Potent Selection of Antigen Loss Variants of B16 Melanoma following Inflammatory Killing of Melanocytes

Luis Sanchez-Perez,1,2 Timothy Kottke,1 Rosa Maria Diaz,1,2 Atique Ahmed,1 Jill Thompson,1 Heung Chong,1 Alan Melcher,1 Sheri Holmen,1 Gregory Daniels,1,3 and Richard G. Vile1,2

1Molecular Medicine Program and Departments of Immunology and Oncology, Mayo Clinic, Rochester, Minnesota; 2Department of Pathology, St. George’s Hospital Medical School, London, United Kingdom; 3Cancer Research UK Oncology Unit, St. James’ Hospital, Leeds, United Kingdom; and 4Van Andel Research Institute, Grand Rapids, Michigan

Abstract

We have reported that i.d. injection of plasmids encoding hsp70 and a suicide gene transcriptionally targeted to melanocytes generates specific proinflammatory killing of melanocytes. The resulting CD8+ T cell response eradicates systemically established B16 tumors. Here, we studied the consequences of that CD8+ T cell response on the phenotype of preexisting tumor. In suboptimal protocols, the T cell response selected B16 variants, which grow extremely aggressively, are amelanotic and have lost expression of the tyrosinase and tyrosinase-related protein 2 (TRP-2) antigens. However, expression of other melanoma-associated antigens, such as gp100, was not affected. Antigen loss could be reversed by long-term growth in culture away from immune-selective pressures or within 96 hours by treatment with the demethylating agent 5-azacytidine (5-Aza). When transplanted back into syngeneic animals, variants were very poorly controlled by further vaccination. However, a combination of vaccination with 5-Aza to reactivate antigen expression in tumors in situ generated highly significant improvements in therapy over treatment with vaccine or 5-Aza alone. These data show that inflammatory killing of normal cells activates a potent T cell response targeted against a specific subset of self-antigens but can also lead to the immunoselection of tumor variants. Moreover, our data indicate that emergence of antigen loss variants may often be due to reversible epigenetic mechanisms within the tumor cells. Therefore, combination therapy using vaccination and systemic treatment with 5-Aza or other demethylating agents may have significant therapeutic benefits for antitumor immunotherapy. (Cancer Res 2005; 65(5): 2009-17)

Introduction

Many tumor-associated antigens of melanoma are unaltered self-proteins of melanocytes (1–3). T cells react to these antigens, which have escaped thymic deletion, have the capacity, if correctly activated, to kill both melanoma cells and normal melanocytes thereby possibly inducing vitiligo (1, 2, 4–10). However, development of antitumor immunity does not necessarily have to generate overt autoimmune disease as well (3). Most previous strategies designed to generate T cell-mediated responses to tumors have used tumor-derived cells, proteins, or peptides as a platform. We hypothesized that it might be possible to reverse the direction of this flow of immunologic information by using the intentional induction of pathologic-like killing of normal cells (melanocytes) to generate immune responses that would be effective against tumor cells (melanoma; refs. 9, 11). We showed that i.d. injections of a plasmid in which the herpes simplex virus thymidine kinase (HSVtk) suicide gene (12) is transcriptionally targeted to melanocytes through the tyrosinase promoter (Tyr-HSVtk) leads to tissue-specific killing of melanocytes on administration of the prodrug ganciclovir (11). However, simple killing of melanocytes was only very poorly effective at generating antimelanocyte/melanoma T cell responses in vivo (11). However, combination of multiple injections of the Tyr-HSVtk plasmid with a plasmid expressing the murine hsp70.7 gene (cytomegalovirus-hsp70; ref. 13) generated localized killing of normal melanocytes within a highly inflammatory environment (11). This was sufficient to generate a CD8+ T cell response, which cleared 3-day established s.c. tumors or 6-day established systemic tumors in the lungs (11) consistent with the known activities of hsp70 as a key molecule mediating the switch between tolerogenic and immunostimulatory cell killing (13–23). However, this CD8+ T cell response was also rapidly suppressed in vivo by a population of putative suppressor cells within the CD25+ T cell population (24), which prevented development of autoimmune vitiligo in tumor-cured mice (11). Autoimmune vitiligo could be induced in treated animals but only if they were not challenged with tumor and this autoimmune disease was significantly more aggressive if the animals were also depleted of CD25+ T cells before plasmid administration (11). These data indicated that inflammatory killing of normal melanocytes primes and expands a CD8+ T cell response that is (a) effective against systemically distributed B16 melanoma; (b) suppressed in vivo by a population of putative suppressor cells within the CD25+ T cell population; and (c) exhausted, or deleted, by the presence of an established tumor mass (11). Presumably, these mechanisms of suppression of anti–T cell responses exist in vivo to safeguard against the development of autoimmune disease in circumstances where pathologic killing of normal cells occurs (24).

