Photothermal ablation of malignant brain tumors by nanoparticle loaded macrophages.

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Abstract

Introduction: Nanoshells are a new class of optically tunable nanoparticles composed of a dielectric core (silica) coated with an ultrathin metallic layer (gold). Since nanoshells are roughly one million times more efficient at converting NIR light into heat than conventional dyes such as indocyanine green , once localized to the tumor and exposed to NIR light, they can generate sufficient heat to induce cell death via thermal ablation. Macrophages are frequently found in and around glioblastomas in both experimental animals and patient biopsies, indicating local synthesis of chemo attractive factors in gliomas and that inflammatory cells loaded with nanoparticles could therefore be used to target tumors.

Materials and Methods: Human brain tumor spheroids were co-incubated with fluorescent labeled mitomycin treated murine Ma (P388-D) loaded with increasing numbers of gold nanoshells. The spheroids were exposed to increasing levels of NIR (810 nm) light, corresponding to the absorption peak of the nanoshells. The effect of the radiation treatment was evaluated by live/dead assay examined by two photon microscopy and the kinetics of spheroid growth in a 2 week period.

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Results: Nanoshell loaded macrophages were capable of invading preformed spheroids following 12hrs of co-incubation. Significant cell death within the spheroids containing nanoshell loaded macrophages was observed following NIR light exposure. Spheroids treated in this manner also showed no tendency to further growth. In contrast spheroids containing macrophages alone (no nanoshell incorporation), exposed to similar levels of NIR light, showed a growth pattern similar to non-treated controls and contained a majority of living tumor cells.

Conclusion: The *in vitro* results of macrophage-mediated delivery of nanoshells into malignant brain tumor spheroids suggest that photothermal ablation of GBM may be possible. Animal experiments on tumor bearing rats are presently underway to examine this possibility further.

Introduction

Primary brain tumors are neoplasms that originate from the parenchymal elements of the brain. There are approximately 17,000 new cases of primary brain tumors diagnosed within the United States every year and an equal number in the EU region. Approximatly 40% of these are of the most malignant variety, glioblastoma multiforme (GBM) Despite substantial improvements in conventional treatments consisting of surgery, radiation therapy and chemotherapy, the prognosis for patients with this disease has not improved significantly over the past four decades: median survival is approximately 12 months. The continued poor prognosis for these patients is mainly due to the aggressive infiltrating nature of these tumors. In most cases glioma cells have already infiltrated 2-3 cm into the surrounding normal brain at the time of bulk tumor resection. One of the major factors that limits the treatment effectiveness for gliomas is the presence of the blood-brain barrier which protects infiltrating glioma cells from the effects of anti-cancer agents. [1] Tumor-associated macrophages (TAMs) are frequently found in and around glioblastomas in both experimental animals and patient biopsies [2,3]. This would indicate local synthesis of chemo attractive factors in gliomas and that inflammatory cells can pass through an intact BBB. Monocyte trafficking into the CNS occurs in a highly regulated fashion and is dependent on cell–cell interactions that involve endothelial cells and astrocytes, as well as the local release of

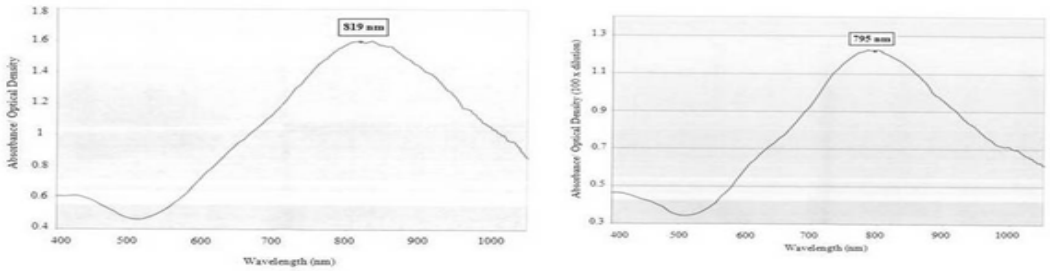
factors that promote BBB permeability. Monocytes or macrophages loaded with drugs, nanoparticles or photosensitizers could therefore be used to target tumors [4,5].

