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A Novel Autocrine Pathway of Tumor Escape from Immune Recognition: Melanoma Cell Lines Produce a Soluble Protein That Diminishes Expression of the Gene Encoding the Melanocyte Lineage Melan-A/MART-1 Antigen Through Down-Modulation of Its Promoter

James T. Kurnick,[1]* Teresa Ramirez-Montagut,* Lenora A. Boyle,* David M. Andrews,* Franco Pandolfi,[†] Paul J. Durda,* David Butera,* Ian S. Dunn,[‡] Elizabeth M. Benson,[‡] Sam J. P. Gobin,[§] and Peter J. van den Elsen[§]

We have observed that malignant melanoma cells produce a soluble protein factor(s), which down-regulates melanocyte lineage Melan-A/MART-1 Ag expression by melanoma cells with concomitant loss of recognition by Melan-A/MART-1-specific T cells. This down-modulation of Melan-A/MART-1 expression, which we refer to as "Ag silencing," is mediated via its minimal promoter, whereas the promoter for the restricting Ag-presenting HLA-A2 molecule is not affected. Significantly, this Ag silencing is reversible, as removal of factor-containing supernatants from Melan-A/MART-1-expressing cells results in up-regulation of the promoter for the gene encoding this Ag, and renewed expression of the protein. We have evaluated over 20 known factors, none of which accounts for the Ag-silencing activity of the melanoma cell culture supernatants. The existence of this autocrine pathway provides an additional novel explanation for melanoma tumor progression in vivo in the presence of CTL specific for this melanocyte lineage Ag. These observations may have important implications for Melan-A/MART-1-specific CTL-mediated immunotherapy of melanoma tumors. *The Journal of Immunology,* 2001, 167: 1204–1211.

The cellular immune response against human melanomas is often characterized by the accumulation of tumor-infiltrating lymphocytes (TIL),[2] which include tumor-specific cytotoxic T cells (1–3). In some patients, TIL show strong in vitro lytic activity that is often directed against HLA-A2$^+$ targets presenting the immunodominant peptide of the melanocyte lineage Ag Melan-A/MART-1 (aa 27–35; AAGIGILTV) (4–7). These observations have led numerous investigators to use T cell-based immunotherapy in attempts to manage this highly malignant tumor (8–11). This is illustrated by the intense ongoing efforts to vaccinate melanoma patients with the immunodominant HLA-A2-restricted Melan-A/MART-1 peptide (aa 27–35) (5, 6, 12). Although it has been demonstrated that such a peptide-based vaccination has led to enhanced cell-mediated immunity against targets expressing this peptide, the escape from T cell recognition of Melan-A/MART-1-negative tumors has limited the efficacy of therapeutic intervention targeting this Ag (13–17).

There is ample evidence to demonstrate that tumor cells use many strategies to evade immune destruction by the host (13–23). A frequent finding is that the HLA-restricting element is lost, sometimes via an allele-specific mechanism that may include loss of heterozygosity or mutations within the coding exons (13, 24–29). In other cases, there is a more global down-regulation of expression of HLA molecules, such as those resulting from mutations in the TAP or β_2-microglobulin genes (14, 30). Recently, the proto-oncogene promyelocytic leukemia-1 has been implicated in the modulation of MHC expression via control of TAP and latent membrane protein genes, thereby providing for tumor escape from T cell recognition (31). In addition, the selective loss of immunodominant tumor Ags will also result in immune escape (19, 32, 33). Such tumor cells are no longer recognized by T cells specific for these elements, and thus are probably responsible, at least in part, for the observed loss of lymphocyte infiltrates in tumors with associated poor prognosis (34). Yet, those cases of melanoma in which progression occurs in the presence of tumor-specific cytotoxic TIL present an enigma (1): clinically apparent tumor cells that evoke a cytotoxic immune response must also be able to implement a mechanism to elude the immune response.

In this study, we describe a novel mechanism of autocrine down-regulation of an immunodominant tumor-associated Ag that explains how a melanoma cell can escape immune destruction in the presence of cytotoxic T cells directed against a widely expressed melanocyte lineage Ag, Melan-A/MART-1. Previously, we have shown that spontaneous down-modulation of Melan-A/MART-1 expression was observed when melanoma cells were grown at high density, whereas this Ag continued to be expressed by tumor cells cultured at low density, and could be rejuvenated by low density culture (35). The reduced expression of Melan-A/

MART-1 protein at high density correlated with decreased T cell recognition (35). These observations suggested that expression of Melan-A/MART-1 could be down-modulated in vitro during melanoma cell growth through an autocrine pathway. In the current work, we demonstrate that several Melan-A/MART-1-deficient melanoma cell lines produce a soluble protein factor(s), the activity of which ultimately results in down-regulation of Melan-A/MART-1 protein expression in autologous and allogeneic melanoma cell lines. This down-regulation, which we shall refer to as melanoma Ag-silencing activity (MASA), prevents immune recognition of Melan-A/MART-1 by suppressing the activity of the gene promoter for this Ag. Of note, neither cell surface expression of HLA class I molecules nor the HLA-A2 promoter was affected by MASA. This loss of a T cell-recognized Ag may have significant biological and therapeutic implications. The reversibility of Ag silencing may hold a key to the maintenance of effective cell-mediated immunity.