In this report, we have characterized the consequences on the phenotype of established tumor of the antitumor immune response that is raised by the inflammatory killing of melanocytes. Our data show that this novel immunotherapeutic regimen raises T cell responses directed against a specific subset of known melanocyte antigens and can lead to the immunoselection of tumor cell variants that have lost expression of these target antigens. However, antigen expression could be reinforced by treatment with 5-azacytidine (5-Aza). Combination therapy of
vaccination plus systemic 5-Aza generated significant increases in efficacy of therapy against antigen loss variants that were otherwise poorly treated by further rounds of vaccination alone. These data suggest that protocols aimed at reinducing expression of tumor-associated antigens may enhance the value of successful vaccination strategies that fail in part because of the emergence of antigen loss variants (25).

Materials and Methods

Cell Lines and Plasmids. Murine melanoma B16.F1, B16ova, and Lewis lung carcinoma cell lines have been previously described (26). B16ova cells were derived from the parental cell lines by transduction with a CDNA encoding the chicken ovalbumin gene (26).

Plasmids used in these studies have been described previously (11). Briefly, the Tyr- HSVtk plasmid uses a hybrid promoter of three tandem copies of a 200 bp element of the murine tyrosinase enhancer (27) upstream of a 270 bp fragment of the tyrosinase promoter (28) to drive expression of the HSVtk gene (29). In CMV-hsp70, the murine hsp70 gene (13) is driven by the CMV promoter in pCR3.1 (Invitrogen, Carlsbad, CA).

Reverse Transcription-PCR. Tumors were explanted and dissociated rapidly on ice (30), and RNA was prepared with the Qiagen RNA extraction kit (Qiagen, Chatsworth, CA). One-microgram total cellular RNA was reverse transcribed in a 20 μl volume using oligo(dT)12-18 as a primer. A CDNA equivalent of 1 ng RNA was amplified by PCR for a variety of murine cytokines or melanoma/melanocyte antigens as described previously (details of the primers upon request; refs. 26, 31).

Melanocyte Killing with Ganciclovir. Melanocyte killing in vivo was achieved as described previously by i.d. injections of the Tyr-HSVtk plasmid (11). Ganciclovir was given i.p. at a concentration of 50 mg/kg.

Demethylation Assays Using 5-Aza. 5-Aza was purchased from Sigma (St. Louis, MO). For in vitro assays of reactivation of tumor antigen expression, 5-Aza was diluted in PBS to obtain the appropriate dilutions (0.1, 1, and 10 μM). For in vivo studies, 5-Aza was given i.p. at a dose of 0.2 mg/kg. Mice were treated with three cycles, each cycle consisting of a daily i.p. injection for 5 consecutive days followed by 2 days rest. In groups treated with both ganciclovir and 5-Aza, i.p. ganciclovir injections were given between 4 and 6 hours before 5-Aza injections.

In vivo Studies. All procedures were approved by the Mayo Foundation Institutional Animal Care and Use Committee. C57BL/6, or T cell-deficient nude, mice were age- and sex-matched for individual experiments. To establish s.c. tumors, 2 × 10^6 B16 cells were injected s.c. (100 μl) into the flank region. Animals were examined daily until the tumor became palpable, after which the diameter, in two dimensions, was measured thrice weekly using calipers. Animals were killed when tumor size was ≥ 1.0 cm in two perpendicular directions. In all experiments, 10 mice per group were used unless indicated otherwise in the figure legends.

Plasmid injections were carried out i.d. injection (11, 32) in a final volume of 50 μl in PBS.

Tumor Treatment Protocols. For protocols aimed at treating established s.c. tumors, 2 × 10^5 B16 cells were seeded s.c. in the right flank of C57BL/6 mice (day 0). At the appropriate day following tumor seeding, 20 μg plasmid DNA was injected i.d. on the contralateral flank consisting of 10 μg of Tyr-HSVtk and 10 μg of CMV-hsp70. For the curative tumor treatment model, DNA injections were given on days 4 to 6, 11 to 13, and 18 to 20; ganciclovir at 50 mg/kg was given i.p. on days 4 to 8, 11 to 15, and 18 to 22. For the suboptimal, noncurative regimen to generate antigen loss variants, DNA injections were given on days 10 to 12 and days 17, 18, and 19; ganciclovir at 50 mg/kg was given i.p. on days 10 to 14 and 17 to 21.