Nanoparticles are structures less than 500 nm in size which have sparked interest with their novel properties (optical, magnetic and thermal) [6]. Gold nanoshells (NS) represent one class of photo-absorbing nanoparticles [7]. They consist of a spherical dielectric silica core (50-500 nm) surrounded by a thin (5–20 nm) gold layer and have a tunable optical absorption within the visible and infrared regions. Since nanoshells are roughly one million times more efficient at converting NIR light into heat than conventional dyes such as indocyanine green , once localized to the tumor and exposed to NIR light, they can generate sufficient heat to induce cell death via thermal ablation. The use of macrophages loaded with gold nanoshells for thermal ablation of GBM tumors is an attractive, relatively safe treatment modality that has not yet been explored.

Materials and methods

Nanoshells

The AU nanoshells used in this study consisted of a 120nm silica core with a 12-15nm gold shell (Nanospectra Biosciences, Inc., Houston, Texas). The resultant optical absorption peak was between 790 and 820 nm for both bare and PEGalated particles. The absorbance curves of bare (**A**) and PEGylated (**B**) nanoshell solutions as supplied by the manufacturer are shown in fig 1.



A B

Fig 1.The absorbance curves of bare (**A**) and PEGylated (**B**) nanoshell solutions as supplied by the manufacturer are shown in fig 1. The solutions were labeled to have an optical density (O.D.) of 1.6 at λ = 819 nm for bare nanoshells (no dilution) and 1.22 at λ = 795 nm for PEGylated nanoshells (100x dilution).

Cell Cultures of Brain Tumor Spheroid and macrophages

The human grade IV glioma cell line (ACBT) and murine Ma P388-D1 (ATCC, CCL-46) were used in all experiments. The cells were cultured in Dulbecco's Modified Eagle Media (DMEM, Gibco, Carlsbad, CA) with high glucose and supplemented with 2 mM L-glutamine, gentamycin (100 mg/ml), and 2% heat-inactivated fetal bovine serum (Gibco). Cells were maintained at 37^{o}C in a 7.5% CO2 incubator.

Generation of tumor cell spheroids

Tumor spheroids were generated with 5×10^{3} cells in 200μl of culture medium per well of an ultra-low attachment surface 96-well round bottomed plate (Corning In., NY). The plates were centrifuged at 1000g for 10 minutes. Immediately following centrifugation the tumor cells formed into a disk shape. The plates were maintained at 37^{0}C in a 7.5% CO2 incubator for 2- 4 days to allow them to take on the usual 3 dimensional spheroid form.

Fluorescence Labeling of Ma

$20x10^{6}$ Ma / ml were labeled with fluorescence cell tracking dye PKH26GLred (SigmaAldrich, St. Louis, MO).

Endocytosis of nanoshells in labeled Ma

Labeled Ma were seeded in 35mm cell a culture dishes at 10^{6} Ma in 2ml of the culture medium. The dishes were incubated overnight to allow the cells to settle and adhere to the plastic. Culture medium was exchanged for 100μl of either bare (3.2×10^{9} particles/ml) or pegalated (2.8×10^{11} particles/ml) NS colloid in 1.9ml of culture medium. The Ma were incubated for 1hour at 37°C, rinsed three times with Hanks' Balanced Salt Solution with calcium chloride and magnesium chloride (HBSS, Gibco, Carlsbad, CA) to wash away the excess of non-ingested nanoshells. Nanoshells laden Ma were then detached with trypsin, washed and counted.

Photothermal treatment (PTT) laser treatment. Individual spheroids in each well of a 96 well round bottomed plate were irradiated with 810 nm laser light (Coherent Inc., Santa Clara, CA) at power densities ranging from 2-28 W/cm^{2} with a laser spot size approximately 3 or 5 mm diameter. Laser treatment lasted for exposure intervals of 1, 5 or 10 min.

Results

The % uptake of PEGylated nanoshells in the macrophages was lower (3.96%) than that of bare nanoshells (15.74%). However, the PEGylated nanoshell solution was available at a much higher concentration since they have a much lower tendency to aggregate compared to bare nanoshells. $5x10^6$Ma incorporated 59 x 10^8 PEGylated nanoshells versus 6.8 x 10^8 bare nanoshells. PEGylated nanoshells were therefore used in all subsequent experiments.

Migration of empty or nanosphears loaded macrophages into preformed spheroids.

ACBT human tumor spheroids were generated as previously described. Forty-eight hours following their generation $2x10^4$/ml PKH26 Red labeled empty or NS loaded Ma were added to the wells and co incubated for 4, 18 and 36 hrs. As can be seen from figs. 2 the number of labeled Ma that had infiltrated into the spheroids increased for the first 12 hrs of co-incubation and then appeared to approach saturation levels.

