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Research article

Novel model of orthotopic U-87 MG glioblastoma resection in athymic nude mice

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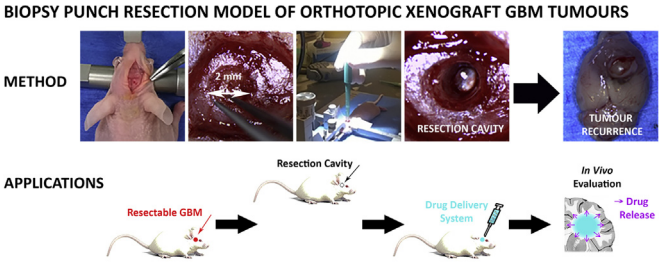
HIGHLIGHTS

- Biopsy punch resection of U-87 MG tumour is effective, affordable and reproducible.
- Biopsy punch cavity suitable for drug delivery system evaluation.
- Clinically relevant model for studying GBM treatments.

GRAPHICAL ABSTRACT



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ABSTRACT

In vitro and *in vivo* models of experimental glioma are useful tools to gain a better understanding of glioblastoma (GBM) and to investigate novel treatment strategies. However, the majority of preclinical models focus on treating solid intracranial tumours, despite surgical resection being the mainstay in the standard care of patients with GBM today. The lack of resection and recurrence models therefore has undermined efforts in finding a treatment for this disease. Here we present a novel orthotopic tumour resection and recurrence model that has potential for the investigation of local delivery strategies in the treatment of GBM. The model presented is simple to achieve through the use of a biopsy punch, is reproducible, does not require specific or expensive equipment, and results in a resection cavity suitable for local drug delivery systems, such as the implantation or injection of hydrogels. We show that tumour resection is well tolerated, does not induce deleterious neurological deficits, and significantly prolongs survival of mice bearing U-87 MG GBM tumours. In addition, the resulting cavity could accommodate adequate amounts of hydrogels for local delivery of chemotherapeutic agents to eliminate residual tumour cells that can induce tumour recurrence.

1. Introduction

Glioblastoma (GBM) is the most common and aggressive malignant tumour of the central nervous system in adults. These tumours show a high proliferation rate with diffuse infiltration of adjacent brain tissue (Bernardi et al., 2009). Conventional therapeutic procedures, aiming at increasing patient life expectancy, focus on maximal surgical resection combined with adjuvant radiotherapy

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and/or chemotherapy by oral delivery of Temozolomide (TMZ) (Stupp et al., 2005). However, tumour recurrences due to residual infiltrative cells at the resection margin are inevitable, leading to a median survival of about 14 months, with a 5 year-life expectancy of less than 10% (Lefranc et al., 2006). In consequence, there is a much unmet medical need that necessitates solving. Innovative drug delivery systems aiming at delivering drugs to the tumour site present a promising approach in treating this disease (Bastiancich et al., 2016). The local drug delivery of cytotoxic agents, using injectable systems in the tumour resection cavity with sustained drug release characteristics, aims at preventing the growth of cancer cells that cannot be resected during surgery.

Local delivery seems very promising for the treatment of GBM for a number of reasons. One is that it allows for bypassing the blood brain barrier through direct administration of a drug into the brain. Another is that sustained drug release, reaching therapeutic concentrations at the tumour site without involving other organs, can be obtained (Bastiancich et al., 2016). The rationale for the use of local delivery strategies in GBM treatment has been highlighted by approval of Gliadel® by the FDA. However, due to some conflicting results being obtained with the use of Gliadel®, and limitations in current treatment options available, novel avenues of treating GBM through local drug delivery strategies need to be investigated (Ashby et al., 2016; Bock et al., 2010).

To evaluate the efficacy of these drug delivery systems on GBM recurrence, a clinically relevant tumour resection model is needed. Despite many preclinical studies, most *in vivo* GBM models do not mimic the clinical scenario of surgical debulking and instead focus on treating solid intact intracranial tumours. Therefore, in light of the central role of tumour resection in clinical therapy, development of rodent models of GBM resection and recurrence are a necessity (Kauer et al., 2011), and indeed, several models do currently exist. Akbar and colleagues were the first to perform an intracranial resection in a rat model of C6–green fluorescent protein (GFP) intracranial glioma model. Through the use of a fluorescent dissecting microscope, they were able to detect the tumour and subsequently guide a suction tip that allowed for the precise microsurgical resection of the tumour (Akbar et al., 2009). This method was also reproduced in nude rats by Denbo and colleagues (Denbo et al., 2011), while Kauer and colleagues further modified it to develop an efficient GBM subtotal resection model in nude mice (Kauer et al., 2011). A simplified technique, using mere aspiration for 5 s to remove a GBM tumour in rats, has also been reported, although it was found less effective in completely or efficiently resecting the tumour tissue, with no difference in survival observed between resected and control animals (Ozeki et al., 2012). Nevertheless, the drawback of these techniques is the need of specific or expensive equipment that are not always available.

