Tumor eradication in rat glioma and bypass of immunosuppressive barriers using internal radiation with $^{188}$Re-lipid nanocapsules

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**A B S T R A C T**

To date, glioblastoma treatments have only been palliative. In this context, locoregional drug delivery strategies, which allow for blood–brain barrier bypass and reduced systemic toxicity, are of major significance. Recent progress in nanotechnology has led to the development of colloidal carriers of radiopharmaceutics, such as lipid nanocapsules loaded with rhenium-$^{188}$ (LNC$^{188}$Re-SSS) that are implanted in the brain. In our study, we demonstrated that fractionated internal radiation using LNC$^{188}$Re-SSS triggered remarkable survival responses in a rat orthotopic glioma model (cure rates of 83%). We also highlighted the importance of the radioactivity activity gradient obtained by combining a simple stereotactic injection (SI) with convection-enhanced delivery (CED). We assumed that the immune system played a role in the treatment’s efficacy on account of the overproduction of peripheral cytokines, recruitment of immune cells to the tumor site, and memory response in long-term survivor animals. Hence, nanovectorized internal radiation therapy with activity gradients stimulating immune responses may represent a new and interesting alternative for the treatment of solid tumors such as glioblastomas.

1. Introduction

Glioblastomas (GBM) are the most common and lethal type of primary brain tumors [1]. Although surgery and external beam radiation therapy, with or without chemotherapy, slightly improve the prognosis, treatments are never curative [2]. Systemic toxicity, normal brain tissue sensitivity, and the blood–brain barrier (BBB) are the main factors responsible for treatment failure [3].

Ionizing radiation is the gold-standard adjuvant treatment for malignant gliomas. Given that, efforts in developing internal radiation have been made in order to prevent harm to healthy tissues. In this context, locoregional drug delivery modalities, such as stereotactic radiosurgery, which allow for blood–brain barrier (BBB) bypass and reduced systemic toxicity, are of major relevance. Clinical trials on GBM patients supported the usefulness of local radiolabeled peptide receptor therapy ($^{90}$Y-DOTATOC [4]) and radioimmunotherapy ($^{131}$I-tenascin antibodies [5] and $^{188}$Re-nimotuzumab [6]). Thus, nanoparticles issued from new technologies hold great promise for developing effective targeted therapies for gliomas. The distribution of the radionuclide will not only depend on its own intrinsic properties but also on those of the vector [7]. Hence, the benefit expected to come from loading the radionuclide is the avoidance of fast elimination after injection.

Colloidal drug carriers have been designed to incorporate radionuclides, such as lipid nanocapsules (LNC). These LNCs are synthesized through a phase inversion process without any organic solvent and consist of a lipid core surrounded by a tensioactive shell [8]. With biomimetic properties, they provide extensive drug encapsulation capacity and exhibit biological effects such as P-gp inhibition [12–14], endo-lysosomal escape [15], and biological barrier crossing [15]. LNCs are implanted in brain tumors using stereotactic injections for locoregional therapy. We recently...
established the feasibility of this technique using 50 nm-LNC loaded with a lipophilic complex of Rhenium-188 (LNC\textsuperscript{\textsuperscript{188}Re}-SSS - half-life: 16.9 h; $\beta^-$ emitter: 2.12 MeV; $\gamma$ emitter: 155 keV) for internal radiation therapy in malignant glioma, demonstrating a median survival of up to 45 days after a single injection of LNC\textsuperscript{\textsuperscript{188}Re}-SSS in an orthotopic 9L-glioma model [9].

In order to optimize internal radiation strategy, we assessed the efficacy of repeated brain administrations of LNC\textsuperscript{\textsuperscript{188}Re}-SSS following 9L cell implantation. As simple stereotactic injections (SI) and convection-enhanced delivery (CED) lead to distinct LNC distribution volumes [16], these two LNC\textsuperscript{\textsuperscript{188}Re}-SSS infusion techniques were chosen to study the impact of the activity gradient.

The current rationale of ionizing radiation is based on its ability to eradicate tumor cells, notably through excessive reactive oxygen species generation [17,18]. Nevertheless, several lines of evidence have established that radiotherapy induces dose-dependent consequences such as adaptive responses, genomic instability, and abscopal effects [19–24]. Hence, the recruitment and activation of biological effectors outside the treatment field, notably inflammatory and immune cells such as macrophages [20,25–27], dendritic cells [28], or T cells, depend on the release of danger signals by irradiated tumor cells and the related microenvironment.

