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Expression of HMP/AN2, a melanoma associated antigen, in murine cerebral gliomas:

potential for radioimmunotargeting

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Abstract

Human melanoma proteoglycan (HMP), a melanoma-associated antigen, is expressed in both human melanomas and gliomas. We used HMP-specific monoclonal antibody (mAb) VT68.2 to investigate whether murine GL261 cerebral gliomas express the HMP homologue AN2 and to determine whether AN2 could be targeted for selective delivery of radiation *in vivo*. HMP-specific mAb VT68.2 stained murine GL261 glioma cells grown in culture and intracerebrally in syngeneic C57BL/6 mice. Positron emission tomography with radiolabeled mAb VT68.2 showed high-contrast, positive images of gliomas against a negative background. At 96 h after injection, glioma uptake of radiolabeled mAb VT68.2 was 10 times greater than that of the isotype control mAb and 20 times greater than that detected in normal cerebral tissue. Our results show murine GL261 cerebral gliomas express AN2 and HMP-specific mAb VT68.2 accumulates selectively and specifically at high concentration and is retained within murine cerebral gliomas. Thus, HMP is a potential target for antibody-mediated selective delivery of radiation to cerebral gliomas *in vivo*.

Keywords

radioimmunotargeting; glioma; human melanoma proteoglycan (HMP); microPET; monoclonal antibodies

Introduction

Despite the recent advances in surgery, chemotherapy and radiation therapy, Patients with malignant gliomas have a very poor prognosis [1]. The potential for immunological intervention to treat malignant gliomas has long been recognized [2]. Nevertheless, a relative lack of glioma-associated antigens that can be effectively targeted presents a major challenge [2,3]. Both melanocytes and glial cells are derived embryologically from the neural ectoderm.

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Their malignant counterparts, melanoma and glioma cells, share a number of common antigens. Chai et al described the expression of melanoma associated antigens (MAA) on glioma cells, including MAGE-1, MAGE-3, gp100, TRP-1, TRP-2 and p97 [4]. The list of MAAs expressed on glioma cells are expanding [4,5]. A number of studies have shown that several of the shared antigens can serve as effective targets for active immunotherapy [6] and antibody-mediated targeted therapy [7-9] in malignant gliomas.

Human Melanoma Proteoglycan (HMP), a membrane bound chondroitin sulfate proteoglycan also known as High Molecular Weight-Melanoma Associated Antigen (HMW-MAA), is expressed in human malignant melanomas as well as in human gliomas [10-14]. HMP is highly conserved through phylogenetic evolution. Studies of the amino acid sequence have identified a high degree of sequence homology among the human HMP [15], rat NG2 [16] and murine AN2 proteins [17]. Each of the AN2 and NG2 shares over 80% amino acid sequence identity with HMP and over 90% amino acid sequence identity with each other. HMP/NG2/AN2 is not only expressed on tumor cells, but also on pericytes in tumor-associated vasculature [11,14]. A growing body of evidence suggests that HMP promotes angiogenesis-dependent tumor growth and its expression is correlated with the invasive phenotype of tumors [11,18]. Because of its presence on both tumor cells and angiogenic vessels, HMP may be a good target for immunotherapeutic interventions [20]. A large panel of monoclonal antibodies (mAb) that recognize distinct and spatially distant determinants on HMP has been developed [10], extensively characterized [20,21] and successfully utilized for targeted delivery of therapeutic or imaging agents to melanomas in experimental animals and in clinical studies [22,23]. HMP-specific mAbs Me1-14 [7] and 9.2.27 [8,9] also showed promising results for delivery of radiation, toxin and immunoconjugate in animals with human glioma xenografts.

To date, the murine homologue of HMP (AN2) has been primarily recognized on glial progenitor cells in the developing and adult nervous systems of mice [17]. One of the most frequently used murine glioma models for immunotherapeutic investigation is established by intracerebral inoculation of GL261 cells in its syngeneic host, the C57BL/6 mouse [24]. GL261 tumors maintain a moderately invasive, but non-metastatic growth pattern, a high tumor take rate, consistent reproducibility, and a brief survival time following tumor implantation [24-26]. The growth progression and vascular changes in this model can be non-invasively monitored using external imaging, making it an attractive model for therapeutic interventions including active and passive immunotherapy in gliomas [27]. In a previous study, Prins et al [6] reported the expression of MAAs such as gp100 and TRP-2 by GL261 glioma cells and demonstrated the ability of gp100-specific cytotoxic T lymphocytes to lyse those cells *in vitro*. Whether GL-261 glioma cells express HMP/AN2 is not known.

