Lack of HLA Class I Antigen Expression by Cultured Melanoma Cells FO-1 Due to a Defect in B_2m Gene Expression

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Abstract

The melanoma cell line FO-1 does not express HLA class I antigens and does not acquire them on the cell surface after incubation with IFN-γ. Immunochemical studies showed that FO-1 cells synthesize HLA class I heavy chain, but do not synthesize β_2-microglobulin (β_2-μ). The latter abnormality is associated with lack of β_2-μ mRNA which remains undetectable in FO-1 cells incubated with IFN-γ. The defect was identified as a genetic lesion in the B_2m gene, since DNA hybridization analysis detected a deletion of the first exon of the 5'-flanking region, and of a segment of the first intron of the B_2m gene. HLA class I antigen expression was reconstituted on melanoma cells FO-1 after transfection with the wild-type mouse B_2m gene, thereby confirming the abnormality of the endogenous B_2m gene.

The defect identified in FO-1 cells is distinct from that underlying the lack of HLA class I antigen expression by lymphoblastoid cells Daudi, but is remarkably similar to that causing lack of H-2 class I antigen expression by mouse lymphoblastoid cells R1 (TL^-). These results suggest that genetic recombination in the 5' region of the B_2m gene is a recurrent mechanism in B_2m gene defects. In addition to contributing to our understanding of molecular abnormalities in HLA class I antigen expression by melanoma cells, FO-1 cells represent a useful model for analyzing the role of HLA class I antigens in the biology of melanoma cells and in their interaction with cells of the immune system. (*J. Clin. Invest.* 1991. 87:284–292.) Key words: transfection • HLA reconstitution • monoclonal antibody • Northern blot • Southern blot

Introduction

A number of recent investigations have shown that HLA class I antigens play a broader role in cell economy than originally described. Besides their well-known function as restricting elements in the recognition of nominal antigen-modified target cells by cytotoxic T cells (1), HLA class I antigens regulate the proliferation of normal and malignant cells (2–4) and modulate the lysis of malignant cells by natural killer (NK)[1] cells (5–7). Interest in the molecular basis for the functional properties of HLA class I antigens has provided the incentive to analyze the structural organization, the biosynthetic pathway, and the expression of these membrane bound molecules. Like their counterparts in other animal species, HLA class I antigens are composed of a 45,000 M_r glycopolypeptide noncovalently associated with the 11,600 M_r β_2-microglobulin (β_2-μ) (8, 9). The cell surface expression of HLA class I antigens is regulated at several levels. Potent transcriptional control of HLA class I genes has been shown to be mediated by the lymphokines immune interferon (IFN-γ) (10) and tumor necrosis factor (11). At the post transcriptional level, association of β_2-μ with the heavy chain controls the transport of HLA class I molecules to the cell surface (8, 9). A defect in β_2-μ synthesis results in lack of HLA class I antigen cell surface expression as observed in lymphoblastoid cells Daudi; the latter cells do not express HLA class I molecules on the cell membrane, in spite of the synthesis of HLA class I heavy chain (12). The defect in Daudi cells has been shown to be a point mutation in the initiation codon of β_2-μ mRNA that causes low efficiency in its binding to ribosomes, with a resultant lack of protein synthesis (13). HLA class I antigen expression by Daudi cells is reconstituted after somatic cell fusion with β_2-μ^+ cells or after B_2m gene transfer (14–17). Reexpression of HLA class I antigens is associated with modifications in cellular immune recognition of the tumor cell line (17).

Malignant transformation of melanocytes may be associated with a reduction or loss of HLA class I antigen expression (18). To date, limited information is available regarding the molecular mechanism(s) underlying the repression of HLA class I antigen expression by melanoma cells. To the best of our knowledge, only Versteeg et al. (19) have described an inverse correlation between the mRNA levels of c-*myc* and HLA class I antigens in a panel of melanoma cell lines. Furthermore, transfection of a melanoma cell line with a c-*myc* expression vector induced a reduction in the level of HLA class I mRNA and in their cell surface expression. Therefore, Versteeg et al. have concluded that HLA class I antigen expression is modulated by the level of c-*myc* expression in melanoma cells.

Abnormalities in HLA class I antigen expression may affect the ability of the host's immune system to interact with melanoma cells, since HLA class I–negative melanoma cells may fail to recruit cytotoxic T cell precursors and/or may escape from immune destruction by cytotoxic T cells (20). Furthermore, the level of HLA class I antigen expression by melanoma cells may modulate their susceptibility to NK cell lysis (21). These phenomena may represent the mechanism(s) underlying the correlation between level of HLA class I antigens in metastatic melanoma lesions in patients with locoregional disease and clinical course of the disease (22).

Characterization of the molecular abnormalities underlying the lack of HLA class I antigen expression by melanoma

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1. *Abbreviations used in this paper:* β_2-μ, β_2-microglobulin; ICAM-1, intercellular adhesion molecule-1; TNF, tumor necrosis factor.

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cells may not only contribute to our understanding of the mechanism(s) controlling the expression of HLA class I antigens, but may also suggest therapeutic approaches to melanoma, since in animal model systems enhancement of class I histocompatibility antigen expression by tumor cells has been found to have a beneficial effect on the course of the disease (for review see reference 23). Therefore, in the present study we have characterized the molecular defect in the melanoma cell line FO-1, which lacks HLA class I antigens (24). Furthermore, we have compared the molecular mechanism(s) underlying the lack of HLA class I antigen expression by melanoma cells FO-1 to those in lymphoid cells Daudi.

Methods

Cell lines. The melanoma cell line FO-1 was cultured in Dulbecco's minimal essential medium (DMEM) (Gibco Laboratories, Grand Island, NY) supplemented with 10% FCS and 2 mM L-glutamine. The T lymphoblastoid cell line MOLT-4, the B lymphoblastoid cell lines Daudi, MANN, and WALK, and the melanoma cell line 3S5 were grown in Opti-MEM (Gibco Laboratories) medium supplemented with 5% FCS and 2 mM L-glutamine. Transfected FO-1 cells were maintained in DMEM supplemented with the neomycin analogue G418 sulfate (Gibco Laboratories) (1 mg/ml). Cells were harvested by vigorous pipetting with PBS supplemented with 1 mM EDTA.

