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Ferrociphenol lipid nanocapsule delivery by mesenchymal stromal cells in brain tumor therapy

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ABSTRACT

The prognosis of patients with malignant glioma remains extremely poor despite surgery and improvements in radio- and chemo-therapies. Thus, treatment strategies that specifically target these tumors have the potential to greatly improve therapeutic outcomes. "Marrow-isolated adult multilineage inducible" cells (MIAMI cells) are a subpopulation of mesenchymal stromal cells (MSCs) which possess the ability to migrate to brain tumors. We have previously shown that MIAMI cells were able to efficiently incorporate lipid nanocapsules (LNCs) without altering either their stem cell properties or their migration capacity. In this study, we assessed whether the cytotoxic effects of MIAMI cells loaded with LNCs containing an organometallic complex (ferrociphenol or Fc-diOH) could be used to treat brain tumors. The results showed that MIAMI cells internalized Fc-diOH-LNCs and that this internalization did not induce MIAMI cell death. Furthermore, Fc-diOH-LNC-loaded MIAMI cells produced a cytotoxic effect on U87MG glioma cells *in vitro*. This cytotoxic effect was validated *in vivo* after intratumoral injection of Fc-diOH-LNC-loaded MIAMI cells in a heterotopic U87MG glioma model in nude mice. These promising results open up a new field of treatment in which cellular vehicles and nanoparticles can be combined to treat brain tumors.

1. Introduction

Malignant gliomas have a poor prognosis despite aggressive treatment with surgery, radiotherapy and chemotherapy (Stupp et al., 2006; Westphal et al., 2003). With many cancer treatments, especially for those used to treat brain tumors, it is difficult to achieve effective delivery of the therapeutic agents to the tumor site and to the infiltrating tumor cells. Many promising treatments involve polymer or lipid nanoparticle (NP) systems for brain tumor therapy (Andrieux and Couvreur, 2009; Huynh et al., 2009; Jain, 2007; Khalil and Mainardes, 2009; Laquintana et al., 2009). These systems protect the therapeutic agent from premature degradation and allow its release to be both sustained and controlled. However, new paradigms must be developed to facilitate tumor-specific targeting and extensive intratumoral distribution.

An alternative method to enhance the delivery of NPs to brain tumors is the use of cellular vectors that have endogenous tumor-homing activity such as mesenchymal stromal cells (MSCs) and can thereby chaperone NP delivery *in vivo* (Kosztowski et al., 2009; Motaln et al., 2010; Roger et al., 2011). In this regard, we obtained recently, proof of concept that a subpopulation of human MSCs, called "marrow-isolated adult multilineage inducible" (MIAMI) cells can be used as NP delivery vehicles (Roger et al., 2011). MIAMI cells were isolated by differential adhesion of iliac crest aspirate of human donors and amplified using particular culture conditions, including low oxygen tension (3%), which mimic the MSC niche (D'Ippolito et al., 2006). These cells are easy to obtain from patients and to handle *in vitro*. Furthermore, they are able to differentiate into neuron-like cells thus representing autologous human cell populations for tissue regeneration of skeletal and nervous system disorders (D'Ippolito et al., 2006; Delcroix et al., 2010; Tatard et al., 2007). We investigated the internalization of two types of NPs into MIAMI cells: poly-lactic acid NPs (PLA-NPs) and lipid nanocapsules (LNCs). These NPs provide considerable drug encapsulation capacity and also exhibit sustained-release functions at the site of action (Huynh et al., 2009; Shah et al., 2009). We showed that PLA-NPs and

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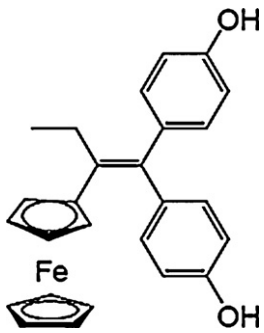

Fig. 1. Chemical formula of Fc-diOH, 2-ferrocenyl-1,1-bis(4-hydroxyphenyl)-but-1-ene (MW = 424.4 g/mol).

LNCs can be efficiently internalized into MIAMI cells without affecting cell viability or differentiation potential. Furthermore, these NP-loaded cells were able to migrate toward the U87MG experimental human glioma in nude mice.

