Microenvironment and Immunology

Vaccination for Invasive Canine Meningioma Induces In Situ Production of Antibodies Capable of Antibody-Dependent Cell-Mediated Cytotoxicity

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Abstract

Malignant and atypical meningiomas are resistant to standard therapies and associated with poor prognosis. Despite progress in the treatment of other tumors with therapeutic vaccines, this approach has not been tested preclinically or clinically in these tumors. Spontaneous canine meningioma is a clinically meaningful but underutilized model for preclinical testing of novel strategies for aggressive human meningioma. We treated 11 meningioma-bearing dogs with surgery and vaccine immunotherapy consisting of autologous tumor cell lysate combined with toll-like receptor ligands. Therapy was well tolerated, and only one dog had tumor growth that required intervention, with a mean follow up of 585 days. IFN-γ–elaborating T cells were detected in the peripheral blood of 2 cases, but vaccine-induced tumor-reactive antibody responses developed in all dogs. Antibody responses were polyclonal, recognizing both intracellular and cell surface antigens, and HSP60 was identified as one common antigen. Tumor-reactive antibodies bound allogeneic canine and human meningiomas, showing common antigens across breed and species. Histologic analysis revealed robust infiltration of antibody-secreting plasma cells into the brain around the tumor in posttreatment compared with pretreatment samples. Tumor-reactive antibodies were capable of inducing antibody-dependent cell-mediated cytotoxicity to autologous and allogeneic tumor cells. These data show the feasibility and immunologic efficacy of vaccine immunotherapy for a large animal model of human meningioma and warrant further development toward human trials. Cancer Res; 73(10); 2987–97. ©2013 AACR.

Introduction

Meningioma is the most common primary brain neoplasm, with more than 100,000 patients diagnosed in the United States between 2004 and 2008 (1). Newly diagnosed tumors are managed by surgical resection alone. Roughly 6,000 patients will need additional treatment in the United States every year due to recurrence (2), which often occurs with invasive or malignant disease (3, 4). Current salvage approaches include reoperation, radiation, radiosurgery, and chemotherapy; there is controversy regarding the perceived clinical benefit of these interventions (5–8). The 3-year recurrence rate in reoperated World Health Organization (WHO) grade 1 meningioma is 50% (9), with risk of recurrence even greater in grade 2 and 3 tumors (10–12). Radiation, although modestly effective in benign disease, has been associated with cognitive deficits, secondary malignancies, and the transformation of the tumor to a higher-grade neoplasm (2).

One challenge in treating aggressive meningiomas is the paucity of animal models with which to test combinations of surgery and systemic therapy in a meaningful manner. Spontaneous meningiomas in dogs make up 45% of primary canine brain tumors (13) and are an underutilized resource for preclinical studies. Canine and human meningiomas share many features, including histologic resemblance, overexpression of growth factor receptors, deletion of chromosomal segments, and losses of function in tumor suppressor genes (14–16). More than 40% of canine meningiomas were atypical.
Materials and Methods

Surgery, vaccination production, and administration

Dogs were enrolled after obtaining owner consent according to an approved protocol from the University of Minnesota (Minneapolis, MN) Institutional Animal Care and Use Committee. A presumptive diagnosis of meningioma was based on the MRI characteristics of a solitary extra-axial mass in the brain, with heterogeneous T1W signal, usually isointense, homogenous T2W signal, sharply defined borders, homogenous contrast enhancement, evidence of a dural tail, that may have associated cysts, peritumoral edema, and falx-shift. Surgical resection was conducted under general anesthesia using the appropriate approach based on tumor location. Dogs were hospitalized with supportive care for 1 to 2 days after surgery. Corticosteroids used to minimize peritumoral edema were discontinued by 48 hours before vaccination. Part of the tumor specimen was used for histologic diagnosis, and the remainder of the tumor was cultured for vaccine production.