Monoclonal antibodies and conventional antisera. The MAb W6/32 to a monomorphic determinant expressed on β_2-μ–associated HLA class I heavy chain (25), the MAb TP25.99 to a determinant expressed on both β_2-μ associated and β_2-μ free HLA class I heavy chain, the antihuman β_2-μ MAb BBM-1 (26) and NAMB-1 (27), the anti–HLA-DR,DQ,DP MAb Q5/13 (28), and the anti–intercellular adhesion molecule-1 (ICAM-1) MAb CL207.14 (29) were developed and characterized as described. The rabbit antiserum R5996-4 to denatured HLA class I heavy chain was developed and characterized following the methodology described by Nakamuro et al. (30). The antimouse β_2-μ xenoantiserum R7038, 59–63 was developed as described by Natori et al. (31).

Purified goat anti–rabbit Ig antibodies were purchased from Jackson ImmunoResearch Laboratories, Inc., West Grove, PA.

MAbs were purified from ascitic fluid by sequential precipitation with caprylic acid and ammonium sulphate (32). MAbs were labeled with ^{125}I using the iodogen method (33). The immunoreactive fraction of the radiolabeled antibodies was at least 50%, as measured by the method of Lindmo et al. (34).

cDNA probes and human and mouse B_2m genes. The cDNA probe for HLA-B7 antigen (35) and the cDNA probe for human β_2-μ (36) were isolated by digestion of the plasmids with restriction endonuclease PstI. The cloned inserts were electrophoresed and excised from low melting agarose gel. Human B_2m gene clone pb2m13 in the vector pEMBL9 (37) was purified on CsCl gradient and digested either with the combination of HindIII and XbaI, with the combination of EcoRI and SmaI or with XbaI alone. 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. Mouse B_2m gene (38) cloned into the vector pSV2ΔHXgpt was purified on a CsCl gradient and linearized with the restriction enzyme XhoI before transfection. cDNA and genomic DNA probes were labeled with α-[^{32}P]dCTP (3,000 Ci/mmol; Amersham Corp., Arlington Heights, IL) by random priming (39, 40) to a specific activity of 10^8 cpm/μg.

Cytokines and chemicals. Recombinant human IFN-γ was obtained from Hoffmann-La Roche, Inc., Nutley, NJ. 5-Azacytidine was purchased from Aldrich Chemical Co., Milwaukee, WI.

Serological assays. The direct binding assay was performed in 96-well microtiter plates (Becton Dickinson & Co., Oxnard, CA). Cells were washed twice with HBSS and seeded at the concentration of 10^5/well in plates that had been coated with BSA by a 1-h incubation at room temperature with PBS containing 1% BSA. After the addition of ^{125}I-labeled antibodies (5×10^5 cpm/well), plates were incubated 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 10^5 cells.

Radiolabeling of cells, indirect immunoprecipitation, and SDS-PAGE. These procedures were performed as described elsewhere (41). Briefly, cells were labeled either with ^{125}I (Amersham Corp.) using the lactoperoxidase method (42) or with [^{35}S]methionine (Amersham Corp.). Cells were then solubilized by incubation for 30 min at 4°C in lysis buffer containing 1% NP-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 Fine Chemicals Piscataway, NJ) One-dimensional SDS-PAGE analysis was performed under reducing conditions in slab gels containing 10% acrylamide and using the buffer system described by Laemmli (43). 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 (44).

RNA hybridization analysis. Total RNA was extracted from cells using the method described by Davis et al. (45). RNA was then size fractionated on a 1% agarose gel containing 2.2 M formaldehyde, 20 mM 3-(*N*-morpholino) propane sulfonic acid, pH 7.0, 5 mM sodium acetate, and 1 mM EDTA (46) and transferred to a nitrocellulose membrane (Bio-Rad Laboratories, Richmond, CA) using 10× SSC (1× SSC: 0.15 M sodium chloride, 0.015 M sodium citrate, pH 7.0) as described by Thomas (47). Blots were baked at 80°C under vacuum for 2 h and prehybridized at 65°C for 3 h in 5× SSC, 5× Denhardt's reagent, 0.2 M sodium phosphate, pH 7.0, and denatured salmon sperm DNA (100 μg/ml). Filters were hybridized for 12–16 h at 37°C in hybridization buffer containing 50% formamide and a ^{32}P-labeled probe. After two washings at 42°C for 30 min with 1× SSC, 0.1% SDS, blots were washed twice at 55°C for 30 min with 0.1× SSC, 0.1% SDS, dried, and autoradiographed at −70°C for 1–3 d using XAR-5 x-ray film (Eastman Kodak Co., Rochester, NY) and an intensifying screen (DuPont Co., Wilmington, DE).

DNA hybridization analysis. Genomic DNA was extracted essentially as described by Perbal (48). DNA was digested with restriction endonucleases for 24 h and then run on a 1% agarose gel containing Tris-borate-EDTA buffer. Blotting was performed according to standard procedures (49). After electrophoresis, the gel was soaked in several volumes of denaturing solution (1.5 M NaCl and 0.5 M NaOH) for 1 h at room temperature and then neutralized in 1 M Tris-HCl (pH 8.0) and 1.5 M NaCl. After gel transfer, the nitrocellulose membrane was baked at 80°C for 2 h. Prehybridization, hybridization, washings, and autoradiography were performed following the same experimental conditions used for RNA hybridization analysis.

Transfection of FO-1 cells with mouse B_2m gene. Transfection of melanoma cells with the 14-kb XhoI fragment containing the mouse B_2m gene and with pSV2neo was performed by electroporation essentially as described (50). Briefly, cells (2×10^7), the 14-kb XhoI 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 DMEM medium supplemented with 10% FCS, seeded in eight 100-mm tissue culture dishes, and incubated at 37°C in 5% CO_2. The following day, G418-sulfate was added to the medium at the final concentration of 3 mg/ml (effective concentration was ~ 1.5 mg/ml). The concentration of G418-sulfate was reduced to 2 mg/ml after 3 d of incubation and to 1 mg/ml after 7 d of incubation. Cells from each plate were allowed to grow to confluence.

