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Laboratory Investigation

The effect of thermotherapy using magnetic nanoparticles on rat malignant glioma

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Summary

Thermotherapy using magnetic nanoparticles is a new technique for interstitial hyperthermia and thermoablation based on magnetic field-induced excitation of biocompatible superparamagnetic nanoparticles. To evaluate the potential of this technique for minimally invasive treatment, we carried out a systematic analysis of its effects on experimental glioblastoma multiforme in a rat tumor model.Tumors were induced by implantation of RG-2-cells into the brains of 120 male Fisher rats. Animals were randomly allocated to 10 groups of 12 rats each, including controls. Animals received two thermotherapy treatments following a single intratumoral injection of two different magnetic fluids (dextran- or aminosilane-coated iron-oxide nanoparticles). Treatment was carried out on days four and six after tumor induction using an alternating magnetic field applicator system operating at a frequency of 100 kHz and variable field strength of 0–18 kA/m. The effectiveness of treatment was determined by the survival time of the animals and histopathological examinations of the brain and the tumor.Thermotherapy with aminosilane-coated nanoparticles led up to 4.5-fold prolongation of survival over controls, while the dextran-coated particles did not indicate any advantage. Intratumoral deposition of the aminosilane-coated particles was found to be stable, allowing for serial thermotherapy treatments without repeated injection. Histological and immunohistochemical examinations after treatment revealed large necrotic areas close to particle deposits, a decreased proliferation rate and a reactive astrogliosis adjacent to the tumor.Thus, localized interstitial thermotherapy with magnetic nanoparticles has an antitumoral effect on malignant brain tumors. This method is suitable for clinical use and may be a novel strategy for treating malignant glioma, which cannot be treated successfully today. The optimal treatment schedules and potential combinations with other therapies need to be defined in further studies.

Introduction

Glioblastoma multiforme (WHO grade IV) is the most malignant intracranial tumor. Median overall survival after first-line therapy does not exceed 12–15 months [1], because the invasive nature of this malicious brain tumor makes a complete resection almost impossible. Despite conventional therapeutic treatment patterns consisting of surgery, radiation and/or chemotherapy, there has been only little increase in overall survival over the last decade.

Although the biological effectiveness of heat in treating cancer is known for decades and the corresponding molecular mechanisms are partly understood, hyperthermia is not yet established in clinical routine. Conventional hyperthermia procedures use different energy sources for the generation of heat within the tissue: externally applied electromagnetic waves (e.g. radiofrequency or microwaves), ultrasound (external or interstitial), current flow between two or more electrodes, electric or magnetic fields between implanted antennas, or electrically or magnetically induced thermoseeds [2].

These conventional techniques have inherent disadvantages that can negatively affect temperature distribution. Especially in hyperthermia of glioma, the main obstacle to clinical efficacy has been the inability to obtain sufficiently high intratumoral temperatures.

A new nanotechnology based thermotherapy using magnetic nanoparticles, also referred to as magnetic fluid hyperthermia (MFH) was developed in our group over more than a decade [3–6]. In contrast to E-field dominant systems used in regional hyperthermia, boundaries of different conductive tissues do not interfere with power absorption, rendering this an attractive new method for thermotherapy of deep-seated tumors.

In our technique, the energy is coupled magnetically to superparamagnetic iron-oxide nanoparticles. After direct injection into the target region, the particles generate heat by Brownian and Néel relaxation processes in an externally applied alternating magnetic field [3]. *In vitro* experiments with magnetic fluids have confirmed their excellent power absorption capabilities [4].

In vivo experiments have documented the feasibility and good overall tolerability of this technique, and significant antitumoral effects in a murine model of

mammary carcinoma [5] and in a rat model of prostate cancer have been described [7–9].

Other groups have evaluated similar techniques of magnetically mediated hyperthermia using nanoscaled magnetites in animal models of melanoma [10,11], breast tumors [12] and prostate cancer [13].

First clinical experiences with thermotherapy using magnetic nanoparticles on prostate carcinoma have been published recently [14].

