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Immunotherapy Can Reject Intracranial Tumor Cells without Damaging the Brain despite Sharing the Target Antigen

Byram W. Bridle,⁎ Jian Li,⁎ Shucui Jiang,† Ruby Chang,† Brian D. Lichty,* Jonathan L. Bramson,* and Yonghong Wan* 

Although vaccines targeting tissue differentiation Ags represent a promising strategy for cancer immunotherapy, the risk of triggering autoimmune damage to normal tissues remains to be determined. Immunizing against a melanoma-associated Ag, dopamine tautomerase (DCT), which normal melanocytes and glial cells also express, allowed concurrent analysis of autoimmune consequences in multiple tissues. We show that vaccination with recombinant adenovirus expressing DCT elicited a strong CTL response in C57BL/6 mice, leading to protection against intracranial challenge with B16-F10 melanoma cells. Both histological analysis and behavioral testing indicated that there was no evidence of neuropathology in vaccinated animals and long-term survivors. Although vitiligo or demyelination could be induced by additional stimuli (i.e., surgery or inflammation) in DCT-vaccinated mice, it did not extend beyond the inflammatory area, suggesting that there is self-regulatory negative feedback in normal tissues. These results demonstrate that it is possible to vaccinate against a tumor embedded in a vital organ that shares the target Ag. The Journal of Immunology, 2010, 184: 4269–4275.

Antigen-specific, active immunotherapy offers a promising approach to target both primary and metastatic cancers of various types including intracranial (i.c.) tumors (1, 2). In this regard, finding Ags exclusively expressed by tumor cells and incorporating them into therapeutic vaccines will not only increase the likelihood of induction of anti-tumor immunity but also reduce the possibility of autoimmune pathology (3). However, development of vaccines directed at tumor-specific Ags is complicated by the fact that each vaccine will likely need to be patient specific (4). As an alternate strategy, efforts have been invested into developing vaccines to target tumor-associated Ags (TAAs) that are typically nonmutated proteins overexpressed in specific classes of tumors (5). Indeed, despite the lack of mutation in these proteins, many cancer patients often display spontaneous reactivity to TAAs. This realization has led to the development of different strategies that can overcome self-tolerance and elicit an immune response against self-Ags.

Whether pathological damage to normal tissues that express TAAs is a requisite outcome of successful anti-tumor immunotherapy remains controversial. Evidence from animal studies has shown that vaccination against melanoma-associated Ags (MAAs), such as tyrosinase-related protein 1 or dopachrome tautomerase (DCT; also known as tyrosinase-related protein 2), is often associated with damage to normal melanocytes, leading to autoimmune vitiligo (6–9). Coupling of anti-tumor immunity and autoimmunity has also been observed in other tumor models, and furthermore, autoimmune symptoms appear to be a positive sign of clinical efficacy in cancer immunotherapy trials (10–13). These results led to the hypothesis that autoimmune destruction is an unavoidable or necessary consequence of effective anti-tumor immunity. However, although the damage to nonessential organs may be acceptable, this hypothesis imposes a conceptual challenge to cancer vaccines targeting Ags that are also expressed by cells in vital organs.

We and others have shown that tumor protection can be achieved in the absence of autoimmune disease (14–17). Specifically, we demonstrated in a murine melanoma model that a high-magnitude CTL response induced by recombinant adenovirus (Ad) expressing human DCT (hDCT) did not result in vitiligo unless there was damage (i.e., trauma or inflammation) to the normal tissue (15). A similar observation was made by Lang et al. (18) that large numbers of CTL specific for pancreatic Ag did not necessarily result in autoimmune diabetes, and overt autoimmune disease occurred only after an inflammatory stimulus that upregulated MHC class I expression in the pancreas. These data identify a concurrent inflammatory event in the target organ as a key factor determining the development of an autoimmune disorder following vaccination against self-tumor Ags.

To directly address the question of whether an effective vaccine imposes a risk to a vital tissue, we extended our melanoma vaccine study from the skin to the brain. Because both melanocytes and CNS tissue differentiate from the neural crest during embryonic development, they share MAAs, including DCT (19–21). This provides an ideal experimental setting to examine the relationship between anti-tumor immunity and autoimmune pathology upon i.c. inoculation with melanoma cells. Furthermore, melanoma commonly metastasizes to the brain in patients (22), and many MAAs are expressed on human malignant gliomas (19, 20, 23); thus, a better understanding of melanoma Ag-based vaccines may have a specific implication in the treatment of brain cancers.