Cultures were established by mincing specimens with scalpels and digestion at 37°C for 15 minutes in suspension with TrypLE Express (Invitrogen/Life Technologies). Cell suspensions were filtered through a 100 µm filter, washed with PBS, and placed in culture in 10 cm culture plates precoated with 1:10 Matrigel (BD Biosciences), in serum-free neural stem cell media consisting of DMEM:F12 (1:1), with l-glutamine, sodium bicarbonate, penicillin/streptomycin (100 U/mL), B27 and N2 supplements (Gibco), and 0.1 mg/mL Normocin (InVivoGen). Semi-weekly, cells were given 20 ng/mL recombinant human EGF and recombinant human FGF (R&D Systems) and were cultured at 5% O2 and 5% CO2. Harvest for vaccination involved scraping cells from one 10 cm dish. Cells were washed thrice in PBS, and underwent 5 freeze-thaw alternations by transfer from warm water bath to liquid N2 followed by radiation (200 Gy). Protein was measured by Bradford assay with standard Coomassie reagent (Pierce), and lysates were stored at −80°C. GMP-grade CpG 685 ODN was provided by SBI Biotech Co., Ltd (Tokyo, Japan), and imiquimod cream was acquired through the University of Minnesota Boynton Health Services Pharmacy. In 5 dogs, CpG (2.0 mg) was mixed with thawed lysates immediately before intradermal injection at 2 sites in the back of the neck. Six other dogs received imiquimod cream (5%; 0.5 g) at 2 intradermal injection sites in the back of the neck 15 minutes before lysate injection. The maximally achievable lysate protein concentration was given to each dog, which varied by tumor volume and the ability of the tumor to proliferate in culture. Lysate doses ranged from 200 to 1,500 µg protein (average of 595 µg), and average dose did not vary significantly between CpG and imiquimod-treated dogs.

Tumor volume measurements

Twenty consecutive surgical human cases (of M.A. Hunt; Department of Neurosurgery, University of Minnesota, Minneapolis, MN) were analyzed for volume by MRI, as calculated by (length × width × height)/2 for tumor and intracranial volume. The same procedure was carried out for the 11 dogs in this study, and the results were compared as described in Supplementary Materials and Methods.
Western immunoblot analysis

Cultured tumor cells or homogenized flash-frozen tumors were used for blotting. Cells were lysed, protease and phosphatase inhibitors (Calbiochem) were added, and for SDS-PAGE, lysates were diluted in Laemmli-reducing sample buffer, heated, and centrifuged. Protein standards (Bio-Rad) were loaded next to each 40 μg lysate and resolved on NuPAGE 4% to 12% Bis/Tris gels (Invitrogen). Proteins were transferred to nitrocellulose (Amersham) at 5 V constant voltage using semidry transfer (BioRad). The membranes were blocked in 5% nonfat dry milk (NFDM)/Tris-buffered saline with 0.1% Tween-20 (TTBS) at room temperature for 1 hour and cut appropriately into identical blots, each with a molecular weight standard (BioRad) run adjacent to lysate. Each membrane was incubated at room temperature for 1 hour in normal, pre or postvaccination sera diluted 1:1000 in 5% NFDM/TTBS, washed 6 times for 10 minutes each in TTBS, followed by room temperature for 1 hour in rabbit anti-canine immunoglobulin G (IgG) horseradish peroxidase (HRP)-conjugated secondary antibody (Jackson ImmunoResearch) at 1:50,000 in 5% NFDM/TTBS. Bands were detected using ECL Western Blotting Detection System (Amersham) and HyBlot CL autoradiography film (Denville Scientific). Densitometry was conducted by the Gel Analysis tool in ImageJ 1.45s software (NIH), and values were normalized by dividing by heavy- and light-chain densities (areas under the curve) from prevaccination lanes.

Detection of tumor cell surface-reactive antibodies

A Becton Dickinson Custom Canto three-laser flow cytometer was used for data acquisition. Tumor cells were removed from Matrigel-coated 10 cm culture plates by scraping or BD Dispase (BD Biosciences), washed with PBS thrice, and incubated with heat-inactivated normal dog,
pre Vaccination, or 3-month postvaccination serum at 1:100 dilutions at 4°C for 30 minutes. Cells were then washed thrice in PBS and incubated with 1 μg anti-canine IgG (H+L)—fluorescein isothiocyanate (American Qualex) at 4°C for an additional 30 minutes before washing and analyzing.