Several HMP-specific mAbs currently available in our laboratory demonstrates cross-reactivity with xenogeneic cells [10]. In the present study, we used the HMP-specific mAb VT 68.2, which recognizes a determinant shared by AN2, to test whether murine GL261 glioma cells express AN2 and whether AN2 represents a useful target for selective delivery of radiation to cerebral gliomas *in vivo*. The results of these studies provide a useful background for the design of experimental strategies for immunotherapy in patients with malignant gliomas.

Materials and methods

Cell line

GL261 murine glioma cells were obtained from Dr. Rolf F. Barth (Ohio State University, Columbus, OH). Cells were grown in 100 mm tissue culture plates in complete Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal calf serum, 5,000 units penicillin/streptomycin, 50µM 2-mercaptoethanol, 25mM HEPES, and 1x non-essential amino acids at 37°C in a 5% CO₂ atmosphere with three media changes per week.

Antibodies

The HMP-specific murine mAb VT68.2, an IgG1, and the isotype matched anti-idiotypic mAb MF11-30 were developed and characterized as described previously [21]. All mAbs were generated from BALB/c mice. Antibodies were purified from ascitic fluid by sequential precipitation with caprylic acid and ammonium sulphate [27]. The purity of the mAb preparations was monitored by SDS-PAGE and their activity was monitored by testing with the corresponding target antigens in binding assays. The PE-conjugated F(ab')₂ fragments and FITC-conjugated F(ab')₂ fragments of anti-mouse IgG antibodies were purchased from Dako (Carpenteria, CA).

GL261 Murine glioma model

All animal studies were pre-approved and performed in accordance with our institutional animal care guidelines and procedures. The procedure for the preparation of the GL261 cerebral glioma model has been described in detail [24-26]. Briefly, male C57BL/6 mice (Harlan-Sprague-Dawley, 6-8 weeks, weight ~20 g) were anesthetized with an intra-peritoneal injection of ketamine (125 mg/kg) and xylazine (25 mg/kg) and fixed in a rodent stereotactic head frame (David Kopf Instruments, Tujunga, CA). A midline scalp incision was made and the bregma was located. Stereotactic coordinates were measured (1.4 mm anterior and 2.0 mm to the right of bregma) for implantation of cells into the head of the right caudate nucleus. A burr hole was drilled and 1×10⁵ GL261 cells suspended in 5 µL of DMEM were injected using a Hamilton syringe with a fixed 25-gauge needle at a rate of 1 µL/mm at a depth of 3 mm relative to the exposed dura mater. The needle was withdrawn at a rate of 1 mm/min and the incision was sutured.

Production of iodine-124 (¹²⁴I)

¹²⁴I has a half-life of 4.2 days and is a suitable radiotracer for positron emission tomography (PET) imaging over a longer period of time after a single injection. ¹²⁴I was produced by the ¹²⁴Te (p, n) ¹²⁴I reaction [28]. A ¹²⁴TeO₂ (tellurium oxide) target was irradiated by 14.1 MeV protons at 24 µA a Cyclone-30 cyclotron (IBA, Louvain-la-Neuve, Belgium). Radioiodine was separated by the dry distillation method and the distilled ¹²⁴I was trapped in 0.1M NaOH. The ¹²⁴I solution was analyzed by HPLC to determine both chemical and radiochemical purity. Separation was performed using a Lichrosorb RP18 analytical column (5 µm, 250 mm × 4.6) (Alltech Corp, Nicholasville, KY). The mobile phase consisted of a mixture of 90% buffer (0.5 M potassium phosphate and 0.002 M tetrabutylammonium hydroxide, pH 7.0) and 10% acetonitrile. The flow rate was 1.0 ml/min and the UV detector was set at a wavelength of 225 nm.

