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Post-mortem studies in glioblastoma patients treated with thermotherapy using magnetic nanoparticles

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ABSTRACT

Patients with glioblastoma multiforme (GBM), the most common primary brain tumor in adults, have still a poor prognosis though new strategies of radio- and chemotherapy have been developed. Recently, our group demonstrated the feasibility, tolerability and anti-tumoral effects of a newly developed therapeutic approach, termed thermotherapy using magnetic nanoparticles or magnetic fluid hyperthermia (MFH), in a murine model of malignant glioma. Currently, the efficacy of MFH is being evaluated in a phase II study. Here, we report on post-mortem neuropathological findings of patients with GBM receiving MFH. In brain autopsies the installed magnetic nanoparticles were dispersed or distributed as aggregates within geographic tumor necroses, restricted in distribution to the sites of instillation. Therefore, our results underscore the need for multiple trajectories of instillation. The typical GBM necrosis with pseudopalisading was free of particles. Dispersed particles and particle aggregates were phagocytosed mainly by macrophages whereas glioblastoma cells showed an uptake to a minor extent. MFH therapy further promotes uptake of nanoparticles in macrophages, likely as a consequence of tumor inherent and therapy induced formation of necrosis with subsequent infiltration and activation of phagocytes. We did not observe bystander effects of MFH such as sarcomatous tumour formation, formation of a sterile abscess or foreign body giant cell reaction. Furthermore, all patients did not present any clinical symptoms related to possible adverse effects of MFH.

1. Introduction

Glioblastoma multiforme (GBM) (WHO grade IV) is the most frequent and malignant astrocytic brain tumor [1]. Despite novel therapy strategies, the prognosis of patients remains poor. Surgical resection is almost always incomplete due to the infiltrating nature of GBM. Therapeutic treatment protocols using surgery, radiation and/or chemotherapy did improve survival rates [2] but the median overall survival after first-line therapy does not exceed 15 months [3]. On population-based data, the prognosis of GBM patients is even poorer [1]. Treatment of GBM has therefore been among the most challenging fields in oncology and has led investigators to develop novel innovative therapies designed to augment local control [4–8].

The biological effectiveness of hyperthermia in cancer treatment has been known for decades but the use of thermal therapy in treating cancer is still not yet established in clinical routine. This is may be due to the lack of obtaining homogeneous intratumoral temperatures [9]. Therefore, our group developed a nanotechnology based cancer therapy, termed thermotherapy using magnetic nanoparticles or magnetic fluid hyperthermia (MFH) [10–13]. Herein, a magnetic fluid is directly injected into a tumor and subsequently heated in an alternating magnetic field. This new technique allows precise heating of almost every part of the body [10]. In vitro experiments with magnetic fluids have confirmed their excellent power absorption capabilities [11]. In vivo experiments have documented the feasibility, good overall tolerability

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and significant antitumoral effects of this treatment in a murine model of mammary carcinoma [12], and in a rat model of prostate cancer [14]. We have recently demonstrated the feasibility of nanoparticle mediated thermotherapy in a murine glioma model [15]. Other groups have evaluated similar techniques involving magnetically mediated hyperthermia using nano-scaled magnetites in animal models of melanoma [16,17], breast tumors [18] and prostate cancer [19]. First clinical experiences with thermotherapy using magnetic nanoparticles on prostate carcinoma [20–22], GBM [23] and other tumor entities [24] have been published recently.

This is the first report of post-mortem neuropathological findings of GBM patients who had undergone injection of magnetic nanoparticles into the tumor and subsequent heating in an alternating magnetic field.

2. Material and methods

2.1. Patient selection

The three patients described were enrolled in a clinical trial on feasibility and efficacy of thermotherapy using magnetic nanoparticles in patients with GBM [23]. Eligibility criteria were: age $\geq$ 18 years and estimated life expectancy >3 months, a tumor diameter of less than 5 cm, absence of multilocular growth and no irremovable metallic parts in the head and neck area.