**Immunohistochemistry staining and quantification of lymphocyte infiltration**

For lymphocyte analysis, 5 μm tissue sections were cut, prepared with standard procedures, and the following antibodies were used: CD3 (AbD Serotec) at 1:2,000, CD20 (Thermo Scientific) at 1:2,000, and IgG (H+L; Jackson ImmunoResearch) at 1:2,000. Tissue sections were incubated with the primary antibodies for 1 hour, rinsed, and a biotinylated secondary antibody was applied for 30 minutes. CD20 and IgG antibodies were followed with undiluted Rabbit Link (Covance), and a biotin-conjugated donkey anti-rat IgG (H+L; Jackson ImmunoResearch) at 1:500 was used with CD3. Sections were rinsed, incubated in hydrogen peroxidase, and a tertiary streptavidin HRP link (Covance) for 30 minutes. The immune complex was visualized using 3,3'-diaminobenzidine as the chromogen. Sections were lightly counterstained with hematoxylin, dehydrated, coverslipped, and scanned using the Aperio Scanscope XT.

All surgical resection specimens and all necropsy blocks containing tumor or inflammation as seen by hematoxylin and eosin stain (H&E) staining were included in counting of CD3+ and CD20+ cells. Samples were blinded, and all 10× fields in slides were captured and saved as image files. Automated cell quantification was conducted with a customized macro and the particle analysis tool in Fiji software (ImageJ 1.46), NIH. Counts underwent statistical analysis as described in Supplementary Materials and Methods before unblinding.

**Antibody-dependent cell-mediated cytotoxicity**

Dog blood from healthy donors was collected in anticoagulant tubes, lysed osmotically, and peripheral blood leukocytes (PBL) were washed thrice with PBS. PBLs were resuspended in complete RPMI-1640 (supplemented with 10% heat-inactivated FBS, 100 U/mL penicillin, 0.1 mg/mL streptomycin, and 0.1 mg/mL Normocin from InVivoGen) in a 96-well plate at 2.5 x 10⁶ cells/mL, and stimulated 14 hours with 30 μg/mL Normocin from InVivoGen (American Qualex) at 4°C for an additional 30 minutes before washing and analyzing.

**Statistical analysis**

Samples were analyzed by unpaired t test (tumor/brain volume, flow cytometry, LDH activity). Histologic cell counts were analyzed by an unpaired t test with Welch’s correction for unequal variances with 95% confidence intervals. Survival was analyzed using a log-rank test with 95% confidence intervals. All statistics were conducted using GraphPad Prism version 4.0c for OS X (GraphPad Software www.graphpad.com).

**Results**

**Canine meningiomas model aggressive human disease**

Meningiomas from dogs treated in our study share histologic features with human tumors of the same subtype (Fig. 1A), in addition to features that signify poor survival in humans. Brain invasion is an independent negative prognostic factor that prompted the reclassification of otherwise WHO grade I meningiomas as grade II (29, 30). Two canine
cases exhibited gross tumor invasion into the brain at resection, and 3 additional cases showed brain invasion upon postmortem analysis (Fig. 1B, left and Supplementary Table S1). Tumor invasion into the skull was also present in 2 cases (Fig. 1B, right). In addition, canine meningiomas occupied more than twice the volume of the brain relative to human tumors (Fig. 1C). All dogs were symptomatic at the time of diagnosis, and in 10 dogs, the presenting clinical sign was seizures. The rapid recurrence and progression of this disease in canines is consistent with the common appearance of invasion into brain, which, in humans, predicts poor outcomes.