To provide a clinically relevant model for studying GBM treatments, we developed a novel approach for resection of U-87 MG mouse intracranial GBM in a validated and reproducible manner, using a biopsy punch. The advantages that our resection technique provides include simplicity, reproducibility, and the lack of necessity for any specific or expensive equipment.

2. Materials and methods

2.1. Animals

All experiments were conducted on six week old, female, specific opportunistic pathogen-free (SPOF) NMRI nude mice (Janvier, France) in accordance with Belgian national regulation guidelines as well as with EU Directive 2010/63/EU. All experiments were approved by the ethical committee of the Université catholique de Louvain (2014/UCL/MD/004). Mice were maintained on standard laboratory food and water *ad libitum*, with a 12 h artificial light/dark cycle.

2.2. Cell culture

U-87 MG glioma cells (ATTC, USA) were cultured in Eagle's Minimum Essential Medium (EMEM; ATTC, USA), supplemented with 10% foetal bovine serum (Gibco, USA), 100 U/mL penicillin and 100 μg/mL streptomycin (Gibco, USA). Cells were cultured as monolayers in 75 cm^2 culture flasks (Sigma, USA) and maintained at 37 °C/5% CO_2.

2.3. Orthotopic U-87 MG human glioblastoma tumour model

For the intracranial glioma model, animals were anesthetised by intraperitoneal injection of ketamine/xylazine (100 and 13 mg/kg, respectively) and positioned in a stereotactic frame. Once immobile, an incision 5 mm long was made along the midline. A burr hole was drilled into the skull at the right frontal lobe, 0.5 mm posterior and 2.1 mm lateral to the bregma using a high-speed drill (Dremel Inc., USA). A 5 μL Hamilton syringe fitted with a 26 gauge needle was used to inject 2.5 μL of complete culture medium containing 3×10^4 U-87 MG glioma cells at the junction between the cortex and striatum at a depth of 2.5–3.0 mm from the outer border of the cranium over a five minute period. After injection, the needle was kept in place for 5 min before slowly being extracted to prevent a vacuum and cell build-up into the needle track. The wound was then sutured and the animals were allowed to awaken under an infrared heating lamp (Danhier et al., 2015). No post-surgery analgesics were administered following the procedure. Animals awoke and were active between 1 and 2 h following surgery and did not display any signs of distress. The presence, volume and localisation of tumours was determined by Magnetic Resonance Imaging (MRI) between day 9 and 12 post inoculation of the U-87 MG cells. Animals were killed when they presented ≥20% body weight loss or 10% body weight loss plus clinical signs of distress (paralysis, arched back, lack of movement).

2.4. Magnetic resonance imaging

MRI was performed using a 11.7 T Bruker Biospec MRI system (Bruker, Germany) equipped with a 1 H quadrature transmit/receive surface cryoprobe after anesthetising animals with 1% isoflurane mixed with air (2.5% for induction, 1% for maintenance). Respiration was continuously monitored while animal core temperature was maintained throughout the experiment by hot water circulation in the cradle. Tumour volume was assessed using rapid acquisition with relaxation enhancement (RARE) sequence (TR = 2500 ms; effective echo time (TE_{eff}) = 30 ms; RARE factor = 8; FOV = 2 × 2 cm; matrix 256 × 256; twenty-five contiguous slices of 0.3 mm, $N_{average}$ = 4). Volumes were calculated from manually drawn region of interest (ROI).

2.5. Biopsy punch resection of tumour mass

On the 13th day post-inoculation of the tumour, mice were randomly assigned into control (no resection, no treatment) or resection (resection, no treatment) groups (n = 11 in each group). For intracranial glioma resection, animals were anaesthetised with ketamine/xylazine as described above before being immobilised in a stereotactic frame. A 7 mm incision was made in the midline along the previous surgical scar. The periosteum was removed revealing the bregma and previous burr hole. A high-speed drill was used to thin the skull area centred around the burr hole, after which fine tip tweezers (Dumont, Switzerland) were used to obtain a 2.1 diameter circular cranial window exposing the brain. A biopsy punch