Materials and Methods

Mice and tumor cells

Age-matched (8–10 wk old at study initiation) female C57BL/6 (H-2b) mice (Charles River Laboratories, Wilmington, MA) were housed in specific pathogen-free conditions. Animal studies complied with Canadian Council on Animal Care guidelines and were approved by McMaster University’s Animal Pathology and Molecular Medicine, McMaster University, Room MDC-5024, 1200 Main Street West, Hamilton, Ontario L8N 3Z5, Canada. E-mail address: wanyong@mcmaster.ca

Abbreviations used in this paper: Ad, recombinant adenovirus; DCT, dopachrome tautomerase; DNFB, 2,4-dinitro-1-fluorobenzene; hDCT, human dopachrome tautomerase; i.c., intracranial; MAA, melanoma-associated Ag; TAA, tumor-associated Ag.

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Research Ethics Board. Murine melanoma (B16-F10) cells were grown in F11-MEM containing 10% PBS, 2 mM l-glutamine, 1 mM sodium pyruvate, and vitamin solution, 0.01 mM nonessential amino acids, 50 μM 2-ME, 100 U/ml penicillin, and 100 μg/ml streptomycin (all from Invitrogen, Grand Island, NY). 

Recombinant adeno virus. Adenoviral vector construction has been described previously (24). Ad-hDCT expresses the full-length hDCT, and Ad-BHG contains no transgene. Viruses were propagated in 293 cells and purified on a cesium chloride gradient.

Peptides

Immunodominant peptides from DCT that bind to H-2K 

Kb SVYDFFVWL tetramer (The Binding Site, San Diego, CA) and I-A 

DCT KFFHRTCCTGNFA (26) were synthesized by Biomer Technologies (Hayward, CA).

Vaccination protocol

Anesthetized mice were immunized by i.m. injection of 1 × 10 

6 PFU of Ad-hDCT in 100 μl PBS (50 μl/hamstring). Control mice received Ad-BHG or PBS.

Stereotactic surgery

To establish brain tumors or local inflammation, mice received i.c. injections of 1 × 10 

9–10 

6 B16-F10 cells or 10 μg LPS in 2 μl saline. Mice were placed in a stereotaxis (Xymotech Biosystems, Mt. Royal, Quebec, Canada), and under both general and local anesthesia, an incision was made in the scalp to expose the skull. A needle mounted on a 10-μl Hamilton syringe (Hamilton, Reno, NV) was positioned over the right hemisphere of the brain, 2.25 mm lateral to Bregma. A small burr hole was drilled through the skull, and the bevel of the needle inserted into the brain parenchyma to a depth of 3 mm. Cells or LPS were injected over a period of 1 min. The needle was left in place for 2 min prior to withdrawal to minimize reflux along the injection tract. The scalp incision was closed with stainless steel clips that were removed 7–10 d later.

Abs

The following mAbs were used in flow cytometry assays: anti-CD16/CD32 (clone 2.4G2) to block FcRs, anti-CD3 (clone 145-2C11), and anti–IFN-γ (clone XMG1.2) (all from BD Biosciences, San Diego, CA) for intracellular staining. Immunodepletion studies were conducted with the mAbs OK1.5 (anti-CD4) and/or 2.43 (anti-CD8) from American Type Culture Collection (Manassas, VA). Purified mAbs (250 μg in 900 μl saline) were injected i.p. 2 d apart and then twice a week thereafter at a maintenance dose of 200 μg/treatment. The efficiency of specific depletion of lymphocyte subsets was >98% as measured by flow cytometry. For phenotyping skin-infiltrating T cells, the following additional reagents were used: PE-conjugated K 

SVYDDFFVWL tetramer (The Binding Site, San Diego, CA) and I-A 

DCT KFFHRTCCTGNFA (26) were synthesized by Biomer Technologies (Hayward, CA).