**Extension of survival following vaccine immunotherapy**

Eleven dogs underwent craniotomy for tumor removal after radiographic diagnosis of an intracranial neoplasm consistent with meningioma. Following histologic confirmation, dogs were administered vaccinations biweekly with lysate derived from their tumors in combination with CpG or imiquimod (Fig. 2A). Mean follow-up time was 585 days, with 36% (4/11) of dogs alive (Supplementary Table S1; Fig. 2B and C). Relative to historic controls, median survival is extended in the immunotherapy cohort (645 vs. 222 days, \( P < 0.05 \)), with no censoring of dogs that died from other causes (Fig. 2B and Supplementary Table S2). Neither vaccination cohort contained a case of frank

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**Figure 3.** Vaccination induces antibody responses to meningioma surface antigens. A, Western immunoblot analysis of autologous tumor cells from culture (cases 10 and 3) or snap-frozen tumor specimen (case 5). Normal dog serum (ND) was used as a control. B, live autologous tumor cells were stained with serum from prevaccination or postvaccination (at 82 days). C, aggregate data of 3 dogs from B. *, \( P < 0.05 \); **, \( P < 0.005 \).
tumor progression, although more deaths occurred in the CpG cohort (Fig. 2C). One CpG-treated dog developed 2 meningiomas, of which the second was not identified before vaccination due to small size. The second tumor was subsequently removed after its growth caused breakthrough seizures, and 4 other vaccines were given using tumor lysate of the second tumor.

**Tumor-reactive antibodies bind cell surface antigens and cross-react with human meningiomas**

Immune responses before and after vaccination were measured in peripheral blood mononuclear cells (PBMC) and sera. IFN-γ–elaborating tumor-reactive T cells were significantly increased in postvaccination PBMCs in 2 of 9 dogs tested, suggesting a low frequency of circulating T cells 3 months after surgery (Supplementary Table S3). In contrast, polyclonal tumor-reactive antibody responses to whole-tumor lysate (Fig. 3A) and cell surface antigens (Fig. 3B and C) were detected in all 11 dogs (Supplementary Table S3). Probing of allogeneic tumor cells and lysates indicated recognition of common tumor antigens among dogs, some of which were present on the cell surface (Fig. 4A–C). Postvaccination antibodies recognized recombinant human HSP60 and HSP60 from autologous and allogeneic tumor (Fig. 4A), showing the common expression and recognition of this antigen. Remarkably, postvaccination canine serum

![Figure 4](image_url)

**Figure 4.** Antibodies recognize allogeneic dog and human meningiomas. A, Western immunoblot analysis of tumor cells from 3 cases and recombinant human HSP60. B, representative histograms of cell surface binding of primary tumor cells from case 7. C, quantification of serum antibody surface binding to an allogeneic papillary (nonstudy dog—recognized by case 3) and a meningothelial meningioma (case 7—recognized by cases 4, 6, 7, and 9). D, Western immunoblot analysis of a human meningioma probed with postvaccination serum from case 3. ***, P < 0.05; ***, ***, P < 0.005.
recognized human meningioma tissue, indicating antigen conservation between species (Fig. 4D).

**Vaccination induces T-, B-, and plasma cell infiltration into peritumoral brain**

We conducted analyses of B- and T-cell infiltration in surgical and necropsy tissue in the 4 cases in which they were available. Three CpG-treated dogs died within 3 weeks of the final vaccination and exhibited robust, focal increases in B-(with relatively less T)-cell infiltration into peritumoral brain (Fig. 5A and B; cases 1, 3, and 5). One imiquimod-treated dog developed acute lymphoblastic lymphoma and was euthanized 25 weeks after the final vaccination. This dog showed mild B-cell infiltration (and no T-cell infiltration) into the brain (Fig. 5B, case 8). As mentioned above, case 3 presented with a second meningioma in the contralateral hemisphere between the fifth and sixth vaccinations, which was subsequently resected. Western blot analysis revealed a profound increase in IgG penetration into the second tumor relative to the first, prevaccination tumor (Fig. 5C and D). Moreover, staining for canine IgG exposed plasma cells in the brains of 3 CpG-treated dogs (Fig. 5E). Although previous studies have reported plasma
cell entry into the brain in antibody-mediated autoimmune (31) and antipathogen (32) responses, this is the first account of induced plasma cell homing into brain tumors. In situ antibody production was further suggested by focal areas of extracellular IgG staining seen in plasma cell-containing areas of brain tissue but not others (data not shown).