Statistics. Data from the animal studies were analyzed by the log-rank test (33). Statistical significance was determined at the level of P < 0.05.

Results

Suboptimal Plasmid Vaccination Selects for Aggressive Tumor Variants. We have reported previously (11) that three rounds of Tyr-HSVtk/CMV-hsp70/ganciclovir treatment (a total of nine i.d. injections) according to the protocol of Fig. 1A cures 70% to 100% of mice bearing 3-day established s.c. B16 tumors on the contralateral flank (11). Because we were interested in the antigen specificity of the CD8+ T cell response that the inflammatory killing of normal melanocytes induces in vivo (11), we repeated these experiments using B16ova tumors that stably express the ovalbumin antigen as a surrogate antigen that is not normally expressed by either melanocytes or melanomas (26). The expression of the oval antigen did not affect the efficacy of this curative vaccination protocol (P < 0.001 compared with control treated animals; Fig. 1B). We have also previously shown that if either the frequency of the plasmid injections was reduced or the size of the tumor at the time of initiation of treatment was increased, or if both occurs, tumor progression could still be significantly slowed; however, fewer animals would be cured long term (11), leading to emergence of tumors that had survived the immune pressure placed upon them by the anti-melanocyte/melanoma T cell response (11) in vivo. Thus, animals bearing 9-day (instead of 3-day) established s.c. B16ova tumors, treated with a suboptimal regimen of plasmid/ganciclovir treatment (two rounds instead of three) survived significantly longer than control treated animals but most eventually succumbed to disease (Fig. 1C and D). In the experiment shown in Fig. 1D, tumors in mice treated by the inflammatory killing of melanocytes in vivo typically were either (a) cured long term (>100 days; 1 of 10 in the experiment in Fig. 1D) or (b) initially well controlled by the CD8+ T cell response but then recurred with an extremely aggressive growth rate (Fig. 1D). Thus, individual tumors could develop from a diameter of 0.2 to 1.0 cm within the space of 3 to 4 days, significantly shorter than a normal B16 tumor would take for the same size expansion. In the experiment shown in Fig. 1D, all tumors were explanted for analysis at day 42; in other experiments, these tumors would not regress further and would lead to rapid sacrifice of the animals.

Antitumor Effector Mechanisms Are Characterized by IFN-γ and Perforin Production. Tumors growing on the contralateral flank to the site of Tyr-HSVtk/CMV-hsp70/ganciclovir treatment were examined at various time points following a single round of plasmid injection (three daily i.d. plasmid and five i.p. ganciclovir, injections). Three days following the first DNA injection, a lymphocytic infiltrate was detectable in the B16ova tumors (Fig. 2A) but only in tumors in which the full combination of Tyr-HSVtk/CMV-hsp70/ganciclovir treatment was given. This infiltrate was also rather transient and disappeared within 14 days of the first plasmid treatment. Using primers specific for a variety of molecules, we were only able to detect reproducible expression of IFN-γ and perforin in these tumors. Neither tumor necrosis factor-α (Fig. 2B) nor interleukin (IL)-2, granulocyte macrophage colony-stimulating factor, IL-12, IL-10, or IL-4 were reproducibly detected at levels above those in normal B16ova tumors growing in control or untreated mice. Once again, the response was highly transient in vivo and had disappeared from tumors by 7 days following the first plasmid injection (Fig. 2B), consistent with our previous
findings that the antimelanocyte/melanoma T cell response induced by inflammatory killing of normal melanocytes is rapidly suppressed in vivo by a population of putative suppressor cells within the CD25+ T cell population (11, 24). We also repeated these experiments using Lewis lung carcinoma tumors on the contralateral flank instead of B16ova. Both the lymphocytic infiltrate and the cytokine profile induced by Tyr-HSVtk/CMV-hsp70/ganciclovir treatment was unique to B16 tumors (Fig. 2B). The ova antigen is not expressed in normal melanocytes, which are the target of Tyr-HSVtk/CMV-hsp70/ganciclovir therapy, but is expressed in B16ova tumor cells. Therefore, we investigated whether ova could constitute a significant immunologic target, either following Tyr-HSVtk/CMV-hsp70/ganciclovir treatment, or within the context of epitope spreading following T cell-mediated B16ova tumor killing in vivo. Using both SIINFEKL tetramers and IFN-γ enzyme-linked immunospot assays, we did not observe any anti-ova immune responses in mice either before or after (up to 40 days following plasmid injection) treatment with Tyr-HSVtk/CMV-hsp70/ganciclovir, irrespective of whether mice had B16ova tumors or not. In contrast, T cell responses specific to the tyrosinase-related protein 2 (TRP-2) antigen were detected from splenocytes of C57BL/6 mice cured of established B16ova tumors by inflammatory killing of melanocytes (Fig. 2C; ref. 11). In contrast, no ova-specific T cells were detectable by either SIINFEKL tetramer staining or by IFN-γ enzyme-linked immunospot analysis in these survivors (Fig. 2C). These data indicate that the in vivo killing of B16ova tumors by the CD8+ T cell response induced by Tyr-HSVtk/CMV-hsp70/ganciclovir treatment does not induce detectable levels of epitope spreading against this particular antigen that is expressed within the tumor cells but which is not expressed in the target melanocytes.

