Lack of HLA Class I Antigen Expression by Melanoma Cells SK-MEL-33 Caused by a Reading Frameshift in β_2-Microglobulin Messenger RNA

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Abstract

The lack of HLA class I antigen expression by the melanoma cell line SK-MEL-33 is caused by a unique lesion in β_2-microglobulin (β_2-μ). Sequencing of β_2-μ mRNA detected a guanosine deletion at position 323 in codon 76 that causes a frameshift with a subsequent introduction of a stop codon at a position 54 base upstream of the normal position of the stop codon in the message. The loss of 18 amino acids and the change of 6 amino acids, including a cysteine at position 80 in the carboxy terminus of β_2-μ, are likely to cause marked changes in the structure of the polypeptide. The latter may account for the inability of β_2-μ to associate with HLA class I heavy chains and for its lack of reactivity with the anti-β_2-μ mAb tested. HLA class I antigen expression on SK-MEL-33 cells was reconstituted after transfection with a wild-type B_2m gene, therefore indicating that the abnormality of endogenous B_2m gene is the only mechanism underlying lack of HLA class I antigen expression by SK-MEL-33 cells. The guanosine deletion in B_2m gene was detected also in the melanoma tissue from which SK-MEL-33 cells had originated. Therefore, the molecular lesion identified in the SK-MEL-33 melanoma cell line is not caused by a mutation acquired during growth in vitro but is likely to reflect a somatic mutation during tumor progression. (*J. Clin. Invest.* 1993. 91:684–692.) Key words: β_2-microglobulin sequencing • transfection • HLA reconstitution • monoclonal antibody

Introduction

Malignant transformation of human melanocytes may be associated with changes in their antigenic profile. The latter include reduction or loss of HLA class I antigen expression, which has been found to occur in ~ 30% of surgically removed melanoma lesions (for review, see reference 1). Abnormalities in HLA class I antigen expression are likely to affect the biology of melanoma cells and their interactions with immune cells because of the role of HLA class I antigens in cell proliferation (2–4), interaction of target cells with cytotoxic T cells (for review see reference 5), and susceptibility of malignant cells to natural killer (NK)[1] cell–mediated lysis (for review see reference 6). Besides contributing to the characterization of the machinery regulating HLA class I antigen synthesis and expression, analysis of the molecular mechanisms underlying reduction or loss of HLA class I antigen expression by melanoma cells provides the necessary background to correct these abnormalities. This information may eventually be used to develop therapeutic approaches to melanoma, if the association between poor prognosis and reduction or lack of HLA class I antigen expression in melanoma lesions (7) reflects their role in the clinical course of the disease.

In a recent study we have shown that melanoma cells FO-1 do not express HLA class I antigens because of a gross deletion of the 5′ region and a portion of the coding sequence of the B_2m gene that prevents its transcription (8). In the present study, we have characterized the molecular defect that accounts for the lack of HLA class I antigen expression by melanoma cells SK-MEL-33.

Methods

Patient. Patient AZ was a 74-yr-old white male who had a lentigo maligna melanoma excised from the skin of his right scapula in 1972. Two years later, in January 1974, a mass appeared in the left axilla. The mass was excised and revealed metastatic melanoma involving regional lymph nodes. A large (5 × 4 × 4 cm), firm mass reappeared at the apex of the left axilla in June 1974. The patient underwent a deep axillary lymph node dissection. Pathological examination showed metastatic melanoma within lymph nodes invading the surrounding subcutaneous tissue, fat, pectoralis minor muscle, brachial nerve plexus, and brachial vein. The melanoma cell line SK-MEL-33 was established from this lesion. The patient remained free of any recurrent melanoma for ≥ 10 yr. He was last seen for follow-up in 1983 when he was 85 yr old. Clinical staging at that time demonstrated no evidence of recurrent melanoma on physical examination, chest x ray, and liver function tests.

Cell lines, tumor specimen, and β_2-microglobulin (β_2-μ). The melanoma cell line SK-MEL-33 was cultured in RPMI 1640 medium (Gibco Laboratories, Grand Island, NY) supplemented with 10% FCS, 2 mM L-glutamine, and 1% nonessential amino acids. The melanoma cell lines FO-1, Colo 38 and 3S5 and the B lymphoblastoid cell line MANN were grown in RPMI 1640 medium supplemented with 10% Serum Plus (Hazelton Biologics, Inc., Lenexa, KS) and 2 mM L-glutamine. Cells were harvested by vigorous pipetting with PBS supplemented with 1 mM EDTA.

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1. *Abbreviations used in this paper:* β_2-μ, β_2-microglobulin; ICAM-1, intercellular adhesion molecule-1; NK, natural killer.

The formalin-fixed and paraffin-embedded specimen of the human malignant melanoma lesion (Clark's level IV), from which the cell line SK-MEL-33 was originally established, was retrieved from the files of the Department of Pathology, Memorial Sloan-Kettering Cancer Center, New York. The tumor had been fixed in 10% formalin (formaldehyde 4% vol/wt, methyl alcohol 1% vol/wt, buffered with phosphate buffer) (Mallinckrodt Chemical Works, NY) for 24 h before embedding into paraffin. One section was cut for standard hematoxylin and eosin staining and three adjacent 20-μm thick sections containing only tumor were cut for DNA isolation. Lesional portions of the two unstained sections were scraped with a stainless steel spatula into a 1.5 ml Eppendorf tube.

β_2-μ was purified from urine as described (9).

mAb and conventional antisera. The mAb W6/32 to a monomorphic determinant expressed on β_2-μ-associated HLA class I heavy chains (10); the mAb TP25.99 to a determinant expressed on both β_2-μ-associated and β_2-μ-free HLA class I heavy chains (8); the anti-human β_2-μ mAb BBM-1 (11), L368 (12), and NAMB-1 (13); the anti-HLA-DR, DQ, DP mAb Q5/13 (14); and the antiintercellular adhesion molecule-1 (ICAM-1) mAb CL207.14 (15) were developed and characterized as described. mAbs were purified from ascitic fluid by sequential precipitation with caprylic acid and ammonium sulphate (16). mAbs were labeled with ^{125}I using the iodogen method (17). The immunoreactive fraction of the radiolabeled antibodies was at least 50% as determined by the method of Lindmo et al. (18).