Materials and Methods

Tumor and TIL

Melanoma cells and TIL from the melanoma tissues were obtained according to approved Massachusetts General Hospital guidelines, and propagated in vitro, as previously described (1). Briefly, tumors were cultured in DMEM medium supplemented with 10% FBS, and TIL were propagated in RPMI 1640 supplemented with 5% human serum containing human rIL-2 at 100 U/ml (Cetus, Emoryville, CA). TIL clones were isolated by limiting dilution, as previously described (1). Tumors MU and EW were obtained from cutaneous metastatic melanoma deposits. Melan-A/MART-1-negative variant MU-X was obtained by culture of MU tumor cells at high density ($>5 \times 10^5$ cells/ml) for several days before immunoselection with Melan-A/MART-1-specific TIL. After 1 wk of coculture of tumor cells and lymphocytes in the presence of human rIL-2, the tumor cells that propagated were collected and maintained in culture in the absence of T cells. These MU-X, Melan-A/MART-1-negative tumor cells have remained negative in the intervening 2-year period. (Melan-A/MART-1 expression was tested as described below.)

Generation of conditioned medium

Conditioned medium from Melan-A/MART-1-deficient melanoma cell lines was generated by culturing cells at a starting concentration of 5×10^5 cells/ml in DMEM medium supplemented with between 1 and 10% FBS. Supernatants were collected after 72 h by centrifugation of the cell cultures and filtration of the medium through a 0.2-μm filter (Millipore, Bedford, MA). Conditioned medium containing 1% FBS was concentrated between 10- and 20-fold by collecting the retentate from a nominal 30-kDa YM membrane (Centriprep; Millipore, Bedford, MA). In addition to tumor cell lines MU-X, EW, and IGR39D, three nonmelanoma cell lines were also used to generate conditioned medium under similar conditions. These human tumor cell lines were Daudi (B cell lymphoma), Jurkat (T cell lymphoma), and MCF-7 (breast carcinoma). These three cell lines were obtained from the American Type Culture Collection (ATCC, Manassas, VA).

Assessment of Ag-silencing activity

To monitor alterations in Ag expression by melanoma cells, MU tumor cells were cultured for varying periods of time in conditioned medium from melanoma cell lines, or purified fractions from such supernatant fluids. MU tumor cells were established at 1×10^5/ml in 1-ml aliquots in individual wells of 24-well culture plates, as previously described (35), which were then incubated at 37°C for between 1 and 7 days, before harvesting cells for fluorescent staining and cytotoxicity assays, as described below. Although production of MASA was constitutive, titers increased as long as tumor cells remained viable, generally proving most potent in supernatants collected after 3–4 days. Promoter assays were performed as described below. The following cytokines were quantified by ELISA (Genzyme Diagnostics, Cambridge, MA): IL-1α, IL-2, IL-12, IL-15, TNF-α, IFN-γ, IL-6, IL-8, and monocyte chemoattractant protein-1 (MCP-1). These assays have a sensitivity of 10 pg/ml when performed according to the manufacturer's protocols. In addition, we evaluated the following recombinant cytokines (Genzyme Diagnostics) for silencing activity: IL-6, IL-8, MCP-1, growth-related oncogene-α, epidermal growth factor, platelet-derived growth factor (PDGF)A, PDGFB, TGFα, TGFβ, nerve growth factor, RANTES,

macrophage-inflammatory protein-1α, LIF, platelet factor-4, neutrophil-activating peptide-2, and α-melanocyte-stimulating hormone obtained from Sigma-Aldrich (St. Louis, MO).

Staining of tumor cells: flow cytometric analysis

To evaluate the expression of cytoplasmic Melan-A/MART-1 Ag in melanoma cell lines, cells were first fixed for 10 s in 1% paraformaldehyde; the cells are pelleted and incubated for 5 min in 0.1% saponin before washing and addition of mAb specific for Melan-A/MART-1, A-103 (36) (a kind gift of E. Stockert and L. J. Old, Ludwig Institute, New York, NY), for 45 min at 22°C. Following two washes, cells were stained for 30 min with FITC-conjugated goat anti-mouse Ig Ab (Dako, Carpenteria, CA) before fixation in 1% paraformaldehyde and analysis by flow cytometry (FACScan; BD Biosciences, Mountain View, CA). Histograms of fluorescence staining were generated for comparison of anti-Melan-A/MART-1 staining of various cell populations. Mean channel fluorescence was calculated using the LYSIS software provided by the manufacturer. Staining for cytoplasmic tyrosinase was performed using mAb T311 (37), also a kind gift of E. Stockert and L. J. Old. Staining of surface HLA-A2 was conducted using the anti-HLA-A2-specific mAb, BB7.2 (ATCC), as previously described (24, 35).

Cytotoxicity assays

TIL were assayed for the ability to lyse melanoma target cells in 4-h ^{51}Cr release assays, as previously described (1). The melanoma target cells with high constitutive expression of Melan-A/MART-1 (MU-Hi) were generated by low density culture (1–2 $\times$ 10^5/ml). These Melan-A/MART-1-expressing cells were compared with respect to their susceptibility to cytolysis with the same cells cultured for 3 to 6 days in the presence of conditioned medium from the Melan-A/MART-1-negative variant, MU-X, to derive target cells with low Melan-A/MART-1 expression. Low Melan-A/MART-1-expressing cells were further assayed after pulsing with Melan-A/MART-1 peptide aa 27–35 (AAGIGILTV) (4–7), by culturing these target cells at 37°C for 2 h in 1 ml medium containing 5 μg peptide before labeling with ^{51}Cr for use in cytolytic assays to demonstrate renewed susceptibility to specific T cell recognition.