In the current study, we evaluated the therapeutic efficacy of MIAMI cells carrying drug-loaded LNCs in a heterotopic glioma model. LNCs were selected as drug carriers because in comparison with PLA-NPs, they are prepared by using FDA-approved constituents in a solvent-free and low-energy process (Beduneau et al., 2006). Furthermore, several studies in our laboratory have demonstrated the prospects of using LNCs to deliver drugs to glioma tumors (Garcion et al., 2006; Sarin, 2009; Shah et al., 2009). LNCs were encapsulated with 2-ferrocenyl-1,1-bis(4-hydroxyphenyl)-but-1-ene (or Fc-diOH), which is an organometallic complex analog of 4-hydroxy-tamoxifen (Fig. 1). It has been shown that substituting a ferrocenyl unit for the phenyl group on hydroxyl-tamoxifen dramatically enhances its cytotoxicity. Indeed, Fc-diOH is cytotoxic to breast tumor cell lines (Nguyen et al., 2008; Vessieres et al., 2010) and melanoma cell lines (Michard et al., 2008). Furthermore, LNCs loaded with Fc-diOH were shown to be specifically toxic to 9L rat glioma cells but not to astrocytes (Allard et al., 2008, 2010). This compound also exhibited considerable antiproliferative activity *in vitro,* with an IC_{50} in the low micromolar range (Allard et al., 2008; Vessieres et al., 2005).

2. Materials and methods

2.1. NP materials

Fc-diOH was prepared by McMurry coupling (Top et al., 2003). Coumarin-6, was purchased from Sigma–Aldrich (St. Quentin Fallavier, France). The lipophilic Labrafac® CC (caprylic-capric acid triglycerides) was kindly provided by Gattefosse S.A. (Saint-Priest, France). Lipoïd® S75-3 (soybean lecithin at 69% of phosphatidylcholine) and Solutol® HS15 (a mixture of free polyethylene glycol 660 and polyethylene glycol 660 hydroxystearate) were a gift from Lipoïd Gmbh and BASF (Ludwigshafen, Germany), respectively. NaCl was obtained from Prolabo (Fontenay-sous Bois, France). Deionized water was obtained using a Milli-Q Plus system (Millipore, Paris, France).

2.2. LNC formulations

LNCs were prepared according to the phase-inversion temperature method (Heurtault et al., 2002). Briefly, Solutol HS15 (9.7%, w/w), Lipoid (1.5%, w/w), Labrafac (24.2%, w/w), NaCl (1.8%, w/w) and water (62.8%, w/w) were mixed and heated to 95 °C on a magnetic stirrer. The mixture underwent three cycles of progressive heating and cooling between 95 °C and 60 °C, and was then subjected to an irreversible shock induced by adding 12.5 mL of 2 °C deionized water to the 80–85 °C mixture. Finally, LNCs were sterilized by 0.2 μm filtration. To formulate Fc-diOH-LNCs, Fc-diOH was dissolved by sonication in Labrafac (40 mg/mL) before being used for formulation (Allard et al., 2008). To formulate coumarin-6-LNCs, coumarin-6 was dissolved in acetone (1.25 mg/mL) and added to Labrafac (400 μL in 2418 mg of Labrafac). Finally, the mixture was mixed and heated to 50 °C on magnetic stirrer to eliminate acetone.

2.3. LNC characterization

2.3.1. Particle size and surface charge

LNCs were characterized for size, polydispersity index and charge distribution by dynamic light scattering using a Zetasizer® Nano Series DTS 1060 (Malvern Instruments S.A., Worcestershire, UK). To ensure accuracy, LNCs were diluted 1:60 (v/v) in deionized water to produce scattering intensity values that were in the midrange of the instrument's detector.

2.3.2. Drug payload

The orange color of the anticancer drug allowed the Fc-diOH payload to be determined spectrophotometrically at 450 nm after the LNCs were dissolved in solvent mixtures. Drug payload was calculated as previously described (Allard et al., 2008). Briefly, an aliquot of the formulation from each batch was filtered using a Minisart® 0.1 μm filter (Sartorius). Three filtered and unfiltered samples from each batch of Fc-diOH-loaded LNCs were prepared by dissolving 250 mg of the LNCs in 2.25 mL of a 22/67/11 (v/v/v) acetone/tetrahydrofurane/water solution. Quantification was achieved by comparing the absorbence of the ferrocenyl-derived LNC samples to a calibration curve made using blank LNCs and Fc-diOH dissolved in the acetone/tetrahydrofurane/water solution. Mean drug payload was calculated in terms of mg of drug per mL of LNC dispersion.