According to the fractionated internal radiotherapy protocol used in our study, different activity gradients may be applied in order to enhance different biologic responses.

In addition, synthetic nano-objects can also function as “danger signals” that activate dendritic cells, potentially inducing subsequent T-cell immunity [29–32]. As gliomas are infiltrative tumors, any modification to the tumor microenvironment via ionizing radiation, associated with synthetic adjuvants, exemplified by nanoparticles, may aid tumor eradication through both direct and immune-dependent cell death.

Accordingly, we investigated the impact of fractionated internal radiation using LNC\textsuperscript{\textsuperscript{188}Re}-SSS on a 9L Fischer rat glioma model. Special attention was given to therapeutic efficiency and the potential involvement of the immune system.

2. Materials and methods

2.1. Ethics Statement

This study was carried out in strict accordance with the French Minister of Agriculture and the European, Communities Council Directive of 24 November 1986 (86/609/EEC). The protocol was approved by the Committee on the Ethics of Animal Experiments of the “Pays de la Loire” (Permit Number: CEEA 201003). All surgery was performed under ketamine/xylazine anesthesia, and all efforts were made to minimize suffering.

2.2. Materials

Lipoid S75-3 (soybean lecithin at 69% of phosphatidylcholine) and Solunol HS15 (a mixture of polyethylene glycol 660 and polyethylene glycol 660 hydroxyystearate) were kindly provided by Lipoid GmbH (Ludwigshafen, Germany) and BASF (Ludwigshafen, Germany), respectively. NaCl and dichloromethane were purchased from Sigma (St-Quentin, Fallavier, France) and 1% antibiotic and antimycotic solution (Sigma, St Quentin Fallavier, France) in a humidified incubator gassed with 5% CO\textsubscript{2} (37 °C) until reaching 80–90% confluence. The number of 9L passages at the time of use for the experiments was between P10–P11.

2.3. Preparation of the \textsuperscript{\textsuperscript{188}Re}-SSS complex

\textsuperscript{\textsuperscript{188}Re} as carrier-free Na\textsubscript{\textsuperscript{188}ReO\textsubscript{4}}\textsuperscript{−} in physiological solution was obtained by saline elution and concentration of \textsuperscript{\textsuperscript{188}W}\textsuperscript{\textsuperscript{188}Re} generator (Institut des Radioéléments, Fleursus, Belgium). The \textsuperscript{\textsuperscript{188}Re}-SSS complex was prepared according to the method developed by Lepareur et al. [33]. In brief, the \textsuperscript{\textsuperscript{188}Re}-SSS complex was obtained by the reaction of the ligand sodium diethiozonebate (Plateform of organic synthesis, Rennes, France) with a freeze-dried formulation containing 30 mg sodium gluco- nate, 30 mg ascorbic acid, 40 mg potassium oxalate, and 4 mg SnCl\textsubscript{2}.2H\textsubscript{2}O reconstituted in 0.5 mL of physiological serum. 1 110MBq of \textsuperscript{\textsuperscript{188}Re}-perchenate (\textsuperscript{\textsuperscript{188}ReO\textsubscript{2}}\textsuperscript{−}) in 0.5 mL was added, and the solution was mixed for 15 min at room temperature. Next, 20 mg of sodium diethiozonebate (in 0.5 mL, pH – 7) was added before being heated at 100 °C for 30 min, which allowed for the formation of the \textsuperscript{\textsuperscript{188}Re}-SSS complex. Due to its precipitation in aqueous media, the \textsuperscript{\textsuperscript{188}Re}-SSS complex was extracted with dichloromethane (1 mL) and washed three times with 1 mL of deionized water. The radiochemical purity (RCP) of the complex was checked by thin-layer chromatography as the ratio of migrated radioactivity to total radioactivity. Thin-layer chromatography was carried out using silica gel 60 F\textsubscript{254} alumina plates (Merck) and a solution of petroleum ether/dichloromethane (6/4; v/v) as an eluant. Radioactivity was assessed with a phosphor-imaging machine (Packard, Cyclone storage phosphor system).