Radiolabeling of mAbs

Labeling of mAbs with ¹²⁴I was performed using a modification of the standard Iodogen method, as described previously [29]. The antibody solution (1mg/mL of PBS; 0.1 mL) was added to 10 µL of ¹²⁴I (1-5 mCi) in 0.1 N NaOH. An Iodogen bead (Pierce Chemical, Rockford, IL) was added to the solution and the two were mixed and incubated at 27°C for 20 minutes. The labeled mAb was isolated by HPLC using a Bio-Sil SEC-125 gel filtration column (BioRad Laboratories, Hercules, CA) with PBS as an eluant. The specific activities of ¹²⁴I-labeled mAb VT68.2 (¹²⁴I-mAb VT68.2) and mAb MF11-30 (¹²⁴I-mAb MF 11-30) were 28.0 and 8.5 µCi/µg, respectively.

Flow cytometry

GL261 cells were fixed in 2% paraformaldehyde, and washed and blocked with 1% BSA in phosphate-buffered saline (PBS). Cells were incubated with mAbs for 1 hour at 27°C, washed and incubated with PE-conjugated F(ab')₂ antibody fragments. After a final wash with PBS,

cells were fixed in FACS buffer containing 1% paraformaldehyde. Samples were then analyzed on a FACScan flow cytometer (Becton Dickinson, Sunnyvale, CA). Data analysis was performed utilizing FCS Express software.

Radioligand binding assay

In vitro radioligand binding assays were performed as described previously [30]. Briefly, a suspension of cultured GL261 cells (2×10^5 cells/100 μ L) was prepared in fresh culture medium; 100 μ L aliquots were added to each well of a 96-well 'V'-bottomed microtiter plates. Radiolabeled antibodies were added in serial dilutions and incubated for 2 hours at 37°C. Three microtiter plates were used to triplicate each of the data points. Plates were then spun (2000 rpm, for 5 minutes, at 4°C) and cells were washed 5 times. Dried plates were then counted for radioactivity on an automated gamma counter (LKB Wallace, Uberlingen, Germany). Each well was counted for 1 minute.

Immunofluorescence staining

GL261 tumors were removed and immediately frozen in isopentane at -70°C. Twelve micron thick tissue sections were prepared using a cryotome. Sections were air-dried, fixed in acetone for 10 minutes at 27°C, washed and blocked with 2% FCS in PBS. Tissue sections were incubated with HMP-specific mAb VT68.2 for 1 hour at 27°C, and then washed and incubated with FITC conjugated F(ab')₂ antibody fragments. After a final washing, tissue sections were analyzed by immunofluorescence microscopy using a Nikon Eclipse microscope with a Spot CCD camera (Nikon Inc, Melville, NY).

PET Imaging

Fifteen days following intracerebral implantation of GL261 gliomas, C57BL/6 mice were divided into 2 groups. Each group of 6 mice received 3.7 MBq (in 0.2 mL normal saline) of either HMP-specific ¹²⁴I-mAb VT68.2, or isotype-matched control ¹²⁴I- mAb MF11-30. Six age-, gender- and weight-matched C57BL/6 mice without tumor received 3.7 MBq of ¹²⁴I-mAb VT68.2 for comparison of the biodistribution with tumor-bearing mice. All injections of radiolabeled mAbs were performed intra-peritoneally into the right lower quadrant of the abdomen of the mouse. A high-resolution dedicated small animal PET scanner (Focus 120@ microPET, Siemens Preclinical Solution, Knoxville, TN) was used to image the mice at 24, 48 and 96 hours after a single injection of the radiolabeled mAb. The performance characteristics of this PET system have been described elsewhere [31]. For each scan, anesthesia was induced with 3% isoflurane gas (Minrad Inc, Bethlehem, PA) in an induction chamber. The mouse was then placed and secured in the scanner bed in the prone position and isoflurane gas inhalation was maintained at 1-2% through a face-mask throughout the scan period. Each scan lasted 20 minutes. Vital signs, including temperature, skin color and respiratory rate, were monitored at regular intervals. Projection data were reconstructed using the standard filtered back projection method. Reconstructed images were displayed in coronal, axial and sagittal slices (0.087 mm/slice). Images were quantified using the in-built ASIPRO® software executed on an IDL Virtual Machine 6.0 platform. Ellipsoid regions of interests (ROIs), 5 × 5 pixel size, were drawn around visible tumors on the right cerebrum and corresponding location on the contralateral left cerebrum. When a tumor was not visible, the ROI was placed in the central part of the right cerebrum. A calibration factor was calculated based on the scanning of a cylindrical phantom of known volume and activity and was applied to convert counts of a ROI to the percentage of the injected dose per gram (%ID/g) of tissue.

