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IN VITRO AND *IN VIVO* EFFECTS OF Fe_3O_4/SALICYLIC ACID MAGNETIC NANOPARTICLES ON THE HUMAN GLIOBLASTOMA CELLS

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We aimed to evaluate *in vitro* and *in vivo* actions of the functionalized Fe_3O_4/salicylic acid magnetic nanoparticles (MNPs) on the human glioblastoma tumoral cells, established from a patient diagnosed with glioblastoma. For *in vitro* study the human glioblastoma cells were cultured and exposed to different concentration of MNPs and cell proliferation was measured. The incubation of the glioblastoma cells with the 0.25 μg/ml, 0.5 μg/ml, and 1 μg/ml MPNs for 72 hours revealed no significant effect induced on human glioblastoma cells *in vitro* proliferation. For *in vivo* study the human glioblastoma cells were transplanted on chick embryo chorioallantoic membrane (CAM). The MNPs were injected into the CAM blood vessels and guided in the tumor area with a strong static magnet. After intravascular MNPs injection the CAM with the human tumoral xenograft were harvested and processed for histological study. The intravascular injected MNPs can be guided under the action of a magnetic field in the glioblastoma xenograft. A decrease in tumor xenograft growth due to intratumoral necrotic lesions and peritumoral vessels nanoblockage induced by MNPs accumulation under magnetic field action were observed on the histological analysis. This behavior suggests the potential of the Fe_3O_4/salicylic acid magnetic nanoparticles in the tumor treatment.

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1. Introduction

Nontoxicity, biocompatiblilty, injectability and high-level accumulation in the target tissue or organ [1] were the important properties of the magnetic nanoparticle that have recomanded its into various fields of biological medicine such as cancer therapy (hyperthermia, targeted drug and genes delivery), [2-8] cancer diagnostics (targeted cancer imaging), [9] or theranostic treatment [10]. Because of their biocompatibility in biological systems [11], superparamagnetic ferric oxide nanoparticles are the most promising magnetic nanomaterials for the treatment of tumors [12]. Functionalized nanoparticles allowed tumor-specific detection and treatment and, in recent years, have used as delivery vectors targeting cerebral gliomas [3]. Glioblastoma is usually highly malignant neuroectodermal brain tumors in adults, with an incidence of 78% of all primary malignant brain tumors [13, 14]. Despite recent improvement in the multimodality approach (combination of surgery, radiation therapy, systemic chemotherapy, photodynamic therapy) and

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diagnostic abilities, the median postoperative overall survival times range between 3 and 16 months [15-17].

The chick embryo CAM model is a well-established method for studying cancer, angiogenesis, photodynamic therapy, human tumoral cell metastasis, microsurgical interventions, cell biology, immunology, drug delivery system and genetics [18-25]. The chick embryo CAM model has already been used to grow glioblastoma samples harvested from the operated patients. Transplanted xenografts of human glioblastomas on chick embryos CAMs survived and exhibited the same features as original glioblastomas [26]. In the present work, we aimed to evaluate the *in vitro* and *in vivo* action of the functionalized Fe_3O_4/salicylic acid MNPs on the human glioblastoma cells (established according to standard procedures from patient diagnosed with glioblastoma) in order to determine their suitability for the treatment of glioblastomas.

2. Experimental

Functionalized Fe_3O_4/salicylic acid magnetic nanoparticles

A modified Massart ferrite salt co-precipitation synthesis was used to obtain an aqueous solution of 60 nm MNPs with well-dispersed properties and homogenous size distribution [27].

In vitro assay

GB3B cell line establishment from glioblastoma tissue

Early passage cell cultures (named GB3B) used in this study, were established from tissue obtained from a patient diagnosed with glioblastoma at the "Bagdasar–Arseni" Emergency Hospital, Bucharest, Romania.