The rabbit antiserum R5996-4 reacting with denatured HLA class I heavy chain and the rabbit anti-human β_2-μ antiserum DP-213456 were prepared by following the methodology described by Nakamuro et al. (19) and by Poulik et al. (20), respectively. Anti-human β_2-μ antibodies were purified from rabbit antiserum DP-213456 by affinity chromatography on purified β_2-μ coupled to AFFI-GEL 10 (Bio-Rad Laboratories, Richmond, CA) (1 mg/ml of gel). Purified goat anti-rabbit Ig antibodies and rabbit anti-mouse Ig antibodies were purchased from Jackson ImmunoResearch Laboratories, Inc. (Avondale, PA).

cDNA probes, B_2m gene, and oligonucleotides. The cDNA probes for human β_2-μ (21) and for HLA-B7 antigen (22) were isolated by digestion of plasmids with restriction endonuclease PstI. The inserts were electrophoresed and excised from low melting agarose gel. Human B_2m clone pb2m13 in the vector pEMBL9 (23) was purified on a CsCl gradient and digested either with XbaI alone or with the combination of HindIII and XbaI or of EcoRI and SmaI. An upstream 5′ fragment (referred to as probe A), the first exon and much of its flanking sequences (referred to as probe B), the second and third exons and their flanking sequences (referred to as probe C), and the fourth exon and its flanking sequences (referred to as probe D) were separated by electrophoresis on a low melting point agarose gel as described (8). cDNA probes were labeled with [α-^{32}P]dCTP (3,000 Ci/mmol; Amersham Corp., Arlington Heights, IL) by random priming (24) to a specific activity of 10^8 cpm/μg. Oligonucleotides were end labeled with [γ-^{32}P]ATP (5,000 Ci/mmol) in the presence of T4 polynucleotide kinase (25).

Oligonucleotides specific for the B_2m gene were synthesized on a DNA synthesizer (BioSearch, MilliGen/Biosearch, A Div. of Millipore, Burlington, MA). A 23-nucleotide oligomer (5′ TAGAAAGACCAGTCCTTGCTGAA 3′) and a 21-nucleotide oligomer (5′ TGTATAAGCATATCAATATTA 3′) complementary to the codon 56–63 and position 467–486 in the 3′ untranslated region of human β_2-μ mRNA, respectively, were used as primers for RNA sequencing. The nucleotide oligomers (5′ GTGGAGCATTCAGACTTGTC 3′ referred to as primer 1 and 5′ GCAGTGCCACTAATCTGATC 3′ referred to as primer 2), which define a region of 300 bp, including codons 49–95 in the second exon of the B_2m gene, were used as primers for PCR. One oligomer (5′ GCATACTCATTTTTTCAGTG 3′) complementary to codon 72–79 in exon 2 of wild-type B_2m gene with a cytosine deletion at the region complementary to codon 76, referred to as probe II, and the other one (5′ TAAGGCCACGGAGCGAGACAT 3′) complementary to codon 1–7 in exon 1 of wild-type B_2m gene, referred to as probe I, were used for DNA hybridization.

Chemicals and cytokines. Actinomycin D was purchased from Sigma Chemical Co., (St. Louis, MO). Recombinant human IFN-γ was obtained from Hoffman-LaRoche, Inc. (Nutley, NJ).

Serological assays. The direct binding assay was performed as described (18) in 96-well microtiter plates (Becton Dickinson Co., Oxnard, CA). Briefly, cells (1×10^5) were incubated with ^{125}I-labeled mAb (2×10^5 cpm per well) at 4°C for 2 h. Then cells were washed five times with PBS and cell bound radioactivity was measured in a gamma counter (LKB-1261; LKB-Wallac, Turku, Finland). Results are expressed as bound cpm per 1×10^5 cells.

Radiolabeling of cells, indirect immunoprecipitation, and SDS-PAGE. These procedures were performed as described elsewhere (8). Cells were labeled with ^{125}I (Amersham Corp.) using the lactoperoxidase method (26) or with [^{35}S]methionine (Amersham Corp.). Then cells were solubilized by incubation for 30 min at 4°C in lysis buffer containing 1% Nonidet P-40, 10 mM Tris-HCl, pH 8.2, 0.5 M NaCl, 1 mM EDTA, 1 mg/ml BSA, and 1 mM PMSF and incubated for 12 h at 4°C with antibodies bound to protein A Sepharose (Pharmacia Inc., Piscataway, NJ). One-dimensional SDS-PAGE analysis was performed under reducing conditions in slab gels containing 12.5% acrylamide and using the buffer system described by Laemmli (27). Gels containing ^{125}I-labeled samples were processed for autoradiography using Kodak XAR-5 film (Eastman Kodak Co., Rochester, NY). Gels containing [^{35}S]methionine-labeled samples were processed for fluorography as described (28).

Preparation of total mRNA and poly A^+ RNA. Total RNA was isolated from cells using the method described by Geliebter et al. (29). Poly(A)-containing RNA was purified by chromatography on oligo(dT) cellulose (Boehringer Mannheim Corp., Indianapolis, IN).

Preparation of DNA from paraffin tissue sections. Tissue sections were incubated in xylene to dissolve the paraffin. The tissue was then pelleted by centrifugation at 12,000 *g* for 5 min (30, 31). The supernatant was discarded and after two additional incubations with xylene the pellet was washed sequentially with 100% and 70% ethanol. The pellet was resuspended in 100 μl of a digestion buffer (0.2 M Tris-HCl, pH 8; 10 mM EDTA, 1% SDS) to which Proteinase K stored at −20°C was added to a final concentration of 0.5 mg/ml. At the end of a 48-h incubation at 55°C, more SDS and freshly thawed Proteinase K were added to the reaction mixture to a final concentration of 2% and 1 mg/ml, respectively. Incubation was continued for an additional 25 h at 55°C. The sample was then heated to 95°C for 6 min to inactivate Proteinase K and was extracted twice with phenol–chloroform. Finally, DNA was precipitated in 0.3 M NaCl and 100% ethanol at −70°C for 12 h and pelleted by centrifugation at 12,000 *g* for 30 min. The pellet was washed twice with 70% ethanol and resuspended in 100 μl of filtered and autoclaved water.

RNA hybridization analysis. Total RNA was size fractionated in a 1% agarose gel containing 2.2 M formaldehyde, transferred to a nitrocellulose membrane, and hybridized with ^{32}P-labeled probes as described (8).

DNA hybridization analysis. Genomic DNA was extracted essentially as described by Perbal (32). DNA was digested with restriction endonucleases, size fractionated, transferred to nitrocellulose membrane, and hybridized as described previously (8). When ^{32}P-labeled specific oligonucleotides for the B_2m gene were used as probes, the hybridization was performed at a temperature 5°C below the calculated melting temperature as described (33).