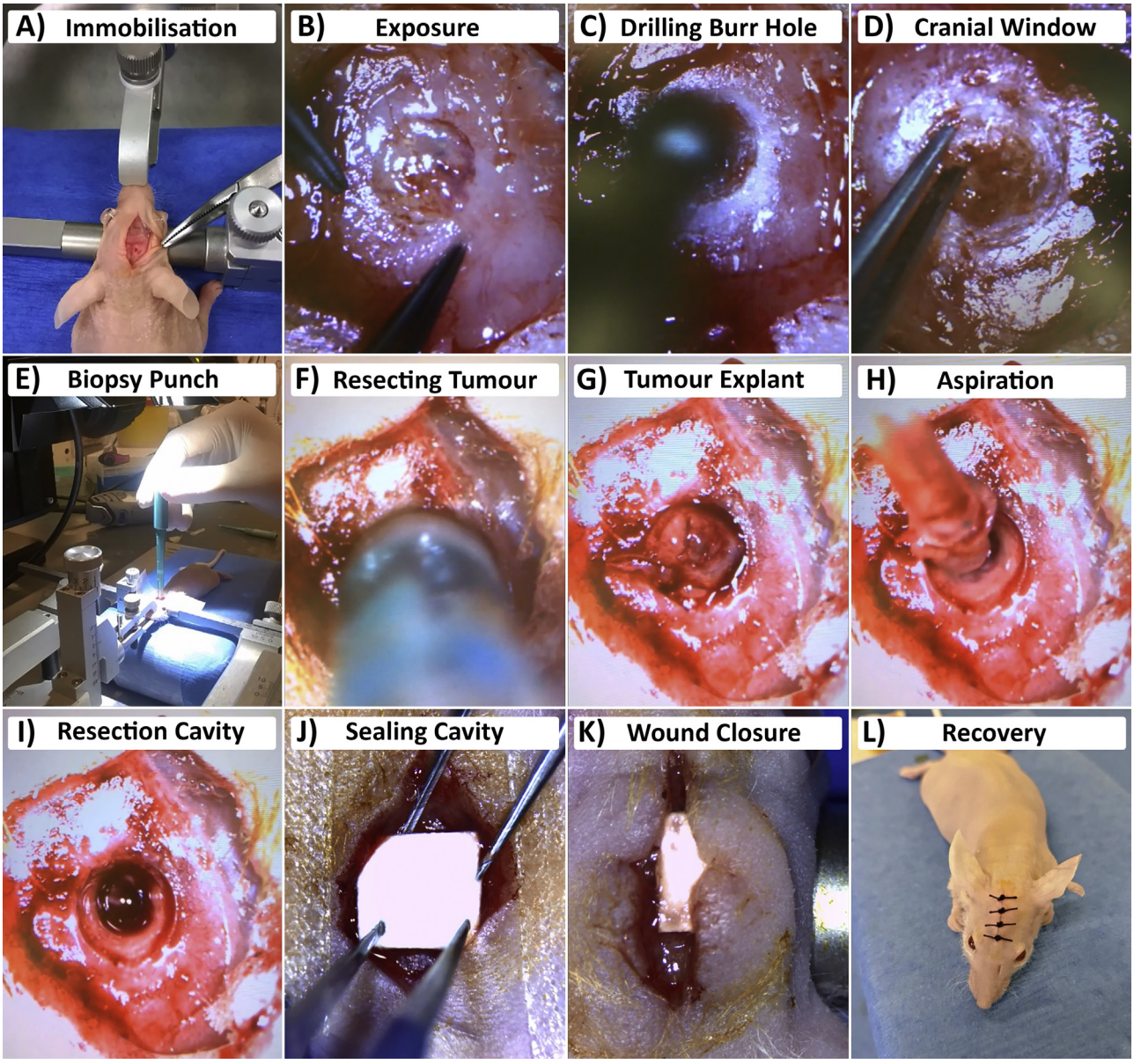

Fig. 1. Orthotopic U-87 MG tumour resection using a 2 mm biopsy punch. A: Immobilised mouse on a stereotactic frame; B: Previous burr hole; C: Drill-assisted widening of previous burr hole; D: Manually expanding cranial window to a 2.1 mm diameter; E: Insertion of biopsy punch, 3 mm deep; F: Resection by twisting for 15 s; G: Tumour explant; H: Aspiration of resected tumour tissue; I: Resection cavity; J: Sealing of cavity with Neuro-Patch®; K: Wound closure; L: Sutured mouse, recovering.