**Inflammatory Killing of Melanocytes Induces an Immune Response that Selects for Antigen Loss B16 Variants.** In the experiment shown in Fig. 1D, B16ova tumors were excised once they started to grow rapidly following the period of in vivo control generated by the suboptimal levels of plasmid injections (Fig. 1D). Of the nine tumors that were explanted, seven (B16ova-VAR1-7) were successfully reestablished in culture (30). Of these seven B16ova explants, all were noticeably depigmented...
immediately after replating (Fig. 3A and B). This was illustrated most effectively by allowing explanted tumor cells to overgrow on a culture plate; cultures of B16ova tumor cells recovered from untreated mice were heavily pigmented compared with the minimal amounts of melanin visible in the B16ova explants derived from vaccinated mice (Fig. 3A and B). Previously, we have shown that splenocytes from Tyr-HSVtk/CMV-hsp70/GCV–treated mice proliferate specifically in vitro following stimulation with the H-2Db–restricted peptide TRP-2180-188 SVYDFFVWL (11), which is the immunodominant epitope from the TRP-2 melanocyte/melanoma antigen (34, 35). Therefore, we used reverse transcription-PCR on cDNA from the B16ova-VAR1-7 cell lines at short periods following explant (<1 week) to screen for expression of melanoma-associated antigens. All seven cell lines expressed the melanoma antigens gp100 (Fig. 3C), MART-1, and TRP-1 (not shown; ref. 1), at similar levels to those expressed in the parental B16ova cell line. In addition, expression of the ova antigen was not affected in the B16ova-VAR1-7 cell lines by previous passage in Tyr-HSVtk/CMV-hsp70/ganciclovir–treated mice (Fig. 3C). In contrast, all seven B16ova-VAR cell explants had lost all detectable expression of TRP-2 mRNA (Fig. 3C). Most had also lost expression of the tyrosinase antigen, although B16ova-VAR3 and 6 retained detectable mRNA but at greatly reduced levels compared with the parental B16ova line (Fig. 3C).

Antigen Expression Is Recovered by In vitro Culture of Antigen Loss Variants. We followed expression of these two tumor antigens with time in culture in the antigen loss B16ova-VAR lines. One month after continuous culture, expression of tyrosinase began to appear in two of the variants (B16ova-VAR1 and 2) and had become progressively more abundant in variants 3 and 6. In addition, low levels of expression of TRP-2 had become apparent in B16-ova VAR1, 2, and 6 (data not shown). Finally, following 3 months of in vitro passage, all seven B16ova-VAR cell lines had restored expression of both TRP-2 and tyrosinase to levels that were directly comparable with those of the parental B16ova cell line (Fig. 3D).

Antigen Loss Variants Grow More Aggressively in Immuno-competent Animals. We studied the effect of loss of these antigens on the growth of the B16ova-VAR cell lines both in vitro and in vivo. In vitro, two representative antigen loss variants, B16ova-VAR1 and 3, grew more slowly in culture at short times post explant (2-3 weeks) than an explanted B16ova tumor from untreated mice (P < 0.02 for B16ova-VAR-3 compared with B16ova; Fig. 4A). To obtain sufficient numbers of cells for in vivo growth studies, it was necessary to expand the variant cell lines in vitro for a minimum of ~21 days following their initial explant. Therefore, cells injected in vivo at this stage were no longer completely negative for expression of TRP-2 or tyrosinase as