**Recognition of nonneoplastic brain and meningeal antigens by postvaccination sera**

Two dogs (cases 3 and 5) were euthanized 7 and 20 days after the most recent vaccination. Both animals presented with uncontrollable seizures and tumor recurrence was assumed. At necropsy, case 3 had a microscopic focus of residual tumor and case 5 had no evidence of tumor. To evaluate vaccine-induced autoantibody production, sera from these and 3 other dogs was probed against normal dog brain, arachnoid/pia mater, and dura mater. Secondary antibody revealed heavy and light chain IgG and IgM deposited in meninges, but not brain parenchyma (Fig. 6A, left). These results are consistent with the physiologic permeability of immunoglobulin into these tissues. Serum from cases 3 and 5 reacted to arachnoid/pia and brain parenchyma, respectively. Consistent with the autoreactivity of case 5 sera, analysis of necropsy brain tissue from this dog revealed IgG accumulation on or in neurons distal to the site of resection (Fig. 6B). The binding of the sera to normal brain antigens in these dogs sets them apart from 2 CpG-treated dogs and one imiquimod-treated dog that remained healthy and had nonreactive sera (Fig. 6A).

**Postvaccination sera are capable of antibody-dependent cell-mediated cytotoxicity**

Antitumor effector activities of antibodies encompass multiple mechanisms, including antibody-dependent cell-mediated cytotoxicity (ADCC). Because antibodies reacted with cell surface antigens (Figs. 3B and C, 4B and C, and 7A and B), and antibody production in situ could enable opsonization of invasive meningioma cells behind an intact blood brain barrier, we tested whether tumor-reactive antibodies could trigger ADCC. PBLs killed few tumor cells when cocultured with meningioma cells, or when tumor cells were preincubated with prevaccination serum; however, ADCC occurred when tumor cells were incubated with postvaccination serum (Fig. 7C). Postvaccination serum also triggered ADCC against allogeneic meningioma cells (Fig. 7D), showing that allogeneic vaccination may be an effective strategy in canines with meningioma.

**Discussion**

As many as 57,000 dogs a year develop meningiomas in the United States (13, 33), and these dogs are an underutilized resource for preclinical study. Because the prognosis for canine meningioma is dismal (34), both dogs and humans could benefit from these studies. Our data show the canine model resembles many histologic subtypes observed in humans and has features associated with poor prognosis in humans. The large size of the canine brain allows for surgery as a component of therapy, enabling a more realistic clinical interpretation of systemic interventions.
The survival outcomes of this study call for further investigation into the predictive potential of the canine model, especially with regard to tumor and immune biology in the 2 species. Different responses to vaccination may occur due to differential antigen expression, TLR expression in leukocyte cell types, tumor growth kinetics, the relationship between histologic subtype, biologic behavior, and clinical outcome.

Addition of lysate/adjuvant vaccination to surgery resulted in favorable survival compared with historical controls, but a prospectively designed, randomized study is required to make firm conclusions on therapeutic efficacy. Nonetheless, only case 3 experienced tumor growth despite treatment. The survival data may underestimate the benefit of vaccination because censoring was not used (Fig. 2B and C). Two dogs included in the analysis died of other malignancies, and 2 dogs lacked postmortem analysis but were assumed to die of age-related causes (Supplementary Table S1). The 3 other deaths were due to euthanasia after dogs presented with uncontrolled seizures assumed to be caused by tumor recurrence. Postmortem analysis in these CpG-treated dogs found minimal or no tumor burden, but immunoreactivity to normal brain structures may have been treatment related and contributed to the acute onset of neurologic symptoms (Fig. 6A and B). Similar seizure activity in glioma-bearing dogs in clinical trials at our institution has been controlled using additional anticonvulsants, corticosteroids, or induction of general anesthesia (data not shown). It is therefore possible that the seizures in meningioma-bearing dogs could have been controlled. Whether CpG or imiquimod is more efficacious is still unresolved from the current study due to the small number of dogs and many non-tumor-related deaths. Given the high level of tumor control and the superior safety profile of imiquimod, however, a prospective randomized trial to compare surgery alone and in combination with vaccines of tumor lysate and a novel TLR 7/8 ligand was initiated.