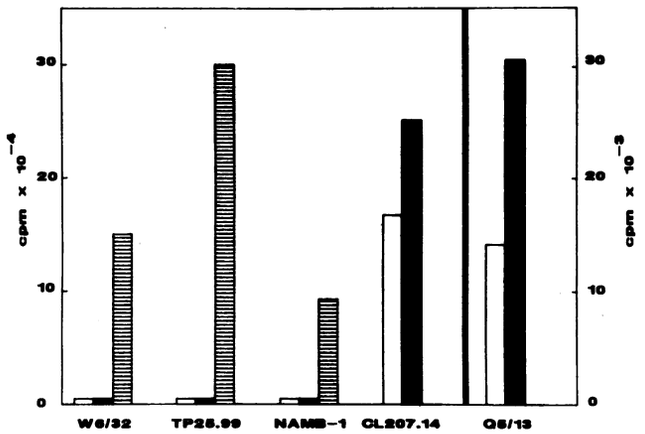

Figure 1. Lack of reactivity of control and IFN-γ–treated cultured melanoma cells FO-1 with anti HLA class I MAb. FO-1 cells were incubated at 37°C for 72 h with IFN-γ (final concentration 1,000 U/ml) (■). Control cells (□) were incubated under the same experimental conditions, but without cytokine. Cells were then harvested, washed twice with HBSS, and tested with either ^{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 or ^{125}I-anti β_2-μ MAb NAMB-1 in the binding assay. ^{125}I–anti-ICAM-1 MAb CL207.14 and ^{125}I-anti HLA class II MAb Q5/13 were used to monitor the susceptibility of FO-1 cells to modulation by IFN-γ. The reactivity of radiolabeled anti HLA class I MAb preparations was monitored by testing with cultured B lymphoblastoid cells MANN (▣) in the binding assay.

Results

Lack of expression of HLA Class I antigens by FO-1 melanoma cells. Testing of melanoma cells FO-1 with radiolabeled anti HLA Class I MAbs in a binding assay showed no reactivity with MAb W6/32 to a monomorphic determinant expressed on β_2-μ–associated HLA class I heavy chain, with MAb TP25.99 to a determinant expressed on β_2-μ–associated and β_2-μ–free HLA class I heavy chain, and with the anti β_2-μ MAb NAMB-1 (Fig. 1). No reactivity with these MAbs was detected even after cells were incubated with IFN-γ (final concentration 1,000 U/ml) for 3 d (Fig. 1) or with 5-azacytidine (final concentration 3 μM) for 7 d (data not shown) before being tested. FO-1 cells are sensitive to the modulating activity of IFN-γ, since treated cells displayed a marked increase in the reactivity with anti–HLA class II MAb Q5/13 and with anti ICAM-1 MAb CL 207.14 (Fig. 1). The lack of serological reactivity of FO-1 cells with anti–HLA class I MAbs was corroborated by the results of SDS-PAGE analysis of antigens immunoprecipitated by anti HLA class I monoclonal and polyclonal xenoantibodies from radiolabeled FO-1 cells (Fig. 2). HLA class I antigens were not detected in the immunoprecipitate either with anti–HLA class I MAb W6/32 or with anti β_2-μ MAb NAMB-1 from an NP-40 extract of ^{125}I surface-labeled (data not shown) or [^{35}S]methionine-labeled control and IFN-γ–treated FO-1 cells. Furthermore, HLA class I antigens were not immunoprecipitated from an NP-40 extract of ^{125}I surface-labeled control and IFN-γ–treated FO-1 cells by MAb TP25.99 reacting with β_2-μ–associated and β_2-μ–free HLA class I heavy chain (data not shown). In contrast, the HLA class I heavy chain was immunoprecipitated from an NP-40 extract of [^{35}S]methionine-labeled FO-1 melanoma cells by the xenoantiserum R5996-4 that recognizes denatured HLA class I heavy chain. When IFN-γ–treated FO-1 melanoma cells were used as an antigen source, the intensity of the component immunoprecipitated by xenoantiserum R5996-4 was increased; furthermore, the HLA class I heavy chain could be detected in the immunoprecipitate with MAb TP25.99. It is noteworthy that β_2-μ was detected neither in the immunoprecipitate from control nor in that from IFN-γ–treated FO-1 cells. To exclude the possibility that the lack of detection of β_2-μ reflected the lack of expression of the determinant recognized by MAb NAMB-1, binding assays were repeated with the anti-β_2-μ MAb BBM-1. Also with the latter reagent β_2-μ was not detected in FO-1 melanoma cells. The