Promoter activity

Assessment of the promoter activity for Melan-A/MART-1 in melanoma cells was made in transient transfection experiments using the 233-bp minimal promoter sequence coupled to the firefly luciferase reporter gene (38) (kindly provided by L. Butterfield, UCLA Medical Center, Los Angeles, CA). A 230-bp HLA-A firefly luciferase construct (pGL3-A230) (39) was transfected in parallel into the melanoma cells. An SV40 renilla-luciferase reporter gene was cotransfected to provide an internal control, using the Dual-Luciferase Reporter Assay System, as described by the manufacturer (Promega, Madison, WI). Transient transfections were conducted using the CaCl$_2$ precipitation method, as previously described (39). After 16 h, the cultures were washed, and after an additional 48 h, cells were lysed and luciferase assays were performed. All assays were performed in quadruplicate, with the SV40 renilla control used to standardize for transfection efficiency. Data shown are means ($\pm$SD) of the corrected luciferase activity, standardized to 100% for optimal promoter activity of control Melan-A/MART-1-expressing tumor cells.

Assessment of mRNA transcripts for Melan-A/MART-1

To determine whether down-modulation of Melan-A/MART-1 Ag expression was accompanied by a concomitant decrease in mRNA transcripts for this protein, we performed RT-PCR analysis of cDNAs transcribed from mRNAs extracted from MU tumor cells before and after addition of MASA-containing supernatants. Assays were set up in a comparable manner with the Ag-silencing assay above in 24-well plates (5×10^4 cells/well). We analyzed and compared the responses of low defined numbers of treated and control cells based as described by Kurokawa et al. (40). Briefly, for extraction of cytoplasmic mRNA, cells were harvested from each well, washed once with PBS, once with 40 mM Tris, pH 8.5, 60 mM KCl, 3 mM MgCl$_2$, and resuspended in ~25 μl of the latter buffer. The cellular concentration of each resuspended pellet was determined, and an equalized number for each experiment (in the range of 10^3–6×10^3 cells) in 10 μl was mixed with 10 μl RNA extraction buffer (to give final 40 mM Tris, pH 8.5, 60 mM KCl, 3 mM MgCl$_2$, 2 mg/ml linear acrylamide, 10 mM DTT, 0.5% Nonidet P-40, 2 U/μl RNase inhibitor (RNAsin; Promega). After a 10-min incubation at room temperature, 20 μl reverse transcription buffer was added (40 mM Tris, pH 8.5, 60 mM KCl, 3 mM MgCl$_2$, 10 mM DTT, 200 μg/ml BSA, 1 mM dNTPs, 8 μM dT$_{18}$ primer, 16 U/μl Moloney murine leukemia virus reverse transcriptase (RNaseH$^-$,

point mutant; Promega). Reactions were incubated for 1.5 h at 42°C, and then for 20 min at 37°C following addition of 1 U RNaseH (Promega), and supernatants were taken after centrifugation and stored at −80°C. Equal volumes (1 μl) of reaction supernatants (taken following centrifugation) were analyzed by PCR, using AmpliTaq Gold (Applied Biosystems, Foster City, CA) in 50-μl reactions with buffering conditions, dNTPs as recommended by the manufacturer, and primers at 300 μM. Cycling conditions were: (95°C/10 min) × 1; (60°C/1 min; 95°C/15 s) × N; (60°C/5 s; 72°C 1 min) × 1, in which N was varied over a range from 25 to 40 cycles. Primers for Melan-A/MART-1 were 5′-CAAGATGCCAAGAGAAGAT GCTCACT (exon 2) and 5′-GCTTGCATTTTTCCTACACCATTCCA (exon 5); and β-actin primers were 5′-GAGATCACTGCCCTGGCAC CCA and 5′-GCTCCAACCGACTGCTGTCACCTTCAC. The authenticity of the PCR products was confirmed in all cases by automated DNA sequencing on both strands.

PCR products were analyzed by gel densitometry scanning. Imaging and densitometric scanning were performed, respectively, with Quantity One and Molecular Analyst computer software (Bio-Rad, Hercules, CA).

Partial purification of MASA and protease digestion

To characterize the active components of the tumor-conditioned medium, we collected supernatants from the most active tumor cell line, the Melan-A/MART-1-negative tumor, EW. The supernatants, which contained 1% FBS, were concentrated by first passing the material over a column of Red-Sepharose (Amersham Pharmacia Biotech, Piscataway, NJ). The majority of the activity bound to Red-Sepharose and was eluted using 1.5 M KCl in 10 mM KPO$_4$ buffer, pH 7. An aliquot of this material was then equilibrated to 1 mM in CaCl$_2$ before loading onto a 1-ml Con A-Sepharose column (Amersham Pharmacia Biotech) at ∼1 ml/min. The column was then washed with 20 mM Tris-HCl, 0.5 M NaCl, 1 mM MnCl$_2$, 1 mM CaCl$_2$, pH 7.4. The bound material was then eluted with 0.5 M α-methyl mannoside in 20 mM Tris-HCl, 0.5 M NaCl, pH 7.4. Before use in assays, the column-passaged material was buffered exchanged vs PBS and concentrated using 15 ml Centriprep YM10 centrifugal filtration devices (Millipore, Bedford, MA), 10-kDa nominal cutoff. Finally, the activity was fractionated on a G-50 Sephadex (Amersham Pharmacia Biotech) sizing column. Fractions were tested for Ag-silencing activity, and active fractions were subjected to nonreducing SDS-PAGE on 12% gels.