Here, we present the results of a systematic analysis of the effects of thermotherapy using magnetic nanoparticles on the RG-2 tumor of rats, an established animal model developed to study gliomas [15].

Materials and methods

Tumor cell line

Rat glioma cells (RG-2) were grown in Dulbecco's Mod Eagle (GIBCO, Gaithersburg, MD) medium with 20% fetal calf serum (Invitrogen) at 37 °C (5% CO_2), until they reached 90% confluence. The medium was removed, and the cells were released by incubation with trypsin (0.04% trypsin/EDTA). The cells were centrifuged at 800 rpm for 5 min, resuspended in Dulbecco's medium to a final concentration of 10^5 cells/10 μl and stored on ice until implantation.

Animals

A total of 120 male F-344 Fisher rats (180–220 g) were obtained from Charles River (Sulzfeld, Germany). Animal care was in accordance with German institutional guidelines. Animals received pellet food and water *ad libitum*.

Tumor model and treatment

The rats were anesthetized with an intraperitoneal injection of ketamine (55 mg/kg b.w.) and xylazine (11 mg/kg b.w.) and placed in a stereotactic head frame (Stoelting Physiology Research Instruments, Wood Dale, IL, USA). The scalp was cleaned with 70% ethanol, incised medially, and the tissue was carefully removed until the bregma was identified. A 1-mm hole was drilled into the skull located 2 mm posterior and 2 mm lateral right from the bregma. A Hamilton syringe with a 26-gauge needle was filled with the cell solution and lowered stereotactically 5 mm below the skull into the thalamus region. Ten microliter of the cell solution was expelled by hand over a time period of 1 min, after which the needle was left in position for 2 more minutes. To minimize suction of the injected solution back up the needle tract, the needle was raised very slowly before removal from the brain. The burr hole was sealed with bone wax, and the scalp was closed.

Animals were randomly allocated to 10 groups of 12 rats each, including four control groups (C) and six treatment groups (T). Four days after tumor cell inoculation mean tumor diameter was 3–4 mm, as confirmed by magnetic resonance (MR) imaging (Signa 3.0T, GE Healthcare, Milwaukee, WS, USA) using a small circularly polarized surface coil (diameter 2 cm; Rapid Biomedical, Wuerzburg, Germany) and a T_1-weighted pulse sequence (magnetization-prepared 3-dimensional gradient-echo, MP-3DGE; repetition time, 13 ms; echo time, 4.0 ms; inversion time, 500 ms; flip angle, 20°; spatial resolution $117\times156\times700$ μm^3) (Figure 1). At this stage of tumor growth intratumoral administration of the magnetic fluids was performed stereotactically under anesthesia. Twenty microliter were injected into the tumor region through the existing burr hole via a 23-gauge needle within 20 min by means of an infusion pump (Precidor, Infors AG, Basel, Switzerland).

Magnetic fluids

Two magnetic fluids with different coatings of the nanoparticles were tested in this study: Carboxydextran-coated DDM128 P6 (Schering AG, Berlin, Germany) with an average core diameter of 3 nm and aminosilane-coated MFL AS (MagForce Nanotechnologies AG, Berlin, Germany) with an average core diameter of 15 nm. The iron concentrations in the applied aqueous solutions were 1.8 (DDM128 P6) and 2.0 (MFL AS) mol/l.

Thermotherapy

A single temperature probe with a diameter of 0.55 mm of a 4-channel fiberoptic thermometry device (Luxtron Corp. Santa Ana, CA, USA) was introduced into the tumor via a 22G catheter. Thermotherapy was carried out under anesthesia using an alternating magnetic field applicator system (MFH 12-TS, MagForce Nanotechnologies, Berlin, Germany) operating at a frequency of 100 kHz and variable field strength of 0–18 kA/m.