Detection of DCT-specific T cell responses

Ag-specific T cell responses were quantified by flow cytometric analysis 7 d postvaccination. Blood was collected from the periorbital sinus and RBCs lysed. To study skin-infiltrating T cells, postmortem damaged and normal shaved skin samples measuring 1 × 5 cm were excised from each mouse. Damaged skin had been injected with CFA 48 h prior to harvest or taken from a surgical lesion. Skin was minced, suspended in HBSS containing 0.1% collagenase (Invitrogen), incubated in a shaker at 37°C for 45 min, and then pressed through a nylon mesh filter with a 40-μm pore size to obtain a single-cell suspension. Mononuclear cells were stimulated with peptides (1 μg/ml DCT 180–188 or 20 μg/ml H-2Kb58–102) in the presence of brefeldin A (GolgiPlug; BD Pharmingen) and/or 2-4E10 Fc chimera (R&D Systems, Minneapolis, MN), biotinylated polyclonal Ab to human IgG (Alexis Biochemicals, Plymouth Meeting, PA), PE-Cy7-conjugated streptavidin (BD Biosciences), and anti-CD194/CCR4 (clone 2G12; BioLegend, San Diego, CA).

Histological analyses

Brains were fixed for 3 d in 10% formalin, transferred to 70% ethanol, paraffin-embedded, sectioned at a thickness of 10 μm, and stained with H&E (Sigma-Aldrich Canada, Oakville, Ontario, Canada). To stain myelin, sections (10–25 μm thick) were dehydrated in graded alcohol and placed in 1% Luxol fast blue at 37°C overnight. Sections were then differenti-
cells (15, 32). We found that i.c. injection of $1 \times 10^3$ B16-F10 cells resulted in formation of lethal tumors within only 13 d of injection (Fig. 1A, left panel). However, immunization with Ad-hDCT 7 d prior to challenge effectively prevented tumor growth (Fig. 1A, right panel), demonstrating the potency of Ad-hDCT as a potential vaccine for brain cancers that express DCT. This efficacy observed at early time points translated into a significant extension of survival (Fig. 1B). Mice treated with PBS (data not shown) or a control Ad vector (Ad-BHG) had a median survival of 15 d posttumor challenge, whereas immunization with Ad-hDCT resulted in a significant survival benefit in all treated mice (median = 64 d; $p < 0.0001$). More importantly, 27% (6 of 22) of vaccinated mice completely rejected implanted tumor cells, offering a unique model for the subsequent analysis of the acute and chronic impact of vaccine-induced autoimmunity in a vital organ.

**Protection against i.c. B16-F10 tumors depended on CD8$^+$ T cells**

To evaluate the induction of Ag-specific T cells by Ad-hDCT, we determined the frequency of both CD8$^+$ and CD4$^+$ T cells that were capable of producing IFN-γ in response to restimulation with their corresponding peptides. At 8 d postvaccination, >3% of blood-derived CD8$^+$ T cells were producing IFN-γ in response to the immunodominant epitope DCT$_{	ext{180-188}}$, shared between mice and humans (Fig. 2A). A significant level of CD4$^+$ T cells (0.4%) specific for hDCT$_{88-102}$, a recently identified helper peptide (26), was also evident by IFN-γ production (Fig. 2B). To determine their relative contribution to tumor protection, immunized mice were depleted of CD4$^+$ and/or CD8$^+$ T cells 2 d before i.c. challenge. Protection was completely abrogated by depletion of CD8$^+$ but not CD4$^+$ T cells, indicating that CD8$^+$ T cells were the primary effectors for i.c. tumor rejection (Fig. 2C). This notion is further supported by histological staining that revealed more immune infiltrates at the tumor challenge site in DCT-vaccinated animals compared with controls (Fig. 2D, upper panels), and many of these infiltrates were CD8$^+$ T cells (Fig. 2D, lower panels).