2.4. Nanocapsule formulation and characterization

The overall study was performed on 50 nm diameter LNCs, which were prepared according to a phase-inversion process described by Heurtault et al. [8]. In brief, 25 mg Lipoid S75-3, 282 mg Solunol HS15, 342.7 mg Labrafac 29.7 mg NaCl, and 987.5 mg deionized water were mixed by magnetic stirring. The \textsuperscript{\textsuperscript{188}Re}-SSS complex, extracted with dichloromethane (1 mL) was then added to the other components of the emulsion. The organic solvent was removed by being heated at 60 °C for 15 min. Three cycles of progressive heating and cooling between 85 °C and 60 °C were then carried out and followed by an irreversible shock, induced by dilution with 4.16 mL of 0 °C deionized water, which was added to the mixture at 70 °C. Afterwards, slow magnetic stirring was applied to the suspension for 5 min LNC\textsuperscript{\textsuperscript{188}Re}-SSS were dia- lyzed during 2 h with deionized water at room temperature by magnetic stirring. The mean diameter and polydispersity index were then determined using a Malvern Zetasizer Nano Série DTS 1060 (Malvern Instruments S.A., Worcester, UK).

2.5. Tumor cells

9L (European Collection of Cell Culture, n° 94110705, Salisbury, UK), a rat gliosarcoma cell line, was maintained in Dulbecco’s modified Eagle’s medium (DMEM, BioWhittaker, Verviers, Belgium) containing 10% fetal calf serum (FCS) (Bio-Whittaker, Verviers, Belgium) and 1% antibiotic and antimitotic solution (Sigma, St Quentin Fallavier, France) in a humidified incubator gassed with 5% CO\textsubscript{2} (37 °C) until reaching 80–90% confluence. The number of 9L passages at the time of use for the experiments was between P10–P11.

2.6. Animals

Female syngeneic Fisher 344 rats aged 9–10 weeks were obtained from Charles River (Larbesle, France). The animals were kept in polycarbonate cages in a room with controlled temperature (20–22 °C), humidity (50–70%), and light (12 h light/dark cycles). Room air was renewed at the rate of 10vol/hour. Tap water and diet were provided ad libitum.

2.7. Intracerebral tumor implantation

Tumor cells for intracerebral implantation were trypsinized, counted, and checked for viability by trypan blue exclusion. Cells were washed twice with Eagle’s minimal essential medium (EMEM, BioWhittaker, Verviers, Belgium) without FCS or antibiotics, and a final suspension of 1 × 10\textsuperscript{5} cells/mL in EMEM was obtained. Animals were anesthetized with an intraperitoneal injection of 0.75–1.5 mL/kg of a solution containing 2/3 of ketamine (100 mg/mL; Clokretam, Vétoquinol, Lure, France) and 1/3 xylazine (20 mg/mL; Rompun, Bayer, Puteaux, France). Using a stereotactic head frame and a 10 mm Hamilton syringe (Hamilton\textregistered glass syringe 700 series RN), 10 mL of 1 × 10\textsuperscript{5} cells were injected into the rat’s right striatum. The coordinates used for the intracerebral injection were 1 mm posterior to the bregma, 3 mm lateral to the sagittal suture (right hemisphere), and 5 mm below the dura.

2.8. External beam radiation and groups

An external beam radiation study was performed using a fractionated regimen of 2 × 8 Gy at D6 and D12 following 9L cell implantation. Two groups were studied: a control group (n = 6) and a treated one (n = 8). We set the therapeutic dose at 16 Gy (2 × 8 Gy) as the maximum tolerated dose (MTD) of 18 Gy (3 × 6 Gy) proved to be effective in the 9L-glioma rat model [34].

2.9. Fractionated internal radiation, protocols, and groups

A fractionated internal radiation study was performed at an early and late stage of tumor progression. In the first study, animals underwent internal radiotherapy with 2.8 MBq of LNCs loaded with rhenium-188 (LNC\textsuperscript{\textsuperscript{188}Re}-SSS) on D6 and D12 following 9L cell implantation. In the second study, the efficacy of LNC\textsuperscript{\textsuperscript{188}Re}-SSS was assessed at a late stage of tumor progression, and the animals therefore received internal radiotherapy on D12 and D18. Two different administration types of LNCs (LNC\textsuperscript{\textsuperscript{188}Re}-SSS) were chosen: a SI with a final volume of 10 μL and a flow of 1 μL/min, and a CED injection with a final volume of 60 μL and a flow of 0.5 μL/min. Depending on the administration technique chosen, four injection protocols were carried out, notably protocol 1: SI at D6 and D12; protocol 2: CED injections at D6 and D12; protocol 3: CED injection at D6 (or D12) and SI at D12 (or D18); protocol 4: SI at D6 (or D12) and CED injection