The cell lines were established according to standard procedures. After dissection into small pieces (<5 mm diameter) the samples were incubated in 0.4 mg/ml DNase (Sigma-Aldrich), 0.25 mg/ml collagenase IV (Gibco/BRL) and 0.5 mg/ml pronase (Boehringer-Mannheim) with gentle stirring in Hank's buffered saline solution for 30 min at 37^0C followed by 30 min at 4^0C. The cell suspension that results from tumor slurry passed through a tissue culture sieve was plated out into tissue culture flasks (Corning). [28-30].

Cell culture and cell treatement

The cells were cultured in minimum essential medium (MEM) containing 10% fetal bovine serum (FBS), 2mM glutamine and antibiotic (100 UI/ ml penicillin and 100 UI/ml streptomycin) (all supplements were provided by Gibco/BRL). The cells were grown in monolayers in tissue culture flasks, maintained in a 95% air/ 5% CO_2 atmosphere at 37°C in a humidified incubator. For experimental propose, cells were seeded in 6-well, culture plates. Cells were seeded at a density of 2,000-3,000 cells/cm^2, and experiments were initiated when they had reached a density of 5,000-10,000 cells/cm^2. Appropriate control groups with diluents only and blank control were included.

Human fibroblast cell line (HDF) was established from normal human tissue (ATCC® PCS-201-010™).

Cell viability

Cells were grown in 6 wells plate, exposed to different concentration of the MNPs and cell proliferation was measured by determining the number of cells attached to the plastic surface of duplicate wells. This was performed by microscopic counting of cells in ink-marked areas on the wells bottom. By repeating the countings after specified time intervals, changes in the number of attached cells could be followed [29].

Statistical analysis

All data are represented as mean ± SD. Data were analysed using ANOVA two-tailed t-test for analysis. $P < 0.05$ values were considered statistically significant.

***In vivo* assay**

Chick chorioallantoic membrane model

20 fertilized White Leghorn chicken eggs were incubated at 37.5ºC and 70% relative humidity. After 3-4 days of incubation, 3-5 ml of albumen was extracted and a 1-2 cm^2 window was cutted at the top of each egg. The window was then resealed with adhesive tape and the eggs

were replaced in the incubator until days 6, when the CAM vessels become capable of sustaining human xenografts. Two or three drops of MEM containing glioblastoma cells was transplanted on top of each 6-day old CAM under sterile conditions and the eggs were returned to the incubator until the day 11. On the day 11, a half of the chick embryo CAM were intravenous injected with 0.2 ml of 0.19% MNPs aqueos solution and 0.18T NdFeB magnete was applied on the CAM tumoral area for 15 minutes. The eggs were returned to the incubator until the day 16, when all the xenografts were harvested together with the surrounding CAM and fixed with 4% paraformaldehyde for 24 hours. After glioblastoma cells transplantation, the CAMs were daily examined and registered by a Zeiss stereomicroscope equipped with a DCM 510 camera system.

Histological analysis

CAM samples with glioblastoma cells xenograft were fixed in 4% formalin, processed for paraffin embedding by standard methods, cut into 5 μm thick sections and stained with Hematoxylin & Eosin for light microscopic examination.

3. Results

In vitro assay

Cell proliferation assays were performed on two cell lines: glioblastoma GB3B cells and human fibroblast HDF cells (Figure 1A, B). Cells were exposed to 0.25 μg/ml, 0.5μg/ml, 1 μg/ml MNPs and the proliferation rates were evaluated at 24, 48 and 72 hours after the treatment. As we seen in the Figure 1, the treatment with 0.25 μg/ml MNPs for 24h, induced an increase by 5% in HDF cell proliferation, while the exposure of the HDF cells for 48h and 72h, resulted in a decrease by 6% and 2% in cell proliferation. The treatment with 0.5 μg/ml MNPs for 48h decreased cell proliferation by 4%. However, the exposure for 24h and 72h did not influence the HDF cell proliferation. The proliferation of the HDF cells increased by 3% and 9% after exposure to 1 μg/ml MNPs treatment for 24h and 48h, while the treatment exposure for 72h resulted in a decrease of cell viability by 11%, compared with the control cells (Figure 1A).