Figure 2. A single round of i.d. plasmid treatment induces lymphocytic infiltrates and IFN-γ expression in contralateral B16 tumors. Mice bearing B16ova tumors seeded 9 days previously were treated with a single round (three total injections) of plasmid injections (days 10, 11, and 12) and given ganciclovir at days 10-14 and 17-21. A, 3 days following the first injection tumors were recovered and examined by H&E staining for the presence of immune cell infiltration. The lymphocytic infiltrate shown here was absent from B16ova tumors in mice treated with PBS, Tyr-HSVtk/ganciclovir, or Tyr-HSVtk/CMV-hsp70/PBS. B, at the times indicated following the first DNA injection, tumors were excised, cDNA prepared, and PCR was done using primers specific for IFN-γ, perforin, tumor necrosis factor-a (8), or IL-2, IL-12, granulocyte macrophage colony-stimulating factor, IL-4, or IL-10 (not shown). C, IFN-γ enzyme-linked immunospot assays (PharMingen, San Diego, CA) were done using splenocytes harvested from C57BL/6 mice that had been cured of established B16ova tumors by treatment with Tyr-HSVtk/CMV-hsp70/ganciclovir. Splenocytes were stimulated in the presence of the synthetic, H-2Dα–restricted peptides hgp10025-33-KVPRNQDWL, TRP-2180-188 SVYDFFVWL, and H-2Kb–restricted Ova SIINFEKL as described (11) in triplicate cultures at a density of 250,000 splenocytes per well. Spot numbers were determined 72 hours later by computer-assisted image analyzer.
described in Fig. 3. Nonetheless, they still only expressed these antigens at very low levels. Both variants grew significantly faster ($P < 0.01$ B16ova-VAR1 and 3 compared with B16ova explanted from an untreated mouse) as s.c. tumors in immunocompetent C57/BL mice (Fig. 4B). The rate of growth of these explanted variants was not, however, as aggressive as was typically seen in vivo following escape from the Tyr-HSVtk/CMV-hsp70/ganciclovir treatment as shown in Fig. 1D. This may be due, in part, to reexpression of the lost antigens during their time in culture before reimplantation (~21 days) and due to a lack of immune selective pressure for them to retain loss of the appropriate antigens during their growth in the mice (a further 20-25 days). This growth differential was also completely lost in nude athymic mice (Fig. 4C).

Antigen Loss Occurs through Reversible Epigenetic Mechanisms. Expression of the TRP-2 and tyrosinase antigens reappeared spontaneously to normal B16ova levels after 3 months in culture (Fig. 3). It is possible that this merely represented the outgrowth, over time, of a few cells that were still antigen positive at the time of B16ova-VAR explant from against a background of cells in which antigen expression had been irreversibly lost due to the immune selection imposed by the antigen-specific T cells raised by the inflammatory killing of melanocytes. To investigate this hypothesis, B16ova-VAR cell lines that had been frozen 1 week after explant were still negative for TRP-2 expression by Northern blot when thawed (Fig. 5A). Treatment of two representative variants (B16ova-VAR1 and 3) with the demethylating agent 5-Aza (36, 37)

![Figure 3](https://example.com/fig3.png)

**Figure 3.** In vivo immune selection of B16ova tumors by inflammatory melanocyte killing leads to loss of pigmentation and expression of at least two melanoma-associated antigens. The B16ova tumors that started to regrow following suboptimal Tyr-HSVtk/CMV-hsp70/ganciclovir therapy in Fig. 1D were explanted and reestablished in six-well culture dishes. One well was allowed to grow until cells were overly confluent at which time maximal pigmentation is observed in B16 and B16ova cultures. Whereas B16ova tumor explants retained their ability to make large amounts of melanin and were heavily pigmented when attached to culture dishes and viewed by normal microscopy (A), B16ova-VAR cell explants (B16ova-VAR-3 shown in B) had lost any detectable pigmentation in vitro. C, 3 days following removal of tumors, cDNA from B16ova explants from untreated mice, or the B16ova-VAR explants 1-7, was screened by PCR for the expression of the antigens gp100, ovalbumin, TRP-2, and tyrosinase. Arrows, weak but detectable PCR products. D, B16ova-VAR1-7 explants were cultured for 3 months in vitro and cDNA was prepared and screened again for expression of TRP-2 and tyrosinase.
restored expression of TRP-2 mRNA to levels comparable with B16ova cells within 96 hours in vitro (Fig. 5A). Cells maintained without 5-Aza did not spontaneously reexpress the antigen over this time (Fig. 5A). These data suggested that the bulk (but not necessarily all) of the B16ova-VAR cell populations consisted of cells in which antigen expression had been downregulated in vivo by epigenetic mechanisms, such as promoter methylation, as a result of immune-selective pressure, but which still retained the capability to express the antigens.