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2.10. Simple stereotactic injection and convection enhancement delivery procedures

The animals were anesthetized with an intraperitoneal injection of 0.75–1.5 mL/kg of a solution containing 2/3 of ketamine (100 mg/ml; Clorketam®, Vétoquinol, Lure, France) and 1/3 xylazine (20 mg/ml; Rompun®, Bayer, Puteaux, France). For the SI, 10 μL were injected into the rat striatum at a flow of 1 μL/min using a 10 μL syringe (Hamilton® glass syringe 700 series RN) with a 32-G needle (Hamilton®). For this purpose, rats were immobilized in a stereotactic head frame (Lab Standard Stereotactic; Stoelting, Chicago, IL). Coordinates were 1 mm posterior to the bregma, 3 mm lateral to the sagittal suture, and 5 mm below the dura. Following the injection, the needle was left in place for an additional 5 min to avoid expulsion of the suspension from the brain during the removal of the syringe.

CED injection was similar, except that the 10 μL Hamilton® syringe with a 32-G needle was connected to a 100 μL Hamilton® 22-G syringe containing the product (Harvard Apparatus, Les Ulis, France) through a cannula (CoExTMPE/PVC tubing, Harvard Apparatus, Les Ulis, France). CED was performed using an osmotic pump PHD 2000 infusion (Harvard Apparatus, Les Ulis, France) by controlling a 0.5 μL/min rate for 2 h.

2.11. Tissue distribution study

A tissue distribution study was carried out using 16 female Fisher rats 6 days following 9L implantation. They were divided into two groups: one injected with LNC188Re-SSS after a SI (n = 8) and one with LNC188Re-SSS following a CED injection (n = 8). In both groups, the animals were sacrificed at post-injection interval times of 24 h (n = 4) and 96 h (n = 4). The organs were removed, washed, and weighed (blood, liver, spleen, kidneys, heart, lung, stomach, small intestine, large intestine, bladder, bone, muscle, brain, and carcass). The content activity of each organ was determined using a gamma counter (Packard Auto-Gamma 5000 series).

2.12. Autoradiography

Female Fisher rats 6 days following 9L cell implantation received 2.8 MBq after SI and CED injections of LNC188Re-SSS (n = 3 per group). Twenty-four hours following the LNC188Re-SSS injection, the brain was extracted and fixed with 4% paraformaldehyde in phosphate-buffered saline 1X (pH = 7.3). Coronal sections (1 mm thick) were prepared from brains on an acrylic brain matrix. Brain slices were then placed on phosphor screens for 1 min and read by the Cyclone Phosphor Imaging System (Packard Instruments).

2.13. MRI

MRI was performed on a Bruker Avance DRX 300 (Germany) machine equipped with a magnet of 7T. Rapid T2-weighted images were obtained using rapid acquisition with relaxation enhancement (RARE) sequence (TR = 2000ms; mean echo time [Tme] = 31.7 ms; RARE factor = 8; FOV = 3 × 3 cm; matrix 128 × 128; nine contiguous slices of 1 mm; eight acquisitions).

2.14. Interleukin-2 (IL-2) and interferon-γ (IFNγ) quantifications

Blood samples were collected from the tail vein using heparinized tubes in each protocol from a fractionated internal radiation study (D6/D12) at D8, D16, and D24 following 9L cell implantation. After centrifugation at 1000g for 20 min, the rat IL-2 and rat IFNγ, ELISA tests (Dusset, R&D Systems Europe, Lille, France) were immediately performed according to manufacturer’s instructions.