The clinical trial was approved by the local ethics committee (Charité, Berlin, Germany). Written informed consent was obtained from each patient prior to enrollment. All procedures were in accordance with the Helsinki Declaration of the World Medical Association. Included in this report are autopsy findings from 3 glioblastoma patients receiving intratumoral nanoparticles. Two of the patients received MFH.

2.2. Nanoparticles

The magnetic fluid MFL AS (MagForce Nanotechnologies AG, Berlin, Germany) used in the studies is subject to European medical devices regulations. It consists of aminosilane coated superparamagnetic iron oxide nanoparticles (core diameter: 15 nm) dispersed in water, with an iron concentration of 112 mg/ml. The particles generate heat in an alternating magnetic field by Brownian and Néel relaxation processes [10].

2.3. Instillation of the magnetic nanoparticles

The procedure of administering the magnetic fluid has been described elsewhere [23]. Briefly, instillation of the magnetic fluid was carried out under neuronavigational control (Stealth Station, Medtronic, Minneapolis, MN, USA) in general anesthesia. Magnetic nanoparticles were injected into non-resected recurrent tumors following presurgical cranial navigation MRI. On the basis of these data, a 3-dimensional reconstruction of the defined tumor volume was generated and the port- and target-points of the magnetic fluid were assigned. To obtain a homogeneous distribution of the magnetic nanoparticles within the target area, as many trajectories as possible were planned, depending on volume, localization and shape of the tumor. Distance between trajectories ranged from 8 mm to 10 mm.

2.4. Thermotherapy

Thermotherapy was performed in the alternating magnetic field applicator MFH® 300 F (MagForce Nanotechnologies AG, Berlin, Germany), operating at a frequency of 100 kHz and a variable field strength of 2.5–18 kA/m. Temperature measurements were performed by means of fiberoptic thermometry probes as part of the therapy device, which can be used for deep heating of any circumscribed tumor within the human body. After completing the therapy, patients were monitored by clinical examination and CT scans at intervals of 3 months.

2.5. Autopsy of patients and MRT examination of brains

Following fully informed consent brain autopsy was performed at the Institute of Neuropathology. Whole brains were fixed in buffered 4% formaldehyde for ten days, after which a CT scan was performed. The brains were subsequently sectioned in horizontal planes comparable to the CT planes. Ten samples from each brain tumor and adjacent tissues as well as samples from the contralateral hemisphere, basal ganglia, hippocampus, brain stem and cerebellum were taken, dehydrated and embedded in paraffin.

2.6. Histology and immunohistochemistry

Brains were postfixed in buffered 4% formaldehyde for 24 h and embedded in paraffin. Samples were subsequently cut into 4 μm sections and further subjected to hematoxylin and eosin (HE), Prussian Blue, periodic acidic Schiff (PAS), silver-impregnation for reticulin or immunohistochemical staining [25].

Sections were dewaxed, rehydrated, and microwaved for 12 min in 10 mM citrate buffer, pH 6.5, at 600 W (Bosch, Germany) for immunohistochemical examinations. 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, followed by the respective primary antibody. We used polyclonal and monoclonal antibodies against glial fibrillary acid protein (GFAP; 1:500; DAKO Cytomation, Germany), S-100 protein (1:500; DAKO), Ki67 (clone MIB-1; 1:100; DAKO) and CD68 (1:200; DAKO) as primary antibodies.

Sections were washed and incubated with rabbit anti-mouse IgG or swine anti-rabbit IgG (1:100; DAKO). The avidin–biotin-complex (ABC; DAKO) and the chromogen 3-amino-9-ethylcarbazole (AEC; DAKO) were used for visualization. Sections were lightly counterstained with hematoxylin. For negative controls primary antibodies were omitted. All histological samples were examined by two experienced, blinded neuropathologists.

We used fifty microscopic fields of 0.0625 μm^2 each for morphometrical analysis of phagocytosing CD68-positive macrophages and S100-positive neoplastic astrocytes [26]. Only immunopositive cells located totally within or on the right or bottom border of the standardized ocular grid were counted.