2.4. Cell culture

MIAMI cells were isolated by differential adhesion of iliac crest aspirates obtained from a human post-mortem organ donor (protocol agreed by the French Agency of Biomedicine) and were cultured *in vitro* under conditions previously described (D'Ippolito et al., 2006). Briefly, iliac crest-derived cells were plated at a density of 10^5 cells/cm^2 in DMEM-low glucose media (DMEM-LG, Gibco, Cergy Pontoise, France), containing 3% fetal bovine serum (FBS, Hyclone, PerbioScience, Bredières, France) and antibiotics (Sigma–Aldrich), on a fibronectin substrate (FN, 10 ng/mL, Sigma–Aldrich) under low oxygen conditions (3% O_2, 5% CO_2 and 92% N_2). Fourteen days after the initial plating, non-adherent cells were removed. For expansion, pooled colonies of adherent cells were selected and plated on a FN substrate at low density (100 cells/cm^2). Cells were expanded in DMEM-LG plus 3% FBS in a humidified incubator at 37 °C in an atmosphere of 3% O_2/5% CO_2/92% N_2 until they were 40% confluent. These cells are negative for hematopoietic lineage markers (CD45, CD34) but are positive for CD13, CD29, CD44, CD73, CD90, CD105, CD140b, CD164 and CD166. Furthermore, MIAMI cells were able to differentiate into osteoblasts and adipocytes under appropriate culture conditions (data not shown).

The human glioma cell line U87MG was obtained from the ATCC (LGC Promochem, Molsheim, France). Cells were maintained in minimum essential medium (Eagle) with Earle's BSS (Lonza, Verviers, Belgium) containing 10% FBS (Lonza), 0.1 mM non-essential amino acids (Lonza), 1 mM sodium pyruvate (Lonza) and 1% antibiotics (Sigma–Aldrich) in a humidified incubator containing 5% CO_2 (37 °C) until they were 80% confluent.

2.5. Cell viability

MIAMI or U87MG cells were plated in 24-well plates at 2.5×10^4 cells/mL. After 48 h, 0–700 μL/mL of blank LNCs or Fc-diOH-LNCs in HBSS (Lonza) was added to the cells for 1 h at 37 °C. Cells were washed in DPBS (Lonza) and culture medium specific for each cell type was added. After three days, cells were incubated in fresh HBSS containing 100 μL of combined MTS/PMS solution (CellTiter 96® AQ_{ueous} non-radioactive cell proliferation assay kit, Promega, Charbonnieres, France) for 3 h at 37 °C. The soluble formazan generated by the live cells was proportional to the number of live cells and the absorbance at 490 nm was recorded for each well using a microplate reader (Multiskan Ascent, Labsystem).

2.6. In vitro uptake of coumarin-6-LNCs by MIAMI cells

MIAMI cells were plated in 8-well labtek chambers at the concentration of 1×10^4 cells per well. After 24 h, MIAMI cells were incubated with 1 mg/mL of coumarin-6-LNCs in HBSS, 1 h at 37 °C. After incubation, cells were washed twice with DPBS and fixed 5 min with 4% paraformaldehyde pH 7.4. Nuclei were counterstained with 4′, 6-diamino-2′-phenylindole dihydrochloride (Sigma–Aldrich). Slides were mounted in a fluorescent mounting medium (Dako, Trappes, France) and analyzed on a fluorescence microscope (Axioscope® 2 optical, Zeiss, Le Pecq, Allemagne).

2.7. In vitro and in vivo toxicity of Fc-diOH-LNC-loaded MIAMI cells on U87MG cells

MIAMI cells (1×10^6 cells) were incubated for 1 h with 2 mL of Fc-diOH-LNCs in HBSS (700 μL/mL of LNCs) which was equivalent to 1.84 mg/mL of Fc-diOH.

Cytotoxicity assay by transwell inserts was used to evaluate the *in vitro* toxicity of secreted factors from Fc-diOH-LNC-loaded MIAMI cells on U87MG cells. U87MG cells (2.5×10^4 cells) were grown in the lower wells of 24-transwell plates with porous inserts (0.4 μm pore size) (Millipore, Guyancourt, France) for 48 h, after which were plated Fc-diOH-LNC-loaded MIAMI cells on the upper wells of the transwell plates with increasing cell numbers (2.5×10^4, 1.25×10^5 and 2.5×10^5 cells). The viability of the U87MG cells in the lower wells was evaluated after 3 days *via* MTS assays as described above.