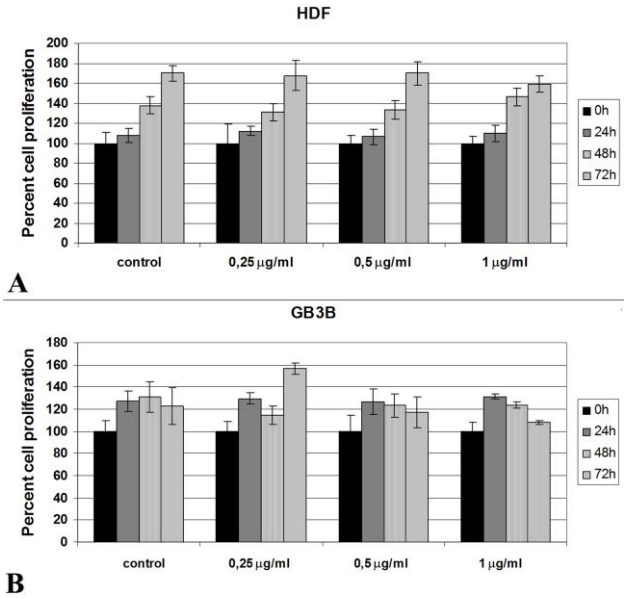

Fig. 1. Effect of MNPs on the human HDF (A) and glioblastoma (B) cells viability. The cells were treated with 0.25, 0.5 and 1 μl/ml MNPs solution and proliferation was determined by microscopic counting of cells 24, 48 and 72 hours after the treatment. Appropriate control groups with diluents only and blank control were included. Results are expressed as percentage of control and the experiments were repeated at times. Data are reported as mean ± SD.

In GB3B cell lines, we calculated 7% increase in cell proliferation after incubation with 0.25 μg/ml MNPs for 24 hours and 8% decrease in cell proliferation after 72 hours incubation. On the cell proliferation, no effect was seen after 48 hours with 0.25 μg/ml MNPs treatment. The treatment with 0.5 μg/ml MNPs for 24 hours did not change the proliferation of the GB3B cells; in contrast, 48 and 72 hours after the treatment decreased cell proliferation by 8% and 5% respectively, compared with the control cells. The treatment of GB3B cells with 1 μg/ml MNPs induces an increase in cell proliferation by 4%, 24 hours after the treatment while the incubation with 1 μg/ml MNPs for 48 hours and 72 hours decreased cell proliferation by 8%, respectively and 15%, compared with the control cells (Figure 1B).

In vivo assay

Samples of MEM with glioblastoma cells were transplanted onto 20 CAMs of 6-day old chick embryos. Two samples of human glioblastoma cells failed to develop (10%). Two chicken embryos died after MNPs intravascular injection (10%).

In vivo stereomicroscopic observation

Daily stereomicroscopic examination of the CAMs with transplantated glioblastoma cells culture showed an intense cell proliferation with appearance of cells clusters on the CAM surface visible at 24 hours from implantation. After 120 hours of the transplantation few cells growths were present on the CAM surface and a marked angiogenesis with new vessels arranged in a wheel-spoke pattern was visible around them (Figure 2 A, B).