RNA sequencing. β_2-μ mRNA was sequenced by following the methodology described by Geliebter et al. (25). Briefly, ^{32}P-labeled oligonucleotide primers (10 ng) and poly A^+ RNA (20 μg) were heated to 80°C for 3 min in 15 μl of annealing buffer (250 mM KCl, 10 mM Tris-HCl, pH 8.3, 1 mM EDTA) and incubated for 60 min at 60°C or at 45°C. The dideoxynucleotides diluted with dH_2O in different ratio and 3.3 μl of reverse transcriptase diluted in extension buffer (24 mM Tris-HCl, pH 8.3, 10 mM $MgCl_2$, 8 mM dithiothreitol, 0.2 mM dATP,

0.2 mM dCTP, 0.2 mM dTTP, 0.2 mM dGTP) were added into RNA primer–annealing solution, mixed, and incubated for 60 min at 50°C. Reactions were terminated by adding 2 μl of loading buffer (100% formamide, 0.3% xylene cyanol F, 0.3% bromophenol blue). Samples were electrophoresed in 6% polyacrylamide, 8 M urea gel at 17–18 V. After electrophoresis, gels were dried and exposed to Kodak XAR-5 film (Eastman Kodak Co.) for 72 h at −70°C. Nucleotide sequences were translated into amino acid sequences by using the software microgenie (Version 7.0, Beckman Instruments, Inc., Fullerton, CA).

In vitro amplification of genomic DNA. Extracted DNA was used as a template for amplification using the Thermus aquaticus (TaqI) heat-stable DNA polymerase as described (34). Amplifications were performed in siliconized tubes (Biomedica, Rutherford, NJ) in a 50-μl solution containing genomic DNA template, 50 mM KCl, 10 mM Tris, pH 8.4, 2.5 mM $MgCl_2$, 300 ng of primer 1 and primer 2, 200 μM of each deoxyribonucleotide triphosphate (dATP, dCTP, dTTP, and dGTP), 100 μg of gelatin/ml, and 3 U of TaqI DNA polymerase (Perkin-Elmer Cetus Instruments, Norwalk, CT). Samples were overlaid with 50 μl of mineral oil to prevent condensation and were subjected to 35 cycles of amplification. The cycling reaction was performed in a programmable heat block (DNA Thermal Cycler; Perkin-Elmer Cetus Instruments) set to heat samples to 94°C for 1 min, cool them to 50°C over 1 min, and heat them to 73°C for 1 min. In the first cycle DNA was denatured at 94°C for 5 min. Amplified DNA was analyzed by nondenaturing agarose gel electrophoresis. 5 μl of the amplification reaction was electrophoresed on a 1.5% Tris–Borate–EDTA agarose gel (FMC Corp., Rockland, ME) in the presence of ethidium bromide.

Cloning and sequencing of PCR products. B_2m PCR products were ligated into plasmid vector PCR (Invitrogen, San Diego, CA) and used to transform competent bacteria (*Escherichia coli* INVαF′). Single colonies of transformed bacteria were selected in medium containing kanamycin (50 μg/ml) and grown to mass culture. Amplified plasmid was extracted according to established protocols (35) and used in direct DNA sequencing reactions using the chain-termination technique (36) with a modified T7 DNA polymerase (37), (Sequenase 2.0; United States Biochemical Corp., Cleveland, OH). Plasmid DNA (2.5 μg) was incubated with 10 ng of M13(−)20 Primer-(5′-GTAAAAC-GACGGCCAGT-3′) (Stratagene, Inc., La Jolla, CA) in 0.25 N NaOH at 20°C for 5 min. Denatured plasmid and annealed primer mixture were then precipitated with 5 M ammonium acetate, pH 4.6, and ethanol and were centrifuged at 15,000 *g* for 15 min. Pellets were washed with 70% ethanol, recentrifuged, dried, and resuspended in 8 μl of water. The annealed primer/template mixture was supplemented with 1 μl 0.1 M DDT, 1 μl 10 mU [α-^{35}S]dATP (1,000 Ci/mmol; New England Nuclear, Boston, MA), 2 μl dilute labeling mixture (1:5), and 3 U of Sequenase 2.0 (United States Biochemical Corp.). The reaction mixture was mixed, incubated for 2 min at room temperature, and aliquoted into four tubes containing 2.5 μl of the respective deoxy/dideoxy termination mixture (80 μM dATP, 80 μM dCTP, 80 μM dGTP, 80 μM dTTP, and 8 μM of the respective dideoxy nucleotide triphosphate). After a 10-min incubation at 37°C, 4 μl of stop buffer (95% formamide, 20 mM EDTA, 0.05% bromophenol blue, and 0.05% xylene cyanol) was added to each tube. Tubes were then heated to 94°C for 2 min. The reaction mixture was loaded on an 8% polyacrylamide–8 M urea gel and run for 4 h at constant wattage.

Transfection of SK-MEL-33 melanoma cells with a wild-type human B_2m gene. The procedure we used previously (8) was followed with minor modifications. Briefly, cells (1×10^7), linearized human B_2m DNA fragment (100 μg), and EcoRI-digested pSV2neo (5 μg) were suspended in 1 ml of cold PBS. An electroporation power supply (Bethesda Research Laboratories, Gaithersburg, MD) applied an electric pulse of 1 kV and 330 μF to the chamber containing cells. Cells were then recovered in RPMI 1640 medium supplemented with 10% FCS, seeded in eight 100-mm tissue culture dishes, and incubated at 37°C in 5% CO_2 for 7 d. On day 8, G418-sulfate was added to the medium at the final concentration of 2 mg/ml. The concentration of G418-sulfate was reduced to 0.4 mg/ml after 3 d of incubation and maintained at this level for an additional 10 d. Cell colonies were detached with HBSS containing trypsin–EDTA, picked up with a plastic ring, and expanded.

Results

Lack of HLA class I antigen expression by SK-MEL-33 melanoma cells. SK-MEL-33 cells did not react in a binding assay with ^{125}I-mAb W6/32 recognizing a monomorphic determinant expressed on β_2-μ–associated HLA class I heavy chains, with ^{125}I-mAb TP25.99 to a determinant expressed on β_2-μ–associated and β_2-μ–free HLA class I heavy chains, and with ^{125}I-labeled anti-β_2-μ mAb NAMB-1 (Fig. 1). No reactivity with these mAbs was detected also after incubation of cells with IFN-γ (1,000 U/ml) for 3 d before testing (Fig. 1). Furthermore, no component was immunoprecipitated from control and IFN-γ–treated ^{125}I-labeled SK-MEL-33 cells by anti-HLA Class I mAb W6/32 and TP25.99 and by anti-β_2-μ mAb NAMB-1. Neither control nor IFN-γ–treated SK-MEL-33 cells reacted with ^{125}I-labeled anti-HLA-DR, DQ, DP mAb Q5/13 (Fig. 1). SK-MEL-33 cells were sensitive to the modulating activity of IFN-γ, since IFN-γ–treated cells displayed a marked increase in the reactivity with anti-ICAM-1 mAb CL207.14 (Fig. 1).