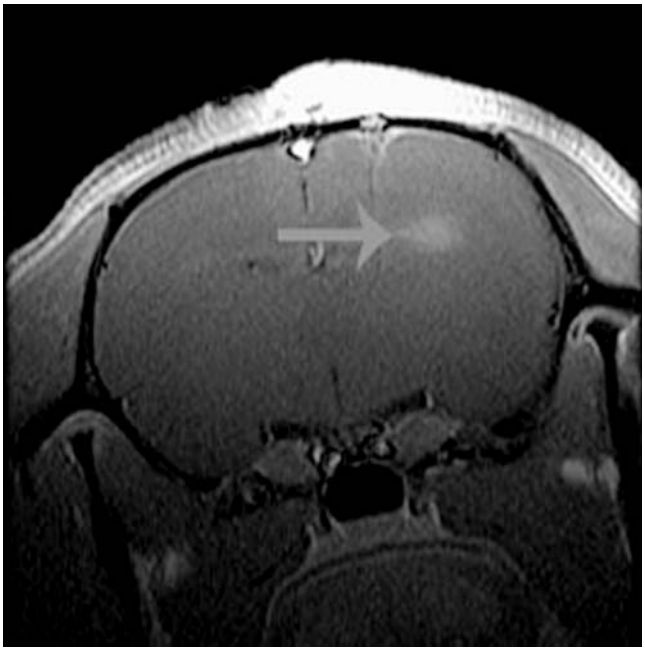

Figure 1. Frontal slice of T_1-weighted contrast-enhanced (gadoteridol) MR image of a rat brain showing the contrast-enhancing RG-2 tumor (arrow). For parameters used for MR imaging refer to the text.

Intratumoral and rectal temperatures were monitored online. Treatments were carried out directly after the administration of the magnetic fluid on day 4 and on day 6 after tumor cell inoculation.

During treatment, the magnetic field strength was gradually increased, starting from 60% of the available maximum and reaching the desired value after 10 min. Treatment duration at this power level was 30 min (Figure 2).

Animals of group C-0 did not undergo any invasive procedure and served as tumor growth controls. Animals of group C-A and C-B received intratumoral injections of the magnetic fluids DDM128 P6 and MFL AS, respectively, but were not subsequently exposed to the alternating magnetic field. Animals of group C-F received 20 μl of normal saline and subsequent exposure to the magnetic field. The treated animals were divided into six groups according to the magnetic fluid applied and the achieved treatment temperatures. Group T-DDM128 P6 received dextran–magnetite and thermotherapy at 39 °C. All other groups (T-MFL AS) were treated at temperatures between 43 °C and 47 °C for 30 min after injection of aminosilane–magnetite.

Histological and immunohistochemical examinations

After deaths of the animals, brains were removed and fixed in 4% formaldehyde. Tissue samples were routinely paraffin embedded, subsequently cut in 4 μm sections and further subjected to HE, Prussian blue (Perl's stain) or immunohistochemical staining. For immunohistochemistry, sections were dewaxed, rehydrated, and microwaved for 12 min in 10 mM citrate buffer, pH 6.5, at 600 W (Bosch, Berlin, Germany). Endogenous peroxidase activity was blocked by incubating sections with 0.6% hydrogen peroxide for 15 min at room temperature. Subsequently, sections were incubated with a 1:20 dilution of normal rabbit or swine serum in PBS for 20 min, and then incubated with either monoclonal mouse anti-rat proliferating cell nuclear antigen (PCNA) antibody (Oncogene, USA) or polyclonal rabbit anti-rat glial fibrillary acidic protein (GFAP) antibody (DAKO, Hamburg, Germany) diluted 1:400 and 1:100, respectively, in 10% fetal bovine serum in PBS. Sections were washed and treated with rabbit anti-mouse IgG or swine anti-rabbit IgG (1:100; DAKO). After detection with the Vectastain ABC Elite kit (Vector Laboratories, Wertheim, Germany), positive cells were visualized with 3-amino-9-ethylcarbazole (AEC; DAKO). Nuclei were lightly counterstained with hematoxylin. For negative controls primary antibodies were omitted. All histological samples were examined by two experienced, blinded neuropathologists.

Statistical analysis

Survival rate was plotted using the Kaplan–Meier method. Statistical significance of survival rate between groups was evaluated using the logrank test. All parametric values are expressed as mean $\pm$ SD and as *P*-values, accordingly.