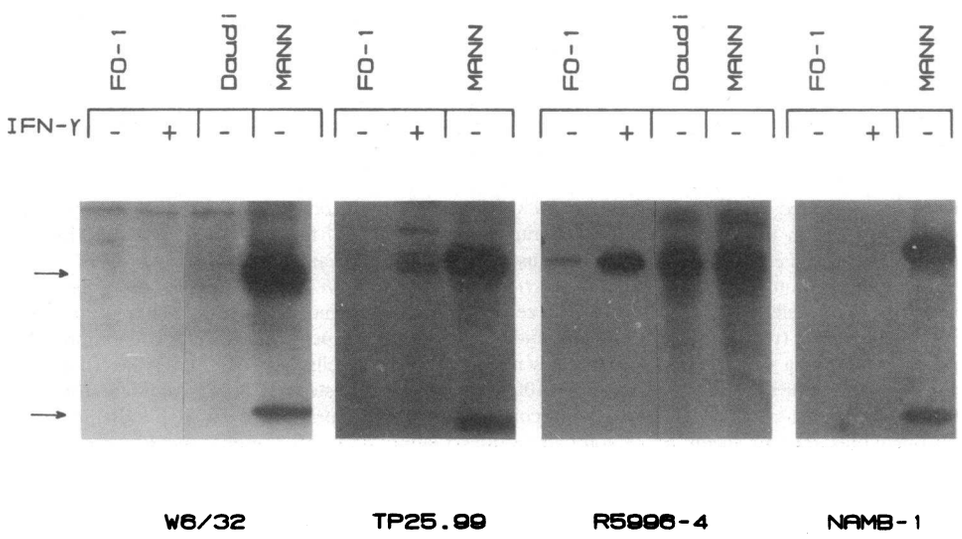

Figure 2. SDS-PAGE analysis of antigens immunoprecipitated from control and IFN-γ–treated cultured melanoma cells FO-1 by anti–HLA class I monoclonal and polyclonal xenoantibodies. After a 72-h incubation at 37°C in medium supplemented with IFN-γ (final concentration 1,000 U/ml), FO-1 cells were starved for 1 h in methionine-free medium and intrinsically radiolabeled with [^{35}S]-methionine. Control FO-1 cells and B lymphoblastoid cells Daudi and MANN 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% NP-40. After indirect immunoprecipitation with MAb W6/32, TP25.99 and NAMB-1, and xenoantiserum R599b-4, antigens were eluted from the immunoabsorbent and analyzed by SDS-PAGE in the presence of 2% β-mercaptoethanol. Gels were then processed for fluorography (44). Arrows indicate the two subunits of HLA class I antigens.

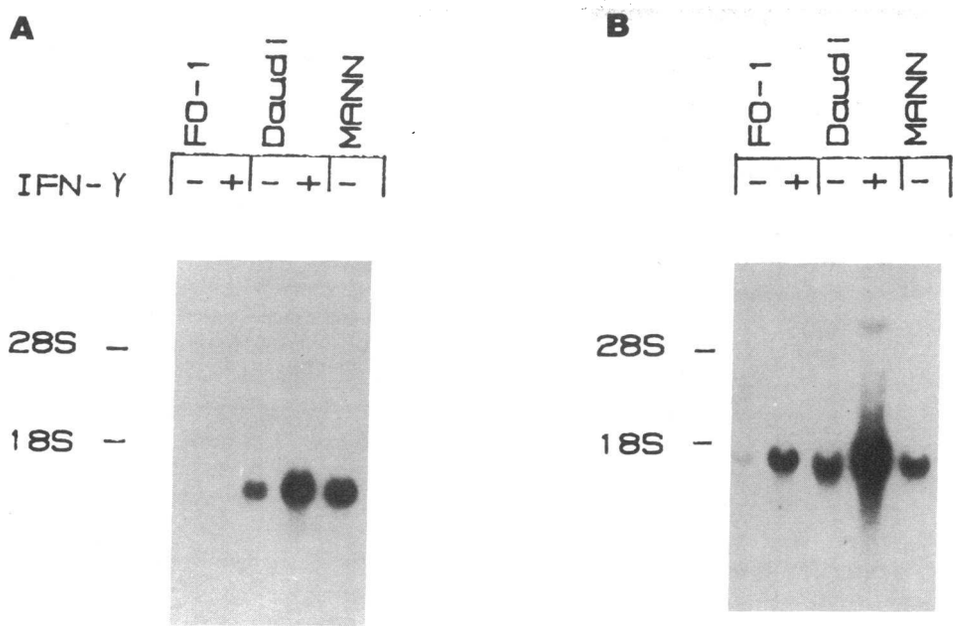

Figure 3. Northern blot analysis of HLA class I heavy chain and β_2-μ mRNA in cultured melanoma cells FO-1. FO-1 cells were incubated at 37°C for 72 h with IFN-γ (final concentration 1,000 U/ml). Control cells were incubated under the same experimental conditions, but without cytokine. At the end of 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*). RNA isolated from control and IFN-γ–treated B lymphoblastoid cells Daudi was analyzed for comparison purposes. RNA isolated from the B lymphoblastoid cell line MANN was used as a positive control.

latter findings parallel those we obtained with Daudi cells, the only other known human cell line that does not express HLA class I antigens because of a β_2-μ defect (12, 13). Representative results are shown in Fig. 2, which also presents the SDS-PAGE profile of components immunoprecipitated from [^{35}S]methionine-labeled cultured B lymphoid cells MANN that were used as a positive control.

Molecular analysis of the lack of β_2-μ expression by FO-1 melanoma cells. To investigate the mechanism(s) underlying the lack of B_2m gene expression, the steady-state level of mRNA for β_2-μ was evaluated by RNA hybridization analysis. β_2-μ mRNA was not detected in FO-1 cells, even when RNA was extracted from cells that had been incubated with IFN-γ (1,000 U/ml) for 72 h. In contrast, RNA hybridizing to the crosshybridizing HLA-B7 cDNA probe was present in FO-1 cells and its level was increased in IFN-γ treated melanoma cells FO-1. These results are presented in Fig. 3, which also shows that a normal level of β_2-μ mRNA is detectable in Daudi cells and is enhanced in Daudi cells treated with IFN-γ (1,000 U/ml) for 72 h.

Abnormal restriction patterns for the B_2m gene in FO-1 melanoma cells. To determine whether the absence of β_2-μ mRNA