Intracellular expression of HLA class I heavy chains and truncated β_2-μ by SK-MEL-33 melanoma cells. To determine whether the heavy chain of HLA class I antigens and β_2-μ are synthesized by SK-MEL-33 cells, an NP40 extract of [^{35}S]-methionine-labeled cells was immunoprecipitated with several anti-HLA class I mAbs, and analyzed by SDS-PAGE. No component was detected in the immunoprecipitate with anti-HLA class I mAb W6/32 and with anti-β_2-μ mAbs BBM-1, L368,

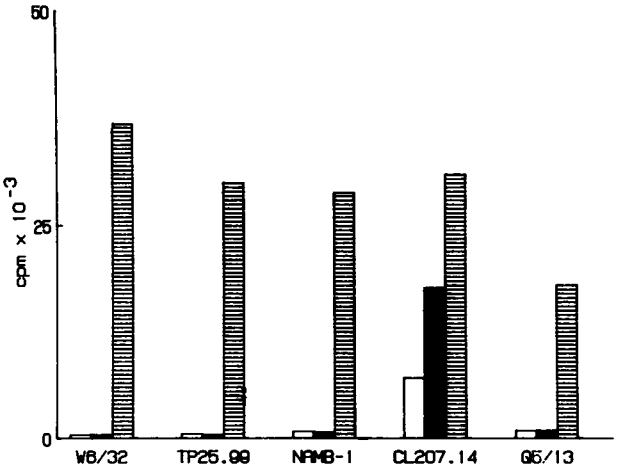

Figure 1. Lack of reactivity of control and IFN-γ–treated cultured melanoma cells SK-MEL-33 with anti-HLA class I mAbs. SK-MEL-33 cells were incubated at 37°C for 72 h with IFN-γ (final concentration 1,000 U/ml) (■). Control cells (□) were incubated under the same experimental conditions, but without cytokine. Cells were then harvested, washed twice with HBSS, and tested with ^{125}I-mAb W6/32 to a determinant expressed on β_2-μ–associated HLA class I heavy chain, ^{125}I-mAb TP25.99 to a determinant expressed on β_2-μ–associated and β_2-μ–free HLA class I heavy chain, ^{125}I-anti-β_2-μ mAb NAMB-1 and ^{125}I-anti-HLA-DR,DQ,DP mAb Q5/13 in a binding assay. ^{125}I-anti-ICAM-1 mAb CL207.14 was used to monitor the susceptibility of SK-MEL-33 cells to modulation by IFN-γ. The reactivity of radiolabeled anti-HLA mAb preparations was monitored by testing with cultured melanoma cells Colo 38 (■) in a binding assay.

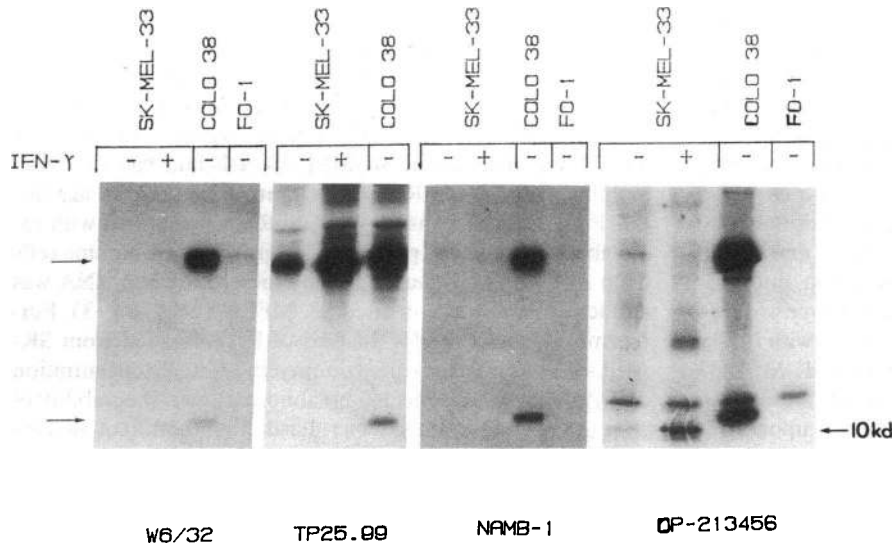

Figure 2. SDS-PAGE analysis of antigens immunoprecipitated from control and IFN-γ-treated cultured melanoma cells SK-MEL-33 by anti-HLA class I monoclonal and polyclonal xenoantibodies. After a 72-h incubation at 37°C in medium supplemented with IFN-γ (final concentration 1,000 U/ml), SK-MEL-33 cells were starved for 1 h in methionine-free medium and intrinsically radiolabeled with [^{35}S]-methionine. Control SK-MEL-33 cells were cultured and radiolabeled under the same experimental conditions but were not exposed to IFN-γ. At the end of the incubation, cells were harvested, washed three times with HBSS, and solubilized with 1% NP40. Antigens were immunoprecipitated with mAb W6/32 to a determinant expressed on β_2-μ-associated HLA class I heavy chains, with mAb TP25.99 to a determinant expressed on β_2-μ-free and β_2-μ-associated HLA class I heavy chains, with anti-β_2-μ mAb NAMB-1, and with anti-β_2-μ polyclonal antibodies isolated from rabbit anti-β_2-μ serum DP-213456. Antigens were then eluted from the immunoabsorbent and analyzed by SDS-PAGE in the presence of 2% β-mercaptoethanol. Gels were then processed for fluorography (27). Arrows indicate the position of HLA class I heavy chain and β_2-μ and of the 10-kD component precipitated from SK-MEL-33 cells. Cultured melanoma cells Colo 38, which express HLA class I antigens and cultured melanoma cells, FO-1, which synthesize HLA class I heavy chains, but do not synthesize β_2-μ (8), were used as controls to monitor the activity of the antibody preparations and the specificity of the immunoprecipitation patterns.

and NAMB-1. Representative results are shown in Fig. 2. In contrast, HLA class I heavy chains were detected in the immunoprecipitate with the mAb TP25.99 and with the xeno-antiserum R5996-4. The latter two reagents react with β_2-μ-free HLA class I heavy chains. Furthermore, a component with the apparent molecular mass of 10 kD was detected in the immunoprecipitate with anti-human β_2-μ antibodies purified from rabbit anti-human β_2-μ serum DP-213456 by affinity chromatogra-