Results

Tumor growth after tumor cell inoculation occurred in 100% of the animals. The presence and size of the tumors were detected by MR imaging. Surgical procedures were well tolerated.

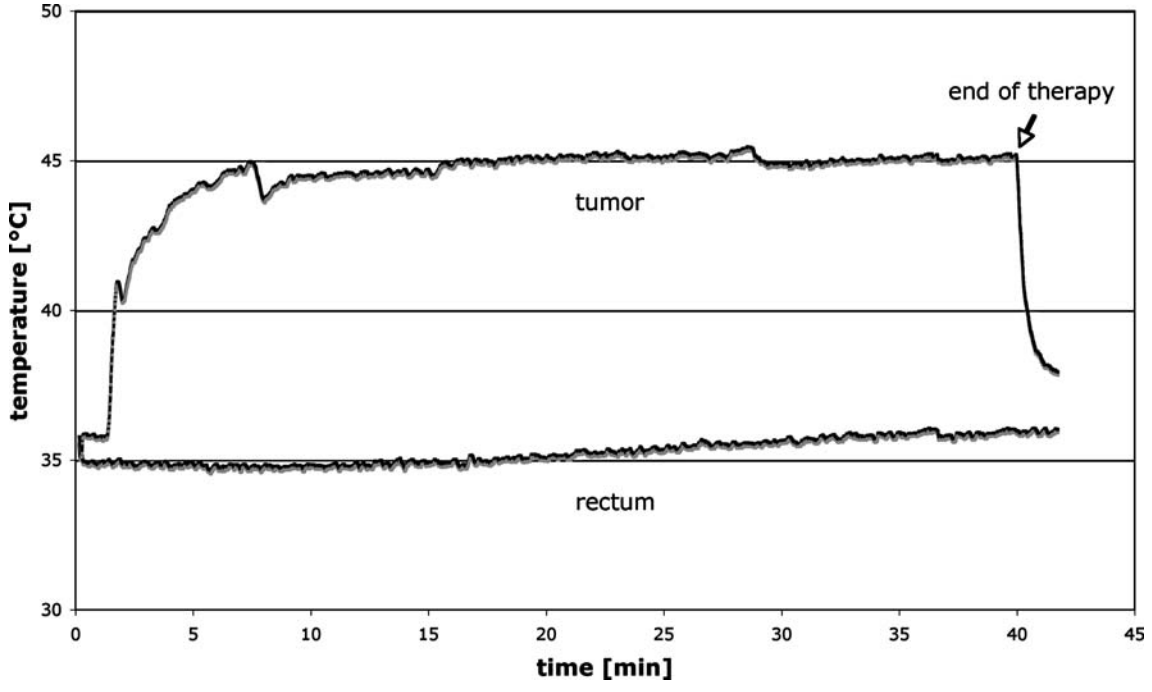

Figure 2. Example of a temperature curve obtained during thermotherapy using magnetic nanoparticles. For measurement of intratumoral and rectal temperatures, a fiberoptic thermometry device was used. Field strength was gradually increased, starting from 60% of the available maximum until the desired temperature was reached.

The implanted glioma cells grew to a round shaped tumor with a diameter of 3–4 mm four days after implantation.

When animals died, the tumors of the control groups and of the animals given thermotherapy did not differ either in dimension or histopathology. Only slight tumor cell invasion into the surrounding tissue was detectable. Beside multiple small, irregularly shaped band-like necroses that did not differ between the various groups, large necrotic areas were observed close to the particle deposits. A difference in vascularization could not be detected in HE sections. Immunohistochemical analysis of PCNA revealed a decreased proliferation rate in the thermotherapy groups treated with aminosilane-coated particles ($48.8 \pm 3.1\%$ versus $79.0 \pm 4.7\%$ of the controls). Brain tissue adjacent to the tumor showed a marked reactive astrogliosis (Figure 3). Other pathological alterations such as neuronal degeneration were not observed.