To demonstrate the protein character of the active material, it was subjected to proteinase K digestion. After buffer exchanging with PBS and concentrating to ∼1 ml, 300 μl of the concentrate was added to the washed pellet from 50 μl of a suspension of solid-phase proteinase K (Sigma, St. Louis, MO). This mixture was incubated at 37°C for ∼18 h, at which time 15 μl 100 μg/ml α-1 anti-trypsin (Sigma) was added and the solid-phase enzyme was pelleted by centrifugation. The supernatant was removed for testing of MASA by FACS, as previously described. As a control, an aliquot of solid-phase proteinase K had protease inhibitors added before addition of the 300 μl of concentrate. This control mixture was stored at 4°C for 18 h before removal of supernatant for MASA evaluation.

Results

Down-modulation of Melan-A/MART-1 expression

Fig. 1 demonstrates that the tumor cell line MU-X has stable, constitutive, low expression of the Melan-A/MART-1 Ag, as indicated by staining with the mAb, A-103 (36). In contrast, the cell line MU displays a high level of Melan-A/MART-1 Ag expression when cultured at 1×10^5/ml. Three additional melanoma cell lines, EW, IGR39D, and A375, were also found to have deficient Melan-A/MART-1 staining (data not shown).

We observed that the cytoplasmic expression of Melan-A/MART-1 in MU cells could be down-modulated by the addition of supernatants collected from any of the four Melan-A/MART-1-deficient tumor cell lines, although EW-conditioned medium was consistently more potent (2- to 4-fold more active on a volume-volume basis) than the other tumor cell supernatants, and was used in most subsequent experiments. As shown in Fig. 2, the down-modulation of Melan-A/MART-1 staining was detectable after 24 h in the presence of EW supernatant (median channel of fluorescence decreased from control level of 60 to mean channel 24), but at 48 h it was more pronounced (mean channel 11), with slightly lower levels of Melan-A/MART-1 staining after 72 h (mean channel 10, data not shown in this figure) in MASA-con-
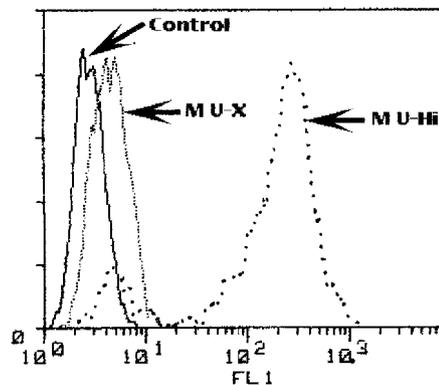

FIGURE 1. Melan-A/MART-1 staining of MU-HI vs MU-X. Melanoma line, MU, is shown in its two variants. MU-Hi, which expresses Melan-A/MART-1, is compared with constitutive minimal Melan-A/MART-1 expressor, MU-X. Tumor cells were stained with mAb to Melan-A/MART-1 (A-103) for cytoplasmic expression of this tumor-associated Ag, followed by FITC goat anti-mouse Ab to allow flow cytometric analysis, as described in the text.

taining supernatants. (Most data in subsequent figures are from 72-h time points.) No modulation of Melan-A/MART-1 staining was observed at any time point using conditioned medium from Daudi, Jurkat, or MCF-7 cells (data not shown). Decreased Melan-A/MART-1 staining was also noted for 136.2 and 453A tumor cells cultured in EW-conditioned medium for 3–6 days (data not shown).

The down-modulation of Melan-A/MART-1 is reversible, as the cytoplasmic expression of Melan-A/MART-1 in MU cells is up-regulated when the tumor cells are removed from the supernatant containing the down-modulatory activity, and grown in control medium (Fig. 3). At the same time, HLA class I expression is not down-regulated following exposure to supernatants from Melan-A/MART-1-negative tumor variants (Fig. 5).

In addition, MU tumor cells were stained for cytoplasmic tyrosinase. Although staining for tyrosinase is much weaker than for Melan-A/MART-1, an approximate 50% reduction in tyrosinase paralleled the reduction in Melan-A/MART-1 (data not shown).

The active material in EW supernatant can be shown to bind to Red-Sepharose and to Con A-Sepharose, which allowed for over 100-fold enrichment of the activity. Red-Sepharose-bound and
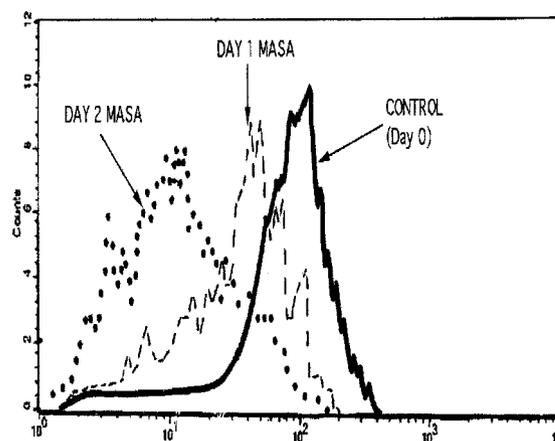