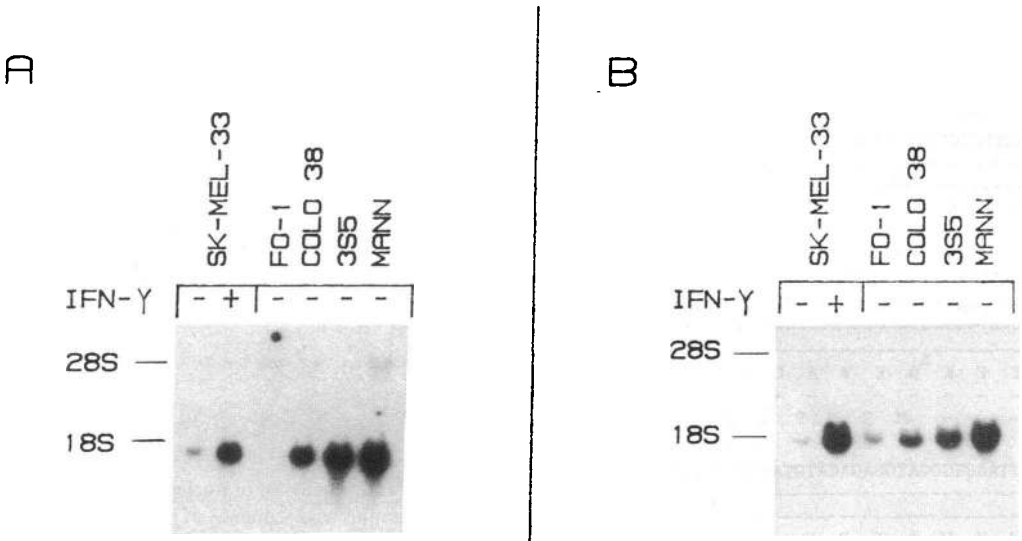

Figure 3. Northern blot analysis of HLA class I heavy chain and β_2-μ mRNA in cultured melanoma cells SK-MEL-33. SK-MEL-33 cells were incubated at 37°C for 72 h with IFN-γ (final concentration 1,000 U/ml). Control cells were incubated under the same experimental conditions, but without cytokine. At the end of the incubation, cells were harvested and total cytoplasmic RNA was extracted and hybridized with ^{32}P-labeled 0.5-kb human β_2-μ cDNA probe (*A*) and with ^{32}P-labeled 1.4-kb HLA-B7 cDNA probe (*B*). Controls analyzed for comparison purposes included RNA isolated from cultured melanoma cells FO-1, which transcribe the HLA class I heavy chain genes, but do not transcribe the B_2m genes, from cultured melanoma cells Colo 38 and 3S5 and from cultured B lymphoid cells MANN, all of which transcribe the HLA class I heavy chain and B_2m genes.

phy on β_2-μ. This component, which has a smaller size than that immunoprecipitated from [^{35}S]methionine-labeled cultured melanoma cells Colo 38, is not associated with HLA class I heavy chains. The immunoprecipitation pattern was specific, since purified rabbit anti-β_2-μ antibodies did not immunoprecipitate the 10-kD component and 12 kD β_2-μ from cultured melanoma cells FO-1, which do not synthesize β_2-μ (8). The intensity of HLA class I heavy chains immunoprecipitated by mAb TP25.99 and by rabbit antiserum R5996-4 and of the 10-kD component immunoprecipitated by rabbit anti-β_2-μ xenoantibodies was increased when the antigen source was represented by SK-MEL-33 melanoma cells incubated with IFN-γ (1,000 U/ml) for 72 h. These results indicate that SK-MEL-33 cells synthesize HLA class I heavy chains and abnormal β_2-μ; the latter had an increased electrophoretic mobility upon SDS-PAGE and lacked the ability to associate with HLA class I heavy chains.

Northern and Southern blot analysis of β_2-μ in SK-MEL-33 melanoma cells. To investigate the mechanism(s) underlying the synthesis of an abnormal β_2-μ, which does not associate with HLA class I heavy chains, the steady state level of mRNA for β_2-μ was evaluated by RNA hybridization analysis. β_2-μ mRNA was detected in SK-MEL-33 cells and had the same mobility as the β_2-μ mRNA from control cells in a 1% agarose gel (Fig. 3). The intensity of β_2-μ mRNA hybridized with radiolabeled β_2-μ cDNA probe was lower than in melanoma cells Colo 38 and 3S5; it was markedly increased when RNA was extracted from IFN-γ-treated SK-MEL-33 cells (Fig. 3). Furthermore, Northern blot analysis of RNA isolated from SK-MEL-33 cells treated with actinomycin D (final concentration 5 μg/ml) for $\leq$ 2 h detected no abnormality in the stability of β_2-μ mRNA (data not shown). Lastly, Southern blot analysis using four DNA fragments isolated from B_2m gene clone pb2m13 (8, 23) as probes detected no difference in the restric-