No evidence of acute neuropathology despite autoimmune targeting of DCT

For evaluation of potential acute autoimmune or bystander damage, mice were immunized with Ad-hDCT and challenged i.c. 7 d later with a high dose of $1 \times 10^6$ B16-F10 cells to ensure survival of tumor cells so malignant lesions could be identified. Rapid dissemination of the melanoma to the ventral surface and brain stem was observed in only 4 d (Fig. 3A, left panel). However, immunization with Ad-hDCT 7 d prior to challenge prevented tumor dissemination (Fig. 3A, right panel), confirming the potency of Ad-hDCT. Four days after the high-
CD8+ T cells are the primary effectors in this autoimmune destruction. CD4 depletion had a significant but incomplete effect, suggesting that before the surgery completely abrogated vitiligo induction, whereas in infiltrating Kb-DCT180–188 E-selectin ligand and CCR4 expression on skin-ramer and Ab staining. A typical contour plot of CFA was used to assess the phenotype of infiltrating, Ad-hDCT–immunized mice 48 h after injection of before i.c. melanoma challenge. (Fig. 4)

**Immunization with Ad-hDCT caused autoimmune pathology in inflamed normal tissues**

We previously reported that i.m. immunization with Ad-hDCT did not cause melanocyte damage (vitiligo) unless there was trauma/inflammation within the skin (15). Consistent with this earlier observation, all Ad-hDCT–immunized mice in the current model developed severe vitiligo along the scalp incision site, whereas this lesion was absent in all sham-treated mice (Fig. 4A). Depletion of CD8+ T cells before the surgery completely abrogated vitiligo induction, whereas CD4 depletion had a significant but incomplete effect, suggesting that CD8+ T cells are the primary effectors in this autoimmune destruction (Fig. 4B). Tetramer staining indicated that a substantial number of DCT-specific CD8+ T cells were indeed recruited into damaged skin and 50.4% (± SE of 3.5; n = 3) of these expressed E-selectin ligand and/or CCR4 (Fig. 4C), a surface phenotype consistent with skin-homing T cells (33). Interestingly, in all cases, the vitiligo was limited to the area immediately surrounding the incision. To further delineate the relationship between skin damage and vaccine-induced vitiligo, a hapten DNFB was used to induce skin sensitization in a controlled fashion. As shown in Fig. 4D, DNFB alone did not damage melanocytes (top panel), whereas the size of the vitiligo lesion directly correlated with the extent of the DNFB-induced skin inflammation in Ad-hDCT–immunized mice (bottom panel). The results reinforce the idea that the number of autoreactive T cells did not limit the extent of vitiligo, but their access to normal skin was apparently controlled by the degree of local inflammation.

To determine whether additional inflammatory stimulation causes autoimmune pathology in the brain, LPS was injected i.c. 14 d after treatment with Ad-hDCT or PBS. Compared with sham injection, LPS elicited more obvious inflammation, although it was limited to the injection site (Fig. 5, upper panels) and resolved in 3 d (data not shown). Considerably more CD8+ T cells were evident in Ad-hDCT–immunized mice (Fig. 5, middle panels), which correlated with localized demyelination (Fig. 5, lower panels; mean integrated density of Luxol fast blue stain = 2.7 ± 1.4 SE, n = 5 for Ad-hDCT + LPS versus 10.2 ± 2.6, n = 5 for PBS + LPS; p = 0.027). Sham injection or LPS challenge alone was insufficient to induce tissue damage (Fig. 5, lower panels). These observations confirm the concomitant requirement of both Ag-specific autoimmunity and local tissue inflammation for the induction of autoimmune sequelae.

**FIGURE 4.** Autoimmune vitiligo is associated with damaged skin, but neural tissue remains intact in long-term survivors. A, Mice receiving Ad-BHG and i.c. challenged with B16-F10 cells never developed vitiligo (top panel). Mice immunized with Ad-hDCT and challenged with B16-F10 cells always developed severe vitiligo limited to the scalp incision area (bottom panel). B, Mice vaccinated with Ad-hDCT had CD8+ (left panel) or CD4+ (right panel) T cells depleted right before i.c. melanoma challenge. C, Skin dissected from Ad-hDCT–immunized mice 48 h after injection of CFA was used to assess the phenotype of infiltrating, DCT-specific T cells by flow cytometry following tetramer and Ab staining. A typical contour plot of E-selectin ligand and CCR4 expression on skin-infiltrating K1–DCT108–1108+ T cells is shown (left panel) along with a paired sample stained with isotype control Abs (right panel). D, Mice treated with Ad-BHG (upper panel) or Ad-hDCT (lower panel) had their backs shaved and the hapten DNFB painted on their skin 7 d later. Only Ad-hDCT–immunized mice developed vitiligo that was limited to the area of inflammation.
Long-term survivors did not have altered behaviors or chronic neuropathology