2.15. Immunohistochemistry

Brains from tumor-bearing animals treated were frozen at D15, D24, and D32 in isopentane cooled by liquid nitrogen and stored at −80 °C. Fourier-micron cryosections were fixed with 4% paraformaldehyde in phosphate-buffered saline 1X (pH = 7.3) and washed three times with phosphate-buffered saline (PBS). In order to block nonspecific binding, sections were incubated 1 h in PBS containing 5% BSA and 10% normal goat serum, and washed twice with PBS. All incubations with primary antibodies (OX18 antibody: mouse; 1:100, BD Sciences; OX6 antibody: mouse; 1:100, BD Sciences; OX26 antibody: mouse; 1:100, BD Sciences; CD161a antibody: mouse; 1:100, BD Sciences; OX42 antibody: mouse; 1:100, BD Sciences; CD4 antibody: mouse; 1:100, BD Sciences; CD3 antibody: mouse; 1:100, BD Sciences; and CD11b isotypes) were performed overnight at 4 °C at a 1:100 final dilution. Primary antibodies were detected using a rat-absorbed biotinylated anti-mouse IgG secondary antibody (BD Biosciences). After 1 h of incubation at 4 °C, the sections were washed twice with PBS containing 4% of BSA. Sections were developed with Alexa 488-conjugated secondary antibody (Streptavidin Alexa Fluor 488 conjugate, Invitrogen) at a final concentration of 2.5 μg/mL after an incubation of 1 h at 4 °C and washed four times with PBS 1X. After immunostaining, DAPI (4′, 6-diamidino-2-phenylindole dihydrochloride D9542, 0.1 μg/mL, Sigma, St Quentin Fallavier, France) was added for 20 min at room temperature to stain the nuclei.

2.16. Re-challenging

Long-term survivors obtained from fractionated internal radiation studies (D6/D12 and D12/D18) were re-challenged with 1000 9L cells in the left striatum. The animals were anesthetized with an intraperitoneal injection of 0.75–15 μL/kg of a solution containing 2/3 of ketamine (100 mg/ml; Clorketam®, Vétoquinol, Lure, France) and 1/3 xylazine (20 mg/ml; Rompun®, Bayer, Puteaux, France). The intracerebral tumor implantation procedure was described above, but the coordinates used were modified: 1 mm posterior to the bregma, 3 mm lateral to the sagittal suture (left hemisphere), and 5 mm below the dura.

2.17. Statistical analysis

Results are expressed as mean ± standard deviation (SD). For the survival study, comparisons between control groups were made using the log-rank test (Mantel–Cox test). For other studies, statistical analysis was performed using the t test. Data was considered to be significant when p < 0.05.

3. Results

3.1. Biodistribution of nanovectorized radionuclide: importance of the administration route

In order to highlight the importance of the administration route, biodistribution of LNC188Re-SSS was assessed. At Day 6 following 9L cell implantation, we examined the usefulness of encapsulating rhenium-188 within LNCs in order to maintain high levels of radiopharmaceutics in the brain. Rhenium-188 entrapping is essential, as only 4% and 65% of the injected dose were eliminated in urine and feces, respectively, 96 h after injecting LNC188Re-SSS and the solution of 188Re-perhenerenate (188ReO₄⁻) (Fig. 1a). Depending on the rhenium-188 formulation, different distributions were obtained, whereas the two administration techniques (SI; CED) had no impact on the elimination process (Fig. 1b,c). This was corroborated by biodistribution studies, with 86% and 78% of the injected dose remaining in the brain 24 h and 96 h post-injection, respectively, regardless of the administration technique used (Fig. 1d).

3.2. Importance of the administration route on the activity gradient

To address the distribution of LNC188Re-SSS within the brain, autoradiography views were performed 24 h after SI and CED injections (Fig. 1e–g). Even if biodistributions were similar using SI or CED injections, the distribution within the brain tissue itself revealed the rhenium-188 spread to be greater with CED than SI administrations, as illustrated by LNC188Re-SSS areas of 34.74 ± 0.72 mm² and 21.57 ± 0.78 mm², respectively (p = 0.0004) (Fig. 1e,f). Relative radioactivity was quantified using OptiQuant software and expressed as the mean radioactivity density (DLU/mm²). Results revealed the radioactivity content to be more concentrated for the SI injection compared to the CED, with 64.54 ± 1.95 DLU/mm² and 23.24 ± 2.68 DLU/mm², respectively (p = 0.0006) (Fig. 1e,g).