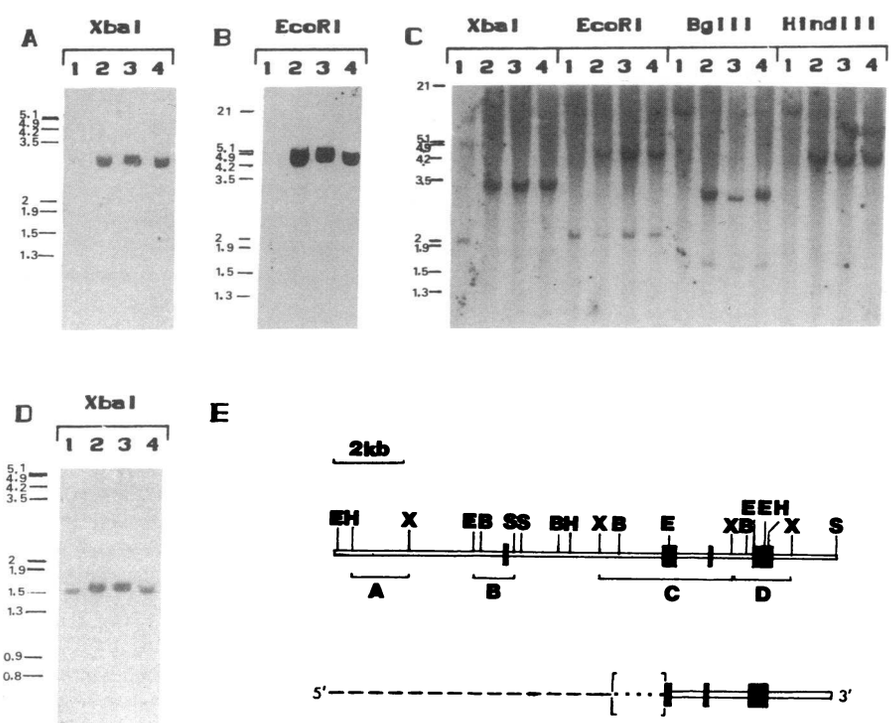

Figure 4. Southern blot analysis of B_2m gene in cultured melanoma cells FO-1. DNA (20 μg) isolated from cultured melanoma cells FO-1 (lane *1*) was digested overnight at 37°C with the restriction enzymes BglII, EcoRI, HindIII, or XbaI. Digested DNA was fractionated on a 1% agarose gel and transferred to nitrocellulose filters. Blots were hybridized with a ^{32}P-labeled 1.4-kb HindIII-XbaI fragment referred to as probe A (*A*), with a ^{32}P-labeled 0.8-kb EcoRI-SmaI fragment referred to as probe B (*B*), with a ^{32}P-labeled 3.5-kb XbaI fragment referred to as probe C (*C*) or with a ^{32}P-labeled 1.4-kb XbaI fragment referred to as probe D (*D*) corresponding to the segments of the human B_2m gene shown in *E*. DNA (20 μg) from cultured B lymphoblastoid cells Daudi (lane *2*) and WALK (lane *4*) and from cultured melanoma cells 3S5 (lane *3*) was used as a control. (*E*) The map of human B_2m gene with the cutting sites of the restriction enzymes BglII (*B*), HindIII (*H*), EcoRI (*E*), SmaI (*S*), and XbaI (*X*) (37). The black blocks indicate the four exons. The broken line indicates the deletion region; the dotted line within brackets indicates the segment within which recombination occurred.

reflected deletion or rearrangement of the B_2m gene, genomic DNA from FO-1 cells was initially analyzed with the human β_2-μ cDNA probe using Southern blot analysis. The restriction pattern of FO-1 cell DNA, digested with several restriction enzymes, was very different from that of the control DNA including cultured B lymphoid cells Daudi and WALK, cultured T lymphoid cells MOLT-4, and cultured melanoma cells 3S5 (data not shown). These results suggested that the defect in HLA class I antigen expression is due to a gross alteration of the B_2m gene or of flanking regulatory sequences.

To define the nature and the extent of the abnormality, DNA from FO-1 cells was digested with XbaI, EcoRI, BglII, or HindIII and hybridized with probes derived from the human pb2m13 clone (Fig. 4, *E* for restriction map according to Güssow et al. [37]). DNA from cultured B lymphoid cells Daudi and WALK and from cultured melanoma cells 3S5 was used as a control. Representative results for the lack of hybridization of probes A and B with the FO-1 cell DNA digested with one of the four enzymes used, are shown in Fig. 4, *A* and *B*, respectively. Hybridization of probe C with FO-1 cell DNA revealed a significant alteration in the restriction pattern as compared with control cell DNA. When digested with XbaI FO-1 cell DNA gave rise to a fragment which is ~ 1.5 kb smaller than that detected in control cell DNA. When FO-1 cell DNA was digested with HindIII, probe C hybridized to a 12.5-kb fragment which is ~ 7 kb larger than the corresponding fragment in control cell DNA. When FO-1 cell DNA was digested with BglII, probe C hybridized to a 12.5-kb fragment, whereas the two fragments detected in control cell DNA were 3.3 and 1.7 kb. Finally, when FO-1 cell DNA was digested with EcoRI, probe C did not hybridize to a 4.5-kb fragment that is present in control cell DNA, but hybridized to a fragment of 2.2 kb that includes the 3′ half of exon 2 and all of exon 3. The hybridization pattern of probe D with FO-1 cell DNA digested with XbaI (Fig. 4 *D*) and with EcoRI (data not shown) was not different from that with control cell DNA.

Induction of HLA class I antigens on FO-1 melanoma cells by transfection with mouse B_2m gene. To prove that the lack of HLA class I antigen expression by melanoma cells FO-1 reflected only the lack of synthesis of the β_2-μ subunit resulting from the genetic defect described above, cells were transfected by electroporation with the mouse B_2m gene. Transfectants were selected by resistance to the antibiotic G418, stained by sequential incubation with anti HLA class I MAb W6/32 and FITC-conjugated antimouse Ig xenoantibodies, and then analyzed by cytofluorometry for an initial screening. FO-1 clones

