Induction of ''Antigen Silencing'' in Melanomas by Oncostatin M: Down-Modulation of Melanocyte Antigen Expression

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Abstract

We previously reported that antigen expression in melanoma cell lines is down-regulated by proteins secreted by antigen-negative melanoma cells. Here we report the purification and characterization of one of these down-regulatory factors, the cytokine, oncostatin M (OSM), which transmits its signal via the gp130 cell surface receptor, resulting in the selective down-modulation of the melanocyte lineage antigens: Melan-A/MART-1, gp100, tyrosinase, tyrosinase-related proteins 1 and 2, and the M isoform of microphthalmia transcription factor. Furthermore, we have found that some melanoma cell lines produce as yet uncharacterized factors distinct from OSM which also down-modulate antigen expression via signaling pathways different from that employed by OSM. These data indicate that there may be several regulatory pathways and molecules involved in the antigen-silencing process which may be related to the state of differentiation of the tumor cell and may affect the outcome of antitumor vaccine immunotherapies.

Introduction

Although there is evidence that it is possible to induce cell-mediated immunity against autologous melanomas, clinical immunotherapy strategies (1–3) achieve clinical responses in only a small fraction of patients. The limited efficacy of immunotherapy is due, at least in part, to the ability of tumors to evade immune destruction (3–7).

In studies of the immune response to melanomas, several melanocyte lineage antigens have been identified as targets of T lymphocytes, including the MAGE antigens, Melan-A/MART-1, gp100, and tyrosinase (3, 8). Recognition that cell-mediated immunity against these antigens could be enhanced *in vivo* by vaccination with immunodominant antigens, such as the HLA-A2-restricted Melan-A/MART-1 peptide epitope (AAGIGILTV) (9), has been exploited in clinical trials by several groups (10, 11). However, escape from T-cell recognition of Melan-A/MART-1-negative tumors has limited the efficacy of protocols targeting this antigen (3–7).

The loss of melanocyte differentiation antigens represents one of several routes by which melanomas can escape immune destruction (3–6, 12, 13). Others include the loss of peptide-presenting HLA antigens (5, 6) and defects in the antigen-presenting machinery (4). Poor immunogenicity of tumors, due in part to lack of co-stimulatory molecules such as B7 (12, 13), has also been noted. Tumor heterogeneity and the variety of effector functions manifested by tumor-infiltrating lymphocytes (TIL) (14–17) are indicative of the complex host-tumor interactions. Nevertheless, there is ample evidence that tumors can be immunogenic, and that it is possible to generate specific antitumor T-lymphocyte responses *in vivo*. In addition to vaccine-enhanced immunity (3–7), our own studies demonstrated that tumor-bearing patients can spontaneously produce tumor-specific T cells that proliferate, migrate, and accumulate in tumor deposits (18). The prognostic implications of an inflammatory infiltrate in melanomas were a seminal observation suggesting the salutary effects of the host response (19). The finding that tumor-specific cytotoxic T lymphocytes (CTL) can often be isolated from antigen-expressing tumors (20) suggests that such TIL may be responsible in part for the positive prognostic implications of intense lymphocytic infiltrates, even in metastatic melanomas. Conversely, the loss of critical tumor-associated antigens could result in the lack of lymphocytic infiltrates that has been associated with more aggressive diseases.

Still, a paradox remains: if the tumor-infiltrating lymphocytes include tumor-reactive T cells, how do tumors continue to grow and metastasize? Our previous finding that tumors can down-regulate expression of the very antigens recognized by infiltrating lymphocytes suggested that tumors have developed the ability to escape immune detection (14). In this report, we show that oncostatin M (OSM) is one of the cytokines controlling Melan-A/MART-1 expression, which may help explain the loss of lytic activity of TIL against autologous tumors. The identification of OSM as an agent that regulates antigen expression has allowed us to evaluate its effect on a panel of melanocyte lineage antigens, including Melan-A/MART-1, tyrosinase, gp100, tyrosinase-related proteins 1 and 2 (TRP-1, TRP-2), and a series of ''control'' proteins including HLA-A2, β-actin, and glyceraldehyde-3-phosphate

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dehydrogenase (GAPDH). We have also evaluated the expression of the melanocyte lineage-specific microphthalmia transcription factor (MITF-M) as compared to the related, but more widely expressed, MITF-A isoform (21–25). Because MITF-M is known to regulate the ability of pigmented cells to develop and survive, modulation of this specific isoform would provide evidence that such regulatory proteins participate in directing antigen expression and tumor cell differentiation (26). Transcription factors targeted by OSM may be responsible, at least in part, for the altered state of differentiation and antigen expression. Cytokine-driven silencing of a series of melanosome-associated antigen-encoding genes, present on different chromosomes, supports the hypothesis that the altered tumor phenotypes result from gene regulatory mechanisms ("antigen silencing"), as opposed to an accumulation of gene deletions and point mutations in each of the melanosome-associated genes.

