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Uniform brain tumor distribution and tumor associated macrophage targeting of systemically administered dendrimers

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ABSTRACT

Effective blood–brain tumor barrier penetration and uniform solid tumor distribution can significantly enhance therapeutic delivery to brain tumors. Hydroxyl-functionalized, generation-4 poly(amidoamine) (PAMAM) dendrimers, with their small size, near-neutral surface charge, and the ability to selectively localize in cells associated with neuroinflammation may offer new opportunities to address these challenges. In this study we characterized the intracranial tumor biodistribution of systemically delivered PAMAM dendrimers in an intracranial rodent gliosarcoma model using fluorescence-based quantification methods and high resolution confocal microscopy. We observed selective and homogeneous distribution of dendrimer throughout the solid tumor (~6 mm) and peritumoral area within fifteen minutes after systemic administration, with subsequent accumulation and retention in tumor associated microglia/macrophages (TAMs). Neuroinflammation and TAMs have important growth promoting and pro-invasive effects in brain tumors. The rapid clearance of systemically administered dendrimers from major organs promises minimal off-target adverse effects of conjugated drugs. Therefore, selective delivery of immunomodulatory molecules to TAM, using hydroxyl PAMAM dendrimers, may hold promise for therapy of glioblastoma.

1. Introduction

Malignant glioma is the most common and most aggressive primary brain tumor [1] and despite the advances in treatment, the median survival remains at 16.4 months [2]. Key challenges faced in the development of effective therapies relate to (a) the ability of systemically delivered chemotherapeutic agents to penetrate the impaired blood–brain tumor barrier (BBTB) and provide coverage across the entire solid tumor [3] and (b) the ability to target specific cells. Although small molecule-based therapeutics can effectively distribute within the tumor tissue, they are limited by rapid tumor clearance [4] and off-target extravasation, potentially leading to adverse effects [3]. Recent advances in nanotechnology have provided selective tumor accumulation. However, the size of most nanoparticles limits extravasation and tumor penetration, thus limiting homogeneous solid tumor coverage [3,5]. Careful tuning of particle size and surface charge has been attempted in order to enhance the nanoparticle distribution profile in subcutaneous tumors [6–9]. Unfortunately, achieving homogeneous coverage of orthotopic brain tumors has been proven even more challenging. This may be attributed to the lower permeability of the BBTB compared to the blood-tumor barrier (BTB) in a subcutaneous tumor, the heterogeneous intervascular spaces and the high

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interstitial pressure in brain tumors. Although, some strategies have attempted nanoparticle delivery through the BBTB via absorptive uptake; passive diffusion through the leaky BBTB fenestrations has only been demonstrated with molecules smaller than 20 nm [10–13] and unhindered diffusion through the BBTB has been achieved with molecules of 7 nm [14], thus limiting the relevance of most nanoparticle-based therapeutics.

Hydroxyl-terminated generation 4 poly(amidoamine) (PAMAM G4-OH) dendrimer is a highly tailorable branched macromolecule with a hydrodynamic size (~4 nm) smaller than conventional nanoparticles and near-neutral surface charge (ζ-potential: +4.5 ± 0.1 mV), physicochemical attributes that may allow for effective blood-brain barrier (BBB) [15] and tumor extra cellular matrix (ECM) penetration [16]. We have previously shown that, without the use of targeting ligands, these dendrimers can target activated microglia/macrophages after passing the impaired BBB in a rabbit model of cerebral palsy [17]. This targeted accumulation resulted in a significant efficacy when the dendrimer was conjugated to N-acetylcysteine [17]. In glioma, tumor associated microglia/macrophages (TAM) have been shown to participate in tumor growth, tumor invasion, angiogenesis and immune system evasion [18]. A variety of microglia/macrophage modulating molecules has been shown to decrease glioma progression and increase survival in preclinical studies [19–22]. Therefore, nanoparticle targeting of TAM has been explored, through the use of different ligands [23–26].