FIGURE 2. Kinetics of down-modulation of cytoplasmic expression of Melan-A/MART-1 in MU tumor cells. MU tumor cells were cultured for 1 or 2 days after addition of MASA-containing supernatant from EW tumor cells (a 1/10 dilution of a 20× concentrate). Control cells (day 0) were never exposed to MASA. Staining and flow cytometric analysis as in Fig. 1.
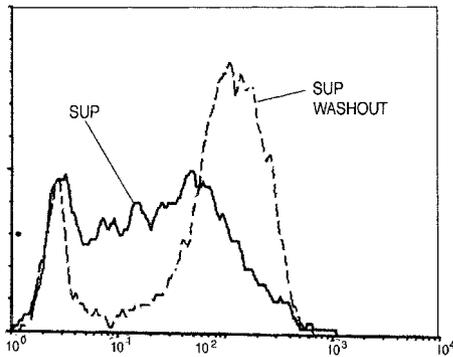
FIGURE 3. Restoration of Melan-A/MART-1 in MU tumor cells. MU tumor cells were treated for 72 h with EW-SUP, as in Fig. 2, to induce diminished Melan-A/MART-1 expression. In parallel, MU tumor cells were subjected to the same EW-SUP for 48 h before washing out the conditioned medium and replenishing with control DMEM/1% FBS medium, to restore expression of Melan-A/MART-1 to levels similar to cells cultured continuously in control medium (data not shown).

Con A-Sepharose-bound and eluted material was then subjected to size fractionation on G-50 Sephadex. The down-modulating activity in fractions collected from the G-50 column comigrated with a single band on SDS-PAGE at ~25 kDa (data not shown). The MASA could be destroyed by treatment of the supernatants at 80°C for 60 min. A similar treatment at 60°C did not influence the activity (data not shown). Treatment of the partially purified MASA (G-50 fractions) with proteinase K destroyed its activity (Fig. 4). Together these observations reveal that the Melan-A/MART-1-deficient tumor cells produce a soluble protein factor(s), the activity of which ultimately results in silencing of Melan-A/MART-1 expression.

To define this silencing activity in more detail, an assessment of cytokine and chemokine production by tumor cells that lacked Melan-A/MART-1 protein expression was performed. These Melan-A/MART-1-silencing supernatants were found to contain several known cytokines, but they lacked TNF-α that has been demonstrated to have a partial down-regulatory activity directed at the Melan-A/MART-1 promoter (38). Moreover, the most prevalent proteins identified, IL-6, IL-8, and MCP-1, did not mimic the Ag-silencing activity of the whole supernatant, either individually over a 3-log dosage, or when recombinant forms of these cytokines were combined to approximate the levels detected in the active supernatants (data not shown). In all, we have assessed over 20
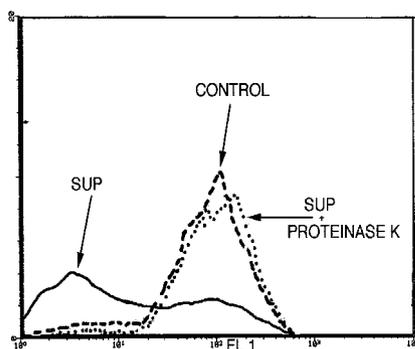

FIGURE 4. Proteinase K sensitivity of MASA. A partially purified fraction of supernatant fluid (SUP) from EW tumor cells that contained MASA is shown to diminish Melan-A/MART-1 staining in MU tumor cells as compared with cells cultured in control medium. When subjected to proteinase K digestion, the Ag-silencing activity of this SUP material was abolished.
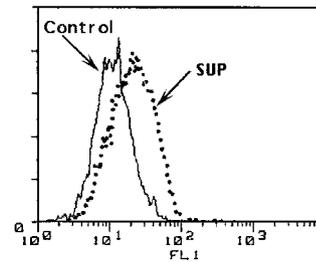

FIGURE 5. Class I MHC expression in μ tumor cells. MU tumor cells were cultured as in Fig. 2 in control medium (DMEM/1% FBS) for 72 h, vs conditioned medium containing MASA. Expression of class I MHC (staining with W6/32) was minimally altered (slightly higher in MASA-containing medium).

proteins, including many known to be secreted by melanoma cells (41–46), for their capacity to mimic the Ag-silencing activity. Other than TNF-α, which was not present in the active supernatants, none of the following proteins impacted cytoplasmic Melan-A/MART-1 expression (using flow cytometric detection of cytoplasmic staining with mAb A-103, as noted above), and promoter activity (using the luciferase assay for promoter activity): MSG/growth-related oncogene-α, epidermal growth factor, PDGFA, PDGFB, TGFα, TGFβ, nerve growth factor, RANTES, macrophage-inflammatory protein-1α, LIF, platelet factor-4, neutrophil-activating peptide-2. The following cytokines were not detected in the active supernatants by ELISA (with sensitivity limits of ~10 pg/ml): IL-1α, IL-2, IL-12, IL-15, TNF-α, and IFN-γ. In addition, heparin, which is known to bind to several growth factors, did not affect Melan-A/MART-1 expression, and did not impact the silencing activity of active supernatants.

Down-modulation of the promoter of the Melan-A/MART-1 gene

To correlate lack of Melan-A/MART-1 Ag expression with a lack of Melan-A/MART-1 gene promoter activity, we have performed transient transfection assays with the 233-bp minimal promoter of the Melan-A/MART-1 gene (38). Using this minimal promoter fused to the luciferase reporter gene, the impact of the Ag-silencing activity on the Melan-A/MART-1 promoter was assessed. As shown in Fig. 7A, there was baseline promoter activity in Melan-A/MART-1-negative tumor cells (MU-X), whereas this promoter showed abundant activity in tumor cells that expressed Melan-A/MART-1. The addition of conditioned medium from Melan-A/MART-1-negative tumor cells (either MU-X or EW) abolished the high level of constitutive Melan-A/MART-1 promoter activity in the MU tumor cells that express high levels of cytoplasmic Melan-A/MART-1. The promoter activity was down-modulated in a dose-dependent fashion. Although these supernatants displayed rather modest activity, as the promoter-silencing activity quickly diluted out, the activity could be concentrated, as it was retained by a nominal 30-kDa cutoff membrane (Millipore, Bedford, MA). This concentrated material retained virtually all of the activity in the starting material, as a 10-fold concentrate could be diluted 10-, 20-, or 40-fold and showed activity almost identical with 100, 50, and 25% supernatant fluids. The fact that the activity could be diluted, so that it constituted only a small proportion of the original cell culture medium, demonstrated that the Ag silencing was not due simply to nutrient deprivation.