The term antigen silencing refers to a form of gene silencing that results in the loss of expression of molecules targeted by the host immune response. There are multiple mechanisms mediating gene silencing in mammals as well as lower species, caused by such diverse processes as DNA hypermethylation (27), double-stranded RNAs (28, 29), altered promoter occupancy by transcription factor complexes (30), and recruitment of co-repressors to target gene promoters (31, 32). These gene-silencing events have diverse outcomes ranging from the prevention of unregulated cell division (33, 34), to the stimulation of cancer cell growth by inhibiting checkpoints to growth (35). Therefore, it is not surprising that some of the genes that are silenced are those related to cellular differentiation, and thereby to expression of lineage-specific antigens. Just as there is evidence that gene silencing is reversible with respect to loss of hormone receptors in tumor cells (36), the prevention and/or reversal of antigen silencing represents a potentially important goal for enhancing clinical immunotherapy.

Results

Purification and Characterization of Melanoma Antigen-Silencing Activity

We previously reported that conditioned medium from tumor cells could down-modulate Melan-A/MART-1 expression, but at that time, we failed to define which cytokine or cytokines were responsible for the antigen down-modulating activity (14), prompting us to purify and identify the responsible proteins. To obtain sufficient quantities of melanoma antigen-silencing activity (MASA)-containing medium for purification, several liters of supernatant fluid were collected from the EW melanoma cell line cultured for 4 days at 5×10^5/ml in DMEM supplemented with 1% fetal bovine serum. Supernatant fluids were stored at 4°C; however, all purification procedures were performed at room temperature. A 500-ml aliquot was subjected to adsorption on a 50-ml (10 g) Red Sepharose column. Fractions of the unbound material were collected and assayed for MASA (*i.e.*, Melan-A/MART-1 antigen down-regulation) to ensure that the column was not over-saturated. The column was washed with 20 mM KPO_4 buffer (pH 7.4) until the absorbance at 280 nm (A_{280}) of the effluent was close to baseline ($\sim$0.03 A_{280} units) and bound material was then eluted with 1.5 M KCl in 20 mM KPO_4 buffer (pH 7.4). Approximately 80% of the original activity was recovered from Red Sepharose in the eluate fractions. The A_{280} of the collected fractions was measured and fractions having an $A_{280} > 0.05$ were pooled, concentrated using a Centriprep YM-10 concentrator, and buffered exchanged with concanavalin A (ConA) binding buffer [20 mM Tris (pH 7.4), containing 0.5 M NaCl, 1 mM $CaCl_2$, and 1 mM $MnCl_2$]. After the Red Sepharose eluate was buffer exchanged and concentrated to 5 ml, it was loaded onto a ConA-Sepharose column. Again unbound material was collected as bulk fractions and the column was washed with binding buffer until the A_{280} was down to baseline. Bound material was eluted using 0.5 M methyl α-D-mannopyranoside in 20 mM Tris (pH 7.4) containing 0.5 M NaCl without divalent cations. Approximately 50% of the original MASA was recovered at this step.

Fractions of the eluate having an $A_{280} > 0.03$ were pooled, concentrated, and buffer exchanged against PBS. Further fractionation by gel filtration on Sephadex G-50 partially resolved the MASA into fractions separate from the major protein peak that is at the void volume of this particular column (see Fig. 1). Antigen down-modulating activity (MASA) chromatographed at approximately M_r 22,000 (fractions 28–30 in Fig. 1B). Active fractions such as #30 contained less than 0.1% of the protein in the starting samples.

The band at $\sim M_r$ 22,000 was cut from gels (*e.g.*, fractions 28, 29, and 30 above) and subjected to high sensitivity, proteolytic, mass spectroscopic, amino acid sequence analysis (LC/MS) by the Harvard University Microchemistry Group, Cambridge, MA. Sequencing results indicated that peptides from seven known human proteins were present in the band that corresponded to the MASA: tissue inhibitor of metalloproteinases; collagen (type V); interleukin 6 (IL-6) precursor; thrombospondin 1 precursor; stanniocalcin; OSM; and tissue factor pathway inhibitor 2 (TFPI2). The peptides corresponding to OSM sequence were QTDLM*QDTSR and SGLNIEDLEK, which correspond respectively to amino acids 45–54 and 126–135 of the OSM precursor. In addition, peptides from three proteins of bovine origin were also found: prostaglandin-H2 D-isomerase precursor; α-1-microglobulin precursor; and prothrombin precursor.

Antigen Silencing by Recombinant Human OSM

Each of the human biomolecules identified by sequencing was tested at four different concentrations for the ability to down-modulate Melan-A/MART-1 expression in MU tumor cells. Only recombinant human OSM (rhOSM) showed antigen-silencing activity. rhOSM at 10–20 ng/ml showed maximal Melan-A/MART-1 down-modulating activity that was comparable to activity present in the unfractionated EW supernatant (see Fig. 2, *first panel versus second panel*). In addition to Melan-A/MART-1, OSM also down-modulated gp100 expression (*fourth panel*, Fig. 2). An ELISA assay for OSM showed that EW supernatants contained approximately 1 ng/ml, while the supernatants from two other antigen-negative melanomas, A375 and IGR39-D, did not contain detectable OSM. It is noteworthy that approximately 10-fold more rhOSM was required to achieve antigen silencing comparable to that