We investigated the use of PAMAM G4-OH dendrimer as a promising therapeutic vehicle for the treatment of malignant glioma. A recently developed fluorescence-based 'quantification' approach and high resolution confocal microscopy were combined to investigate the kinetics, biodistribution and clearance of these dendrimers in a 9L gliosarcoma intracranial tumor model. We also characterized the dendrimer's intrinsic ability to selectively target TAM.

2. Materials and methods

2.1. Materials and reagents

The following agents were purchased: hydroxyl terminated ethylenediamino-core PAMAM dendrimer (referred to as dendrimer throughout, unless otherwise specified) (Dendritech, Midland, MI), Methanol (HPLC grade), DMF (HPLC grade), stainless steel beads (Fisher Scientific, Waltham, MA); and Cyanine 5 (Cy5) (GE Healthcare Life Science, Pittsburgh, PA). For confocal microscopy: nuclei counterstain, 4′,6-diamidino-2-phenylindole, dihydrochloride (DAPI), Alexa Fluor® 594 Goat Anti-Rabbit IgG (H + L) Antibody (Molecular Probes, Eugene, Oregon); Fluorescent mounting media (Dako, Santa Clara, CA); Anti-Iba1, Rabbit (Wako, Osaka, Japan); Lectin from Bandeiraea simplicifolia (BSI-B4) (Sigma–Aldrich, St. Louis, MO); Anti-GFAP 488 (eBioscience, San Diego, CA); Fluorescein isothiocyanate–dextran (FITC-dextran), molecular weight 70,000 Da (Sigma Aldrich, St. Louis, MO).

2.2. Synthesis of dendrimer Cy5 (D-Cy5) conjugates

D-Cy5 was prepared through two steps following a previously published method [27]. Briefly, hydroxyl-terminated PAMAM dendrimer was surface-modified with amine groups to make a bifunctional dendrimer. We used 6-(Fmoc-amino) caproic acid to produce a Fmoc-protected bifunctional dendrimer intermediate that was eventually de-protected by re-dissolving in a mixture of piperidine/DMF. Cy5 dye with N-hydroxysuccinimide monoester was reacted with amine groups on the surface of bifunctional dendrimer. The 'crude' products were further extensively purified by dialysis. The final D-Cy5 conjugate was characterized using 1H NMR, high-performance liquid chromatography (HPLC) and gel permeation chromatography (GPC). The conjugate was stored as a solid powder at −20 °C and reconstituted at 10 mg/mL with sterile 0.9% NaCl solution on the day of administration.

2.3. Tumor inoculation

Female Fischer 344 rats, weighing 125–175 g each (Harlan Bioproducts, Indiana, IN), were housed in standard facilities and given free access to food and water. 9L gliosarcoma intracranial implantation was performed as previously described [28]. Briefly, the 9L gliosarcoma (obtained from the Brain Tumor Research Center, UCSF, San Francisco, CA) maintained in the flank of F344, was surgically excised sectioned into 1 mm^3 pieces and placed in sterile 0.9% NaCl solution on ice for intracranial implantation. Rats were anesthetized and a midline scalp incision was made to identify the sagittal and coronal sutures. A burr hole was made 3 mm lateral to the sagittal suture and 5 mm posterior to the coronal suture. The dura was incised, and using a surgical microscope and gentle suction a small cortical area was resected. A tumor piece was placed in the resection cavity and the skin was closed using surgical staples. All animals were treated in accordance with the policies and guidelines of the Johns Hopkins University Animal Care and Use Committee.

2.4. D-Cy5 administration for quantification and immunofluorescence

Animals were injected in tail vein with a 3 mg/300 μL dendrimer conjugated-Cy5 (D-Cy5) solution. For imaging of dendrimer and dextran distribution, 3 animals were co-injected with a 0.9% NaCl solution of 2 mg D-Cy5 and 2 mg dextran-FITC in 300 μL.

To study the dynamics of dendrimer accumulation in the tumor brain, D-Cy5 was injected into 27 tumor inoculated rats when the average tumor size was 6 mm in diameter and then animals were sacrificed at fixed time points (15 min, 1 h, 4 h, 8 h, 24 h, and 48 h). Magnetic resonance imaging was used to measure intracranial tumor size. Blood was drawn through cardiac puncture and immediately centrifuged to collect plasma. Brains were harvested and flash frozen on dry ice for fluorescence spectroscopy based quantification or placed in 4% formalin solution for immunofluorescence.