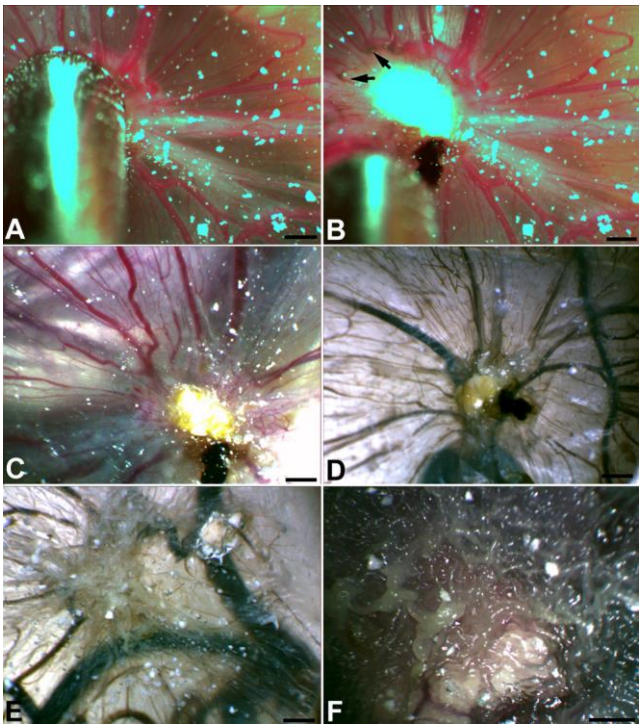

Fig. 2. In vivo study on the CAM model. A: NdFeB magnete applied on the 120h-old glioblastoma cells growth after MNPs intrvascular injection; B: MNPs visible in the CAM blood vessels (black arrows) after magnet removing; C: glioblastoma cells growth appearance, 96h after MNPs intrvascular injection; D: CAM and xenograft harvested and formalin fixed 192h after MNPs intrvascular injection; E, F: formalin fixed martor CAM with 192h old glioblastoma xenograft. Bar scale = 1mm.

After intravenous injection of MNPs in the CAM vessels and for 15 minutes static magnetic field application (Figure 2 A), MNPs becam visible in the CAM vessels surround glioblastoma cells growth (Figure 2 B). In the martor CAMs with glioblastom xenografts the glioblastoma cells growths continued to develop and joined to form a cerebriform-like area on the

CAM surface (Figure 2 E). The CAM under transplanted region was notably thickened, opaque and its blood vessels were not visible on the stereomicroscope examination. In the CAMs with glioblastom xenograft and intravascular injected MNPs the glioblastoma cells growths ceased to develop (Figure 2 C, D) but continue to exert an angiogenic effect on the CAM blood vessels. Around the nanoblocked xenograft, the CAM was thin, transparent and its blood vessels were very well visible on the stereomicroscope examination.

Histological analysis

The histological study of the glioblastoma xenografts on the CAMs not injected with MNPs revealed a thick CAM whith glioblastoma cells growths wich continued to develop, visible on the CAM surface, and clusters of glioblastoma cells in the CAM mesenchymal layer (Figure 3A).