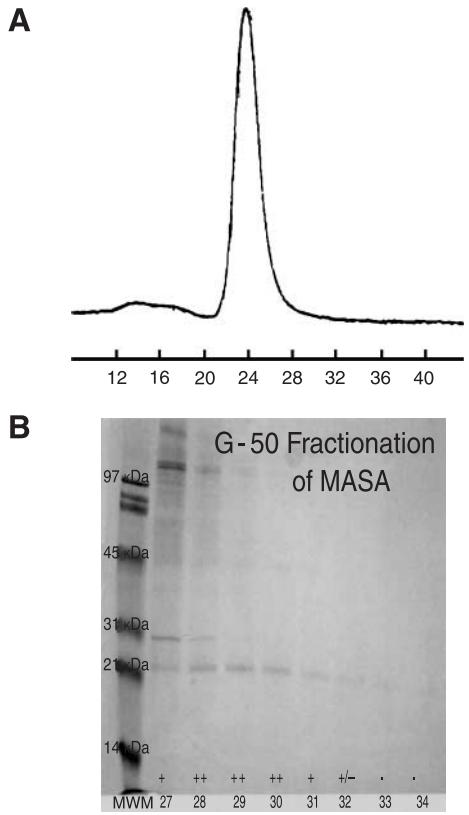

FIGURE 1. Fractionation of MASA. **A.** A_{280} profile of MASA fractionated on Sephadex G-50. *Abscissa,* fraction numbers. *Ordinate,* A_{280} units (full scale = 0.4 A_{280} units). **B.** SDS-PAGE profile and MASA profiles of fractions from G-50 fractionation. Molecular weight markers are shown in the track on the *far left*. Fraction numbers (27–34) are shown along the *bottom* of the figure. Relative MASA for each fraction is indicated as *pluses* (+) above the fraction number.

effected by EW supernatant containing 1 ng/ml. The EW-produced OSM was the only "natural" OSM we tested, but differences in bioactivity between recombinant and natural cytokines have frequently been observed (37–39).

A monoclonal antibody (mAb) specific for human OSM (hOSM; tested at 10 μg/ml) completely blocked the antigen-silencing activity of 10 ng/ml rhOSM, but this mAb maximally blocked only approximately half of the activity present in the EW supernatant. Furthermore, approximately one half of the Melan-A/MART-1 modulating activity (*i.e.*, hOSM) from EW supernatant could be removed by, and eluted from, an anti-rhOSM immunoaffinity column. Thus, the unbound, hOSM-depleted supernatant still contained antigen down-modulating activity, equivalent to approximately half of the activity of the unfractionated EW supernatant. We confirmed that the unbound material from the immunoaffinity column contained no ELISA-detectable OSM. Other supernatants, such as those from the A375 cell line, demonstrated similar ability to effect down-modulation of Melan-A/MART-1 expression, even though there was no detectable OSM by ELISA, and these antigen-silencing activities could not be inhibited by antibody (Ab) to OSM. Thus, it is clear that antigen silencing can be induced by more than one melanoma-produced cytokine.

OSM Production by Melanomas

As we have shown that Melan-A/MART-1-deficient melanoma cell lines, such as A375 and EW, produce soluble factors that down-modulate antigen expression in cells that would otherwise be constitutively positive for antigen expression (14), we determined the natural repertoire of gene expression of OSM in a series of antigen-positive and antigen-deficient cell lines. We assessed four Melan-A/MART-1-expressing melanoma cell lines (136.2, 453A, MM96L, and MU), and an additional four melanoma lines that have weak or virtually no Melan-A/MART-1 expression (MU-X, EW, IGR-39D, and A375), and the phorbol 12-myristate 13-acetate-activated myelomonocytic cell line U937 that secretes abundant OSM. Among the melanomas, only EW secretes measurable amounts of OSM protein (as determined in ELISA), but an additional three cell lines show detectable OSM mRNA expression [by reverse transcription (RT)-PCR], albeit weaker than EW (Fig. 3). On the other hand, MM96L, 453A, and A375 appear to be deficient for significant levels of OSM mRNA transcripts (Fig. 3). Also presented in Fig. 3 are data on gp130 and OSM receptor expression. All of the melanoma cell lines contained mRNA coding for those two proteins which constitute the OSM receptor complex (40). The control cell line Ramos showed gp130 but not OSM receptor expression. Primers encompassing the entire OSM coding sequence (confirmed by sequencing) also allowed amplification of a product from EW cells and stimulated U937 cells (data not shown).