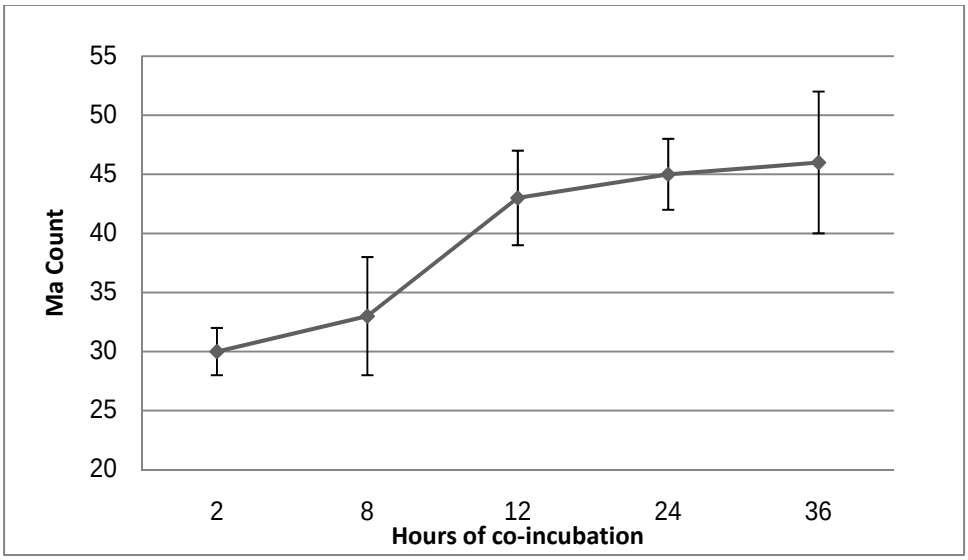
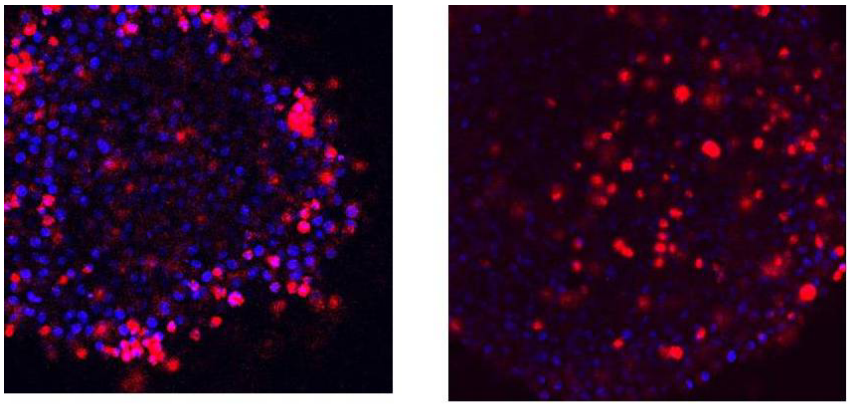


Figure 2. Infiltration of empty Ma into glioma spheroids with increasing co-incubation time. $2x10^4$ Ma incubated for; 2,8,12,24 and 36hr. together with 700um diameter tumor spheroids. The counts were obtained from two photon microsections from a 15μm slice located 80μm into the spheroid. The values shown represent the average Ma counts of 4 spheroids +/- SD

Loaded macrophages appeared to infiltrate at an increased initial rate and penetrated deeper into the spheroid during the first 2 hrs of co-incubation (Fig 3a,b). The majority of empty Ma were found in the outer rim of the spheroid (fig3a) while in contrast to this, NP loaded Ma had a much deeper penetration during this initial incubation period (Fig 3b). By the 12 hr point no significant differences between empty and loaded Ma could be determined when comparing either number of Ma or their penetration depth.



Spheroid+Ma

Spheroid+Ma +NP

Figure 3 Infiltration of empty or NS loaded Ma into glioma spheroids. $2x10^4$ Ma incubated for; 2 hrs. together with 700μm diameter tumor spheroids.

Effects of PTT on Ma infiltrated spheroids.

Spheroids containing either empty or NP loaded Ma were exposed to NIR irradiation of increasing power densities. Figure 4 shows a size comparison of the control (no laser treatment) and the laser exposed spheroids 12 days following PTT.