In vivo toxicity of Fc-diOH-LNC-loaded MIAMI cells on U87MG cells was performed in a heterotopic glioma model. Female athymic Swiss nude mice, 8–9 weeks old, were obtained from Charles River (L'Arbresle, France). The experiments were conducted according to the French Minister of Agriculture and the European Communities Council Directive of 24 November 1986 (86/609/EEC). For tumor growth analysis, animals received subcutaneous injections (s.c.) of 5×10^5 U87MG cells into the upper right hind leg. The length and width of each tumor were measured at regular intervals using a digital caliper, and tumor volume was calculated as follows: $(\pi/6) \times \text{width}^2$ (l) × length (L). When tumor volumes reached $50 \pm 5\ \text{mm}^3$, mice received an intratumoral injection (100 μL) of one of the following treatments: HBSS saline solution ($n = 10$), 5×10^5 MIAMI cells ($n = 10$) or Fc-diOH-LNC-loaded MIAMI cells ($n = 10$). After treatment, tumor volumes were calculated every day. At day 15 after the treatment injection, mice were sacrificed and the weight of each tumor was measured.

2.8. Statistical analysis

Results are given as mean ± standard error of mean (SEM). To describe statistical differences with controls, the Dunnet test was used. For tumor growth analysis, a two way ANOVA was performed to compare curve profiles. Differences were considered statistically significant for $P < 0.05$.

3. Results and discussion

3.1. Fc-diOH-LNC characterization

Fc-diOH is one of the most active molecules in a new class of organometallic drugs, and has shown *in vitro* antiproliferative effects on breast cell lines and the 9L glioma cell line (Allard et al., 2008; Nguyen et al., 2008). To improve the bioavailability of this hydrophobic compound and to prepare it for incorporation into cells, we encapsulated it into LNCs (Allard et al., 2008). The physicochemical characteristics of blank LNCs, coumarin-6-LNCs and Fc-diOH-LNCs (particle size, polydispersity index and zeta potential) are presented in Table 1. Blank-, coumarin-6- and Fc-diOH-loaded LNCs formulated by the phase-inversion temperature method have mean diameters of 96 ± 2 nm, 88 ± 4 and 76 ± 2 nm, respectively, and a polydispersity index less than 0.1. In all cases, the zeta potential was slightly negative. The amount of drug loaded into Fc-diOH-LNCs was 2.6 ± 0.1 mg Fc-diOH per mL of LNC suspension.

3.2. In vitro uptake of LNCs by MIAMI cells

Coumarin-6-LNCs were efficiently taken up by MIAMI cells after 1 h of incubation at 37 °C and were localized throughout the cytoplasm (Fig. 2). Due to their small size, LNCs can be incorporated by cells in the absence of any transfection agents (Gratton et al., 2008; Lorenz et al., 2006; Mailander et al., 2008). Moreover, as previously described, LNC uptake was concentration- and time-dependent (Roger et al., 2010). In addition, LNCs persisted inside the cells for at least seven days after incorporation. Internalization of LNCs did not inhibit proliferation or prevent the differentiation of MIAMI cells into osteoblasts and adipocytes (Roger et al., 2010).

3.3. In vitro survival of MIAMI and U87MG cells after Fc-diOH-LNC internalization

To use MIAMI cells as cellular carriers of drug-loaded LNCs, it was necessary to determine that internalization of drug-loaded LNCs did not lead to MIAMI cell death. Three days after Fc-diOH-LNC uptake, MIAMI cell viability remained unchanged irrespective of the Fc-diOH-LNC concentration used (Fig. 3). In contrast, U87MG cell viability decreased to less than 65%, 3 days after they had taken up Fc-diOH-LNCs. Blank LNCs were only toxic to U87MG cells at a high concentration (700 μL/mL). These results agree with previously published results in which Fc-diOH-LNCs were only slightly

Table 1
Physicochemical characteristics of blank LNCs, coumarin-6-LNCs and Fc-diOH-LNCs (mean of three samples ± SEM).