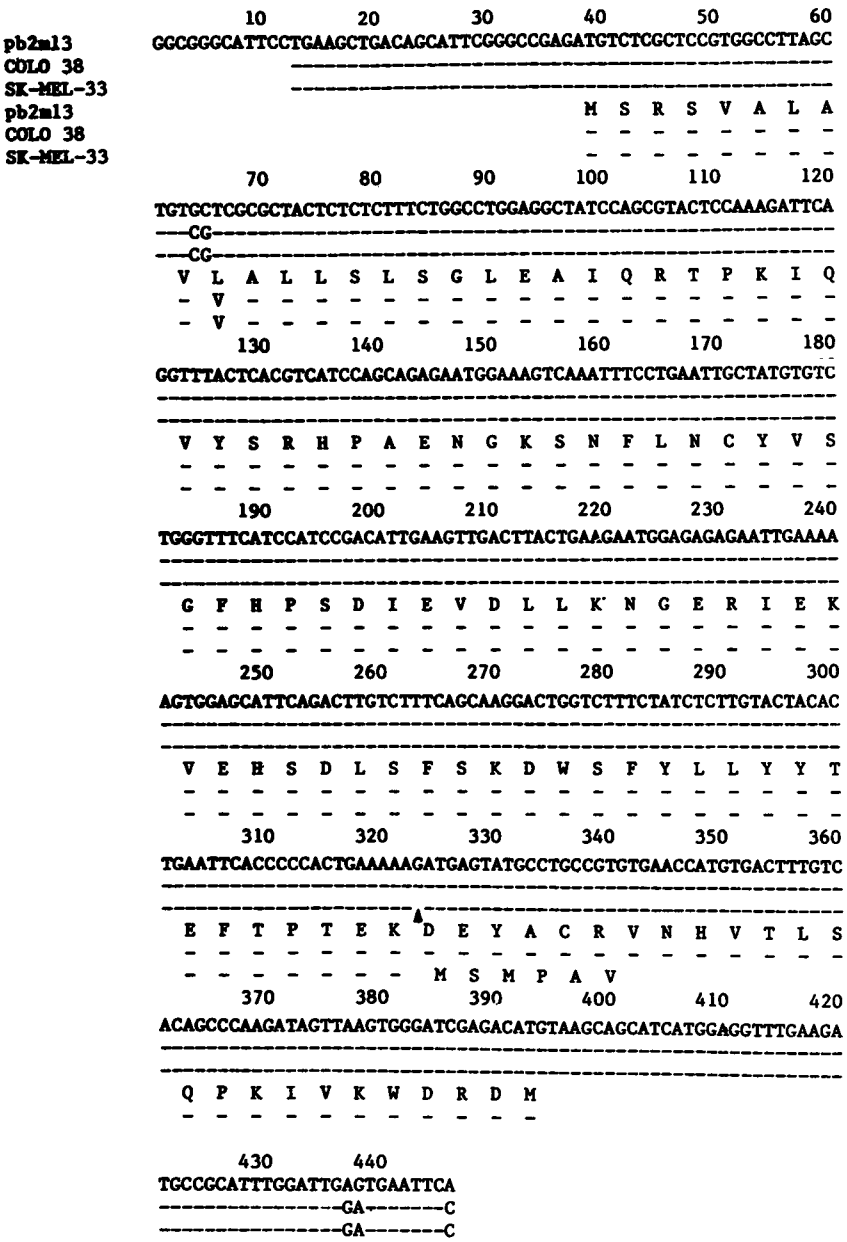

Figure 4. Comparison of nucleotide and deduced amino acid sequence of β_2-μ mRNA from SK-MEL-33 melanoma cells with that of β_2-μ mRNA from Colo 38 melanoma cells and with the published sequence of pb2m13. Dashes indicate sequence identity. The arrow indicates the base deletion detected in SK-MEL-33 cells.

tion pattern of the genomic DNA between SK-MEL-33 cells and melanoma cells Colo 38 (data not shown).

Reading frameshift mutation in β_2-μ mRNA of SK-MEL-33 melanoma cells. To determine the molecular basis for the synthesis of an abnormal β_2-μ by SK-MEL-33 cells, the β_2-μ mRNA sequence was determined. β_2-μ mRNA from Colo 38 melanoma cells, which express HLA class I antigens, was sequenced as a wild-type control. The sequences of the β_2-μ mRNA from the two melanoma cell lines displayed a 99.8% homology between themselves and of at least 98.6% with the published sequence of pb2m13 (Fig. 4). The SK-MEL-33 and Colo 38 β_2-μ mRNA sequences differ from that of pb2m13, with transition of CG to GC at positions 64 and 65, AG to GA at positions 437 and 438, and A to C at position 446. All of these nucleotide substitutions were silent except that occurring at position 65, which resulted in a switch from valine to leucine at position −11 within the leader peptide. No alteration was detected in the leader sequence and in the initiation codon of SK-MEL-33 β_2-μ mRNA. However, the base guanosine was deleted at position 323 (in codon 76) of the sequence of β_2-μ mRNA in SK-MEL-33 cells (Fig. 5). To confirm the base deletion in B_2m gene of SK-MEL-33 cells, a Southern blot analysis was performed using an oligonucleotide probe complementary to the codon 72–79 sequence of β_2-μ mRNA of SK-MEL-33 cells with the deletion of a cytosine at the region complementary to codon 76 (referred to as probe II). For this analysis an oligonucleotide complementary to the codon 1–7 of wild-type human β_2-μ (referred to as probe I) was used as a control. A temperature 5°C below the melting temperature was used for

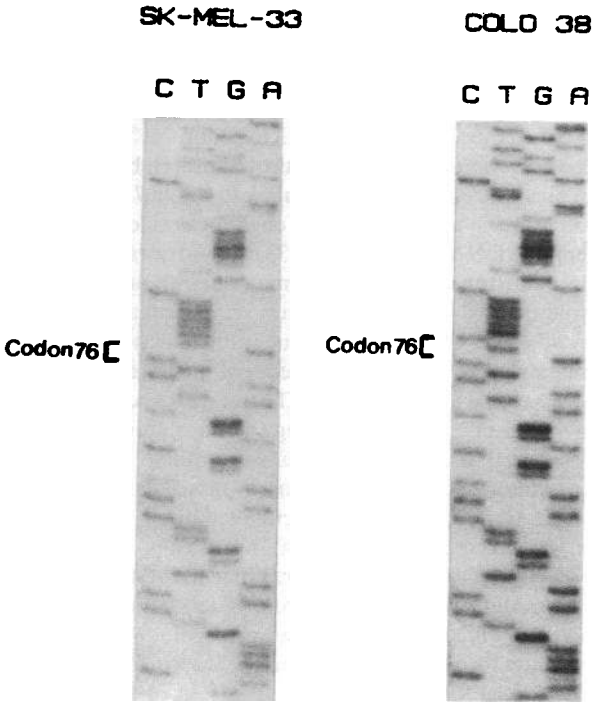

Figure 5. Sequencing gel of β_2-μ mRNA in SK-MEL-33 melanoma cells. RNA sequencing was primed with an oligonucleotide complementary to the β_2-μ mRNA at position 467–486 of 3′ untranslated region. The sequence, when read bottom to top, is complementary to the sense strand in 3′ → 5′ orientation. The sequence complementary to codon 76 is indicated. β_2-μ mRNA from cultured human melanoma cells Colo 38 was sequenced as a control.

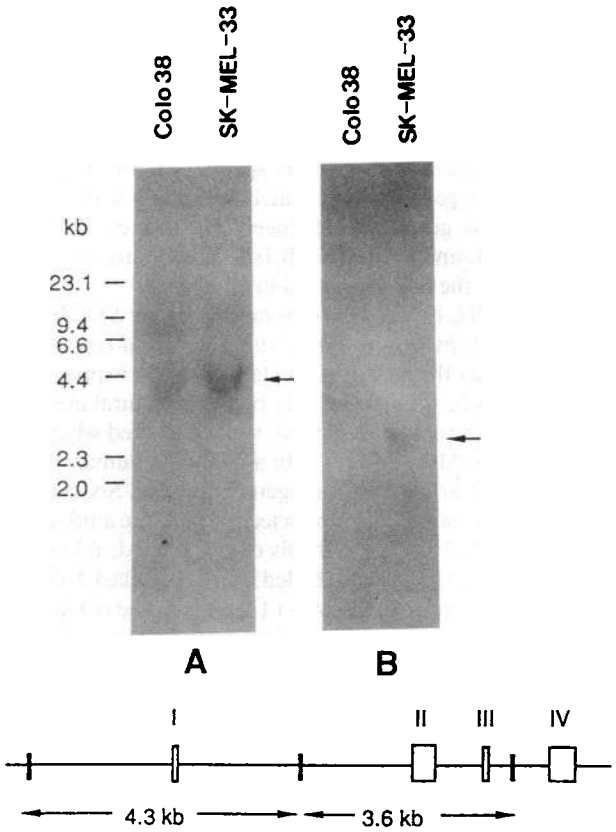

Figure 6. Southern blot analysis with oligonucleotides of B_2m gene in cultured melanoma cells SK-MEL-33. DNA (30 μg) isolated from cultured melanoma cells SK-MEL-33 (lane *2*) was digested overnight at 37°C with the restriction enzyme Xbal. Digested DNA was fractionated on a 1% agarose gel and transferred to nitrocellulose filters. Blots were hybridized with a ^{32}P-labeled oligonucleotide probe I complementary to codon 1–7 of wild-type human β_2-μ mRNA (*A*) and with a ^{32}P-labeled oligonucleotide probe II with a cytosine deletion at the region complementary to codon 76 (*B*). DNA (30 μg) from cultured melanoma cells Colo 38 (lane *1*) was run as a control. The marker lane contained λ phage DNA digested with HindIII. The bottom shows the map of the B_2m gene with restriction enzyme Xbal sites indicated by black blocks. The open blocks indicate the four exons.