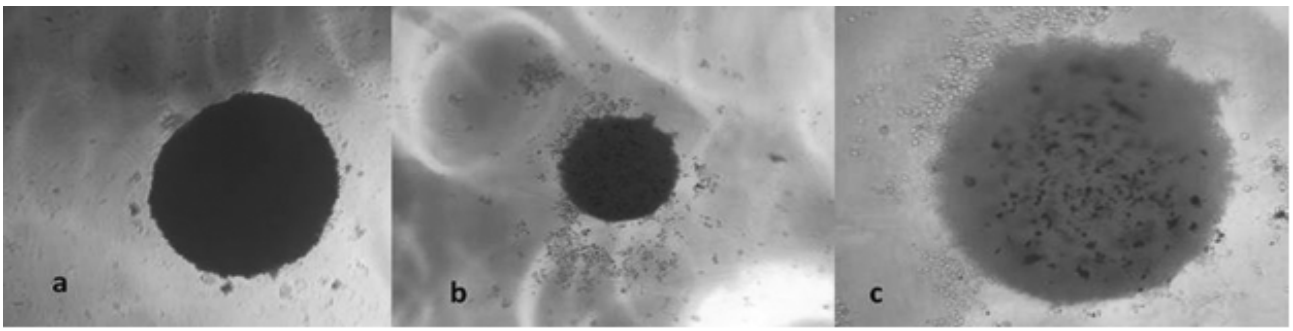


Fig 4. Phase contrast micrograph of control (a) and PTT treated (b,c) hybrid spheroids. Treated spheroids showed no growth during the 12 days of incubation. Images a,b x4, image cx10. The NP loaded Ma are clearly seen in c. PTT 14W/cm^2, 10min.

The control spheroids had clearly shown growth increasing their average diameter from 700 to 1100, corresponding to an increase in volume of 6 -7 times in contrast to the PTT treated spheroids which diminished in size. At higher magnification the NP labeled Ma inside the treated spheroid are clearly visible. Spheroids were allowed to grow in culture for a period of 14 days to determine their viability. PTT was administered at a power density of 14W/cm^2 for 10 min. The kinetics of their growth pattern is shown in fig.5.

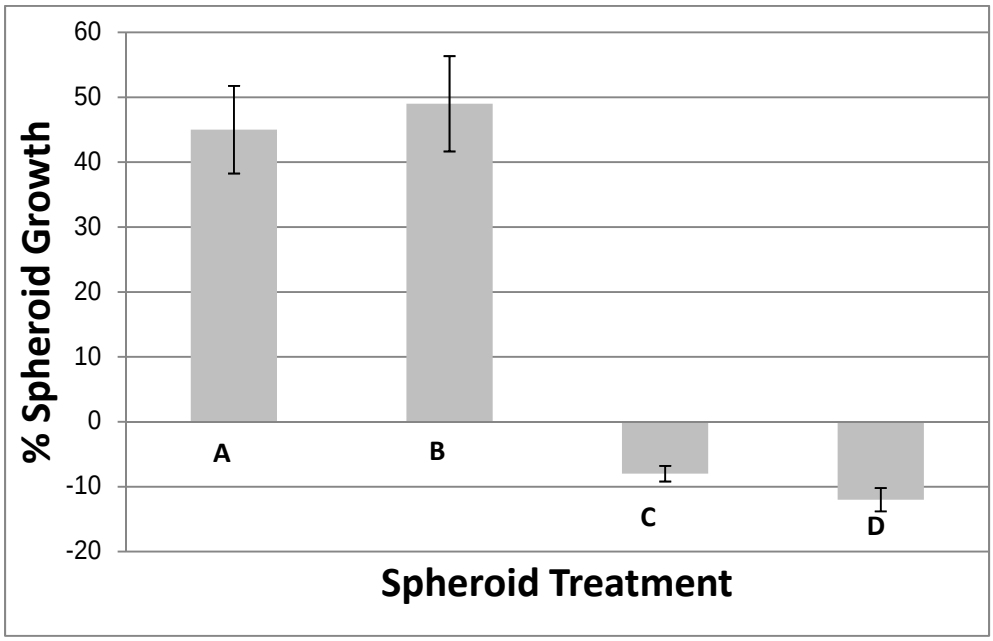


Fig. 5. Kinetics of spheroid growth as a % initial size. Spheroids were formed containing 5x10^3 tumor cells. 48 hrs after formation, individual spheroids were coincubated with 2x10^4 empty Ma for 18hrs (**b**) or NS loaded Ma for 2 hrs. (**d**) or 18 hrs. (**a, c)** in the wells of round bottomed 96 well plates. The spheroids were washed and transferred to new wells to remove non-incorporated NS and evaluated for size 14 days post PTT. PTT, 14 W/cm2, 10 min. (**b,c,d**).