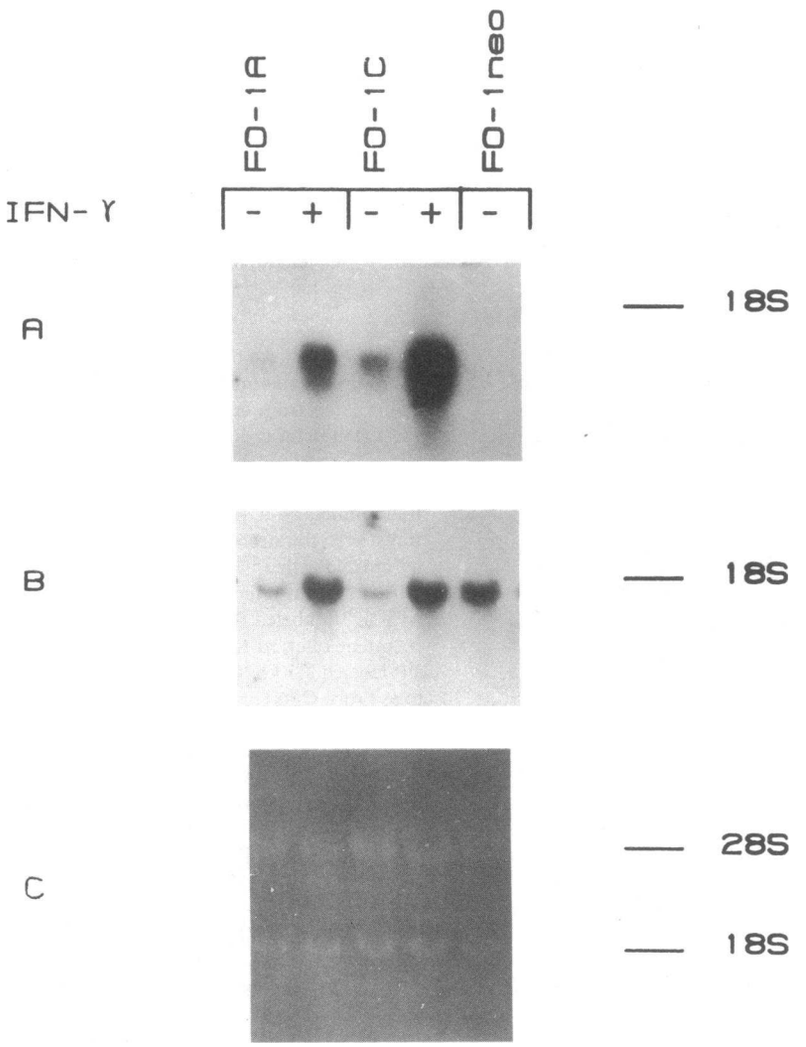

Figure 5. Northern blot analysis of β_2-μ mRNA in cultured melanoma cells FO-1 transfected with mouse B_2m gene. Clones FO-1A and FO-1C transfected with the mouse B_2m gene were incubated at 37°C for 72 h with IFN-γ (final concentration 1,000 U/ml). Control FO-1A and FO-1C clones and FO-1 cells transfected with pSV2neo gene 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 a ^{32}P-labeled 14-kb XhoI mouse B_2m gene fragment (*A*) and with ^{32}P-labeled 1.4-kb HLA-B7 cDNA probe (*B*). (*C*) The ethidium bromide staining of RNA applied to each lane of the gel before transfer.

A and C, which were weakly stained, were expanded and characterized. To confirm co-transfection, Northern blot analysis was performed using the mouse B_2m gene as probe. Both FO-1 clones A and C expressed β_2-μ mRNA which was not detected in FO-1 cells transfected with the pSV2neo gene alone (Fig. 5). In a direct binding assay, FO-1 clones A and C displayed a weak reactivity with ^{125}I-labeled anti-HLA class I MAb W6/32 and TP25.99 (Fig. 6). Furthermore, SDS-PAGE analysis of immunoprecipitates with the MAb W6/32 (Fig. 7) from ^{125}I-labeled FO-1 clones A and C detected two components with the characteristic electrophoretic mobility of the heavy and light chain of HLA class I antigens.

The reactivity of MAb W6/32 and TP25.99 with FO-1 clones A and C reflects the transfection of B_2m gene, since the two MAbs did not react in binding assays with FO-1 cells transfected with pSV2neo gene alone (Fig. 6) and did not immunoprecipitate the two subunits of HLA class I antigens from these latter cells. After incubation with IFN-γ (1,000 U/ml) for 3 d FO-1 clones A and C displayed a marked increase in the level of β_2-μ mRNA (Fig. 5), in their reactivity with ^{125}I-MAb W6/32 and TP25.99 in binding assays (Fig. 6) and in the intensity of HLA class I antigens detected by SDS-PAGE in the immunoprecipitates with MAb W6/32 (Fig. 7). The increase induced by IFN-γ in the synthesis and expression of HLA class I antigens by FO-1 clones A and C is specific, since it induced only an increase in the HLA class I heavy chain mRNA in FO-1 cells transfected with the pSV2neo gene alone (results not shown) and did not change their lack of reactivity with anti-HLA class I MAb W6/32 and TP25.99 (Fig. 6).

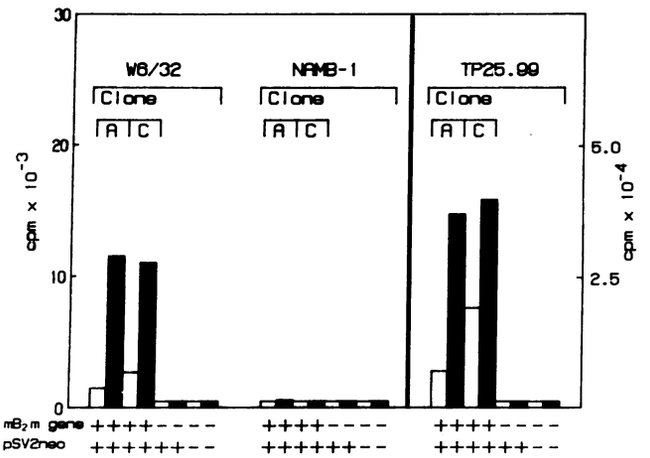

Figure 6. Reactivity with anti HLA class I MAbs of control and IFN-γ-treated FO-1 clones A and C transfected with mouse B_2m gene. FO-1 clones A and C, FO-1 cells transfected with pSV2neo gene, and untransfected FO-1 cells were incubated at 37°C for 72 h with IFN-γ (final concentration 1,000 U/ml) (■). Control cells were incubated under the same experimental conditions, but were not exposed to cytokine (□). At the end of the incubation, cells were 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, and with ^{125}I-MAb TP25.99 to a determinant expressed on β_2-μ–associated and on β_2-μ–free HLA class I heavy chain in the binding assay. ^{125}I-anti-human β_2-μ MAb NAMB-1 was used as a negative control.