Long-term survivors generated in our study provided an opportunity to analyze potential neural damage beyond static pathology. Twenty-one mice that survived i.c. challenge with B16-F10 cells and 22 age-matched naive mice were enrolled in a behavior study to assess multiple sensory motor skills. This analysis included tests that evaluated olfactory, visual, and auditory functions, all of which reflect the integrity of CNS functionality. As summarized in Table I, mice that survived their i.c. tumor challenge did not show any deficits in the tested aspects, compared with controls. Furthermore, brains harvested from these mice did not show any evidence of tumor cells, suggesting these were completely eradicated instead of their growth simply being suppressed. Notably, there was no evidence of any neuropathology in these survivors, despite immunization with Ad-hDCT and tumor destruction in the brain (data not shown). Because DCT-immunized mice challenged with LPS had evidence of acute demyelination at the injection site, we repeated the study to determine whether there were any signs of chronic damage. At 90 d post-LPS challenge, these mice passed all behavioral tests (Table I) and did not have any demyelination (mean integrated density of Luxol fast blue stain = 241.1 ± 1.0 SE, n = 5 for naive controls versus 240.2 ± 1.9, n = 5 for LPS challenge).

Ad-hDCT vaccination provided a potent therapeutic effect

We next extended the analysis to evaluate the therapeutic potential of Ad-hDCT in mice bearing pre-existing tumors in the brain. In this regard, mice were injected i.c. with 1 × 10^5 B16-F10 cells and then treated with Ad-hDCT 5 d later. Eleven days after treatment, the whole brains were harvested from both naive (tumor-free) and treated (tumor-bearing) mice for quantitative analysis of efficacy. Tumor burden was determined by subtracting the mean weight of tumor-free brains from those in the tumor-bearing groups. Fig. 6A shows that the mean tumor weight in Ad-hDCT–immunized mice was 4.7-fold less than that in mice treated with a control Ad vector (47.1 ± 21.3 versus 9.9 ± 2.8 mg; p < 0.05). Furthermore, mice treated 5 d postengraftment had a significantly longer median survival time following Ad-hDCT treatment, compared with sham-treated controls (Fig. 6B). An additional study extended this observation of Ad-hDCT–mediated therapeutic efficacy by demonstrating that immunization of mice with 7-d-old brain tumors increased median survival to 27 d versus only 15 for sham-treated controls (p = 0.0067) (Fig. 6C). As had been observed in the prophylactic challenge model, vitiligo was evident in all mice treated in the therapeutic setting (Fig. 6D). However, these lesions were less severe than those observed in the prophylactic model (Fig. 4A), presumably due to altered intervals between the peak of the anti-self-immune response (10–14 d after immunization) and surgery-induced skin damage (1–2 d after surgery). Specifically, immunization was carried out several days after tumor inoculation, such that a significant amount of wound healing had occurred by the time the CTL response was fully activated.

**Discussion**

Strategies to eliminate cancer cells by breaking tolerance to self-Ags have raised an issue regarding the potential risk for collateral autoimmune damage to normal tissues (34–36). Using a murine melanoma model, we and others have demonstrated that the development of effective anti-tumor immunity indeed results in autoimmune vitiligo, especially when there is a concurrent inflammatory event in healthy tissues that express Ags carried by the vaccine (8, 15, 37, 38). Results from several clinical trials also indicate that development of

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Mice vaccinated with Ad-hDCT were challenged i.c. with B16-F10 cells or LPS 7–14 d later. Some mice completely rejected the implanted tumor cells. Twenty-one long-term survivors of the tumor challenge (>90-d survival), 5 mice at 90 d post-LPS challenge, and 27 age-matched naive controls were enrolled in a series of behavior studies designed to assess general health and neurophysiological functions. Absence of brain tumors in these mice was confirmed by postmortem gross and histological examination following testing.

aMeans (± SEM) were not significantly different.

bThe mouse that failed these tests was the longest-term survivor and severely overweight.

cThe whiskers on two of these mice were absent because of barbering.
autoimmune toxicities correlated with tumor regression (13, 39–41). These observations point to the possibility that autoimmune pathology may be an unavoidable or even favorable outcome of cancer immunotherapies.