Mean survival of untreated animals was 8.9 days. This was shorter than described in the literature [16,17], but this discrepancy might be explained by slightly

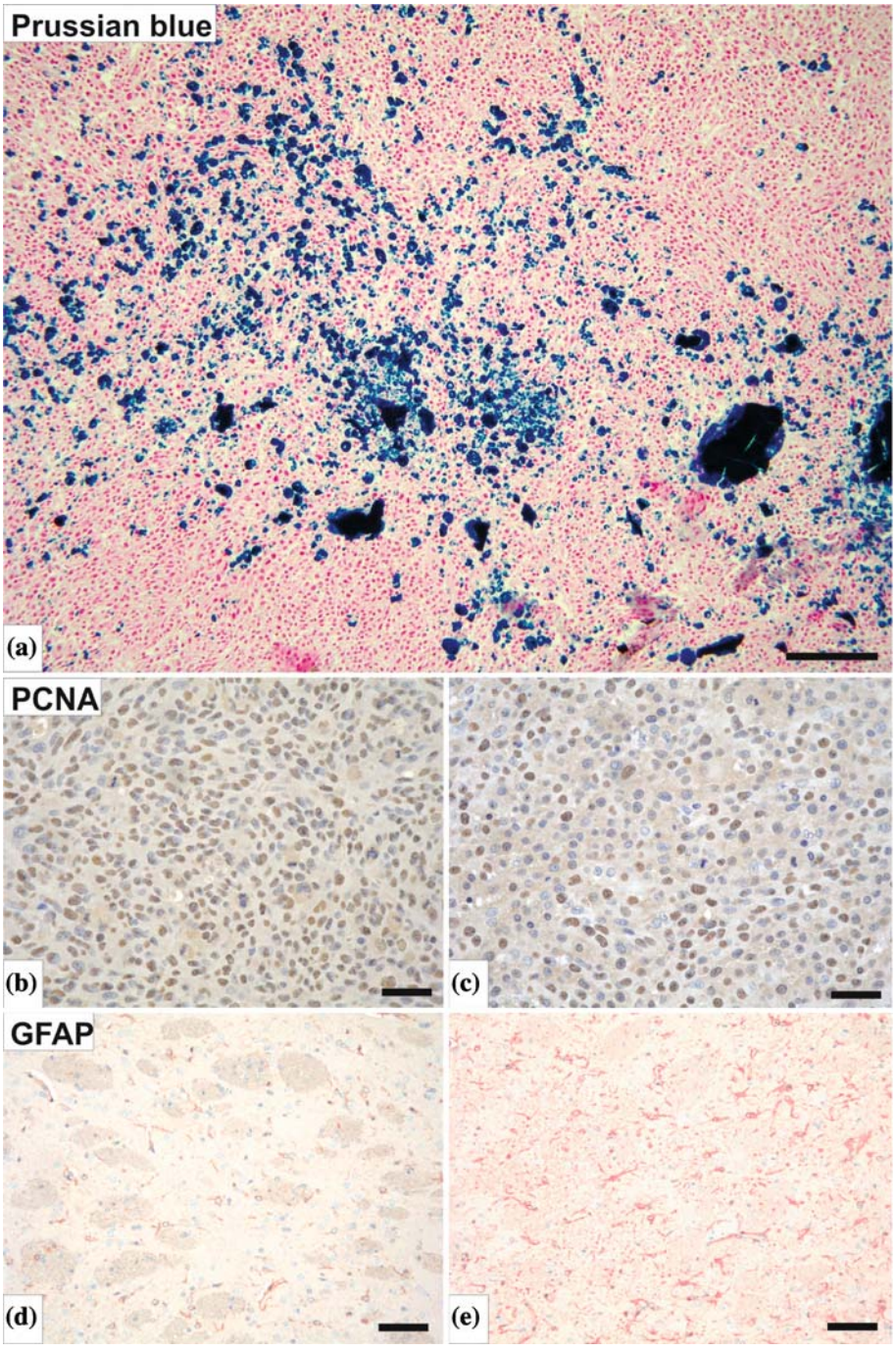

Figure 3. Micrographs of RG-2 tumors of rats. (a) Distribution pattern of magnetic nanoparticles (blue) after two thermotherapy treatments (Prussian blue staining); Immunohistochemistry with antibodies to proliferating cell nuclear antigen (PCNA) and glial fibrillary acidic protein (GFAP) in controls (b, d respectively) and animals after thermotherapy using aminosilane-coated particles. The proliferation rate was decreased in the treatment groups (c). GFAP staining revealed a marked astrogliosis in brain structures (e) adjacent to the tumor (e.g. thalamus region as shown here). Bar 50 μm.

altered growth characteristics of the cell-line due to differences in cultivating and passaging protocols. Data on survival of all animals are shown in Table 1.