2.7. Case reports and autopsy findings

Patient 1 (P1), a 41-year-old male with left frontoparietal GBM had received first-line therapy including surgery, external beam radiation with 60 Gy and chemotherapy with temozolomide (1 cycle). At tumor recurrence (volume: 36.3 ml) the patient received irradiation with 30 Gy (single dose of 2 Gy, 5 times a week) and 6 hyperthermia treatments following injection of 4.5 ml of the magnetic fluid distributed over 5 instillation channels. A maximum intratumoral treatment temperature of 49.5 °C was measured. Survival after injection of the particles was 7.9 months. At autopsy, the brain weight was 1780 g. There were signs of transtentorial and subfalcial herniation (Fig. 1).

The tumor of patient 2 (P2), a 57-year-old male with a primary, untreated left temporo-occipital GBM had a volume of 14.0 ml. 4.2 ml of magnetic fluid was injected over 7 channels. The patient died of pneumonia 14 days after the application without having undergone MFH or any other treatment. His brain weight was 1610 g and showed signs of transtentorial herniation (Fig. 1).

Patient 3 (P3), a 69-year-old male with left temporal GBM, received first-line therapy consisting of surgery and external beam radiation with 34 Gy. At tumor recurrence chemotherapy with temozolomide was given (3 cycles). Thereafter, the patient received 6 hyperthermia treatments following injection of 4.6 ml of magnetic fluid distributed over 8 channels (tumor volume: 50.0 ml). Maximum intratumoral treatment temperature reached 65.6 °C. Survival after injection of the particles was 2.1 months. At autopsy, the brain weight was 1640 g. There was a severe generalized edema with transtentorial and tonsillar herniation (Fig. 1).

The CT scans and brain sections show the corresponding areas of the lesions in all three brains (Fig. 1A–C). Nanoparticles appear as hyperdense areas within each lesion due to the high X-ray attenuation of metallic components (Fig. 1A). The cortico-medullar contrast of the preparations in general is clearly inferior compared to that seen in diagnostic in vivo scans. This difference is due to the formaldehyde fixation.

The cut surfaces of all brains showed moderately delineated tumors measuring $7 \times 9 \times 9.5$ cm (P1), $4.5 \times 5.5 \times 6$ cm (P2, left hemisphere) and $1 \times 3.5 \times 4.5$ cm (P2, right hemisphere), and $4 \times 4 \times 4.5$ cm (P3) involving cortex, subcortical white matter, the basal ganglia and thalamus (Fig. 1A–C). The lesions showed aspects of solid tumor, necrosis and hemorrhages. All showed multifocal deposits of magnetic nanoparticles, mainly within geographic necrosis.

2.7.1. Histological, immunohistochemical and morphometrical analysis

All glioblastomas shared similar morphological appearance regarding tumor cells and architecture. Tumor cells were small with marked nuclear pleomorphism. Scattered gemistocytes and multinucleated giant cells were seen. Intermediate expression of GFAP was present in about one third of the tumor cells, with regional differences. Most tumor cells expressed S100. Prominent subpial aggregation of tumor cells was seen in all of the three cases. Mitotic figures were frequent with an average mitosis rate of $1.2 \pm 0.1/0.0625\ \mu m^2$ (P1), $1.3 \pm 0.3/0.0625\ \mu m^2$ (P2) and $1.2 \pm 0.3/0.0625\ \mu m^2$ (P3). The average proliferation rate was $10.3 \pm 1.4\%$ (P1), $15.3 \pm 2.5\%$ (P2) and $11.8 \pm 1.8\%$ (P3). There were no differences compared to the surgical biopsies available in patients 1 and 3 regarding mitosis and proliferation rate. Extended areas of necrosis with pseudopalisading and geographic necrosis with thrombosis of blood vessels were observed. Nanoparticles were dispersed or distributed as aggregates within geographic necrosis (Fig. 2A–D). In contrast, circumscribed necroses with pseudopalisading, characteristic of GBM, were free of particles. The aggregates of nanoparticles were partly surrounded by a rim of phagocytes with a cross-section dimension range of 0–250 μm, then followed by tumor cells. Areas of hemorrhages of different ages were seen multifocally, especially along the canals of the instillated nanoparticles. Tumor-induced microvascular proliferation included marked endothelial proliferation and many glomerulum-like