However, because the potential destruction of normal brain tissue could have severe consequences, the balance between anti-tumor immunity and autoimmune pathology in the case of cerebral malignancies needed to be investigated. The tumor model and vaccination approach presented in this study provided several unique features to address this issue. First, the potency of the Ad-hDCT vaccine allowed us to achieve treatment efficacy against i.c. growth of B16-F10 melanomas and provided an opportunity to analyze pathological consequences both in early stages and in long-term survivors. Second, the potential autoimmune destruction could be concomitantly evaluated in the skin and brain, both of which express the target Ag. Third, i.c. challenge with B16-F10 cells did not only provide a brain tumor model but also served as a local inflammatory stimulus within the brain.

We previously reported that vitiligo lesions following melanoma immunotherapy required two events: Ag-specific immunity plus damage to the skin (15). The current study corroborates this. Specifically, vitiligo was associated with the scalp incision site in Ad-hDCT–immunized mice. Moreover, vitiligo was severe in the prophylactic model where immune responses were peaking at the time of injury to the skin. In contrast, vitiligo severity was reduced in the therapeutic model where >1 wk of wound healing occurred before significant DCT-specific T cell responses were detectable. These observations reinforce our hypothesis that autoimmune pathology following effective cancer vaccination is the result of secondary trauma to normal tissues, and its severity is affected by the degree and persistence of normal tissue damage. We speculate that tissue inflammation/trauma is required to recruit autoreactive T cells, as demonstrated in Fig. 4C, and to modulate expression of MHC molecules and target Ags. Our results are consistent with the finding by Lang et al. (18) that highly activated CD8+ T cells could coexist with pancreatic b islet cells expressing the relevant autoantigen; however, overt autoimmune disease occurred when an inflammatory response was coupled.

Despite the presence of robust DCT-specific CD8+ T cells and the evidence that DCT is expressed in normal brain (19–21, 23, 42), we never saw any neuropathology in Ad-hDCT–immunized mice. This may be explained by the inherent immune-privileged nature of the brain (43) or the lack of inflammatory signals to recruit autoreactive T cells into sites distal to the i.c. tumors. Our results confirm that Ag-specific vaccines are capable of inducing an anti-tumor response within the immunologically privileged brain and that the CNS is accessible to systemic immunotherapy (44). More importantly, similar to what we observed in skin, immune destruction appeared to only focus on the inflammatory area (i.e., tumor) and did not extend beyond the site of tumor growth. Furthermore, the lack of behavioral anomalies or demyelination in normal brain tissue by long-term survivors provides further evidence that cerebral cancer can be eliminated by autoreactive T cells while leaving the CNS unharmed. The linkage between tissue inflammation and autoimmune pathology is further supported by deliberate i.c. injection of LPS where only Ad-hDCT–immunized animals exhibit localized, acute demyelination, presumably due to the recruitment of CD8+ T cells that recognize DCT expressed by normal glial cells. The fact that demyelination was no longer evident at 90 d post-LPS challenge suggests the cells that were acutely destroyed could be renewed following cessation of inflammatory stimulation. Taken together, these results clearly demonstrate that the inflammation is tightly regulated in normal tissues, which limits the initiation and progression of autoimmune destruction in the vaccinated host.

It is possible that additional stringent mechanisms may also be operational in the brain to regulate local immune responses to avoid autoimmune pathology. For instance, although most tumors are inherently inflammatory and the integrity of the blood-brain barrier is locally compromised allowing extravasation of activated T cells, their trafficking may be more limited in normal parenchyma (45). However, it remains to be determined whether the local environment functionally influences brain-infiltrating lymphocytes. The fact that immunotherapy is less efficacious in i.c. versus s.c. compartments using the same vaccination strategy suggests anatomical location of the tumor can influence the outcome of cancer vaccines, with the brain being a particularly difficult site (46).

Overall, our findings provide proof-of-principle that self-Ags can safely be targeted by cancer immunotherapy, even if the tumor is embedded within a vital tissue sharing the same Ag. Compelling evidence supports the concept that anti-tumor immunotherapy will only spill over into autoimmune pathology if linked by inflammation. Given the demonstration of MAA expression by gliomas, our findings on vaccination efficacy and the relationship between anti-tumor immunity and autoimmune pathology have significant implications for the design of therapies directed not only against brain melanoma but also potentially for primary brain cancers.

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Disclosures
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