(7 mm long, 2 mm Ø, Kai Medical, Germany) was limited to and inserted 3 mm deep and twisted for 15 s to cut the brain/tumour tissue. Once withdrawn, a Pasteur pipette connected to a diaphragm vacuum pump (Vaccubrand GMBH + CO KG, Germany) was used to remove the explant and blood build up. Residual blood was removed by allowing an absorbable haemostatic triangle (Fine Science Tools, Germany) to rest in the formed cavity. Once stabilised, the dural window was repaired by covering with a 4 mm × 4 mm square piece of Neuro-Patch® (Aesculap, Germany) impregnated with a reconstituted fibrin hydrogel (25 mg/mL fibrin, 10 IU/mL thrombin, equal volumes; Baxter Innovations, Austria). The wound was closed with 3-0 Vicryl sutures and the animals allowed to recover. No post-surgery analgesics were administered following the procedure. Animals awoke and were active between 1 and 2 h following surgery and did not display any signs of distress. Each step of the biopsy punch resection procedure is outlined in Fig. 1. Weight and behaviour were monitored over time as described above. To evaluate tumour growth and the efficacy of tumour resection, at least 3 mice were sacrificed at day 13 before and after resection. Once experimental endpoints of survival had been reached, the brains were extracted and fixed in 10% formalin solution (Merck, Germany) overnight. The brains were then rinsed in PBS, cryoprotected with 30% sucrose solution for 24 h, snap frozen and stored at −20 °C until analysed.

2.6. Histological analysis of recurrent tumours

Brains were sectioned at 12 μm using a Leica CM 1950 cryostat (Leica Biosystems Nussloch GmbH, Germany) and stored at −20 °C until used. For histological analysis, slides were allowed to dry at room temperature overnight before being subjected to haematoxylin & eosin (H&E) staining or immunofluorescence. For H&E staining, samples were processed using a Sakura DRS 601 automated slide stainer (Sakura Finetek Europe, The Netherlands). For immunofluorescence, brain sections were rehydrated and blocked for 1 h at room temperature in a blocking solution consisting of

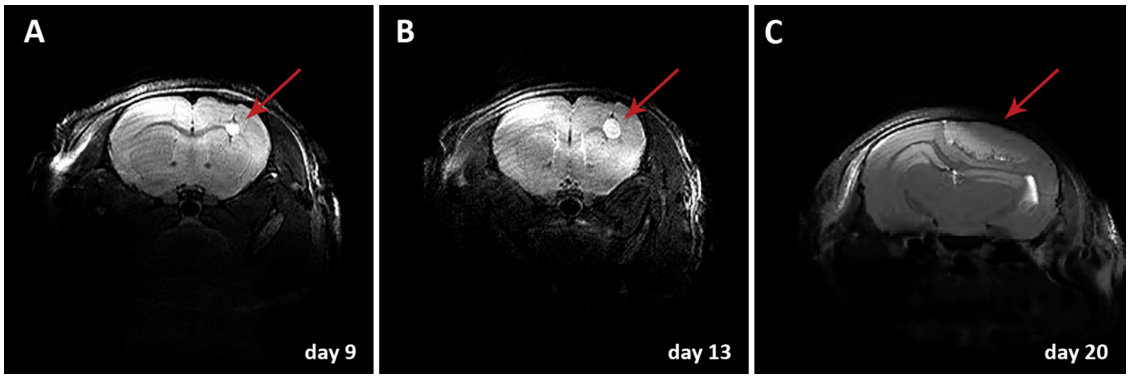

Fig. 2. Coronal (T_2-weighted) images of U-87 MG tumours obtained through MRI that monitor tumour growth at day 9 (A), day 13 (B), and day 20 (C) post implantation. Lighter zones indicated by red arrows show tumour location within the brain high in the right striatum and lower cortex of U-87 MG inoculated mice. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