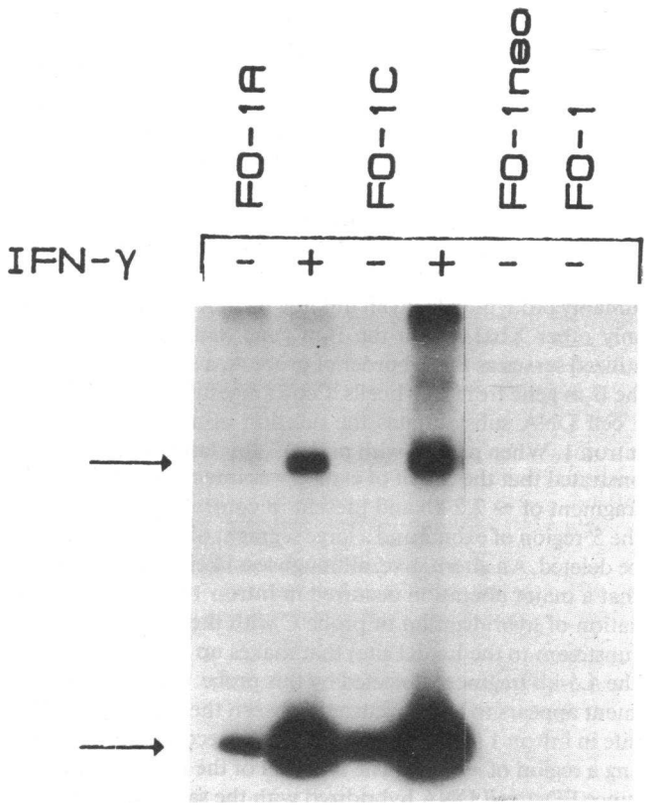

Figure 7. SDS-PAGE analysis of antigens immunoprecipitated by anti-HLA class I MAb W6/32 from control and IFN-γ–treated FO-1 clones A and C transfected with mouse B_2m gene. After a 72-h incubation at 37°C in medium supplemented with IFN-γ (final concentration 1,000 U/ml), transfected cells were harvested. Control cells were cultured 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, labeled with ^{125}I, and solubilized with NP-40 lysis buffer. After indirect immunoprecipitation with the anti-HLA class I MAb W6/32 antigens were eluted from the immunoadsorbent and analyzed by SDS-PAGE in the presence of 2% β-mercaptoethanol. Gels were then autoradiographed. Arrows indicate the two subunits of HLA class I antigens.

Discussion

Using a combination of immunochemical and recombinant DNA approaches we have characterized the molecular mechanism(s) underlying the lack of HLA class I antigen expression by melanoma cells FO-1. These cells synthesize HLA class I heavy chain, but display a B_2m gene defect that precludes production of this protein. Genomic DNA hybridization analysis has shown that the B_2m gene from FO-1 cells sustained a genetic alteration in its 5′ region, since no hybridizing bands were detected in FO-1 cells when probed with a DNA fragment corresponding to exon 1 of the B_2m gene. On the 5′ side of exon 1 the deletion extended at least −3 kb relative to the transcription start site. On the 3′ side of exon 1, the limit of the deletion appears to extend to a region within or immediately 5′ of exon 2, since several restriction sites between exon 1 and 2 had been lost. Specifically, when probe C containing exons 2 and 3 was

hybridized to XbaI-digested FO-1 cell DNA, a 2-kb fragment was identified, while a 3.5-kb fragment was detected in control cell DNA. Since the 3′ XbaI site is intact (based upon hybridization with probe D, corresponding to exon 4), this places the 5′ upstream XbaI site immediately upstream or within exon 2. That the 5′ XbaI site present in the wild-type B_2m gene was deleted in FO-1 cells was shown by BglII digestion, which identified a deletion of a 1.7-kb BglII fragment present in intron 1; the XbaI site maps to this DNA segment. Thus, the 5′ boundary of XbaI-digested FO-1 cell DNA probed with probe C is presumably brought in from an unknown DNA segment, since the only other XbaI site in the B_2m gene that could have been utilized serves as the 3′ border of probe A, a segment deleted in the B_2m gene from FO-1 cells. EcoRI digestion patterns of FO-1 cell DNA substantiates the deletion of a major section of intron 1. When probed with probe C, this latter digestion demonstrated that the 3′ half of exon 2 was intact (represented by a fragment of $\sim$ 2.2 kb and present in control cell DNA), while the 5′ region of exon 2 and a large segment of intron 1 appear to be deleted. An alternative, although less likely, interpretation is that a major alteration occurred in intron 1 causes a destabilization of hybridization of probe C with the portion of exon 2 (upstream to the EcoRI site) that makes up a small segment of the 4.5-kb fragment detected by this probe. Thus, a rearrangement appears to have occurred between the downstream BglII site in intron 1 and the EcoRI site in the second exon, comprising a region of $\sim$ 2 kb. The 3′ region of the B_2m gene is intact, since FO-1 cell DNA hybridized with the same pattern as control cell DNA with a probe corresponding to exon 4. The deletion, therefore, comprises a region of DNA of at least 6 kb based on the restriction maps of Güssow et al. (37).

DNA hybridization analysis with the probe derived from exons 2 and 3 (probe C, Fig. 4) of the B_2m gene detected no hybridizing fragment with the same size in XbaI-, BglII-, or HindIII-digested FO-1 and control cell DNA, and one hybridizing band with the same size in EcoRI digests. According to the size, the latter component is likely to contain part of exon 2, the second intron, exon 3, and intron 3. These data suggest that FO-1 cells have a single copy of the B_2m gene or that both copies have the identical lesion. Karyotype analysis indicates that only one copy of chromosome 15, on which B_2m gene maps (51), is present in FO-1 cells. Thus, unless this gene has undergone duplication on the existing chromosome, we favor the possibility that a single B_2m gene exists in FO-1 cells and that this gene has sustained a deletion of the 5′ coding region.