3.3. Treatment efficacy of fractionated internal radiotherapy at Day 6 and Day 12 following tumor implantation

In addition to characterizing the LNC188Re-SSS distribution, the efficacy of fractionated internal radiation therapy was studied. Rats were treated with stereotactic injections of 2.8MBq of LNC188Re-SSS 6 days (D6) and 12 days (D12) after 9L cell implantation. Depending on the administration technique (SI or CED), four injection protocols were used, notably protocol 1: SI at D6 and D12; protocol 2:
ced at D6 and D12; protocol 3: CED at D6 and SI at D12; protocol 4: SI at D6 and CED at D12. In control group animals, the median survival time was close to 30 days for $^{188}$ReO$_4^-$ and 28 days for both blank LNC and saline solutions (Fig. 2a–d). There were no significant differences between the control groups (p > 0.05), regardless of the injection protocol used. Treatments with LNC$^{188}$Re-SSS were associated with an increased median survival time (IMST) of 37.5% and 35.7% for protocols 1 and 2, with 13% and 0% of long-term survivors, respectively (Fig. 2a,b). Long-term survivors were defined as animals that survived for more than 120 days following 9L cell implantation [35]. Magnetic resonance imaging [36] corroborated this observation, with no tumor progression evolving with a slightly delayed tumor progression. In contrast, protocols 3 and 4, which provided the best survival results during prior treatment, were used. As expected, no significant differences between the control groups were detected, with a median survival close to 28 days. However, with protocols 3 and 4, five out of six rats (83%) were long-term survivors (Fig. 2f,g). MRI confirmed these results, with a tumor lesion at D9 following 9L cell implantation, which grew up until D25 and then regressed, long-term survivor animals being free of brain tumors (Fig. 2h).

3.5. Effect of LNC$^{188}$Re-SSS on the production of peripheral cytokines

As over-expression of interleukin-2 (IL-2) and interferon-γ (IFN-γ) cytokines produced by T cells are important for anti-tumoral brain immune responses [37], these cytokines were quantified at D8, D16, and D24 in blood of control and LNC$^{188}$Re-SSS-treated animals for protocols 3 and 4 (Fig. 3a,b). No significant differences between the control groups were observed (saline solution, blank LNC, and $^{188}$ReO$_4^-$ solution); hence results of control groups were expressed.
Fig. 2. Efficacy of fractionated internal radiation with LNCs loaded with rhenium-188 a–d: Kaplan–Meier survival curves of rats treated at D6 and D12, 5.6MBq of LNC\(^{188}\)Re-SSS (n = 6), 5.6MBq of \(^{188}\)ReO\(_4\) (n = 4), blank LNC (n = 4), and saline solution (n = 4). a: Protocol 1, SI at D6 and D12. One in six rats was a long-term survivor (>120 days). b: Protocol 2, CED injections at D6 and D12. c: Protocol 3, CED and SI at D6 and D12. Three in six rats were long-term survivors. d: Protocol 4, SI and CED injections at D6 and D12. Four in six rats were long-term survivors. e: T2-weighted images of control rats and LNC\(^{188}\)Re-SSS in each protocol of the D6/D12 fractionated internal study. f–g: Kaplan–Meier survival curves of rats treated at D12 and D18, 5.6MBq of LNC\(^{188}\)Re-SSS (n = 6), 5.6MBq of \(^{188}\)ReO\(_4\) (n = 4), blank LNC (n = 4), and saline solution (n = 4). f: Protocol 3, CED and SI at D12 and D18. Five in six rats were long-term survivors. g: Protocol 4, SI at D12 and CED injection at D18. Five in six rats were long-term survivors. h: T2-weighted images of control rats and LNC\(^{188}\)Re-SSS in each protocol of the D12/D18 fractionated internal study.

Fig. 3. Peripheral cytokines (interleukin-2 and interferon-γ) quantification after nanovectorized internal radiotherapy Concentrations of interleukin-2 (IL-2) (a) and interferon-γ (IFNγ) (b) for control group and LNC\(^{188}\)Re-SSS of each protocol. Results are expressed in pg/ml of IL-2 and IFNγ, mean ± SD. Comparison of IL-2 content in LNC\(^{188}\)Re-SSS groups versus control groups; **p < 0.01; ***p < 0.001.
as a mean ± standard deviation of all control groups data. LNC\textsuperscript{188}Re-SSS treatment resulted in an overproduction of peripheral cytokines, as major increases in IL-2 and IFNγ were observed in LNC\textsuperscript{188}Re-SSS groups.