Our study is a starting point for investigation of immunotherapy for meningioma. The data indicate that B-cell activation and antibody production is the predominant mechanism of immunity induced by vaccination. Tumor-reactive antibodies were detected in the serum of all dogs regardless of lysate dose, showing feasibility of autologous lysate/TLR vaccine production. Antibodies exhibited considerable intercase and species cross-reactivity (Fig. 4A and D and Supplementary Table S3). B-cell infiltration outnumbered T-cell infiltration in postmortem brain tissue adjacent to the resection cavity (Fig. 5B). In contrast, only 2 dogs had increased tumor-reactive T-cell responses as measured by IFN-γ ELISpot (Supplementary Table S3). B-cell infiltration outnumbered T-cell infiltration in postmortem brain tissue adjacent to the resection cavity (Fig. 5B). In contrast, only 2 dogs had increased tumor-reactive T-cell responses as measured by IFN-γ ELISpot (Supplementary Table S3). However, it is likely that reactive CD4 T cells were primed following vaccination because antibody responses often require CD4 T-cell help. Tumor-reactive T cells in the blood may not represent what occurs in the tumor-draining lymph nodes or tumor site. A limitation of our study was that ELISpot was carried out only in prevaccination and 3-month postvaccine blood samples. Nevertheless, infiltrating CD3 cells were observed in dogs that died within 2 weeks of the final vaccination (Fig. 5A and B), suggesting some T-cell activation. Future studies will examine T-cell responses in greater detail.

Tumor antigens in lysate vaccines could include overexpressed normal antigens, mutated (neo) antigens, oncofetal...
antigens (expressed during development), or tumor-specific carbohydrates, glycoproteins, or lipoproteins. That 2 dogs with severe postvaccine seizure activity had autoreactive sera to brain and meninges suggests that severe autoreactivity is possible but infrequent (Fig. 6A and B). Lysates may be depleted of these autoantigens to yield greater tumor specificity and less risk. Because most dogs had no autoreactivity or refractory seizures, risk of autoimmunity must be weighed against the threat to life posed by aggressive meningiomas.

Identification of meningioma antigens recognized by postvaccination sera will enable the discovery of crucial epitopes for fully synthetic vaccine strategies in more widespread application. We identified HSP60 as one antigen recognized by sera following vaccination. HSP60 can translocate to the cell surface upon stress or malignant transformation (35), but it was not expressed on the tumor cell surface of case 3 (data not shown), arguing against cell surface protein as the functional target of this antibody. Sera that recognized HSP60 also reacted with a human meningioma, and reactivity was observed at 60 kDa; however, more study is needed to determine whether HSP60 is a relevant target in human meningioma.

Antibody-mediated autoimmunity is well characterized, with autoimmune conditions in the central nervous system (CNS) such as multiple sclerosis being re-evaluated in light of autoimmune conditions in the central nervous system (CNS). For the first time the induction of plasma cells homing to the brain also indicates that vaccination can induce a “Trojan horse”-like infiltration of plasma cells that can in turn trigger ADCC, and thus create excitement for translation of this approach to human meningioma and other CNS cancers.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

Authors’ Contributions
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): B.M. Andersen, G.E. Pihlak, C. Seiler, K.S. SantaCruz, M.G. O’Sullivan, R.T. Bentley, R.A. Packer, S.A. Thromovsky, D. Faissler, M.A. Hunt

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References

Immunotherapy for Spontaneous Canine Meningioma

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