Biodistribution study

After final PET scan (18 days after implantation of tumor cells), mice were euthanized with an intra-peritoneal injection of 100 mg/kg body weight of sodium pentobarbital (Vortech,

Dearborn, MI). Cerebral gliomas, brain tissues and other organs were harvested and weighed. Blood was collected directly by cardiac puncture immediately before euthanasia. Each specimen was counted for 1 minute using an automated gamma counter (LKB Wallace, Uberlingen, Germany) in reference to the counts of standard samples prepared from aliquots of the injected doses. The results were expressed as: a) % ID/g of tissue (weight-adjusted, background-subtracted counts of tumor or tissue divided by the counts of the injected dose), b) glioma-to-cerebral count ratio, c) specificity index (ratios between ^{124}I -mAb VT68.2 counts and ^{124}I -mAb MF11-30 counts in tumor or tissue) and d) localization index: (^{124}I -mAb VT68.2 counts in tumor or tissue/ ^{124}I -mAb VT68.2 counts in blood) / (^{124}I -mAb MF11-30 counts in tumor or tissue/ ^{124}I -mAb MF11-30 counts in blood) [32].

Statistical analysis

Data are expressed as mean values $\pm$ SD. Comparisons between the paired data within a group were made using Student's paired t test. Comparisons between more than 2 groups were made by Analysis of Variance with Bonferroni's correction. *P* values ≤ 0.05 were considered statistically significant.

Results

In vitro expression of HMP/AN2 by GL261 cells

GL-261 cells were cultured *in vitro* and incubated with mAbs, VT68.2 and MF 11-30. Flow cytometric analysis of these cells show that HMP-specific mAb VT68.2 binds AN2 on murine GL261 cells across a range of antibody concentrations; in contrast, the binding with control mAb MF11-30 was minimal (Fig 1A). The quantitative binding of radiolabeled and unlabeled mAb VT68.2 at the same concentration was comparable suggesting that mAb VT68.2 bound to AN2 on GL261 cells retained its immunoreactivity following radio-iodination. This conclusion is further supported by the results of the *in vitro* radioligand binding assay (Fig. 1B). The radioactivity of ^{124}I -labeled HMP-specific mAb VT68.2. bound to GL261 cells were high, whereas radioactivity of ^{124}I labeled control mAb MF11-30 was at the level of background radioactivity measured in blank tubes.

In vivo expression of HMP/AN2 in murine cerebral gliomas

Brain sections of C57BL/6 mice with GL261 glioma implants were stained with mAb VT68.2 and analyzed by immunofluorescence microscopy (Fig. 2). These studies showed a relatively homogenous staining of the cell membrane within gliomas by mAb VT68.2. In contrast, gliomas as well as brain adjacent to the tumors remained unstained by the control mAb MF 11-30. These results support that GL261 gliomas express AN2, which is recognized by the cross-reactive HMP-specific mAb VT68.2. The lack of glial fibrillary acid protein (GFAP) reactivity, a marker of the intermediate filament specific for astroglial-derived cells, as observed in Fig 2E is a well-known characteristic of GL261 tumors [33].

In vivo imaging of cerebral gliomas with radiolabeled VT68.2

PET imaging of the glioma-bearing mice was performed at 24, 48 and 96 hours after the injection of ^{124}I -labeled mAbs. The images show gradually increasing accumulation of ^{124}I -mAb VT68.2 (HMP-specific) in cerebral gliomas (Fig. 3A). No extratumoral uptake of ^{124}I -mAb VT68.2 was visible within the brain. Outside the brain, visible uptake was limited to heart (blood pool) and liver. The hepatic uptake is likely to reflect the binding of the whole antibody with the Fc-receptor on reticuloendothelial cells. When ^{124}I - mAb MF11-30 (control) was injected to mice bearing cerebral gliomas, no accumulation was detected in gliomas or any other part of the brain at any of the time points (Fig. 3B). The quantitative accumulations of radiolabeled HMP-specific VT68.2 were significantly higher than those of control mAb MF

11-30 ($P<0.01$ for all time points) (Figure 3C). At 96 hours after injection, glioma-to-cerebral counts ratio of ^{124}I -mAb VT68.2 (4.5 ± 1.6) calculated from images was significantly greater than that of ^{124}I -mAb MF11-30 (1.2 ± 0.2 ; $P<0.005$). Serial PET imaging of mice without any glioma showed no accumulation of radiolabeled mAbs within the brain following injection of ^{124}I -mAb VT68.2 suggesting that mAb did not penetrate the intact blood-brain barrier.

