by the low level of TAP1 and the reduced function of the peptide transporters in these cell lines. These abnormalities are not unique to SK-MEL-29 cell lines because they have been described in several types of tumor cell lines (for review, see 57). One might wonder about the functional significance of the down-regulation of HLA class I antigens and of the antigen-processing machinery in SK-MEL-29 cell lines. Previous studies (58) have shown that HLA class I antigen down-regulation may become a limiting factor in target cell recognition by CTLs, when antigens are available in limited amount. This mechanism may account for the low killing efficiency of SK-MEL-29.1 cells by MAA-specific, HLA-B45-restricted CTLs (9).

Our results have shown for the first time that multiple defects may underlie HLA class I antigen abnormalities in melanoma cells. This information represents a useful background to design effective strategies to screen melanoma lesions for HLA class I antigen down-regulation and to correct them.

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Molecular Analysis of the HLA-A2 Antigen Loss by Melanoma Cells SK-MEL-29.1.22 and SK-MEL-29.1.29

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