The differences in survival between the control groups were not statistically significant. Thermotherapy with aminosilane-coated nanoparticles significantly ($P < 0.01$) increased the life span of tumor-bearing rats over that observed for non-treated controls (Figure 4). We recorded different intratumoral temperatures in these therapy groups between 43 °C and 47 °C. The gain of survival was correlated with the intratumoral temperatures, leading to a 4.5-fold prolongation after treatment at 47 °C.

Contrarily, intratumoral temperatures in the group T-DDM128 P6, treated with dextran-coated nanoparticles, did not exceed 39 °C despite of equal magnetic field application compared to the aminosilane-coated particles. The difference in survival against the control group C-0 was not significant.

During exposure to the magnetic field in the group C-F (intratumoral injection of normal saline) no temperature increase beyond 37 °C was observed. Rectal temperatures in all animals did not increase more than 1 °C during therapy.

Instillation of the aminosilane-coated nanoparticles led to the formation of stable deposits (Figure 5), thus

Table 1. Survival rate after thermotherapy using magnetic nanoparticles

Treatment groups	Mean survival (days) ± SD*	Significance	Factor[a]
C-0	8.9 ± 3.1		
C-A	8.0 ± 1.6		0.9
C-B	7.6 ± 0.7		0.9
C-F	10.1 ± 1.1		1.1
T-DDM128 P6, 39 °C	10.3 ± 2.1		1.2
T-MFL AS, 43 °C	15.4 ± 6.3	$P < 0.01$	1.7
T-MFL AS, 44 °C	28.3 ± 7.4	$P < 0.01$	3.2
T-MFL AS, 45 °C	34.7 ± 6.8	$P < 0.01$	3.6
T-MFL AS, 46 °C	37.8 ± 2.2	$P < 0.01$	4.3
T-MFL AS, 47 °C	39.7 ± 3.5	$P < 0.01$	4.5

[a]Factor describes prolongation of survival in correlation to group C-0 = tumor growth control, C-A = application of magnetic fluid DDM128 P6, no magnetic field treatment; C-B = application of magnetic fluid MFL AS, no magnetic field treatment; C-F = application of normal saline and magnetic field treatment; T-DDM128 P6 = application of dextran-coated nanoparticles, treatment temperature 39 °C; T-MFL AS = application of aminosilane-coated nanoparticles, treatment temperature 43–47 °C.