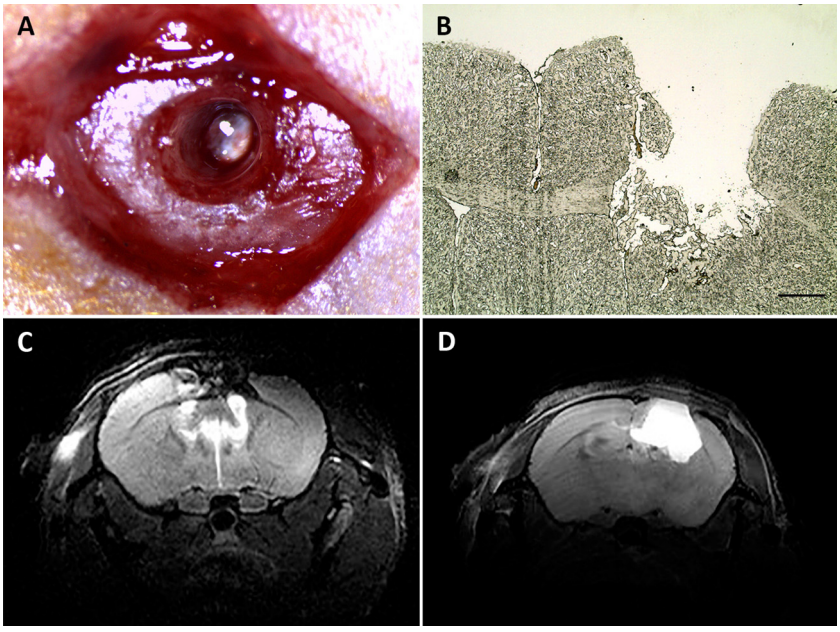

Fig. 3. A: Cavity, immediately post-resection and cleared of blood, showing a defined edge; B: Cryosectioned brain immediately following resection; C: MRI scan following resection at day 7 showing the extent of tissue removal; D: MRI scan at day 7 post resection, showing fluid build-up within the resection cavity, indicated by the hypersignal. Scale bar in B = 400 μm.

10% normal goat serum, 2% bovine serum albunin (BSA), and 0.01% Triton X-100 in PBS. A rabbit polyclonal anti-Glial fibrillary acidic protein (GFAP) (1:800 in blocking solution, Abcam, UK) antibody was used to identify normal brain tissue, while a mouse monoclonal anti-human mitochondria (1:800 in blocking solution, Abcam, UK) antibody was used to identify U-87 MG cells by incubation for 1 h at room temperature. Sections were then rinsed with PBS (3 × 5 min washes) after which Alexafluor antihost IgG antibodies (1:400 in PBS containing 0.5% BSA, Invitrogen, USA) were applied for 1 h at room temperature and away from light. Sections were rinsed again with PBS (3 × 5 min washes) before cell nuclei were counterstained through staining with DAPI (1 μM in PBS, Sigma, USA) for 10 min and away from light following the secondary antibodies. Sections were mounted with Vectashield hard set mounting medium (without DAPI, Vector Laboratories) and stored away from light until analysed. Digital images were acquired using an EVOS fluorescent microscope. For survival, statistical significance was analysed using the Wilcoxon test in GraphPad Prism (GraphPad Software, USA) and determined based on $p < 0.05$. Images were processed using Adobe Photoshop.

3. Results

3.1. Tumour implantation

A modification of a previously described U-87 MG cell inoculation protocol to induce tumours in nude mice was used in this study (Danhier et al., 2015). MRI analysis of tumours at different time points after cell implantation showed that developing tumours had volumes of 0.2 ± 0.1 μL at day 9, and 0.4 ± 0.2 μL at day 13 ($n = 11$), with a median survival of 24 days being observed in animals in which tumour development was left unhindered. Considering these results, we determined that the optimal day for resection using a 2 mm biopsy punch was 13 days post inoculation. Before day 13, tumours were considered inadequate in size, therefore all tumour tissue could be removed, resulting in tumour recurrences not being visualised. Beyond day 13, the exponential development of tumours resulted in overgrowth that could not be excised adequately, leaving the majority of tumour intact following resection (Fig. 2).

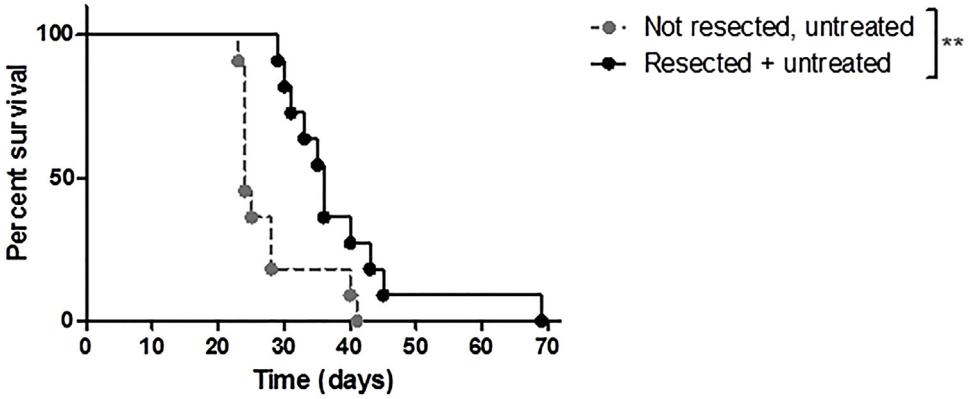

Fig. 4. Kaplan-Meier survival curve for resected, untreated animals and non-resected, untreated animals ($n = 11$ for both groups).