Localization of mAb VT68.2 in murine cerebral gliomas

Consistent with the imaging data, *ex vivo* gamma counting showed that the accumulation of ^{124}I -mAb VT68.2 in glioma tissue was 10 times greater than that of the ^{124}I -mAb MF11-30 (Fig. 4A). A relatively higher uptake in the right cerebrum relative to the normal left cerebrum reflected some residual activity in the brain tissue immediately adjacent to the tumor edge. The glioma-selective uptake of ^{124}I -mAb VT68.2 is illustrated by a 20 times higher uptake in glioma tissue compared to that in normal cerebral tissue (5.43 ± 0.74 vs. 0.27 ± 0.06 ; $P=0.0007$). The accumulation of ^{124}I -mAb VT68.2 in normal left cerebrum in glioma-bearing mice was not different from that in normal mice that had intact blood-brain barrier (BBB). The distribution of ^{124}I -mAb VT68.2 in different organs of glioma-bearing mice and normal mice were comparable (Fig. 4B). The specificity and localization indices in gliomas and other organs are summarized in Table 1. The accumulation of the HMP-specific mAb VT68.2 relative to the control antibody MF11-30 in gliomas as well as that in comparison to the blood was many times higher.

Discussion

The results of the current study provide two important findings. First, we report for the first time the expression of AN2, the murine homologue of HMP, on GL261 glioma cells cultured *in vitro* and in an orthotopic intracerebral tumor model in syngeneic mice. This adds to the growing list of MAAs expressed by GL261 cells [6]. Second, we demonstrated that a HMP-specific mAb VT68.2 has the potential to be utilized for selective delivery of radiation to cerebral gliomas *in vivo*.

HMP, also known as HMW-MAA, is expressed in over 80% of human melanoma lesions and in the majority of melanoma cell lines [10]. In the central nervous system, the rat homologue of HMP, NG2, was originally identified on neural cell lines and subsequently demonstrated to be a marker of multipotent glial progenitor cells [34]. Loss of NG2 expression on these cells correlates with their terminal differentiation into oligodendrocytes or astrocytes [35]. Subsequently, AN2, the murine homologue of HMP and NG2, has been identified; suggesting that it is evolutionary conserved [17]. In the current study, we have taken advantage of the cross-reactivity of the HMP-specific mAb VT68.2 with its xenogeneic counterpart and identified the HMP homologue in murine GL261 glioma cells *in vitro* and *in vivo*. These results can be compared with a number of previous studies demonstrating expression of HMP on human glioma cells. Chekenya et al reported a differential expression of HMP by biopsy-derived human glioma cells in culture, where high expression was evident in high grade glioblastoma multiforme (GBM) cells and reduced expression with decreasing malignancy suggesting its association with proliferative activity [13]. In a subsequent study by the same group, HMP expression was found on both the tumor cells as well as on pericytes in tumor associated vasculatures in 20 out of 28 surgically resected human brain tumor samples that include not only GBM but also a variety of other malignant gliomas including oligodendroglioma and ependymoma [14]. None of the meningiomas in that study showed expression of HMP. In another study, Shoshan et al [12], utilizing HMP-specific mAb 9.2.27, identified HMP on tumor cells in 7 out of 7 oligodendrogliomas, 3 out of 3 astrocytomas and 1 out of 5 GBM obtained from surgical samples. Along this line, Schrappe et al [11] reported the variable expression of HMP by human glioma cells *in vitro* and *in vivo*, whereas there was

a notable lack of reactivity with glia, neurons and normal cerebral grey and white matter. Our findings in GL261 glioma paralleled those observations in human samples (Fig. 2). The exact explanation for HMP expression on neoplastic glial cells is not clear. The variable presence of HMP on neoplastic glial cells raises the possibility that some gliomas may originate from immature progenitor cells retaining the markers [12]. Alternately, HMP expression may be a dynamic process and glioma cells may acquire HMP immunoreactivity as they undergo malignant transformation, thus exposing unique antigenic determinants not presented by normal cells of the CNS.