Tumor recurrence, possibly as a result of the emergence of antigen loss variants following initial control, is a common clinical observation with many types of therapy. Therefore, we hypothesized that we might exploit this mechanism of antigen down-regulation/immune escape by combining vaccination with systemic treatment with 5-Aza to reinduce lost antigen expression in vivo, thereby making escape variants sensitive once again to antigen T-specific T-cell killing. B16ova-VAR3 cells, which had been cultured in vitro for a total for 21 days following explant and therefore expressed only very low levels of TRP-2 or tyrosinase antigens, were transplanted s.c. back into immunocompetent mice. As expected, because of their greatly reduced levels of expression of the appropriate target antigens, the resultant tumors were only very poorly controlled by an otherwise curative vaccination protocol of inflammatory melanocyte killing (P < 0.0001 compared with simple treatment by PBS; Fig. 5B and C). Treatment of the tumors with 5-Aza alone, in the absence of concomitant vaccination, had no antitumor effect (Fig. 5B and C). In contrast, vaccination combined with 5-Aza treatment led to highly significant antitumor effects on the B16ova-VAR tumors. Tumor development was significantly slowed relative to PBS (P < 0.0001), vaccination (P < 0.0002), or 5-Aza treatments alone (Fig. 5B and C). To investigate the reasons for these effects, 22 to 24 days after tumor seeding, the largest three tumors from each group were explanted and used to prepare RNA. Northern blotting and reverse transcription-PCR for expression of TRP-2 and tyrosinase mRNA showed that all three B16ova-VAR tumors grown in the absence of any immune selective pressure (No vaccination) had largely restored expression of TRP-2 consistent with our earlier findings (Fig. 5D and E, lanes 1-3). In contrast, two of three B16ova-VAR tumors grown in the presence of immune selection applied by inflammatory killing of melanocytes were, once again, heavily reselected for decreased levels of TRP-2 expression (Fig. 5D, lanes 4-6) or tyrosinase (Fig. 5E). However, if systemic 5-Aza was given during the vaccination regimen, TRP-2 and tyrosinase expression was maintained to levels similar to those in non-vaccinated animals PBS i.p. (lanes 10-12) or in mice treated only with 5-Aza (lanes 7-9).

**Immune Reactivity to a Non-Melanocyte-Derived Tumor Antigen following Inflammatory Melanocyte Killing.** Consistent with the immunologic monitoring data for ova (Fig. 2C), we did not observe any changes in levels of expression of ova in B16ova tumors during, or after, treatments with inflammatory melanocyte killing. Levels of ova expression in tumors that grow out following suboptimal Tyr-HSVtk/CMV-hsp70/ganciclovir treatment were unaffected relative to a B16ova tumor, which had not been subjected to any in vivo, vaccine-mediated immune-selective pressures (Fig. 3C). In addition, levels of ova expression were also unchanged (relative to those in B16ova tumors not subjected to in vivo immune selection) in B16ova variant tumors that were delayed in growth in response to vaccination + 5-Aza treatment in vivo (Fig. 5E). Finally, B16ova variants that do not regress in vivo in response to Tyr-HSVtk/CMV-hsp70/ganciclovir treatment, because they had low levels of expression of target TRP-2 and tyrosinase antigens, still expressed normal levels of ova (Fig. 5D and E). Taken together, these data, along with those of Fig. 2C, suggest that inflammatory killing of melanocytes...
Figure 5. Inflammatory melanocyte killing can be combined with systemic 5-Aza. A, B16ova explants from untreated mice, or B16ova-VAR-1 or B16ova-VAR-3 explants, were frozen 1 week after tumor removal. Thirty-six hours following thawing of these cells, they were grown in varying concentrations of 5-Aza (37) for 96 hours; RNA was prepared and probed for expression of TRP-2 mRNA. B, mice bearing B16ova-VAR3 tumors seeded 3 days previously were treated with three rounds (nine total injections) of plasmid or PBS i.d. injections (days 4, 5, 6 and 11, 12, 13 and 18, 19, 20) and given ganciclovir, 5-Aza, or PBS i.p. at days 4-8, 11-15, and 18-22. Mean tumor volumes in each group were measured with time (n = 12-13; B). C, growth rates of individual tumors in each group. D, at days 22-24 following seeding of B16ova-VAR3 tumors as described in B, tumors were explanted from three animals in each group and RNA was prepared and probed for presence of mRNA of TRP-2. Lanes 1-3, RNA from tumor explants from mice treated with PBS i.d. and i.p. (No Vaccination); lanes 4-6, mice treated with Tyr-HSVtk/CMV-hsp70 i.d. + ganciclovir i.p. (Vaccination); lanes 7-9, mice treated with 5-Aza alone; lanes 10-12, mice treated with plasmid injection + ganciclovir + 5-Aza. Equal loading was confirmed by probing for β-actin (not shown). E, reverse transcription-PCR was also used to screen these tumors for expression of the tyrosinase (Tyr) and ova (Ova) antigens as shown (lanes as in D).
raises CD8+ T cell responses that focus predominantly upon melanocyte-derived antigens and that a non-melanocyte-derived antigen within the tumor cells is not targeted in vivo as a result of the therapy.