	Mean particle size (nm)	Polydispersity index (PDI)	Zeta potential (mV)	Drug payload (mg/mL of suspension)
Blank LNCs	96 ± 2	0.06 ± 0.0005	−5 ± 1	–
Coumarin-6-LNCs	88 ± 4	0.06 ± 0.02	−4 ± 2	–
Fc-diOH-LNCs	76 ± 2	0.03 ± 0.007	−7 ± 2	2.6 ± 0.1

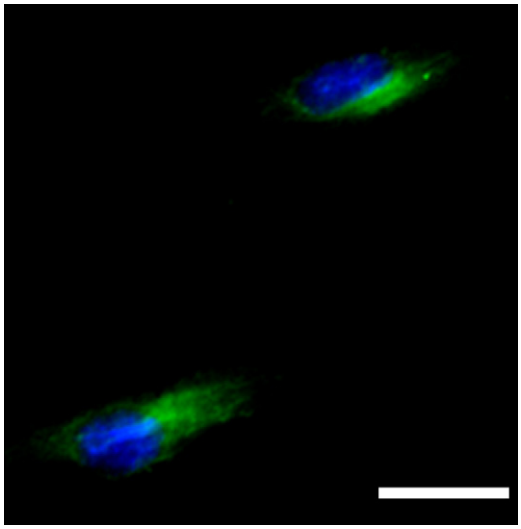

Fig. 2. MIAMI cells containing coumarin-6-LNCs detected by fluorescence microscopy. MIAMI cells were incubated with LNCs (1 mg/mL) for 1 h at 37 °C and then visualized by fluorescence microscopy. The green fluorescence represents coumarin-6 and nuclei stained with Dapi are blue (scale bar = 25 μm).

toxic to healthy astrocytes but exerted a cytostatic effect on rat 9L glioma cells (Allard et al., 2008, 2009, 2010). Our data indicated that MIAMI cell viability was unaffected by the incorporation of Fc-diOH-loaded LNCs and that these cells could be promising drug-loaded LNC carriers.

3.4. *In vitro and in vivo toxicity of Fc-diOH-LNC-loaded MIAMI cells on U87MG cells*

A second condition for using MIAMI cells as cellular carriers of drug-loaded LNCs in brain tumor therapy was to verify that MIAMI cells loaded with Fc-diOH-LNCs were cytotoxic to glioma cells. To test this principle, we plated U87MG cells on the bottom of culture wells and seeded upper transwell inserts with either unloaded MIAMI cells or Fc-diOH-LNC-loaded MIAMI cells (Fig. 4A). This coculture experiments on inserts with 0.4 μm diameter pores allowed determining the toxicities of secreted factors from Fc-diOH-LNC-loaded MIAMI cells such as Fc-diOH but not membrane factors. We found that Fc-diOH-LNC-loaded MIAMI cells induced a