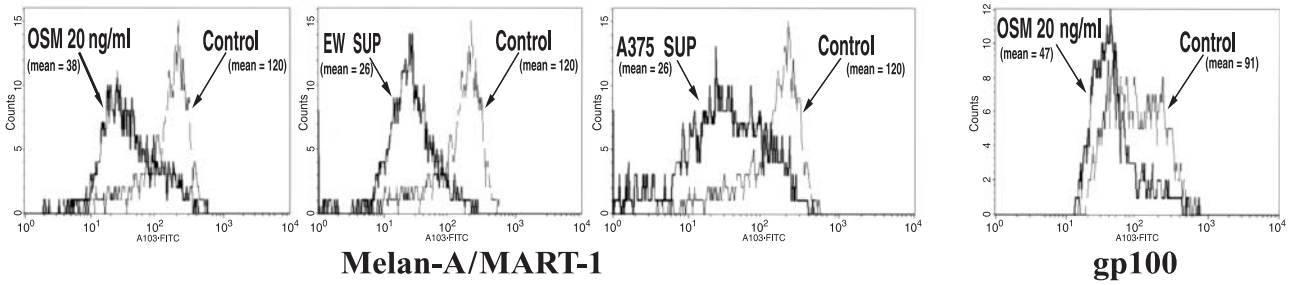

FIGURE 2. Down-modulation of antigen expression in melanoma cell line MU. MU tumor cells were cultured for 3 days in control medium, in medium containing 20 ng/ml of OSM (*first* and *last panels*), or in supernatants from the EW (contains OSM) (*second panel*) or A375 (does not contain OSM) (*third panel*) tumor cell lines. MU cells were stained for cytoplasmic expression of Melan-A/MART-1 protein (*first three panels*) or gp100 (*last panel*) and assayed by flow cytometry. Mean channel of fluorescence for each curve is indicated in *parentheses* within each panel. Each curve is based on 2000 gated cells. *Ordinate,* number of cells per channel (maximum 15). *Abscissa,* $\log_{10}$ of fluorescence (4 logs shown).

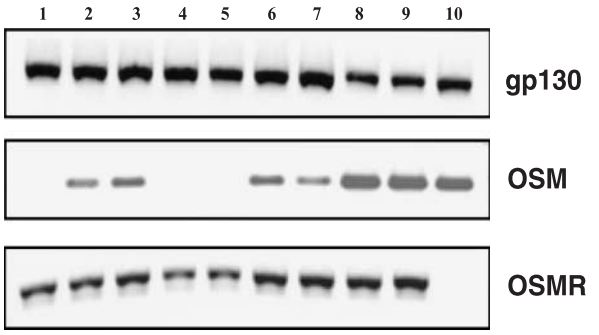

FIGURE 3. RT-PCR analyses: OSM, OSM specific receptor (*OSMR*), and gp130 in cell lines. RNA was extracted and reverse transcribed ("Materials and Methods") from the following melanoma cell lines: (1) A375, (2) 136.2, (3) IGR-39D, (4) 453A, (5) MM96L, (6,7) MU, and (8,9) EW. *Lane 10* shows the B-cell line RAMOS, as a control. PCR analyses were performed with appropriate primers with standard conditions ("Materials and Methods").

Distinct Molecules and Activation Pathways Induce Antigen Silencing

As OSM appears to transmit its signal via the gp130 chain shared among receptors for the IL-6 family of cytokines (41), we tested the ability of a gp130-neutralizing mAb, clone B-K5 (41), to inhibit the rhOSM down-modulating effect on Melan-A/MART-1 expression (Fig. 4). This Ab significantly blocked the activity of rhOSM, but the anti-gp130 mAb did not inhibit the effect of A375 supernatant, and only partially blocked the down-modulation of Melan-A/MART-1 expression in cells treated with EW supernatant. Together, these data indicate that OSM, and as yet undetermined additional factors produced by melanoma cells, which we call "MASA-2," are capable of down-modulating Melan-A/MART-1 expression.

Because OSM exerts its effects through the gp130 chain of the "IL-6 family" receptors, we evaluated the other members of this family: IL-6, IL-11, leukemia inhibitory factor (LIF), CNTF, and cardiotrophin for antigen-silencing activity. Among these IL-6 family members, only OSM displayed any antigen-silencing activity when tested at concentrations between 100 and 0.1 ng/ml.

Down-Modulation of Multiple Melanocyte Lineage Antigens

In addition to the down-modulation of cytoplasmic protein expression of Melan-A/MART-1 and gp100 (Fig. 2), OSM down-modulated steady-state mRNA levels for these antigens as well as other melanocyte lineage proteins, including tyrosinase, TRP-1, and TRP-2 (see Fig. 5). These effects, monitored by RT-PCR, were highly reproducible (20 independent experiments). Titration of target amounts consistently showed reduction of product levels (for melanocyte lineage antigens) from OSM-treated cells relative to control cells (Fig. 5, *bottom four panels*). On the other hand, with titration of control targets such as MITF-A (shown in Fig. 5, *lowest right panel*) and β-actin (not shown), no effect of OSM was observed. A similar OSM-induced differential was seen when amplification cycle number was systematically reduced until the threshold of product detection (not shown). These data indicate that OSM signaling results in negative modulation of mRNA levels for the melanocytic markers under scrutiny. However, OSM did not influence steady-state levels of mRNA for control genes: β-actin and GADPH (Fig. 5). It is noteworthy that OSM induces selective down-modulation of the melanocyte-associated MITF-M isoform, but does not impact the level of the more widely expressed MITF-A isoform (see Fig. 5), thus indicating a level of specificity to the targeting of a melanocyte-specific transcription factor (42) by OSM. Titrations of MITF-M *versus* MITF-A target quantities in 2-fold decrements from untreated *versus* OSM-treated MU cells are shown in the *bottom two panels* of Fig. 5. Because it is known that dysfunction of Mitf in mice impacts the development and survival of melanocytes (21, 23–26, 43), it is not surprising that an agent that can down-modulate expression of this transcription factor would have far-reaching effects on the state of differentiation of cells that lose expression of this protein. In addition, we found that Melan-A/MART-1-deficient cells: EW, IGR-39D, MU-X, and A375 are also relatively deficient in protein and mRNA for gp100 and tyrosinase, and are often lacking in TRP-1 and TRP-2 as well (not shown). We have also noted selective absence of MITF-M but retention of MITF-A expression in several of the Melan-A/MART-1-negative tumor lines, including EW, IGR-39D, and MU-X (not shown).