hybridization. The ^{32}P-oligonucleotide probe II hybridized to a 3.6 kb Xbal genomic DNA fragment from SK-MEL-33 cells but did not hybridize to that from Colo 38 cells. On the other hand, the ^{32}P-oligonucleotide probe I hybridized to a 4.3 kb Xbal genomic DNA fragment both from Colo 38 cells and from SK-MEL-33 cells (Fig. 6). These results corroborate the sequencing data that guanosine was deleted at position 323 (in codon 76) in β_2-μ mRNA of SK-MEL-33 cells.

The guanosine deletion causes a frameshift with a subsequent introduction of a stop codon at a position 54 bases upstream of the normal position of the stop codon in the message (Fig. 4). This results in the translation of a truncated protein that is 18 amino acids shorter than the wild-type β_2-μ protein. Furthermore, the changes from codon 76 to codon 81 result in the substitution of the corresponding six amino acids. Of great significance in terms of the overall structure of β_2-μ, is the replacement with an alanine of a cysteine that is at position 80 in the β_2-μ polypeptide.

Detection of a guanosine deletion in β_2-μ in melanoma tissue from which the SK-MEL-33 cell line had originated. To determine whether the base deletion detected in SK-MEL-33 cells was present in the lesion from which the cell line had originated, DNA extracted from the melanoma lesion was used for PCR amplification with primers specific for the second exon of the human gene. Approximately 200 bases of the amplified region of this gene were sequenced. The guanosine deletion in codon 76 found in the SK-MEL-33 melanoma cell line was found also in the melanoma lesion (Fig. 7).

Induction of HLA class I antigen expression on SK-MEL-33 melanoma cells by transfection with human wild-type B_2m gene. To prove that the lack of HLA class I antigen expression by SK-MEL-33 cells was caused only by the structural abnormality of β_2-μ that we have described, we determined whether transfection of SK-MEL-33 cells with a wild-type human B_2m gene could induce HLA class I antigen expression. Six clones were isolated after transfection and selection with the antibiotic G418-sulfate. Cells from five of the six clones reacted, although to a different extent, with ^{125}I-labeled anti-HLA class I mAb W6/32 and anti-β_2-μ mAb NAMB-1 (Table I). The reactivity is specific since the transfected cells continued to be unreactive with the anti-HLA-DR, DQ, DP mAb Q5/13. Furthermore, neither mAb W6/32 nor mAb NAMB-1 reacted with melanoma cells SK-MEL-33 transfected with the pSV2neo gene alone.

*Table I. Reactivity with Anti-HLA Class I mAb of SK-MEL-33 Melanoma Cells Transfected with Human Wild-Type B_2m Gene**

Cells	^{125}I-labeled mAb		
	W6/32	NAMB-1	Q5/13
		cpm × 10⁻³	
T_3-1‡	24.2**	18.7	0.2
T_3-2	5.2	4.7	0.1
T_3-3	0.2	0.6	0.2
T_3-4	5.3	4.6	0.8
T_3-5	18.0	17.7	0.2
T_3-7	23.2	17.8	0.1
SK-MEL-33 neo§	0.2	1.4	0.7
SK-MEL-33‖	0.2	0.6	0.1
Colo 38¶	21.3	15.9	9.1

* Cells were tested with ^{125}I-mAb W6/32 to a determinant expressed on β_2-μ–associated HLA class I heavy chains and ^{125}I-anti-β_2-μ mAb NAMB-1 in a binding assay. ^{125}I-anti-HLA-DR, DQ, DP mAb Q5/13 was used as a specificity control. ‡ Clones isolated after transfection of SK-MEL-33 cells with linearized human B_2m DNA fragment and EcoRI-digested pSV2 neo and selection with the antibiotic G-418 sulfate. § Clone isolated after transfection of SK-MEL-33 cells with EcoRI–digested pSV2 neo and selection with the antibiotic G-418 sulfate. ‖Untransfected cells. ¶ Cultured melanoma cells Colo 38 were used to monitor the reactivity of radiolabeled anti-HLA mAb preparations in a binding assay. ** cpm × 10^{-3}/1 × 10^5 cells.

Discussion

Serological and immunochemical analysis with monoclonal and polyclonal xenoantibodies has shown that cultured melanoma cells SK-MEL-33 do not express HLA class I antigens on their cell surface and do not acquire them after incubation with IFN-γ. This abnormality reflects the synthesis of a truncated β_2-μ that does not associate with HLA class I heavy chains. Since association with β_2-μ is required for the transport of HLA class I heavy chains to cell membrane (38, 39), HLA class I heavy chains that are synthesized by melanoma cells SK-MEL-33 remain in the cytoplasm and are not expressed on the cell surface. This conclusion is supported by the results of immunoprecipitation experiments performed with an extract of intrinsically radiolabeled melanoma cells SK-MEL-33 and anti-HLA class I mAb with distinct specificities. HLA class I heavy chains are immunoprecipitated by mAb TP25.99, which recognizes a determinant expressed also on β_2-μ–free HLA class I heavy chains (8), but are not immunoprecipitated by mAb W6/32, which requires the association of the HLA class I heavy chain with β_2-μ for the expression of the corresponding determinant (40).

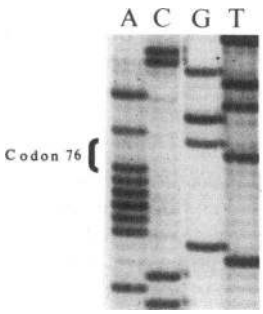

Figure 7. Direct sequence analysis of the region surrounding codon 76 of the B_2m gene in the surgically removed melanoma lesion from which the cell line SK-MEL-33 was originated. DNA was extracted from paraffin-embedded lesion, amplified in vitro using PCR. The PCR products were ligated into plasmid vector pCR and used to transform *E. coli* INVαF. The plasmid amplified from single colonies was extracted and used in direct DNA sequencing reactions using the chain-termination technique. The sequence, when read bottom to top, is the sense strand in 5′ → 3′ orientation. The sequence shown corresponds to the stretch from codons 73 to 79.