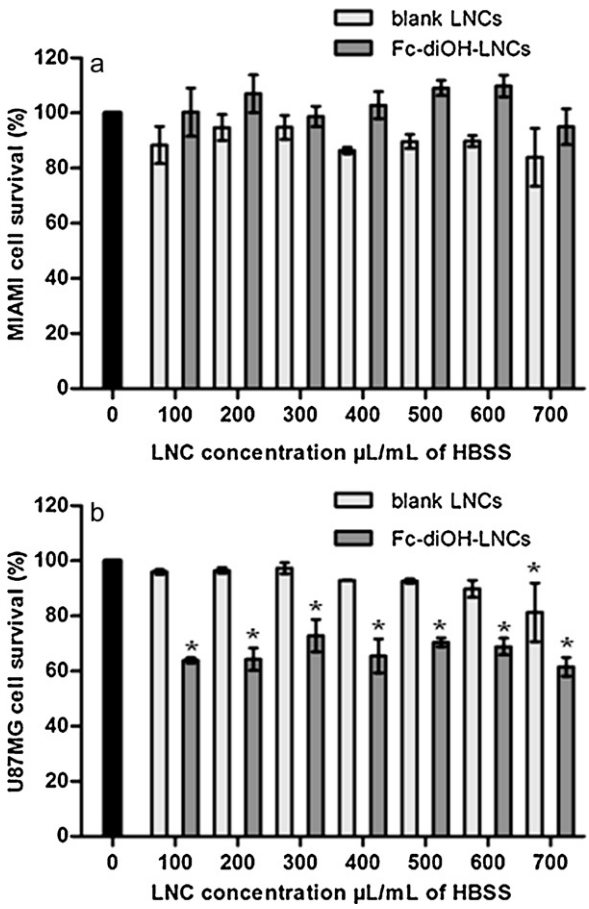

Fig. 3. Cell viability following LNC uptake. The viability of U87MG cells (A) and MIAMI cells (B) was assessed three days after incorporating various concentrations of blank LNCs or Fc-diOH-LNCs. Cell survival was expressed as the percentage of the total number of viable control cells that contained no NPs (100%). Data are expressed as the mean of four wells ± SEM ($n = 3$) (*$P < 0.05$, versus control without LNCs).

significant dose-dependent inhibition of U87MG cell proliferation (Fig. 4B), which suggested that MIAMI cells were able to deliver Fc-diOH or Fc-diOH-LNCs into the cell culture medium. This cytotoxic effect was confirmed *in vivo* in the heterotopic U87MG glioma model. Intratumoral injection of Fc-diOH-LNC-loaded MIAMI cells

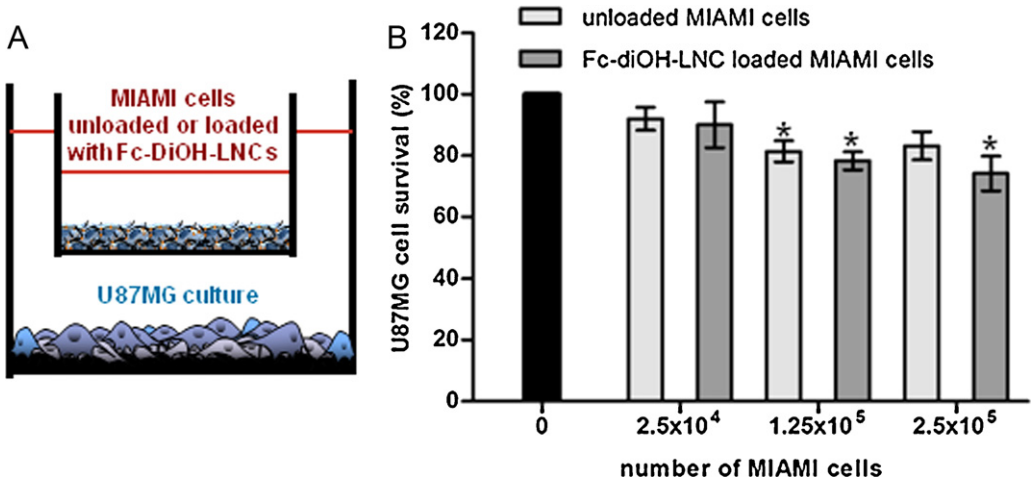

Fig. 4. (A) Schematic principle of coculture experiments to assess the U87MG cell cytotoxicity of Fc-diOH-LNCs delivered by MIAMI cells. The pore size of the insert (0.4 μm) allowed the passage of LNCs. (B) Cell viability analysis of U87MG glioma cells after three days of being cocultured with MIAMI cells, either unloaded or loaded with Fc-diOH-LNCs. Three doses of MIAMI cells were tested. Cell survival was expressed as the percentage of surviving U87MG cells that were not cultured with MIAMI cells (100%). Data are expressed as the mean of four wells ± SEM ($n = 3$) (*$P < 0.05$, versus control without MIAMI cells).