Although OSM showed strong antigen down-modulating activity on several melanoma cell lines, it did not influence antigen expression in the MM96L cell line (not shown); MM96L is known to be defective in its responsiveness to other cytokines (44). Likewise, although OSM down-modulated a series of melanocyte lineage antigens in cell lines such as MU, the OSM-containing supernatant fluid from the EW cell line showed strong down-modulation of Melan-A/MART-1 expression, but had minimal effect on gp100 expression in this cell line

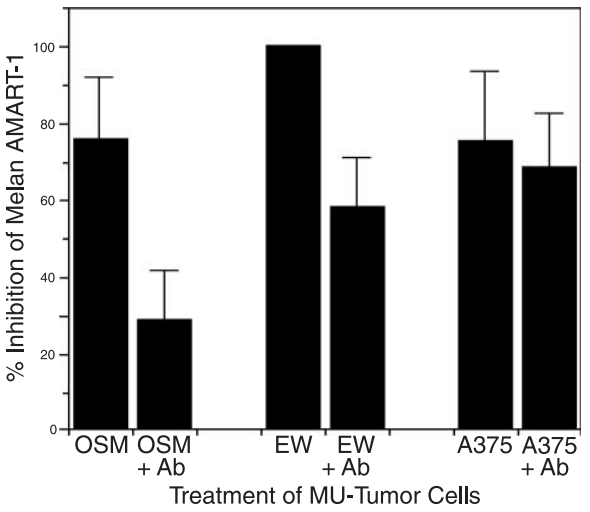

FIGURE 4. Effect of neutralizing anti-gp130 Ab on antigen silencing. MU tumor cells (three identical samples) were pretreated with anti-gp130 Ab, clone B-K5, at 10 μg/ml for 1 h. Ab-treated and untreated samples were then cultured for 3 days with OSM (10 ng/ml) or with supernatants from the EW and A375 melanoma cell lines. The data presented are the averages of five separate experiments. One hundred percent inhibition was defined as that achieved using the EW supernatant. Because this was 100% in each experiment, the SE was zero, and thus, no error bar is observed with the EW supernatant treated sample (*third bar from the left*).

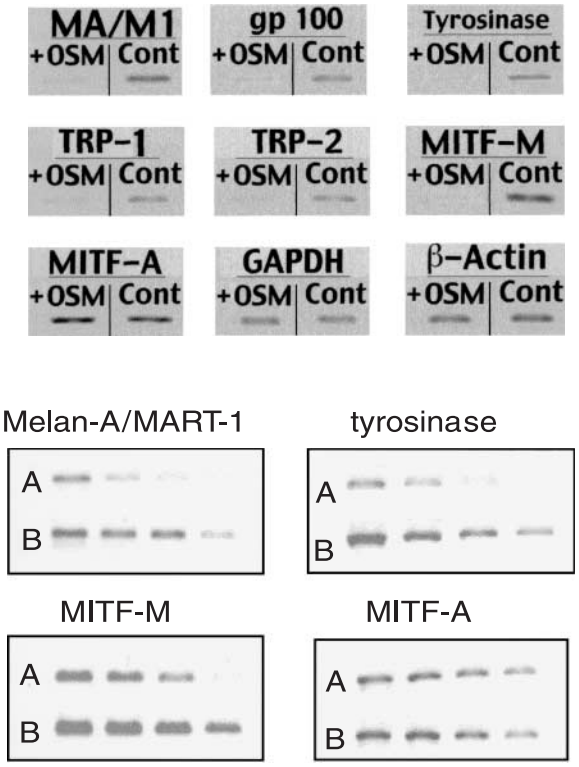

FIGURE 5. Effect of OSM on melanoma gene expression in MU tumor cells. *Top nine panels,* MU cells were cultured with or without 20 ng/ml OSM (3×10^5 cells/well in six-well plates, 3 ml medium/well) for 72 h and then total RNAs harvested and quantified, and amplified by RT-PCR using primers as indicated in the "Materials and Methods." Product identities were confirmed by automated sequencing. Note: Melan-A/MART-1 = MA/M1. All PCR products shown were generated from a starting RNA/sample of 0.39 ng, except GADPH and β-actin that were at 24.4 pg, and TRP-1 at 15.6 ng. *Bottom four panels,* titration of PCR product formation with (**A**) and without (**B**) OSM treatments, in successive 2-fold decrements beginning with 3.13 ng at the *left*. *Top rows* of products (**A**) for each panel, +20 ng/ml OSM; *bottom rows* (**B**) for each panel, untreated controls.