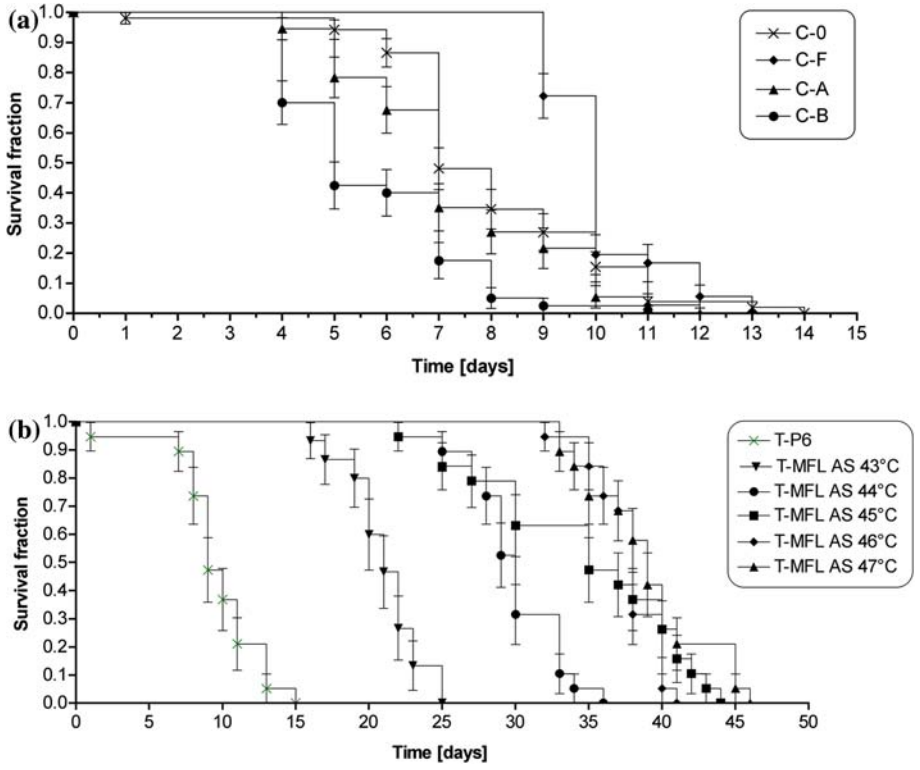

Figure 4. Survival rate after thermotherapy using magnetic nanoparticles (Kaplan–Meier method) C-0 = tumor growth control, C-A = application of magnetic fluid DDM128 P6, no magnetic field; C-B = application of magnetic fluid MFL AS, no magnetic field; C-F = application of normal saline and magnetic field treatment; T-DDM128 P6 (P6) = application of dextran-coated nanoparticles, treatment temperature 39 °C; T-MFL AS = application of aminosilane-coated nanoparticles, treatment temperatures 43–47 °C.

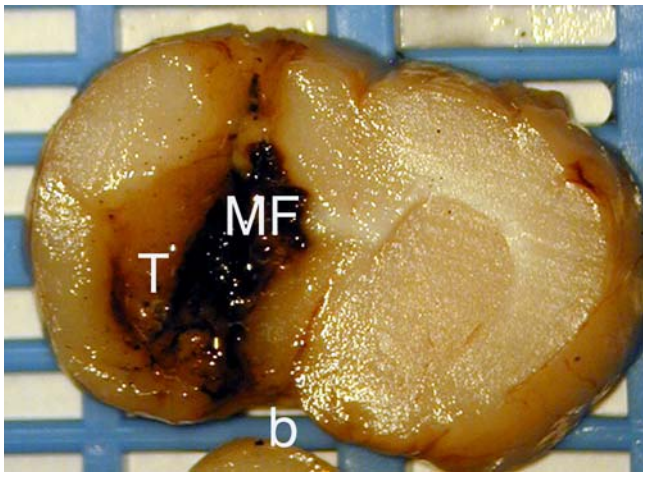

Figure 5. Frontal section of the rat brain with the implanted RG-2 tumor (T) and magnetic fluid (MF), b = base side.

allowing repeated magnetic field treatments without repeated application of the particles.

Differences in the distribution pattern of the two different magnetic fluids were striking. While the dextran-coated nanoparticles were almost undetectable at the time of death, the aminosilane particles were distributed throughout the tumor (Figure 3a).

Discussion

The aim of the present study was to analyze the effects of thermotherapy using magnetic nanoparticles on local tumor growth of the RG-2 glioma of the rat, a well-established model for human malignant glioma [18]. In our experiments, we used a standardized procedure of tumor induction. As confirmed by MR imaging, reproducibly located tumors with similar sizes were obtained at the time of instillation of the magnetic fluids. The nanoparticle infiltration into the tumors proved to be technically feasible and safe.

Thermotherapy using magnetic nanoparticles represents an innovative technique to heat deep-seated tumors. It may have several advantages over conventional techniques employed for regional hyperthermia to date such as radiofrequency, microwave, or ultrasound methods, which are often limited by their inability to selectively target the tumor tissue and by their inhomogeneous temperature distribution.

Our approach has the potential to overcome these limitations by nearly unlimited tumor SAR (specific absorption rate) and biologic specificity due to the particle coating [6]. In our method, heat can be delivered selectively to the targeted volume, while interference of the magnetic field with healthy tissues is kept negligible.