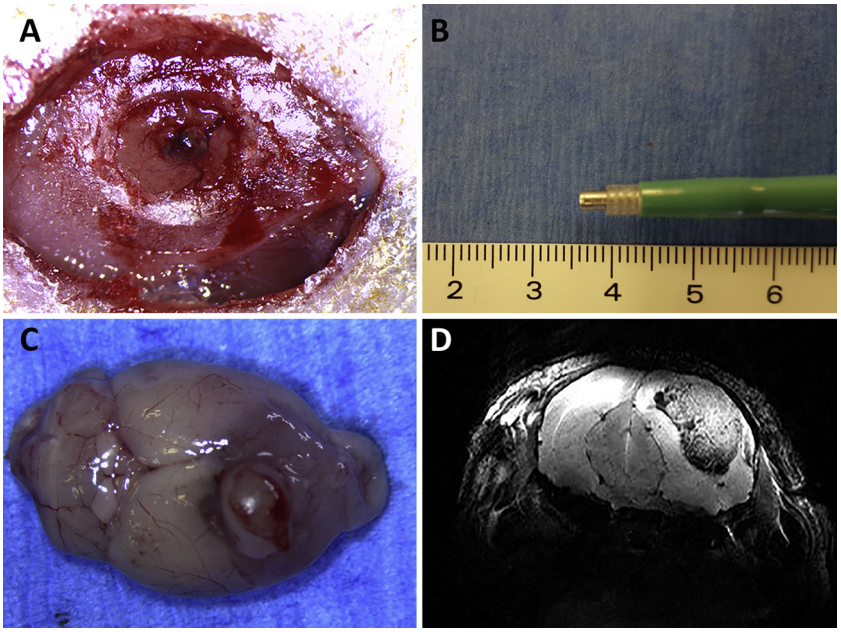

Fig. 5. A: A cranial window at 13 days post inoculation. The dark, blood vessel rich central area corresponds to the tumour; B: A disposable 2 mm Ø biopsy punch limited to 3 mm in length used for tumour resection; C: Tumour recurrence at day 36; D: MRI scan showing the extent of recurrence at 31 days post resection.

3.2. Tumour resection using a biopsy punch

The biopsy resection procedure can be fully completed within 25–30 min per animal. The two main difficulties that could arise during this procedure are swelling of the brain parenchyma, restricting cavity size and formation, and excessive bleeding. Swelling is the brain parenchyma, thus potentially constricting the formed cavity, occurred to varied degrees in all animals undergoing the procedure. Tumour location, and in particular the presence of large blood vessels above its location, was a main factor that influenced the extent of bleeding during resection. Bleeding usually abated within 2–3 min following resection. No hemiplegia was observed due to the resection procedure.

3.3. Visualisation of cavity post resection

Following resection, a number of animals were immediately sacrificed to observe cavity formation. We observed that a cavity with clearly defined borders forms (Fig. 3A). The theoretical volume of the cavity, when using a 2 mm biopsy punch, equals to 9.42 μL. Once bleeding was abated, 5 μL of liquid or gel could fit adequately within the freshly formed cavity (not shown). Analysis of cryosectioned brains bearing a cavity also confirmed the formation of defined borders and resection volume (Fig. 3B). Animals that had undergone resection were also imaged using MRI 7 days after resection (Fig. 3C, D). Scans showed that the cavity remained intact, and could be distinguished at 1 week post resection. MRI scans also revealed that fluid accumulation within the cavity can occur in some animals (hypersignal in T_2-weighted image, Fig. 3D), although they did not reveal when they commence or if the fluid diffuses back into the surrounding parenchyma. Nevertheless, the presence of fluid did not influence survival or behaviour of the animals, and most importantly, did not influence recurrence.

3.4. Survival following U-87 MG tumour resection

A Kaplan-Meier survival curve showed a significant increase in survival ($p = 0.0021$) of mice that had undergone biopsy punch resection (median survival 36 days) to those mice not receiving surgical resection (median survival 24 days) following U-87 MG inoculation and tumour formation (Fig. 4). Resection did not result in any observable side effects or deficits in neurological function. Once recurrence had taken hold, the health of the animals deteriorated rapidly to include marked weight loss, hunching of the