(see Fig. 6), suggesting that antigen up-regulating factors may also be present in supernatants, because OSM alone down-regulated both antigens on the MU cells. OSM-deficient supernatant from the A375 cell line also down-modulated Melan-A/MART-1 expression, but had minimal effect on gp100 expression. OSM also down-modulated antigen expression in the MO cell line, as did supernatants from A375 and EW, but the latter supernatants induced minimal down-modulation of gp100 expression (not shown). An additional cell line, MM96L, shows no alteration in either Melan-A/MART-1 or gp100 with any of the treatments, showing that some cell lines are not responsive to these antigen-silencing treatments, although a subset of the MM96L tumor cells spontaneously lost expression of the whole array of melanosome antigens, suggesting that this cell line is also susceptible to gene regulatory events. Thus, while some agents such as OSM affect a series of antigens in concert, other agents or tumor cell supernatant "cocktails" may show selective activity on different antigens in different cell lines.

Discussion

The preceding data demonstrate that human melanoma cell lines produce antigen-silencing factors that down-regulate the expression of a series of melanocyte-lineage antigens, leading to the simultaneous loss of differentiation antigens targeted by T lymphocytes. The first of the factors displaying MASA to be purified and characterized is identified herein as the cytokine OSM, which induces a series of phenotypic changes in melanoma cell lines, including altered antigen expression and cell morphology.

OSM was originally reported to be a product of mononuclear inflammatory cells (45). That OSM is also a product of melanoma cells has recently been reported in a hamster model where unpigmented melanoma cells with enhanced malignant properties were shown to produce OSM, in contrast to their more indolent pigmented counterparts (46). To date, our report is the only other finding of OSM production by melanomas, and the only one in human melanomas. In fact, the anti-proliferative effects of OSM have been proposed as the basis for using OSM as a possible therapeutic agent (45, 47), although more aggressive tumors have been shown to lose responsiveness to OSM (*i.e.*, they no longer slow their growth in the presence of OSM) (48). In contrast, our findings show that OSM may down-modulate a series of melanocyte-lineage antigens targeted by CTL, effectively producing cells with a phenotype expected to be more resistant to immune cytotoxic therapies. In addition to the tumor-produced cytokine, infiltrating inflammatory cells can also produce OSM (45). Ironically, cytokine from such inflammatory cells might actually decrease the expression of antigens that originally attracted them to the site of the tumor. The suggestion that inflammatory cells may influence antigen expression on melanomas was also noted by Giacomini *et al.* (49) and Le Poole *et al.* (50), who found that INF-γ could down-modulate melanosome antigens. Our own studies confirmed that interferon gamma (IFN-γ) could down-modulate Melan-A/MART-1 on 136.2 tumor cells, but did not impact antigen expression on five other melanomas we tested (data not shown), indicating that this cytokine has a differential effect of individual melanomas.

As shown in our previous report, when the MASA-containing conditioned medium was removed from the Melan-A/MART-1-expressing tumor cells, cellular antigen expression was restored (14). Similar recovery of antigen expression was noted following removal of OSM (not shown). Furthermore, as was previously shown using unpurified MASA (14), OSM down-modulation of Melan-A/MART-1 for 3 days results in the cells being less susceptible to T-cell cytolysis (not shown). The addition of Melan-A/MART-1 peptide restores CTL recognition of antigenically down-modulated tumor targets (14), indicating that cytokine-mediated reduction in antigen expression renders these tumor cells less susceptible to recognition by CTL.

Although we have shown that OSM acts through the gp130 receptor, we have not yet confirmed that the binding also requires a second receptor chain as described previously (40). We have, however, tested the other cytokines which signal via the IL-6 receptor gp130 chain (*i.e.*, IL-6, IL-11, CNTF, LIF, and cardiotrophin), and found that none demonstrated antigen down-modulating activity.

In addition to OSM, there are obviously other factors that can regulate the expression of melanocyte differentiation

antigens. We have previously shown that EW tumor cell line supernatants manifest antigen silencing by down-modulating the activity of the promoter for the Melan-A/MART-1 gene (14); OSM induces similar promoter down-modulation using the identical luciferase reporter system used for assessment of EW supernatants (not shown). The ability of additional OSM-deficient melanoma supernatants to down-modulate antigen expression, acting via receptor interactions distinct from the gp130 chain of the OSM receptor, demonstrates that there is more than one entry point in the antigen-silencing pathway. As we have not yet purified the molecule(s) present in MASA-2, we do not yet know if there is any structural relationship to OSM, but the lack of activity of MASA-2 via gp130 (shown by the failure of gp130 neutralizing Ab to inhibit MASA-2) suggests that MASA-2 is not a member of the IL-6 family of cytokines that includes OSM (41). Development of more than one mechanism for regulating differentiation and antigen expression indicates the potential importance of antigen silencing for survival of tumor cells in the face of the host immune response.

The data we have presented indicate that tumor cell phenotype is affected by cytokines produced by the tumors themselves, and by host inflammatory cells. Importantly, the OSM-induced loss of antigen expression appears to be the result of regulatory events that lead to the down-modulation of promoter activity for genes encoding T-cell-recognized structures. As the removal of OSM and other cytokines results in the restoration of antigen expression (14), we suggest that potentially reversible regulatory events, rather than presumably irreversible mutational events, lie at the heart of antigen silencing and the immunoselection than ensues.