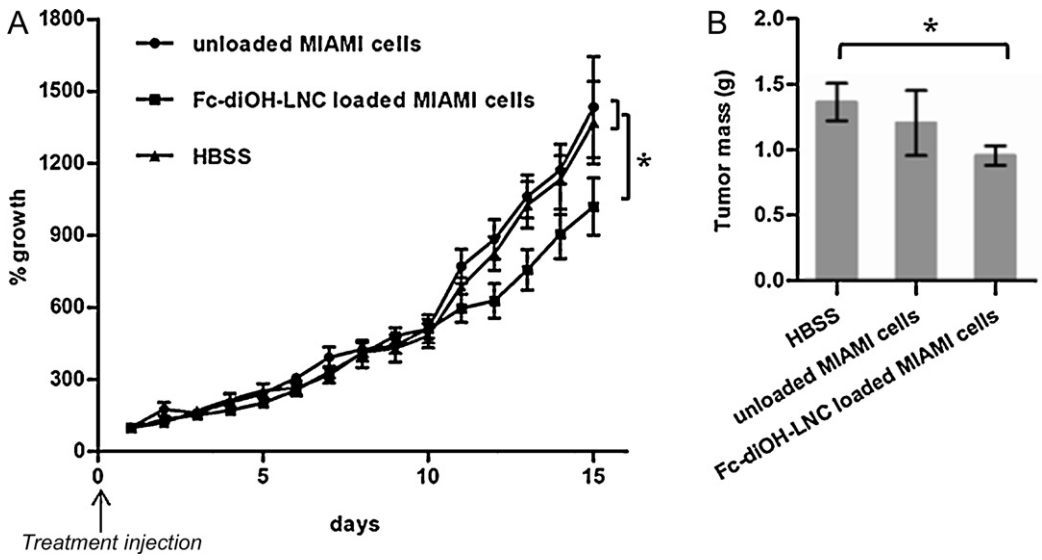

Fig. 5. *In vivo* effects of Fc-diOH-LNC-loaded MIAMI cells on the growth of heterotopic U87MG glioma tumors. (A) Percentage (±SEM) of tumor growth after the injection treatment. The tumor size on the day of the injection treatment was considered as 100%. (B) Weight of the s.c. tumors (±SEM) on day 15 after the injection treatment (*$P < 0.05$).

resulted in significantly greater reductions in final tumor volume and tumor weight than did intratumoral injection of HBSS (Fig. 5). Similar results on tumor growth were obtained for the intratumoral injection of unincorporated Fc-diOH-LNCs in a heterotopic 9L glioma model (Allard et al., 2008).

The precise mechanism underlying Fc-diOH-LNC cytotoxicity is not fully understood. It has been suggested that organometallic ferrocifens induce significant ROS production in tumor cell lines, which is associated with cell cycle arrest and senescence (Vessieres et al., 2010). This may explain why we observed only slowed tumor growth, rather than tumor shrinkage or a static effect. In our study, we did not compare the effects of Fc-diOH-LNC-loaded MIAMI cells with those of Fc-diOH-LNCs alone because the principal aim of this study was to determine whether MIAMI cells were able to efficiently internalize drug-loaded LNCs, maintain sufficient viability afterward, and induce a cytotoxic effect on glioma cells and solid tumors. Comparing the efficiency between these two drug delivery systems would require conducting additional studies to determine the dose of the drug contained in MIAMI cells.

In the transwell experiment, coculturing U87MG cells with 1.25×10^5 unloaded MIAMI cells also significantly inhibited U87MG cell growth, but this effect did not increase with a higher dose of MIAMI cells (Fig. 4B). However, unloaded MIAMI cells injected into U87MG tumors *in vivo* did not affect tumor growth (Fig. 5). Contradictory results have been reported concerning the effect of MSCs on tumor cell growth. Some studies have indicated that MSCs promote tumor development (Djouad et al., 2003; Karnoub et al., 2007; Yu et al., 2008), whereas other studies have demonstrated an opposite effect (Lu et al., 2008; Nakamura et al., 2004) or no effect at all (Bexell et al., 2009; Komarova et al., 2006; Kucerova et al., 2007; Sasportas et al., 2009).

4. Conclusion

The combination of NPs and stem cells has been used to deliver growth factors to enhance angiogenesis (VEGF) (Yang et al., 2010) and to induce osteogenic (bFGF, BMP-2, BMP-2/BMP-7) or chondrogenic (TGF-beta1, THF-beta3) stem cell differentiation (Jung et al., 2009; Kim et al., 2008; Park et al., 2009a,b; Wu et al., 2010; Yilgor et al., 2009, 2010). We demonstrated for the first time that MSCs were able to deliver drug-loaded LNCs into solid tumors. The efficacy of this strategy should be confirmed in an orthotopic glioma model. In addition, this strategy could be improved by combining it with external radiotherapy. Indeed, it has been reported that local irradiation might enhance the migration and engraftment specificity of MSCs (Francois et al., 2006), in addition to potentiating the cytotoxic action of Fc-diOH (Allard et al., 2010). To summarize, the combination of MSCs and drug-loaded NPs represents a promising new treatment strategy for malignant gliomas.

Acknowledgments

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