The reversibility of the promoter down-modulation was demonstrated by the return of promoter activity when cells were removed from the presence of the MASA. As a specificity control to determine whether promoter activity is generally down-modulated
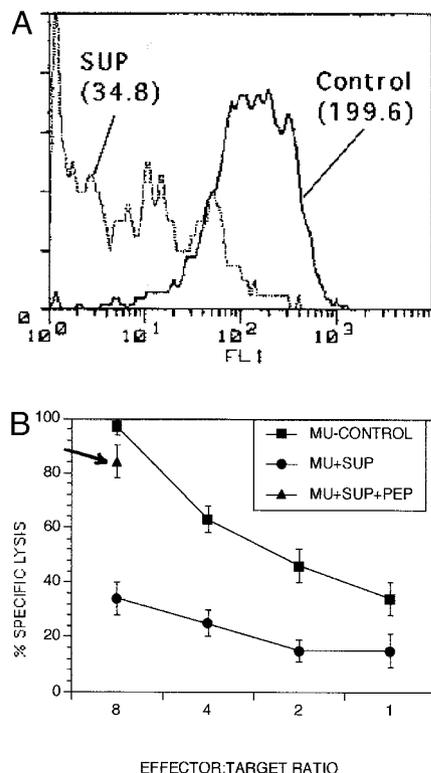
FIGURE 6. Down-modulation of Melan-A/MART-1 Ag with loss of cytotoxic T cell recognition of MU tumor cells. MU tumor cells were cultured at a beginning concentration of 1×10^5 cells/ml in control medium or in conditioned medium from Melan-A/MART-1-negative melanoma cell line EW for 6 days before labeling with ^{51}Cr and assay in a 4-h cytolysis assay with autologous anti-Melan-A/MART-1-specific TIL clone, MU-79. An aliquot of the low Melan-A/MART-1-expressing cells was pulsed with Melan-A/MART-1 peptide, AAGIGILTV, before labeling with ^{51}Cr. Each point is represented with SE bars. *A*, Flow cytometric analysis of Melan-A/MART-1 staining of control MU tumor cells vs MU tumor cells cultured in the presence of EW-conditioned medium (SUP). (Shown in parentheses is the mean channel of fluorescence of tumor cells in each condition.) *B*, Cell-mediated cytotoxicity against MU tumor cells cultured in control medium (MU-Control) vs EW-conditioned medium (MU+SUP). ▲ (see arrow), Lysis of MU+SUP cells to which Melan-A/MART-1 peptide has been added (MU+SUP+PEP).

by MASA, we also assessed the promoter activity for genes encoding HLA class I molecules. The promoter for HLA-A2 Ag, required for T cell recognition of the Melan-A/MART-1-derived peptide (AAGIGILTV), was not down-modulated (Fig. 7*B*).

Down-modulation of T cell recognition

The diminution of expression of the Melan-A/MART-1 Ag correlated with reduced susceptibility of the target cells to lysis by HLA-A2-restricted, Melan-A/MART-1-specific CTL (Fig. 6). Thus, as the level of Melan-A/MART-1 Ag expression by tumor cells diminished, whereas levels of HLA-A2 cell surface expression did not decrease, the T cell recognition of these targets decreased. However, when the target cells were pulsed with the Melan-A/MART-1 peptide (aa 27–35, AAGIGILTV), normal levels of cell lysis were observed, indicating that the tumor cells had not become resistant to cell-mediated lysis.

Down-modulation of Melan-A/MART-1 mRNA transcript levels

As shown in Fig. 8, the down-modulation of Melan-A/MART-1 protein expression, as reflected in decreased cytoplasmic staining with mAb A-103, was accompanied by a decrease in the steady state levels of mRNA encoding this Ag in MU tumor cells treated for 3 days with EW supernatants before extraction of mRNA and RT-PCR amplification. A pronounced diminution in the amounts of Melan-A/MART-1 product was seen in the supernatant-treated cells, which was not observed for the β-actin controls. This effect was independent of cycle number (from the threshold of detection up to product saturation) and was repeated in four independent experiments, indicating real differences in steady state levels of the Melan-A/MART-1 target mRNA between the treated and untreated cellular populations. The Melan-A/MART-1 mRNA diminution mediated by the EW supernatants was detectable on day 1 post treatment (but was less pronounced than at day 3) and still notable 7 days post treatment (data not shown).

Discussion

The autocrine modulation of Melan-A/MART-1 may be central to the effectiveness of a cellular immune response against this T cell-defined Ag, thereby allowing the escape of melanomas from immune destruction. We hypothesize that the variable expression of Melan-A/MART-1 among tumor cells in vivo (13, 19, 33) may be the result of a mechanism that affects Melan-A/MART-1 promoter activity, as we have described in these in vitro studies. The finding that four different Melan-A/MART-1-deficient tumor cell lines (EW and IGR39D (which were established directly from melanoma patients), the ATCC-provided A375, and the immunoselected Melan-A/MART-1-deficient cell line, MU-X) were found to produce this Melan-A/MART-1-silencing activity is in strong support of such a hypothesis. This MASA is the result of melanoma cell-produced soluble 25-kDa glycoprotein (binding to Con A) factor that is destroyed by proteinase K.