Although many kinds of radiolabeled molecules have been explored for targeted radiotherapy of cancers, studies in glioma patients have almost exclusively utilized mAbs as the targeting vehicle. Studies with radiolabeled mAbs targeting the extracellular matrix-associated glycoprotein tenascin [36] and the receptor tyrosine kinase epidermal growth factor receptor [37] have shown promising results in clinical trials. The results of those studies suggest that antibody-mediated radiotherapy of gliomas may be possible with appropriate selection of target antigen and its corresponding antibody. The differential expression of HMP on neoplastic glia but not on normal brain tissues offer the opportunity of selective and targeted delivery of therapeutic agents to malignant gliomas. We investigated this potential in the second part of the study. We initiated our imaging experiments 15 days after implantation of tumor cells so that tumor size (~3 mm) was adequate for monitoring by PET imaging. Perfusion magnetic resonance imaging shows that the BBB is disrupted relatively early (i.e. within 1-2 weeks) in GL261 tumors [26]. Thus, tumors were permeable to antibodies at the time of imaging and entry of the mAb VT68.2 may have been facilitated by disruption of the BBB. Subsequent glioma-specific accumulation and retention of the radiolabeled mAb was demonstrated by the high specificity and localization indices in paired labeled imaging experiments. The ratio of accumulation of HMP-specific mAb antibody and control antibody is much higher in gliomas compared to other organs, including liver and blood (Table 1). In addition, accumulation of mAb VT68.2 was 20-fold greater in glioma than in contralateral cerebral hemisphere. Radioactivity in the normal cerebral hemisphere of glioma-bearing mice was minimal and equivalent to the background radioactivity in brain that was present in mice with an intact BBB. Thus, specific binding of VT68.2 to distinct antigenic determinants present on GL261 cells is likely to be the principal mechanism for the accumulation and retention of labeled mAb VT68.2 within cerebral gliomas. In a previous study, Colapinto et al have reported a high level of glioma-selective accumulation of ^{131}I -labeled F(ab)'₂ fragment of mAb Me1-14, which targets an epitope on HMP and treatment with this antibody prolonged the survival of nude rats bearing human glioma xenografts [7]. Treatment with chemo-immunoconjugate [8] and toxin [9] that is bound to HMP-specific mAb 9.2.27 also have been shown to prolong survival and suppress growth of gliomas, respectively, in xenograft model in immunodeficient animals. One fundamental difference between our study and those studies is that our study was done in an immunocompetent syngeneic model. Nevertheless, collectively these data suggest that HMP can serve as a target for antibody-mediated delivery of radiation or other therapeutic agents *in vivo*.

One challenge is to achieve a sufficient level of radiolabeled antibody within the tumor so that it is effective for therapeutic or imaging applications. Even though the BBB is partially disrupted in the presence of gliomas, access of antibodies to tumor tissues may be limited by high interstitial fluid pressure in both tumor tissue and in the surrounding cerebrum. Consequently, a very low level of radiolabeled mAb is generally achieved in gliomas after systemic administration. In this study, the degree of localization of ^{124}I -mAb VT68.2 within GL261 gliomas is high enough to yield excellent tumor-to-background contrast. This contrast (~5) is sufficient to provide high-quality positive contrast images of the glioma in a negative background (Fig. 3A). Considering the difficulties inherent in attaining high antibody concentrations in intracerebral tumors, uptake of ~5% of a systemically injected dose of ^{124}I -

VT68.2 per gram of tumor tissue is notable. A further increase in antibody delivery may be desirable for therapeutic efficacy. Riva et al showed that higher accumulation resulting in better response rate can be achieved by the loco-regional administration of radiolabeled antitenascin mAbs in patients with high-grade malignant gliomas [38]. In addition, concomitant external beam radiation therapy has been shown to open the blood brain barrier, potentially giving greater access of mAbs to cerebral gliomas [39]. In another approach, Petronzelli et al have shown that targeting gliomas with administration of two different anti-tenascin antibodies resulted in additive accumulation within the tumor [40]. This approach is likely to be relevant in the development of HMP-targeted therapy against glioma, since a large panel of well characterized HMP-specific mAbs that recognize unique and spatially distinct antigenic determinants are already available [10,16]. While we have shown that HMP can be targeted with high-degree of selectivity and specificity relative to the control mAb (Table 1), one limitation of our study is that we used a whole antibody which resulted in some accumulation of radioactivity in nontumoral organs due to nonspecific clearance of antibody and radioactivity. The application of which can be improved by using antibody fragments and techniques such as multistep pre-targeting in order to improve the delivery of antibodies to tumors and accelerate the clearance from blood and non-tumoral organs [41,42]. These established delivery techniques are not the focus of the current study; here we have instead focused upon proof of principle data that HMP can be effectively targeted in glioma.