**Discussion**

We investigated the consequences of exposure to a potent CD8+ T cell response, induced by a novel form of antitumor vaccination involving the hsp70-mediated, inflammatory killing of normal melanocytes in situ (11) on the phenotype of an established tumor. This strategy can cure both established s.c. or lung metastatic B16 melanomas (11). However, under circumstances where the vaccination strategy itself is not enough to clear established tumor, tumor cells that escape clearance undergo stringent selection for loss of expression of a particular subset of known melanocyte-associated antigens. These immune escape variants grow extremely aggressively in vivo following immune-imposed selection. On explant from the host, seven of seven B16ova variants had lost all, or the majority of, detectable expression, of TRP-2 and tyrosinase (1, 38). However, expression of other known melanoma differentiation antigens (1), such as gp100, MAGE, or TRP-1, as well as an artificial (ova) antigen, which has nothing to do with melanocyte or melanoma biology, were not affected by in vivo selection. Loss of the melanogenic enzymes TRP-2 and tyrosinase (1, 38), or other as yet unidentified antigens, slowed the in vitro growth rate of these variant cell lines compared with similarly explanted, unselected B16ova parental cells. However, in the presence of an intact immune system, a selective growth advantage was conferred on the variant cells, an advantage that was lost in mice lacking T cells.

We observed reinduction of TRP-2 and tyrosinase antigen expression in the B16ova variant explants under two separate experimental conditions. In the first, long-term culture of the cell lines away from immune-selective pressure led to full reexpression within a period of ~3 months. In the second, treatment with even low doses of 5-Aza restored TRP-2 antigen expression rapidly within 96 hours. 5-Aza is a potent inhibitor of DNA methylation, which can, therefore, reinduce gene expression from promoters that have been shut down by methylation of critical sequences (36, 37). Taken together, these results suggest that antigen loss in vivo probably occurs via a reversible epigenetic mechanism involving selection-imposed shutoff of gene expression rather than loss of the encoded genes. This raises the intriguing possibility that the activity of cytotoxic T cells within a tumor may initiate signals that actively lead to down-regulation of antigen gene expression within neighboring (non-killed) tumor cells rather than simply a passive selection of cells that have already low levels of expression of the relevant antigens.