3.6. Recruitment and activation of immune and inflammatory cells within the central nervous system after LNC\textsuperscript{188}Re-SSS treatment

In order to evaluate immunostimulating effects of LNC\textsuperscript{188}Re-SSS versus blank LNC, the immunostaining of central nervous system (CNS) infiltrating or resident immune cells was assessed and illustrated for protocol 4, with results similar to those observed in protocol 3 (Fig. 4a,b). Immunostaining of brain cryosections at D15 demonstrated a stronger activation of monocyte-macrophage-microglia in LNC\textsuperscript{188}Re-SSS treated animals, as proven by the ameboid shape of OX42-positive cells [38,39]. In addition, an improved recruitment of natural killer (CD161a) and dendritic cells (OX62) was observed from D15 to D25, with a slight decrease at D32.

MHC class II (OX6) over-expression in LNC\textsuperscript{188}Re-SSS-treated rats confirmed the recruitment and activation of inflammatory and immune cells in the CNS. Strong induction of MCH class I (OX18), whether present on the glioma cells themselves or on antigen-presenting cells, provided evidence in favor of an improved capability to develop an antitumor immune response. As effectors of the antitumor immune response, such as CD4 and CD8 positive cells, were absent at D15, they were progressively recruited in the CNS tumors at D25 and D32 (Fig. 4a,b).

3.7. Rechallenge in long-term survivors reveals immune protection

To validate this immune response, long-term animal survivors obtained with protocols 3 and 4 were re-challenged with implantation of 1000 9L cells in the left striatum. Regardless of the fractionated internal radiation timing used (D6/D12, Fig. 5a; D12/D18, Fig. 5b), median survival was significantly improved (from 35 to 37 days) when compared to control animals (25 days). Moreover, one long-term survivor was obtained for treatment at D6/D12 with protocol 4 and for treatment at D12/18 with protocols 3 and 4, thus representing three of 17 animals included in the study.

3.8. Treatment efficacy of fractionated external beam radiation at Day 6 and Day 12 following tumor implantation and its immune system effect

External beam radiation was performed and its related-biological effect assessed in order to compare our internal radiation strategy with routine treatment. Rats were treated with 2 × 8 Gy regimen at Day 6 and Day 12 following 9L cell implantation. External beam radiation efficacy resulted in a slight increase with a median survival of 26.5 ± 2.1 days and 33.5 ± 1.5 days for control and treated animals, respectively (Fig. 6a,b). Meanwhile, the immunostaining of CNS infiltrating or resident immune cells revealed a weaker recruitment of immune cells, in particular natural and dendritic cells, which are crucial in adaptive immune responses (Fig. 6c,d).

4. Discussion

In this study, we evaluated fractionated internal radiation therapy using LNC\textsuperscript{188}Re-SSS in an orthotopic 9L Fischer rat glioma model. Survival and immune-related effects induced by the variation in the \textsuperscript{188}Re-activity gradient within the brain parenchyma were investigated.

The first part of this work highlights the advantages of using LNC for entrapping Rhenium-188 as physico-chemical properties of the nanocarrier prevail over those of Rhenium-188. Hence, our data supported that most of rhenium-188 activity from LNC remained confined to the brain until its disintegration.

The originality of our strategy was to use two modes of stereotaxic injections during the fractionated treatment in order to modulate \textsuperscript{188}Re distribution within the brain. Thus, a remarkable survival benefit was only revealed when SI injection was combined with CED, indicating that the \textsuperscript{188}Re-activity gradient is of major significance. This therapeutic effect can be explained by the cellular heterogeneity and the related microenvironment of the tumor mass. Solid tumors are indeed heterogenous from a histology point of view with inflammatory infiltrates and vascular structures [40]. Different subpopulations of cancer cells are hierarchically and topographically organized, with radioresistant cancer initiating cells [41] within either hypoxic or vascular niches [42]. Thus, we can assume that the injection of LNC\textsuperscript{188}Re-SSS by the combination of SI and CED injections targets different types of radiosensitive and radioresistant sub-cellular populations within the tumor mass. As it is more difficult to apply an activity-gradient irradiation within the tumor mass through external beam radiation, being the gold-standard adjuvant treatment for gliomas, these possibilities are important to consider. Corroborating this idea, the fractionated external beam radiation used in this study triggered weaker therapeutic efficiency as compared with internal radiotherapy.