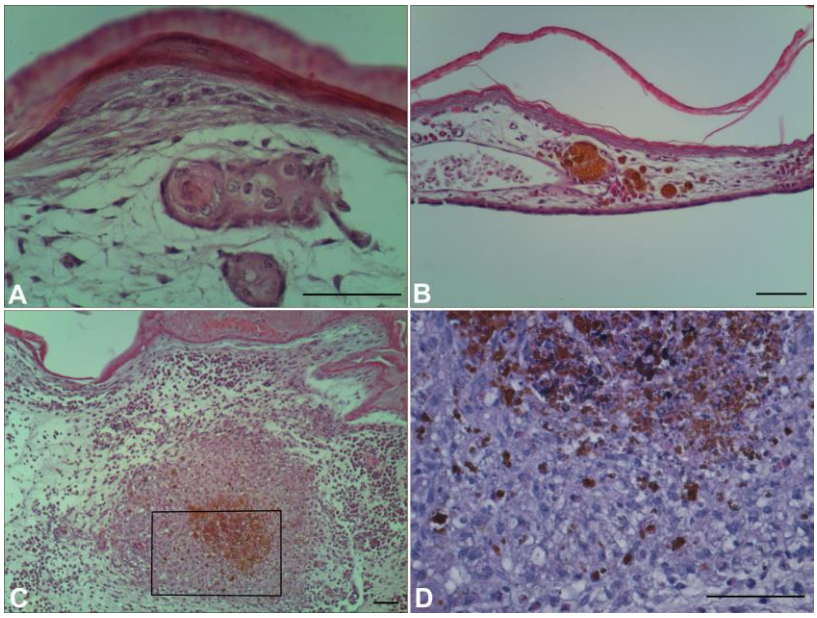

Fig. 3. A: thick CAM with glioblastoma cells clusters and chorial metaplasia in the non-treated specimen; B: thin peritumoral CAM with nanobloked vessels in the treated specimen; any glioblastoma cells was not identified in that CAMs areas; C: intratumoral MNPs acumulation under static magnetic field action; D: necrotic lesions and apoptotic bodies in the MNPs acumulation area. HE staining; Bar scale = 50 μm.

The histological study of the glioblastoma growths on the MNPs intravascular injected CAMs revealed magnetic induced MNPs aggregations in the central area of the tumoral growth producing cell necrosis (Figure 3 C, D). In the peritumoral areas a normal-thickness CAM with same blood vessels blocked by MNPs aggregations can be seen (Figure 3B). No glioblastoma cells growths were visible in these regions.

4. Discussion

Recent research validated CAM as a viabil model for *in vivo* study of the human glioblastoma xenograft [26, 31]. The use of the iron oxide nanoparticles as theranostic agents at the patients with glioblastoma tumors seems to be a promising study direction given their ability to crossing the blood-brain barrier [3], their cytotoxic tumoral effect at high concentration [12, 32] and contrast enhanced effect in diagnostic imaging [9].

Several studies have shown the safety of low concentrations of functionalized MNPs [33-36]. The oxidative stress and subsequent apoptosis induced by functionalized MNPs were time-dependent and dose-dependent. The toxicity of the functionalized MNPs was not significant at

concentrations up to 100 μg /ml in murine macrophage cells [32]. The viability of the human glia and breast cell lines is unaffected up to 10 μg/ml MNPs and it is reduced at 100 μg/ml MNPs [37]. Low concentrations of ferrite nanoparticles (up to 1 μg/ml) were used for *in vitro* study to evaluate cytotoxic effect on the human glioblastoma cells, the same concentrations obtained by intravascular injection of 0.2 ml MNPs into CAM vessels. *In vitro* study revealed no marked cytotoxic effects on the human glioblastoma or fibroblast cells at this concentration of the Fe_3O_4/salicylic acid MNPs. Intra and extravascular tumor deposits with high concentration of Fe_3O_4/salicylic acid MNPs were obtained under the action of a static magnetic field by placing a 0.18T NdFeB magnete on the CAM xenograft area for 15 minutes. The extravascular accumulation of the MNPs observed in the tumor vessels were absent in the CAM vessel and is probably due to the larger endothelial fenestrations (more than 60 nm in diameter), transcellular holes, (about 600 nm in diameter), and intercellular openings, (about 1700 nm in diameter) specific of the tumor vessels [38-40]. Under physiological conditions, oxygen and nutrient can diffuse efficiently into the tissue up to 200 μm [41]. These intratumoral deposits of Fe_3O_4/salicylic acid MNPs act as an effective barrier against oxygen and nutrient supply which would explain the tumor necrotic lesions observed around the tumoral nanoblocked vessels. In addition, the vessels nanobloking effect seems to prevent the perivascular dissemination of the glioblastoma cells on the CAM vessels.

5. Conclusions

Up to 1 μg/ml Fe_3O_4/salicylic acid MNPs, statistical analysis of the *in vitro* cells proliferation revealed no significant effect induced on human glioblastoma or fibroblast cell lines used in this study ($p>0.05$). Low concentrations of Fe_3O_4/salicylic acid nanoparticles aqueous solution injected into the CAM vessels can be guided under static magnetic field action inside the glioblastoma xenografts. At this level the MNPs have accumulated and produced intratumoral necrotic lesions, peritumoral vessels nanoblockage and prevented the perivascular metastasis. This behavior suggests the potential of the Fe_3O_4/salicylic acid functionalized nanoparticles in the treatment of the glioblastoma.

Acknowledgments

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