Tumor recurrence following initial immune-mediated control is a common observation with many types of antitumor vaccination therapy (25). However, the mechanisms involved can be varied including development of functional tolerance (2, 39, 40) due to exhaustion of effector cells (41), induction of tolerance to target antigen (40, 42–45), low levels of antigen expression in peripheral tissues (antigenic ignorance; refs. 46–49), and/or down-regulation of molecules associated with antigen presentation (50–52). In addition, tumors can alter their antigen expression either qualitatively—for instance, through the mutation of antigenic epitopes leading to modulation of MHC: peptide interaction and TCR binding (antigenic drift; ref. 53)—or quantitatively with complete loss of expression of antigen (54–56). Therefore, we hypothesized that it might be possible to exploit our ability to restore antigen expression in the previously immunoselected B16ova variants by systemic treatment with 5-Aza, thereby making escape variants sensitive once again to antigen-specific T cell killing induced by concomitant vaccination (25). We transplanted B16ova-VAR3 cells back into immunocompetent animals to test the efficacy of the inflammatory melanocyte killing protocol against these antigen loss variants in mice, which were fully competent to raise anti-melanocyte T cell responses. Induction of an antimelanocyte/melanoma immune response exerted continued immune-selective pressure to maintain very low levels of TRP-2 antigen expression in these tumor cells during in vivo growth and, consequently, a moderate control of tumor growth rate compared with PBS treatment. Systemic treatment with 5-Aza had no therapeutic effect. However, a combination of vaccination with 5-Aza treatment generated highly significant improvements in delaying tumor development. Of the few tumors that did emerge, expression of the TRP-2 antigen was present at levels similar to those in nonvaccinated animals or in mice treated only with 5-Aza, and at significantly higher levels than in animals in which immune selective pressure was applied without concomitant enforced induction of TRP-2 expression. These data show that chemotherapy with 5-Aza, at concentrations where it has no cytotoxic effect on actively growing tumors, produces highly significant therapeutic enhancements when used in combination with a vaccination strategy that has minimal efficacy against a highly selected population of antigen loss variants. Our data supports a hypothesis in which antigen loss is a major, but not exclusive, reason for the failure of suboptimal regimens of inflammatory melanocyte killing. This is based on our observations that antigen-specific CD8+ T cell responses are absolutely required and responsible for antitumor effects (11); that tumors that escape clearance by this T cell response have lost expression of these melanocytic antigens but still retain expression of a potentially immunogenic nonmelanocytic antigen; and that in vivo treatment of antigen loss variants with 5-Aza reinduces expression of these antigens and restores sensitivity to inflammatory melanocyte killing. However, other factors will, inevitably, also be important in the efficacy of this strategy such as upregulation of MHC I (57) and TNFR (58). Thus, in the presence of 5-Aza + vaccination, B16ova antigen loss variants that do grow, despite antigen reinduction and vaccination, express mRNA for TRP-2 and tyrosinase at near-normal levels (Fig. 5D and E). We observed that retreatment of these tumors with a further round of 5-Aza in vivo does not have any further impact on treatment, consistent with the fact that they already have full reinduction of TRP-2/tyrosinase expression. We believe that these antigen-positive tumors reemerge because, at least in part, the antimelanocyte CD8+ T cell response is also rapidly suppressed in vivo by a population of putative regulatory T cells in the CD4+CD25+ population, as described by us previously (11). 5-Aza analogs have already been used in melanoma patients with some level of success (59), and are currently FDA approved to treat myelodysplastic syndrome. We would suggest, therefore, that many different immunotherapeutic approaches may benefit from both the administration of adjuvant chemotherapies designed to reinduce antigen expression within tumor cells, and from the use of strategies aimed at modifying negative immune effector mechanisms to restore sensitivity to vaccine-induced, immune-mediated tumor control.
Acknowledgments

Received 9/7/2004; revised 12/9/2004; accepted 12/22/2004.

Grant support: Mayo Foundation and NIH grants BOI C491480 and P50CA91956.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734.

We thank Toni Higgins for expert secretarial assistance.

References

1. Engelhard VH, Bullock TN, Colella TA, Sheasley SL, Mullins DW. Antigens derived from melanocyte differ-
188:136–46.

2. Overwijk W, Theoret M, Finkelstein S, et al. Tumor regres-


5. Dudley ME, Wunderlich JR, Robbins PF, et al. Cancer re-

6. Hodi FS, Mihm MC, Soiffer RJ. Biologic activity of cyto-
toxic T lymphocyte-associated antigen 4 antibody blockade in previously vaccinated metastatic melano-

7. Phan GQ, Yang J, Sherry RM, et al. Cancer revers-
ion and autoimmunity induced by cytotoxic T lymphocyte-
associated antigen 4 blockade in patients with meta-


Potent Selection of Antigen Loss Variants of B16 Melanoma following Inflammatory Killing of Melanocytes *In vivo*

Luis Sanchez-Perez, Timothy Kottke, Rosa Maria Diaz, et al.


**Updated version**  
Access the most recent version of this article at:  
http://cancerres.aacrjournals.org/content/65/5/2009

**Cited articles**  
This article cites 57 articles, 24 of which you can access for free at:  
http://cancerres.aacrjournals.org/content/65/5/2009.full.html#ref-list-1

**Citing articles**  
This article has been cited by 18 HighWire-hosted articles. Access the articles at:  
/content/65/5/2009.full.html#related-urls

**E-mail alerts**  
Sign up to receive free email-alerts related to this article or journal.

**Reprints and Subscriptions**  
To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

**Permissions**  
To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.