Spheroids containing empty Ma displayed the same growth pattern following PTT as that observed for spheroids containing NS loaded Ma but received no light treatment (fig5 a,b). PTT had a pronounced effect on NS loaded spheroid survival with a complete cessation of growth in all of the spheroids examined. Following either 2 or 18hrs of co-incubation sufficient numbers of

NS loaded Ma infiltrated into the spheroid to completely inhibit their growth following PTT (fig.5c,d).

Discussion

One of the many obstacles to effective treatment of malignant brain tumors is limited transport of anti-tumor agents through brain and brain tumor capillaries due to the blood–brain barrier (BBB) and the blood-brain tumor barrier (BBTB), the latter which retains many BBB characteristics. The use of macrophages as a cell-based delivery vehicle for nanoparticles stemmed from the observations that the iv administration of paramagnetic nanoparticles for MRI, were ingested by endogens macrophages that subsequently migrated and accumulated in and around tumors [8] This was also the case when exogenous *in vitro* labeled macrophages were injected iv. [9]

The primary objective of this study was to examine the ability macrophages to act as vehicles that could deliver a sufficient quantity of gold nanoshells into glioma tumor spheroids for efficient PTT to take place. Our results clearly indicate that macrophages can efficiently incorporate pegalated gold nanoshells, nanoshell-loaded macrophages could infiltrate glioma spheroids following co-incubation to the same degree as empty NP, and NIR laser irradiation of nanoshell loaded Ma infiltrated spheroids would result in significant growth inhibition while spheroids infiltrated with empty macrophages would be undamaged .

Among the various polymers used to prevent NS removal from the circulation, poly(ethylene glycol) (PEG) is currently the most popular and the most effective in prolonging circulation time of nanoparticles [10] On the other hand the strategy of employing in vitro loading of macrophages vectors would dictate a maximum uptake of nanoparticles by the macrophages. We found that the % uptake of bare or PEGylated nanoshells in the murine macrophages used in this study was 16% and 4% respectively. However, PEGylated nanoshell were available in higher concentration since they have a much lower tendency to aggregate compared to bare nanoshells. The total amount of PEGylated nanoshells taken up by the macrophages was more than 8 times that of bare nanoshells. The incorporation of gold nanoshells into the macrophages used in this study did not appear to inhibit the infiltrative nature of the cells but in fact seemed to increase their ease of spheroid penetration compared to empty Ma (Fig. 3). The majority of empty Ma were found in the outer rim of the spheroid while, in contrast to , NP loaded Ma had a much deeper penetration during the first hours of the incubation period. By the 12 hr point no

significant differences between empty and loaded Ma could be determined when comparing either the number of Ma or their penetration depth.

Spheroids containing NS loaded Ma were clearly effected by exposure to NIR laser light as is evident in fig 5 (c,d) which completely stopped their further growth. In general normal tissues along the laser path should be optically transparent at the wavelengths employed and should therefore suffer minimal thermal damage, which is not the case for radiofrequency (RF) or magnetic thermal ablation. Most biological tissue lack NIR-absorbing chromophores, thus permitting transmission of NIR light (700–1,000 nm) with minimal direct thermal effects and good penetration depth. [11,12]. Spheroids containing empty Ma were not affected by even the highest laser power densities tested ($28W/Cm^2$) and showed a growth pattern unchanged compared to untreated controls (fig 5a and b). In principle PTT should be effective on both well oxygenated and hypoxic cancer cells providing adequate heating occurs i.e. temperatures over 50C for a sufficient time interval. In many of the laser exposed cultures the spheroids containing loaded Ma were transformed to an amorphous mass indicating that the collagen in the extracellular matrix in the tissue had undergone hyalinization, a clear heat induced process. The results of this study provide proof of concept for the use of macrophages as a delivery vector of NP into gliomas for photothermal ablation and open the possibility of developing such regimens for patient treatment. Animal experiments on tumor bearing rats are presently underway to examine this possibility further.

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