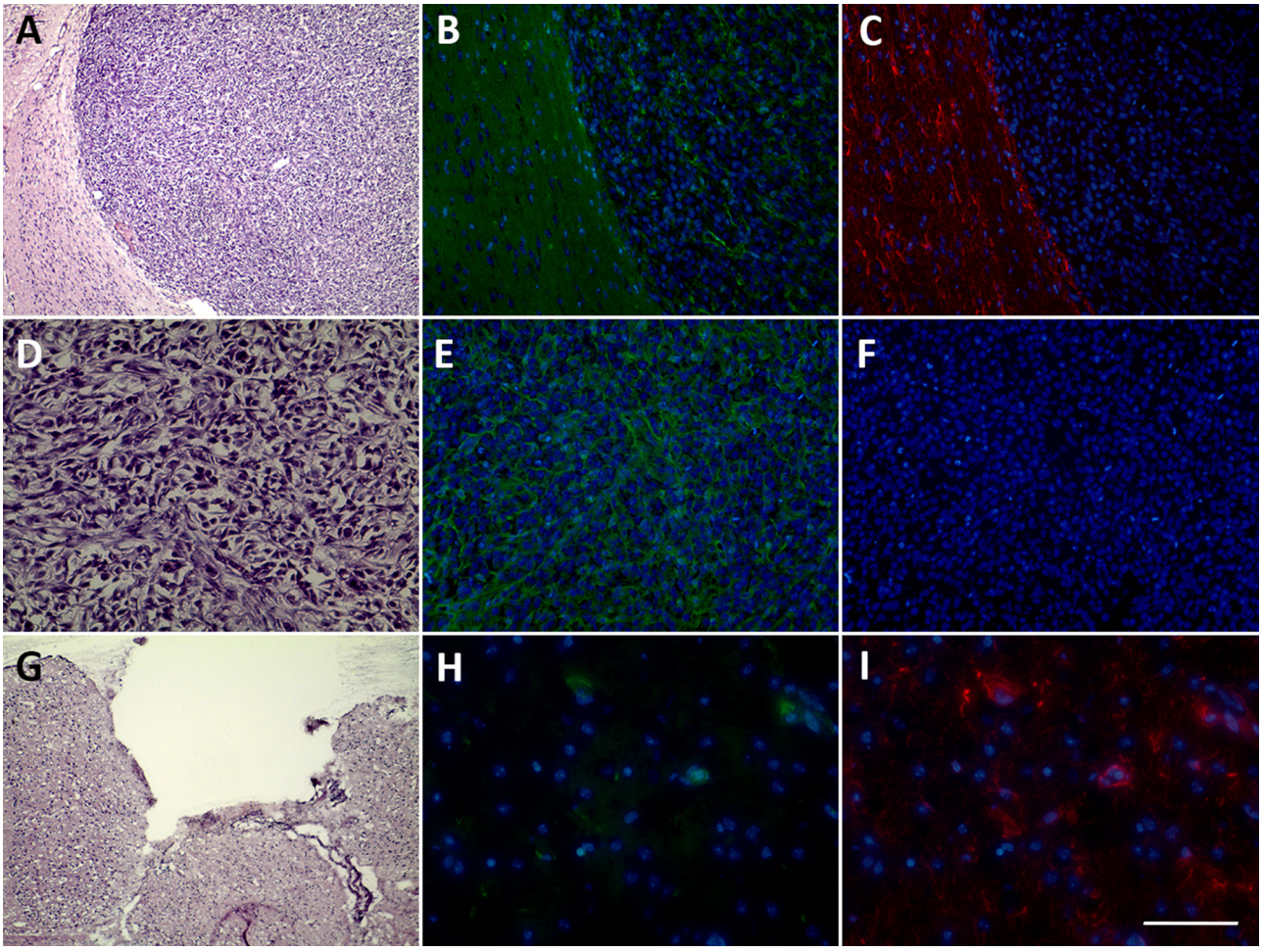

Fig. 6. A: H&E staining showing a recurred tumour and the distinct border between cancerous and normal tissue; B: Anti-human mitochondria staining of U-87 MG cells within the tumour tissue; C: Anti-GFAP staining showing normal brain parenchyma next to the tumour mass; D: H&E staining of within the recurred tumour mass; E: Anti-human mitochondria staining within the recurred tumour mass; F: No GFAP staining observed within the tumour mass; G: H&E staining of resected tumour; H: Anti-human mitochondria staining of tissue bordering resection cavity; I: Anti-GFAP staining of tissue bordering resection cavity. Scale bar: A, G = 400 μm; B, C, E, F = 200 μm; D, H, I = 100 μm.

back, disorientation, ultimately resulting in death or sacrifice. Paralysis was observed in a number of mice early during the recurrence growth of the tumour.