Although the Ag-silencing activity, as measured by cytoplasmic staining with Ab, is apparent after 24 h of culture, maximal effect is not seen until 72 h. A similar kinetic was noted for diminution in mRNA transcript levels for Melan-A/MART-1. The transfection assay with the luciferase-linked promoter construct indicates that down-modulation of the promoter is maximal by the earliest time point we could examine, namely 24 h. We interpret these data to indicate that the promoter is turned off rather quickly, but protein expression is altered more gradually, perhaps as a reflection of the $t_{1/2}$ of the protein, or continued synthesis by a stable mRNA.

We hypothesize that, at any given time, some of the tumor cells are producing Melan-A/MART-1-positive progeny, which continue to stimulate infiltration with Melan-A/MART-1-specific T cells. However, at the same time, some of the tumor progeny down-modulate Melan-A/MART-1, rendering them resistant to immune recognition by Melan-A/MART-1-specific T cells. Unfortunately, when the T cell response against such melanocyte Ags is enhanced, as in vaccine trials, the resulting outgrowth of Ag-negative variants could be a frequent outcome (13, 15, 19). As tyrosinase expression is also reduced by the same supernatants, it is possible that the changes in Melan-A/MART-1 expression reflect an alteration in the differentiation status of the tumor cells, suggesting that multiple lineage Ags may be altered by the activity we describe.

As we have shown, the in vitro expression of Melan-A/MART-1 is regulated, at least in part, by a soluble protein factor (or factors) that is produced by tumors with low Melan-A/MART-1 expression. In vivo such protein(s) could down-modulate Melan-A/MART-1 expression on neighboring cells. Our in vitro data suggest that these tumor variants are not mutants that have lost the Melan-A/MART-1 gene, but rather cells in which Melan-A/MART-1 expression is down-regulated, perhaps reversibly, by targeting the promoter for this gene. It is noteworthy that the four Melan-A/MART-1-deficient melanoma cell lines we studied all

A Melan-A/MART-1 Promoter
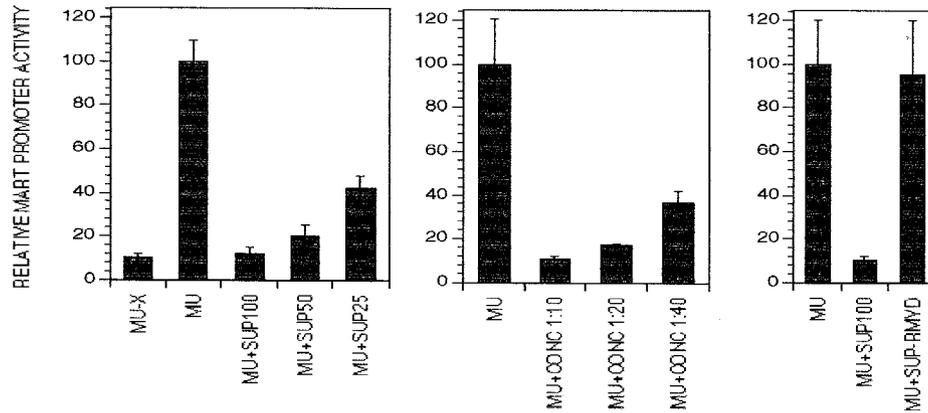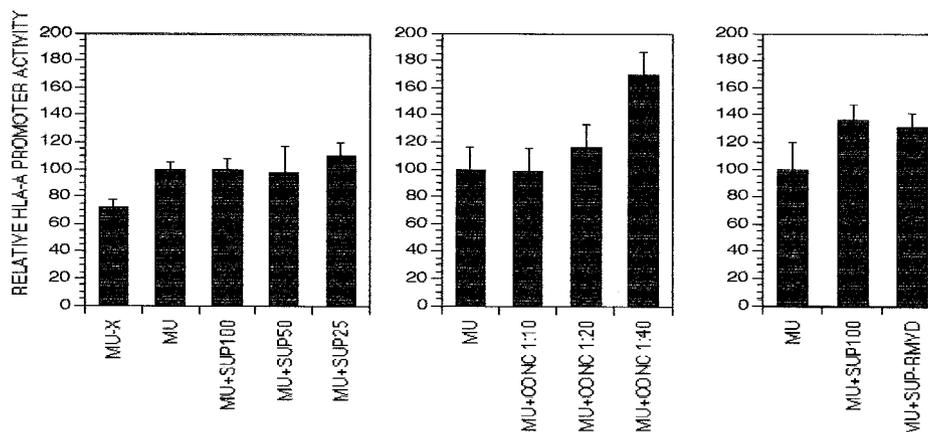

B HLA-A2 Promoter.