Furthermore, tumors that have spontaneously lost Melan-A/MART-1 expression generally are low or deficient in other melanocyte-associated antigens, suggesting that these genes (and others) may be controlled in concert. Indeed, the finding that MITF-M (but not MITF-A) can be down-modulated by OSM indicates that selective targeting of the melanocyte-restricted isoform of this transcription factor may prove to be one of the critical regulatory proteins involved in antigen silencing. This is further supported by the finding that melanocyte-restricted transcription factor MITF-M (21–25) is selectively absent in several antigen-negative tumor cells, but expressed among the antigen-positive tumor cells. While the altered differentiation induced by OSM affects several melanocyte lineage markers, it is important to note that this cytokine-induced antigen silencing has specificity, because MITF-A, as well as several "housekeeping" genes and HLA Class I antigens, appear to be unchanged. Although there are undoubtedly additional regulatory interactions resulting from OSM-induced changes, it is tantalizing to suggest that diminished MITF-M expression could play a central role in subsequent phenotypic changes in the tumor cells we have studied.

The ability to intervene with agents known to block OSM-activation pathways may provide potential targets for pharmacological intervention to restore melanocyte differentiation to enhance tumor cell antigen expression, and hopefully to improve the outcome of immunotherapies.

Materials and Methods

Cell Lines

All of the cell lines used in this report have been previously described, including melanoma tumor cells lines that were established at the Massachusetts General Hospital: MU; MU-X; and EW (14). A375 and RAMOS were purchased from the American Type Culture Collection (Manassas, VA). IGR-39D, 453A, and 136.2 were originally provided by Dr. Peter Schrier, Leiden University, Leiden, The Netherlands. MM96L was originally provided by Dr. P.G. Parsons, Queensland Institute of Medical Research, Herston, Australia. The U937 myelomonocytic cell line was isolated by Dr. Kenneth Nilsson, Uppsala University, Uppsala, Sweden.

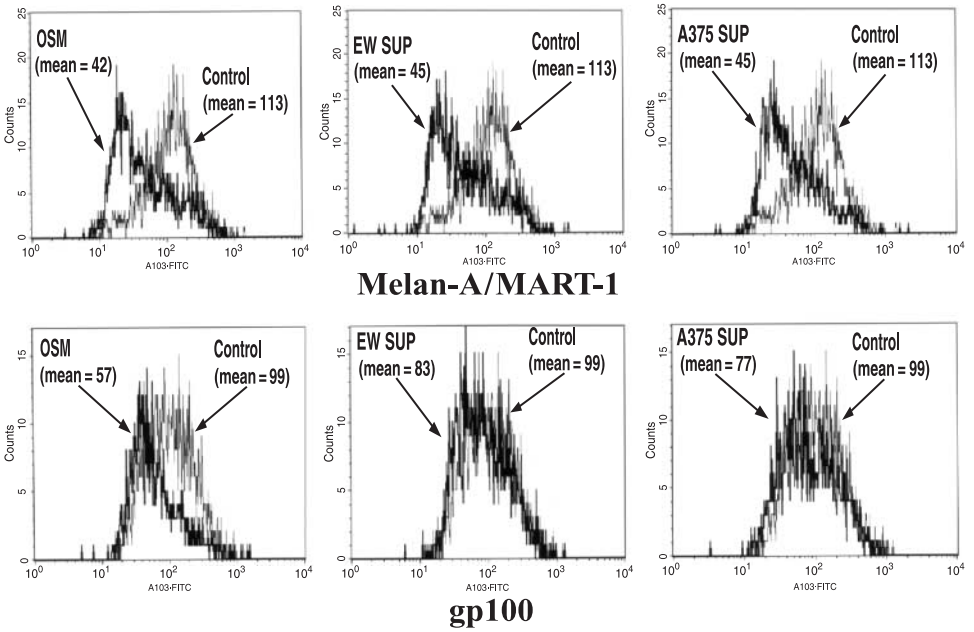

FIGURE 6. Differential response of Melan-A/MART-1 and gp100 antigens in MU tumor cells. MU melanoma tumor cells were treated for 3 days with OSM (20 ng/ml) or with supernatant fluids from EW (*EW SUP*) or A375 (*A375 SUP*) tumor cell lines, before staining with Ab to either Melan-A/MART-1 (*upper panel*) or gp100 antigen (*lower panel*). (Mean channel fluorescence is shown in *parentheses* for each curve.)

Reagents

Antibodies: anti Melan-A/MART-1 (clone A103) (51) was generously provided by Dr. Lloyd J. Old, Ludwig Institute of Cancer Research (New York, NY); anti-gp100 Ab (HMB45) was from DAKO Corp. (Carpinteria, CA); monoclonal anti-hOSM (#MAB295) was from R&D Systems (Minneapolis MN); and anti-gp130 (clone B-K5) was from Cell Sciences (Norwood, MA). rhOSM, LIF, CNTF, IL-6, IL-11, and the Quantikine ELISA for measuring OSM were from R&D Systems. Blue Sepharose CL-6B, Red Sepharose CL-6B, activated CH-Sepharose 4B and ConA linked to Sepharose, Heparin-Sepharose, and G-50 Sephadex were from Amersham-Pharmacia Biotech (Piscataway, NJ). Chemicals and other reagents were Analytical Grade from Sigma-Aldrich (St. Louis, MO). Ultra-filtration membrane units, Centricon Plus-20 (with various molecular weight cutoffs) and Centriprep YM-10 and YM-30 were from Amicon/Millipore (Bedford, MA). Oligonucleotides (listed below under "PCR Analysis") were obtained from Sigma-Genosys (The Woodlands, TX).