Although one cannot exclude that FO-1 cells transcribe an abnormal mRNA species that is unstable and degraded more rapidly than the normal β_2-μ mRNA, the transcriptional block of the B_2m gene is likely to be caused by the lack of a major portion of its coding sequence and of upstream transcriptional regulatory sequences. This possibility is corroborated by the expression of HLA class I antigens on FO-1 cells transfected with the wild-type B_2m gene. The latter finding also indicates that the HLA class I heavy chains synthesized by FO-1 cells are not aberrant and can be expressed on the plasma membrane in association with β_2-μ. Furthermore, these data are consistent with the general conclusion that the expression of HLA class I antigens on the cell membrane requires the association of heavy chain with β_2-μ (8, 9). This is in striking contrast to the mouse MHC class I products encoded by the H-2D/H-2L region genes, which are unique in their capacity for transport to the cell surface in the apparent absence of β_2-μ (52–54). The reasons for this disparity remain an enigma.

Several cell lines without detectable HLA class I antigen expression have been characterized. Different molecular mechanisms have been shown to underlie this abnormality and each is different from the B_2m gene defect we have identified in FO-1 cells. The small cell lung cancer cell lines analyzed by Doyle et al. (55) showed a marked reduction in the transcription of HLA class I heavy chain and B_2m genes. A similar defect was found in the melanoma cell lines X3C-5, 453A, and 136-2 described by Versteeg et al. (19). At variance with FO-1 cells, both the small cell lung cancer cell lines and the melanoma cell lines 453A and 136-2 acquired HLA class I antigens after incubation with IFN-γ. Although the nature of the molecular defect(s) in these cell lines was not characterized, it appears to be unrelated to that identified in FO-1 cells, since the latter do not acquire HLA class I antigens after incubation with IFN-γ. Gamma irradiation of B lymphoblastoid cells followed by selection for HLA class I antigen loss by antibody and complement has resulted in the loss of genes encoding the HLA class I heavy chains or of transacting elements that regulate a post-transcriptional step in the expression of HLA class I antigens (56–58). However, no mutant was isolated with a structural abnormality of B_2m gene. Daudi cells, which are the only previously described human cell line with a spontaneous lack of β_2-μ synthesis (8, 12, 14), transcribe the B_2m gene, but do not translate β_2-μ mRNA (13, 59) because of a point mutation in the initiation codon (13). Furthermore, Daudi cells differ from FO-1 cells in their susceptibility to modulation by IFN-γ of the mRNA encoding the two subunits of HLA class I antigens: IFN-γ enhances the level of HLA class I heavy chain and β_2-μ mRNA in Daudi cells, but upregulates only the level of HLA class I heavy chain mRNA in FO-1 cells. However, in both Daudi (16) and FO-1 cells, HLA class I antigen expression was reconstituted by transfection with the mouse B_2m gene.

Although not identified previously in human cell lines, the deletion of exon 1 from the B_2m gene we have described is not unique of FO-1 cells, since a similar defect has been identified in four mouse cell lines derived from the C58 mouse thymoma cell line R1.1 by chemical or irradiation mutagenesis and subsequent selection with anti-TL antisera and complement (38, 60, 61). Like FO-1 cells, the four mouse cell lines synthesize H-2 class I heavy chain but do not express H-2 class I antigens on the cell membrane, since defects in B_2m gene preclude transcription of functional β_2-μ mRNA. Furthermore, like FO-1 cells, two mutants have only one copy of the chromosome which carries the B_2m gene, and one mutant expresses H-2 class I antigens after having reacquired a functional B_2m gene by hybridization with an antigenically distinct cell line (61). Although different genomic rearrangements underlie the B_2m gene abnormality in the four mutants, in each of them the defects in at least one B_2m gene map to the same small DNA segment within the first intron. Thus, there may exist a hypermutable site within the first intron of mouse B_2m gene. What is the mechanism responsible for the deletion of B_2m exon 1 DNA from the FO-1 cell line? It has been established that the 5′ region of the human B_2m gene is a region of clustered CpG dinucleotides (37). Such regions may be prone to genetic recombination. Thus, the CG-rich nature of the B_2m gene may have contributed to the genetic rearrangement that has occurred in melanoma cell line FO-1. Since a similar genetic alteration has

occurred in unrelated tumor cell lines with a known defect in B_2m gene expression, recombination in the 5′ region of the B_2m gene may be a frequent mechanism for defective B_2m gene expression.

The defect we have found in FO-1 cells is not a general mechanism for the lack of HLA class I antigen expression in melanoma cells. Besides the inverse relationship between HLA class I antigen mRNA and c-*myc* mRNA described by Versteeg et al. (19, 21, 62), we have recently shown that the melanoma cell lines SK-MEL-19 and SK-MEL-33 are defective in HLA class I gene expression due to phenotypically distinct alterations (unpublished observation). Post-transcriptional abnormalities result in the arrest of HLA class I heavy chain and β_2-μ biosynthesis in SK-MEL-19 and in SK-MEL-33 cells, respectively. Characterization of the molecular mechanisms underlying the lack or reduction of HLA class I antigen expression by these melanoma cell lines may suggest approaches to analyze abnormalities in HLA class I antigen expression which are frequently found in surgically removed melanoma lesions (18).

Conflicting results have been reported regarding the relationship of class I histocompatibility antigen expression and susceptibility of malignant cells to NK-mediated cytolysis (for review see reference 63). To the best of our knowledge, only Lobo and Spencer (64) and Versteeg et al. (21) have investigated this phenomenon in human melanoma. The results have not been conclusive, since only the latter investigators reported a role of HLA class I antigens in NK cell–mediated lysis of melanoma cells and the experimental conditions used do not exclude the interference of unrelated molecules in the phenomenon. Furthermore, the conclusions drawn from experiments performed with other types of malignant cells might not be applicable to melanoma cells, since they frequently express HLA class II antigens (for review see reference 65) and adhesion molecules (66, 67) which are involved in NK cell mediated lysis (64; Maio, M., and S. Ferrone, unpublished results). Therefore, the HLA class I–negative and HLA class I–reconstituted FO-1 melanoma cells, which express also HLA class II antigens and ICAM-1, represent a useful model to investigate the role of HLA class I antigens in NK cell–mediated lysis of melanoma cells. This information in conjunction with the characterization of the molecular mechanisms underlying abnormal HLA class I antigen expression in melanoma lesions may suggest novel therapeutic strategies to melanoma.

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