FIGURE 7. Promoter activity for Melan-A/MART-1 (*A*) and a 230-bp HLA-A (*B*), in MU tumor treated with supernatant (SUP) from Melan-A/MART-1-negative melanoma cell lines. *A*, Effect of MASA-containing SUP on Melan-A/MART-1 promoter activity. Melanoma cell lines from patient MU were transiently transfected with the 233-bp minimal promoter for Melan-A/MART-1 coupled to a firefly luciferase reporter to assess Melan-A/MART-1 promoter activity. (Data shown are standardized to 100% for the Melan-A/MART-1-expressing line, MU.) In the first frame, MU-X (first bar) have minimal promoter activity in comparison with MU tumor cells (second bar). MU were cultured in dilutions of MASA-containing supernatants (SUP; from MU-X) (100%, third bar; 50%, fourth bar; and 25%, fifth bar). Data show almost complete suppression of the promoter activity in high concentrations of SUP, with slightly increased promoter activity in dilutions. In the second frame, control MU tumor cells (first bar) were treated with a 10× concentrate of MASA-containing supernatant collected from EW tumor cells. Dilutions of concentrate show suppression of promoter activity similar to that seen in SUP in the first frame (bars 2, 3, and 4). In the third frame, Melan-A/MART-1 promoter activity was tested after removal of MASA-containing supernatant (100%). The first bar shows promoter activity of control MU tumor cells. Second bar shows suppression of promoter activity when cells were cultured in SUP. The third bar demonstrates restoration of promoter activity in MASA-treated cells when they were restored to control medium at the time of transfection, indicating that this suppression of promoter activity is reversible. *B*, Effect of MASA-containing SUP on HLA-A promoter activity. MU tumor cells were treated as in each of the frames of *A*, but transiently transfected with the 230-bp HLA-A promoter coupled to a firefly luciferase reporter. Similar promoter activity for HLA-A is demonstrated in all culture conditions.

produced strong Ag-silencing activity, whereas nonmelanocyte tumors, such as Daudi (B cell lymphoma), Jurkat (T cell lymphoma), and MCF-7 (breast carcinoma), did not produce MASA (data not shown). Thus, in nonmelanocyte cells, there is no selective advantage to actively down-regulate the promoter for Melan-A/MART-1, whereas the melanocytes that normally express this Ag must be down-regulated to shut off transcription of this protein. In a tumor variant that has lost the gene or its promoter, there would be no selective advantage for the cell to continue to produce an Ag-silencing factor. Thus, whether such a factor is involved in differentiation of the melanocyte lineage, or perhaps maintenance of a less mature phenotype, the active production of MASA seems to be characteristic of tumors that have lost expression of Melan-A/MART-1.

A variety of autocrine and paracrine factors has been described that alter the phenotype and behavior of melanoma cells (47), but the activity described in our study, which selectively down-regulates the expression of a tumor-related Ag critical to T cell recognition, represents a heretofore unrecognized mechanism for immune escape by tumors. Admittedly, this Ag-silencing pathway does not account for all of the pathways of immune escape manifested by melanoma cells, but the spontaneous, reversible nature of this tumor Ag expression represents a novel pathway for possible therapeutic intervention. Further characterization of the regulatory pathways involved in this autocrine down-modulation of T cell-recognized Ags will perhaps allow for the design of novel therapeutics to limit tumor progression or to enhance the effectiveness of antimelanoma vaccines (15, 18, 48, 49). Further studies
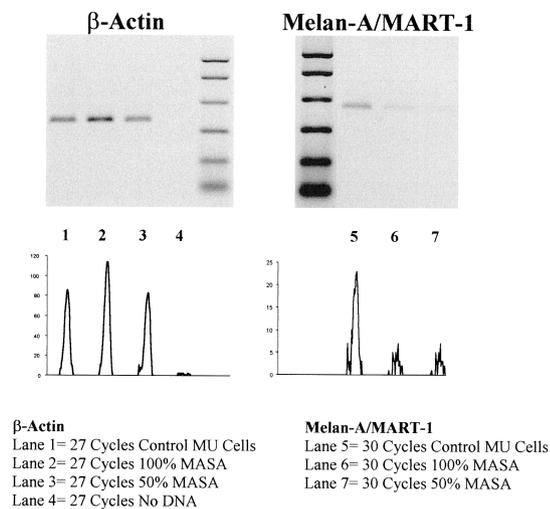
FIGURE 8. Diminished Melan-A/MART-1 mRNA transcripts following MASA treatment. MU tumor cells were cultured in 24-well plates in control medium or in MASA-containing EW-conditioned medium for a period of 72 h before extraction of RNA, as described in *Materials and Methods*. The extracted RNA was subjected to RT-PCR amplification using the Melan-A/MART-1 and β-actin primers. Data shown (1.4% agarose gels) are for 27–30 rounds of amplification. *Lanes 1–4*, Results for β-actin after 27 rounds of amplification. *Lane 1*, Control MU cells; *lane 2*, MU cells treated with 100% MASA; *lane 3*, MU cells treated with 50% MASA; *lane 4*, no DNA template control. The m.w. ladder is in the *far right lane* of this first gel. *Lanes 5–9*, 30 rounds of amplification for Melan-A/MART-1, with the m.w. ladder in the *far left lane* of this gel. *Lane 5*, Control MU cells; *lane 6*, MU cells treated with 100% MASA; *lane 7*, MU cells treated with 50% MASA. Beneath each lane is a graphic showing the relative density of each of the bands. (Note: Scales for the densitometry tracings are different for the β-actin and the Melan-A/MART-1 products, as there is considerably more β-actin product. The exposure of the gels is adjusted to optimize visualization of the bands in the respective gels.)

will clarify whether this Ag-silencing mechanism for tumor escape is characteristic only of specific melanoma-associated Ags, such as Melan-A/MART-1, or whether it is also relevant for other melanoma-associated Ags, as well as in the immune escape and progression of other types of tumors.

Acknowledgments

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