The treatment temperatures are dependent on the amount and the distribution of the magnetic nanoparticles injected into the target area and on the applied magnetic field strength.

While maximal intratumoral temperature is an important variable in clinical hyperthermia, intratumoral temperature homogeneity is also critical for a successful outcome [19,20]. Homogeneous intratumoral temperatures are only obtained with an even infiltration and thus an even intratumoral distribution of the magnetic fluid. Nevertheless, such an optimal infiltration may be difficult to achieve in human treatment, because access to the tumor, anatomy of the organ and regional factors may be limiting.

Since in our experiments only a single probe could be inserted for temperature measurements due to the location and the small size of the tumors, intratumoral temperature homogeneity could only be measured in one representative axis.

The two different magnetic fluids used in the study displayed different behavior with respect to their distribution patterns within the target area, resulting in different intratumoral treatment temperatures. Only the aminosilane-coated particles displayed a stable interstitial deposition followed by a homogeneous distribution within the tumor tissue after the heating process. Intratumoral temperatures during thermotherapy could be adjusted to values between 43 °C and 47 °C by varying the magnetic field strength. Such temperatures are appropriate to inflict direct cell damage. Serial thermotherapy treatments without need for repeated magnetic fluid injections were possible due to the very stable intratumoral deposition of the nanoparticles.

The dextran-coated nanoparticles revealed a distinctly different behavior after injection into the target area. The particles spread into the surrounding tissue shortly after administration, leading to a reduction of the magnetic material in the tumor region. Consequently, a temperature rise of only 2 °C was observed during the first thermotherapy treatment, while during the second no temperature rises could be detected anymore.

This behavior of the dextran-coated nanoparticles is due to their chemical composition of the shell, originally designed for high colloidal stability in physiologic media and in blood after intravenous application. Aminosilane-coated particles specifically interact with the tumor cells facilitating the formation of deposits immediately after injection and, ultimately, their interstitial distribution in the tumor tissue.

Differences in survival of the different treatment groups reflect differences in intratumoral treatment temperatures. While two thermotherapy treatments with aminosilane-coated particles resulted in a 1.7–4.5-fold prolongation (6–10 °C temperature rise) of survival time, the dextran-coated particles only caused a single, short-lived temperature rise of 2 °C and had no significant effect on survival.

The prolonged survival in animals treated with aminosilane-coated particles may be explained on the one hand by the markedly decreased proliferation rate of the heat-treated tumor cells and on the other hand by the existence of widespread necrotic areas close to the nanoparticle deposits, indicating a reduction of tumor growth.

To our knowledge, there are only very few reports in the literature on successful treatment of malignant glioma by magnetic nanoparticles. Our findings are in accordance with Ohno et al. [21], who achieved similar results treating malignant glioma of rats using stick type carboxymethylcellulose–magnetite.

Yanase et al. investigated the effect of hyperthermia on rat glioma using cationic magnetic liposomes and observed a high percentage of complete tumor regression [22]. Their excellent results might be explained by less cooling effects of the blood stream due to minor perfusion of their heterotopically implanted tumors (solid glioma tissue formed subcutaneously in the femoral region).

In our current study, thermotherapy treatment was started on day 4 after tumor cell inoculation. Nevertheless, considering the fast tumor growth in the RG-2 tumor model, the optimal time to start local treatment may be even earlier.

Summarized, this study shows that thermotherapy using aminosilane coated magnetic nanoparticles caused a significant prolongation of survival in glioma-bearing rats. Further preparations of ferrofluid formulas with both optimization of intratumoral distribution and stable deposition are under development. To exploit possible synergistic effects, thermotherapy combined with external radiation is currently being evaluated in the RG-2 tumor model.

Conclusion

Our data suggest that in the human brain, given adequate intratumoral application and distribution of nanoparticles, thermotherapy using magnetic nanoparticles should be able to yield hyperthermal temperatures. Since our results were strongly dependent on the type of the shell of the nanoparticles, further experimental studies will have to investigate the effect of new magnetic nanoparticle preparations with both stable intratumoral deposition and homogeneous distribution patterns.

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