In conclusion, we have found that murine GL261 glioma cells express HMP homologue AN2 *in vitro* and *in vivo*. Our results also show selective and specific accumulation and retention of a HMP-specific radiolabeled mAb VT68.2 in murine GL261 cerebral gliomas *in vivo*. Thus, HMP can be utilized as a potential target for antibody-mediated targeted delivery of radiation to gliomas. GL261 intracerebral gliomas in its syngeneic murine host can serve as a useful model for this purpose.

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Fig. 1. Immunoreactivity of GL261 glioma cells. (A) Flow cytometry results showing relative saturation of binding sites on GL261 cells by mAbs. (a-c) Comparative binding with 0.5 μg of unlabelled VT68.2 (a), ^{124}I -mAb VT68.2 (HMP-specific) (b) and ^{124}I -mAb MF11-30 (isotype matched control) (c). (d-f) The binding with 0.25 μg (d), 2 μg (e) and 8 μg (f) of ^{124}I -mAb VT68.2. (B) Results of radioligand binding assay showing ^{124}I -mAb VT68.2 radioactivity (Filled circle) and ^{124}I -mAb MF11-30 radioactivity (empty circle) on GL261 cells as a function of mAb concentration. Triangles represent background counts obtained from blank tubes. Each symbol represents mean ($\pm$ SD) of values for each dilution of mAb.

Fig. 2. (A-B) Hematoxylin stained GL261 glioma sections showing tumor and brain adjacent to tumor (BAT) at 10x (A) and 20x (B) magnifications. (C-D) Immunofluorescence microscopy of GL261 gliomas stained by HMP-specific mAb VT68.2 showing BAT (C) and central tumor (D). (E) Minimal glial fibrillary acidic protein (GFAP) reactivity of GL261 tumor. (F) Negative control M 11-30 staining of GL261 tumor and BAT.

Fig. 3. Sequential microPET images of two glioma-bearing mice injected with ^{124}I -mAb VT68.2 (A) and ^{124}I -mAb MF11-30 (B). High-contrast positive images of glioma (yellow arrow) reveal gradual accumulation of HMP-specific ^{124}I -mAb VT68.2. In contrast, no visible accumulation of isotype-control ^{124}I -mAb MF11-30 within glioma is found at any of the time points (red arrow). The gross appearance of the corresponding gliomas at autopsy is displayed in the column at far left. Mice were imaged in the prone position. The left side of the image represents the left side of the corresponding mouse brain. (C) Quantitative accumulation of ^{124}I -labeled mAb VT68.2 (Filled circle) and mAb MF11-30 (empty circles) into gliomas expressed as the

percent injected dose per gram (%ID/g) of tissue over time. Each symbol represents the mean of values ($\pm$ SD) obtained from 6 mice.

Fig. 4.

Biodistribution of radiolabeled antibody obtained by *ex vivo* gamma counting of dissected tissues 96 hours after injection. Counts are displayed as the percent injected dose per gram (% ID/g) of tissue. (A) Accumulation of ^{124}I -mAb VT68.2 (Filled bar) is significantly greater than that of ^{124}I -mAb MF11-30 (empty bar) in glioma (* $P < 0.0005$) and tumor-adjacent right cerebral hemisphere (RC) († $P < 0.001$). Accumulation of ^{124}I -mAbs VT68.2 and MF11-30 are not different in normal left cerebral hemisphere (LC) ($P = 0.18$). (B) Accumulation of ^{124}I -mAb VT68.2 in different organs in glioma-bearing mice ($n=6$) and normal mice ($n=6$). Comparison of paired values did not reach statistical significance in any of the organs. Each bar represents mean (+SD) of values obtained from 6 mice in the corresponding group.

Table 1

Specificity and localization indices in gliomas and non-tumoral organs

Tissue	Specificity index	Localization index
Glioma	9.41	7.66
Left cerebrum	0.87	0.70
Thyroid gland	1.47	1.19
Liver	1.46	1.19
Kidneys	1.27	1.03
Muscle	1.77	1.44