In this study, animals were treated with a combination of SI and CED at Days 6/12 and 12/18 following 9L cells implantation. As no significant differences were noted between early- and late-care of the tumor, tumor size and proliferation gradient did not appear to influence treatment response. This could be explained by a \textsuperscript{188}Re-activity gradient that is sufficient for direct eradication of the entire tumor mass in the two situations (early- and late-care). In addition, the \textsuperscript{188}Re-activity gradient might induce an indirect immune response likely to affect all types of tumor cells. Thus, we have investigated whether an adaptive immune response was involved in tumor regression. According to the scientific literature, radiation after external beam radiation exposure produces an immunogenic death of the most radiosensitive subset of cancer cells [43]. Recent evidence has highlighted the involvement of calreticulin and high-mobility group protein B1 (HMGB1) in the mechanism by which the irradiated tumour can become a source of antigen [19,44]. Our data demonstrated that while there was a recruitment of immune cells with both internal and external radiotherapy, the intensity of this response was weaker after external than internal radiation. In addition, our internal radiation strategy induced a memory anti-tumor response as long-term survivors were partially or totally immunized after re-injecting 9L cells in contrast to naïve animals. As no long-term survivor animals were obtained with external beam radiation, the intensity of the immune response depending on the irradiation mode may play a role. Radiation has been reported to induce up-regulation of MCHI and other pro-
imunogenic effects at the irradiated site [21,45]. As MCHI expression was more important after internal radiotherapy compared with external radiation modality, we assume that tumor cell recognition by the immune system is improved in this context. Moreover, Dewan et al. have shown that two external radiation regimens had similar effects on tumor growth, but led to different synergistic effects when associated with immunotherapy [46]. The conditions of irradiation are of major significance, and the $^{188}$Re-activity gradient used in our study may well play a role by enhancing a particular type of cell death (apoptosis, autophagy, or necrosis), thus leading to tumor eradication or not [47].

The nano-object used for internal radiotherapy can interact with immune responses. As previously shown in scientific literature, the transporters associated with antigen processing (TAP) and multidrug resistance efflux pumps share a significant degree of homology among their transmembrane domains, which are

![Fig. 5. Rechallenge in long-term survivors obtained from the nanovectorized internal radiation studies a: Kaplan-Meier survival curves of re-challenged long-term survivors from the D6/D12 fractionated internal radiation study. b: Kaplan-Meier survival curves of re-challenged long-term survivors from the D12/D18 fractionated internal radiation study.](image)

Fig. 5. Efficacy of fractionated external beam radiation at D6 and D12 following tumor implantation and its immune system effects. a: Kaplan-Meier survival curves of rats treated at D6 and D12 with 2 x 8 Gy (n = 6) and control animals (n = 6). b: T2-weighted images of control and treated rats of the D6/external beam radiation study. c: Immunohistochemistry staining of macrophage cells (OX42), natural killer cells (OX61), major histocompatibility (class I - OX18; class II - OX6), dendritic cells (CD161a), and T lymphocytes cells (CD4 and CD8) of the D6/D12 external beam radiation study. d: Semi-quantitative results of immunohistochemistry. Results are expressed as % of immunostaining area after their determination with MetaMorph software.
thought to be the primary determinants of substrate specificity [48,49]. As nanoparticles interfere with P-gp and reverse multidrug resistance in glioma cells [12], they could promote pro-immunogenic conditions by increasing antigen processing based on interactions with TAP transporters. LNC may also cross biological barriers [12] with endo-lysosomal escape [15], which may impact autophagy cell death [50]. This could be crucial as migrating glioblastoma cells have been shown to be resistant to apoptosis [50].

5. Conclusion

Fractionated internal radiotherapy using LNC 188Re-SSS induced a remarkable survival benefit in rat glioma model, with an unprecedented increase in the number of long-term survivor animals. Those observations are mainly ascribed to 188Re-activity gradient leading to a bypass of immunosuppressive barriers, thus demonstrated by total or partial immunity of rechallenged animals. Hence, the present work strengthens the interest of developing new anti-glioblastoma strategies based on internal radiotherapy using 188Re-lipid nanocapsules associated with immunotherapy.

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