Sequencing of β_2-μ mRNA from SK-MEL-33 detected a deletion of guanosine at position 323 in codon 76. This finding has been corroborated by the results of Southern blot analysis with an oligonucleotide complementary to codon 72–79 of SK-MEL-33 cells β_2-μ mRNA. This oligonucleotide hybridizes with genomic DNA from SK-MEL-33 cells but does not hybridize with that from Colo 38 melanoma cells without detectable abnormalities in β_2-μ. The base deletion causes a reading frameshift in β_2-μ mRNA with a subsequent introduction of a stop codon in codon 82 (54 bases upstream of the normal position of the stop codon) in the message. Therefore, the codons from 82 to 99 are not translated, causing the translation of a truncated protein that is 18 amino acids shorter than the wild-type protein. This accounts for the apparent molecular mass of 10 kD of the component immunoprecipitated by polyclonal anti-human β_2-μ xenoantibodies from intrinsically labeled SK-MEL-33 cells. Furthermore, the reading frameshift causes a missense mutation in the codons from 76 to 81 and a change in the corresponding amino acids. The latter include replacement of an alanine for cysteine at position 80, thereby disrupting an intrachain disulfide bond in β_2-μ. The loss of this disulfide bond is likely to have dramatic effects on the structure of β_2-μ in SK-MEL-33 cells, since β_2-μ has been shown to have a β-sandwich structure composed of two antiparallel β-pleated sheets joined by a disulfide linkage (41). The lack of this disulfide bond in SK-MEL-33 cells, the introduction of 6 amino acid substitutions, and the loss of 18 amino acids in the carboxy terminus of β_2-μ are likely to cause marked changes in the

conformation and structure of the polypeptide. The latter may account for the lack of association of β_2-μ with HLA class I heavy chains and the lack of reactivity with mouse anti–human β_2-μ mAb. An alternative, but not exclusive possibility for the lack of reactivity with mouse anti–human β_2-μ mAb may be the loss of the corresponding determinants located on the carboxy terminus of β_2-μ, since the most significant difference in the sequence of human and mouse β_2-μ is located at the carboxy terminus of the molecule (42). Therefore, this moiety is likely to be the most immunogenic when mice are immunized with human β_2-μ.

Karyotype analysis indicates that SK-MEL-33 cells contain two copies of chromosome 15 (unpublished results) where the B_2m gene maps (43). No rearrangement of the B_2m gene was found by Southern blot analysis of SK-MEL-33 cell DNA with fragments of wild-type B_2m gene as probes. Nevertheless, biochemical analysis demonstrated that SK-MEL-33 cells synthesize only a truncated β_2-μ polypeptide. Therefore, it is likely that a wild-type allele of β_2-μ was deleted or is nonfunctional, since melanoma cells in metastatic lesions are genetically unstable and are characterized by widespread chromosomal alterations, including genetic deletions (44). The mechanisms for loss of a functional β_2-μ allele include, (*a*) hemizygosity, with loss of a wild-type allele and duplication of the mutant B_2m gene; (*b*) deletion of the wild-type allele without duplication of the mutant allele; or (*c*) transcription of only the mutant copy of the B_2m gene due to mutations in coding or noncoding regions that are undetectable with the probes we have used. Whatever is the mechanism underlying the lack of translation of a functional β_2-μ in SK-MEL-33 cells, abnormal β_2-μ is the only defect responsible for lack of HLA class I antigen expression by SK-MEL-33 cells, since their transfection with a wild-type human B_2m gene induced their expression.

SK-MEL-33 cells are not the first example of human cells without detectable HLA class I antigen expression because of a β_2-μ abnormality. Cultured lymphoblastoid Daudi cells, which are the most extensively characterized human cells without detectable HLA class I antigen expression, transcribe the B_2m gene but do not translate β_2-μ mRNA (20, 45) because of a point mutation in the initiation codon (46). Cultured melanoma cells FO-1 do not transcribe the B_2m gene because of a deletion of the first exon, of the 5′-flanking region, and of a segment of the first intron of the B_2m gene (8). A similar defect has been identified in four mouse cell lines derived from the C48 mouse thymoma cell line R1.1 by chemical or irradiation mutagenesis and subsequent selection with anti-TL antisera and complement (47–49). Interestingly, a similar treatment of cultured human B lymphoblastoid cells has caused the mutations of genes located in the HLA class II region which control the HLA class I antigen presentation pathway (50, 51). On the other hand, no abnormalities in the structure of β_2-μ polypeptide and/or in mechanisms that regulate transcription or translation of the gene have been induced. SK-MEL-33 cells are the first example of cells with a structurally abnormal β_2-μ because of a frameshift mutation due to deletion of a single nucleotide. Furthermore, SK-MEL-33 cells represent the first example in which the same HLA class I molecular abnormality present in the cell line could be detected in the original melanoma lesion from the patient. These findings show that the lesion we have identified in the SK-MEL-33 cell line is not caused by a mutation acquired during growth in vitro, but reflects an in vivo abnormality. The lack of HLA class I antigen expression may have provided the mutant clone with a mechanism to escape from lysis by HLA class I cytotoxic T cells. The abnormality in β_2-μ is likely to reflect a somatic mutation during tumor progression, since the allospecificities HLA-A1, Aw33, B5, B14, Bw4, Bw6 were detected on patient's lymphocytes by using the complement-dependent cytotoxic assay (unpublished results). Immunohistochemical techniques could not be used to prove the lack of HLA class I antigens in the melanoma lesion, since only the formalin-fixed, paraffin-embedded tissue was available. Anti β_2-μ mAbs reacting with formalin-fixed, paraffin-embedded tissues are not available to us. Furthermore, mAbs recognizing determinants expressed only on β_2-μ associated HLA class I heavy chains stain only frozen tissue sections.

The clinical course of patient AZ was more favorable than expected, although tumor progression in patients with metastatic melanoma can be variable (52). The estimated 5-yr survival was < 20%, based on the presence of extensive regional metastases. If the lack of HLA class I antigen expression by melanoma cells played a role in the clinical course of the disease in this patient, two contrasting scenarios may be envisioned. Loss of HLA class I antigen expression could have selected for a clone of AZ melanoma cells that escaped immune recognition by HLA class I–restricted cytotoxic T lymphocytes. If so, surgery removed all melanoma cells lacking HLA class I antigens. Alternatively, lack of HLA class I antigen expression could have enhanced the susceptibility of AZ melanoma cells to NK cell–mediated lysis, thus facilitating the clearance of residual melanoma cells after the surgical excision of nodal metastases. We favor the latter possibility, since in preliminary experiments we have found a reduction in susceptibility to NK cell–mediated lysis of melanoma cells SK-MEL-33 associated with the induction of HLA class I antigens by transfection with a wild-type human B_2m gene.

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