Tissue inhibitor of metalloproteinase 1 (TIMP-1) was obtained from Oncogene Research Products (Boston, MA). Collagen (type V) was purchased from Research Diagnostics, Inc. (Flanders, NJ). Thrombospondin constructs consisting of residues 1–356 and 1–359 were obtained from Dr. Deane Mosher of the University of Wisconsin, Madison, WI. These constructs are predicted to contain all of the thrombospondin 1 precursor peptides found on sequencing. Stanniocalcin was the generous gift of Dr. Roger R. Reddel, of the Children's Medical Research Institute, Westmead, Sydney, Australia. rhOSM and recombinant human IL-6 were purchased from R&D Systems. Recombinant human tissue factor pathway inhibitor 2 (TFPI-2) was graciously provided by Dr. Stavros Topouzis of Zymogentics (Seattle, WA).

Solid-phase anti-hOSM was produced by linking anti-rhOSM mAb (R&D Systems) to activated CH-Sepharose 4B according to the manufacturer's protocol.

Treatment of Cells for Evaluation of Antigen Expression

The cell line MU was cultured in DMEM containing 5% fetal bovine serum (14); concentrated supernatants, cytokines, or other biomolecules were added to the medium such that their maximum contribution to the volume was 10%. Cytokines or other biomolecules (the presence of which was determined by protein sequencing) were tested at serial 10-fold dilutions as follows: human thrombospondin (2000 ml to 2 ng/ml); human TIMP-1 (200–0.2 ng/ml); human collagen type V (2000–2 ng/ml); human IL-6 (200–0.2 ng/ml); human TFPI-2 (1–0.01 μg/ml); human stanniocalcin (1–0.01 μg/ml); and hOSM (100–0.01 ng/ml). The other cytokines that signal through the IL-6 family receptor, IL-6, LIF, CNTF, IL-11, and cardiotrophin were tested at 100–0.1 ng/ml.

Medium supplemented 1 part in 10 with PBS (the maximum concentration of this diluent for the various agents tested) was always included as a control.

Flow Cytometric Analysis

Flow cytometric analysis of cytoplasmic Melan-A/MART-1 expression was performed as described previously (14). Analyses for gp100 and tyrosinase were performed similarly.

Electrophoretic Analysis

SDS-PAGE was performed under reducing conditions; reagents and 12% gels from Bio-Rad (Hercules, CA) were used according to the manufacturer's directions. Colloidal Coomassie Blue Stain from Novex (San Diego, CA) was used to stain gels to visualize protein bands.

PCR Analysis

Equal quantities of oligo-$(dT)_{18}$ reverse-transcribed RNAs were subjected to RT-PCR analyses, as previously described (14), using multiple dilutions to establish conditions where initial amounts of control mRNAs resulted in subsaturating amounts of products, with representative template concentrations shown. Primers were designed from appropriate Genbank mRNA and genomic entries and designed to be intron spanning to prevent simultaneous amplification of traces of genomic DNAs. Primer sequences: (Forward {sense}/reverse {antisense} pairs) β-actin (14);

Melan-A/MART-1:	CAAGATGCCAAGAGAAGATGCTCACT/GCTTGCATTTTTCCTACACCATTCCA
β-Actin:	GAGATCACTGCCCTGGCACCCA/GCTCCAACCGACTGCTGTCACCTTCAC
gp100/Pmel17	CTGATTGGTGCAAATGCCTCCTTCT/AGGAAGTGCTTGTTCCCTCCATCCA
gp130:	CGCGGCCTGAGTGAAACCCAA/CCCTTCCACCATCCCACTCACACCT
Tyrosinase:	CAGCCCAGCATCATTCTTCTCCTCT/GCAGTGAGGACGGCCCCTACCA
TRP-1:	TGTTGCCCAGACCTGTCCCCT/GCAACATTTCCTGCATGTCTTTCTCCA
TRP-2:	CCTAGTGAACAAGGAGTGCTGCCCA/CGCTGGAGATCTCTTTCCAGACACAAC
MITF-M:	TCTCTCACTGGATTGGTGCCACCT/CATGCCTGGGCACTCGCTCTCT
MITF-A:	GGGAGCCATGCAGTCCGAATC/CATGCCTGGGCACTCGCTCTCT
GAPDH	TGAAGGTCGGAGTCAACGGATTTGGT/CTGCAAATGAGCCCCAGCCTTCT
OSM (peptide):	CAGACAGATCTCATGCAGGACACCA/CTCCAAGTCCTCGATGTTCAGCCCA

OSM (full coding sequence):	CATGGGGGTACTGCTCA/TACCGGGGCAGCTGTCCCCT
OSM receptor β-subunit:	GAATCCGACAACTTCGCAGCCCA/CAATGCAGAACCTGGTTCCACTTCACA

Note that MITF-M and MITF-A share a common reverse primer owing to their shared mRNA 3′ regions. Product identities were confirmed by automated sequencing.

Cell-Mediated Lysis

For assessment of Melan-A/MART-1 specific lysis, MU tumor cells were labeled with ^{51}Cr, and cocultured for 4 h with varying numbers of cloned CTLs specific for this antigen, as previously described (14).

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Molecular Cancer Research

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