3.5. Visualisation of tumour recurrence

After inoculation with U-87 MG cells, and following 13 days of growth, a thickened and vascularised periosteum could be observed above and around the original burr hole. Upon creation of the cranial window, the majority of animals presented a vascularised growth centred on the injection site that corresponded to the underlying tumour (Fig. 5A). A biopsy punch, limited to 3 mm in length with surgical tape (Fig. 5B) was then centred above the tumour and used to make the resection cavity. Tumour recurrence, observed in all resected animals, was robust and aggressive, often extending from the resection cavity borders into other brain regions through growth and compression of healthy brain tissue (Fig. 5C, D).

3.6. Histological evaluation of U-87 MG orthotopic tumour and resection

H&E and immunofluorescence were used to further confirm the efficiency of resection using a biopsy punch (Fig. 6). As U-87 MG tumours lack glial fibrillary acidic protein (GFAP) immunoreactivity, an anti-GFAP antibody was used to distinguish normal brain tissue versus cancerous tissue. The human origin of U-87 MG allowed for the use of an anti-human mitochondria antibody to identify cancerous growth. A clear and distinct border could be seen between normal and cancerous tissue (Fig. 6A–C). It is clearly seen that GFAP positive cells are only present in normal brain tissue. Although some auto-fluorescence of the brain tissue is present, human mitochondria stained cells can be seen within the GFAP negative tumour. Cancerous tissue was shown to exhibit distinctly different morphology and density to normal mouse brain tissue, showing profuse human mitochondria staining, while being negative to GFAP (Fig. 6D–F). H&E staining did not show presence of tumour at or around the resection site (Fig. 6G). Even though U-87 MG is not an invasive cell type (Jacobs et al., 2011), sparse staining of anti-human mitochondria cells could be observed at the resection border (Fig. 6H), although GFAP staining was abundant (Fig. 6I). It could be implied that, even though not invasive, enough U-87 MG cells were left unresected following our resection to induce tumour recurrence in our model.

4. Discussion

Aggressive surgical resection is an important factor for improved outcomes when treating gliomas. Increased volumetric extent of resection has been directly correlated with improved sur-

vival in low as well as in high grade gliomas such as GBM (Chaichana et al., 2014; Fukui et al., 2016; Sanai et al., 2011). To this day, surgical resection remains as the main component in treating GBM. However, the focus of most preclinical models is on treating established tumours, with only a limited number of animal models currently available that focus on resecting tumours. In view of this shortage, we developed a novel, simplified and reproducible intracranial resection mouse model. Even though we used the non-invasive U-87 MG cell for tumour formation, the purpose of this study was to develop a resection and recurrence model that could be implemented in varying treatment modalities for GBM. For example, the resulting resection cavity will allow us to investigate novel treatment strategies based on local delivery of bioactive molecules. One strategy that has shown potential in treating GBM is the use of hydrogels for local delivery of anti-cancer agents, permitted by their unique properties. Because hydrogels have a hydrophilic but cross-linked structure, they are able to absorb large amounts of water or biological fluid without the dissolution of the polymer (Hoare and Kohane, 2008). Following surgery, drug-loaded hydrogels could be administered directly into the brain, either within the tumour or following resection (Bastiancich et al., 2016), overcoming technical issues such as the limitations of the blood brain barrier.

It has previously been established that GBM recurs within 2 cm of the resected tumour site in 90% of cases (Hochberg and Pruitt, 1980). Our resection model, although not invasive, is subtotal and thus leads to tumour occurrence. We chose to resect at day 13 post inoculation, and observed a median post resection survival of 23 days. If a strategy using bioactive molecules would be investigated, this survival period provides ample time to assess the effects of anticancer drugs in eliminating tumour cells that have remained after resection, in turn delaying tumour recurrence. As mentioned above, U-87 MG cells do not infiltrate deep into the brain after inoculation. However, our model could also be applied to other xenograft models that could mimic a wider range of clinical hallmarks associated with GBM, including brain invasion.

All mice that had a confirmed tumour at day 13, as seen by MRI, achieved 100% recurrence following resection. Given that surgery in GBM patients is clearly beneficial with its primary goal of maximising the extent of resection while minimising injury, mortality and morbidity was not observed in our model during the biopsy punch resection procedure. A few mice died shortly after inoculation with U-87 MG cells within the brain, while one mouse was sacrificed (at day 55 post resection) due to a cutaneous infection not related to the inoculation or resection. Overall, the resection and recurrence model we have developed was well tolerated, and could serve as a template for future studies investigating treatment options for GBM.

5. Conclusions

We have established a reproducible surgical resection and recurrence mouse model of GBM devoid of injurious neurological outcomes following surgery. This model has applications in investigating intracavity mediated distribution of bioactive materials for local delivery of anticancer agents in the treatment of GBM in a pre-clinical model.

Conflict of interest

The authors report no conflicts of interest

Acknowledgments

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