To study the dendrimer cell uptake, D-Cy5 injection was performed in 3 tumor inoculated rats and 3 healthy rats, and animals were sacrificed 24 h after the injection. Brains were harvested and placed in 4% formalin for immunofluorescence study.

To study the pharmacokinetics and biodistribution of dendrimer in plasma and systemic organs D-Cy5 was injected into 15 tumor-inoculated rats which were placed in metabolic cages for urine collection and animals were subsequently euthanized at fixed time points (15 min, 1 h, 4 h, 8 h, 24 h, and 48 h). Organs were harvested and flash frozen on dry ice for fluorescence spectroscopy-based quantification or placed in 4% formalin for immunofluorescence.

2.5. Fluorescence spectroscopy

Fluorescence-based quantification of D-Cy5 conjugates followed our previously published protocol [27]. Briefly, 100–150 mg of frozen tissue was homogenized in 1 mL of methanol using a homogenizer (TissueLyser LT, Giagen) in 2 mL DNA LoBind Eppendorf tubes and subsequently sonicated. Suspensions were diluted to 100 mg/mL and centrifuged at 15,000 rpm for 15 min at 4 °C. The resulting supernatants were subjected to fluorescence spectroscopy. Importantly, prior studies showed that D-Cy5 was stable in plasma, and could be recovered from the tissue intact, without appreciable release of the conjugated Cy5 [27].

For brain tissue, precise dissection of the tumor was performed and the peritumoral area was defined as up to 1 mm away from the tumor dissection plane. In the contralateral hemisphere 100 mg of the caudate/putamen with the surrounding white matter area was dissected and used for analysis. For plasma and urine samples, a sample of 100 μL of plasma and urine was mixed with 900 μL of phosphate buffer (0.1 M) and analyzed by fluorescence spectroscopy.

Fluorescence spectra of D-Cy5 conjugates and that obtained from tissue extracts were recorded using a Shimadzu RF-5301 Spectrofluorophotometer (Kyoto, Japan). D-Cy5 calibration curves were constructed, following every experiment, under different slit widths using the maximum emission wavelength of 662 nm after recording spectra from 650 nm to 720 nm with excitation wavelength of 645 nm. The D-Cy5 concentration was measured in methanol or phosphate buffer (0.1 M) in solutions ranging from 1 ng/mL to 100 μg/mL. The slit width was chosen based on the observed fluorescence level of different sample sets. For biological samples with low levels of D-Cy5 (i.e. brain, lung, heart), the excitation and emission slit width was set at 10; for biological samples with high levels of D-Cy5, (i.e. urine and kidney) excitation and emission slit width were set at 3. For the remaining biological samples, excitation slit width of 5 and emission slit width of 10 were used. All calibration curves exhibited linearity with $R^2 \sim 0.99$. Fluorescence registered from tissue of non D-Cy5 injected healthy and tumor inoculated rats was subtracted from the values observed from samples of D-Cy5 injected tissue in order to account for tissue autofluorescence.

Concentration of D-Cy5 conjugate in the brain was expressed in μg per g of tissue. The concentration of D-Cy5 conjugate in the other organs was expressed in percentage (%) of injected dose per gram of tissue or % of injected dose per organ. Concentrations of the D-Cy5 conjugate in urine and blood were expressed in % of injected dose per mL or % of injected dose in total amount of urine or plasma. Total plasma concentration was calculated based on the weight of the animal. The brain and plasma quantification data were analyzed to calculate the area under curve (AUC) and the brain to serum ratio.

The permeation constant (Kin) and the initial volume of distribution (Vi) were calculated in the brain tumor [29]. The brain to serum ratio and the area under the curve of the serum (AUC(serum) (t)) to serum concentration (Serum(t)) were calculated for each time point and linear regression analysis was performed in order to get the Kin and Vi based the following equation:

$$\frac{Brain(t)}{Serum(t)} = K_{in}\frac{AUCserum(t)}{Serum(t)} + V_i$$