Annals of Warsaw University of Life Sciences – SGGW
Animal Science No 52, 2013: 29–35
(Ann. Warsaw Univ. of Life Sci. – SGGW, Anim. Sci. 52, 2013)

Changes in glioblastoma multiforme ultrastructure after diamond nanoparticles treatment. Experimental model *in ovo*

MARTA GRODZIK
Department of Animal Nutrition and Feed Science, Warsaw University of Life Sciences – SGGW

Abstract: *Changes in glioblastoma multiforme ultrastructure after diamond nanoparticles treatment. Experimental model in ovo.* Glioblastoma multiforme (GBM) is the most common primary malignancy in the brain and confers a uniformly poor prognosis. Despite decades of research on the topic, limited progress has been made to improve the poor survival associated with this disease, new therapeutic strategies are still needed. The application of nanotechnology to disease treatment, diagnosis, monitoring, drug delivery platform and to the control of biological systems is promising, also in cancer therapy. Diamond nanoparticles (DN) are bioactive substance toward glioma tumour cultured on the chicken embryo chorioallantoic membrane (CAM). DN reduce tumor mass and volume and inhibited new blood vessel development in glioma tumor. In the present experiment we additionally observed, that DN caused changes in the tumor ultrastructure testify to the ongoing process of cell death, probably carried out by autophagy.

Key words: autophagy, diamond nanoparticles, glioma, *in ovo* culture, transmission electron microscope

INTRODUCTION

Malignant glioma cells like glioblastoma multiforme (GBM) have rapid growth rate and an aggressive nature. This cancer is the one of the most common brain tumors and is known for the relatively high morbidity. The short time prognosis for patients with GBM is associated with intratumoral heterogeneity on the genomic and cytopathologic level and general lack of the successful therapy. Therefore, it is important to identify potential drugs and explore more efficient therapeutic strategies for the treatment of malignant gliomas (Sathornsumetee and Rich 2008, Jain 2013). Animal models for cancer experiments, such as chick embryo chorioallantoic membrane, are successful and powerful tools to investigate therapeutic aspects of glioma (angiogenesis, metastasis) that cannot be studied in 2D cell culture systems (Strojnik et al. 2010).

Increasing usage of nanomaterials (also nanoparticles) in the past decades, for various biological and medical applications (such as imaging, diagnosis, therapy and drug delivery), shows that nanotechnology is the important tool for developing modern life sciences (Roco 2003). The unique potential of nanoparticles is due to their size in nanoscale and physicochemical properties that allow to overcome the limitation of using the bulk materials of the same composition as a traditional therapeutic and diagnostic agents (Zhang et al. 2008). Among various nanoparticles, diamond nanoparticles (DN) are the one with promising prospects in applications that require optical transparency, chemical inertness, hardness and low cytotoxicity toward living organism. Moreover, DN are the most bioactive nanoparticles among all

the allotrope forms of carbon, including C60, fullerenes, carbon black, and single and multi-walled carbon nanotubes (Huang et al. 2007, Liu et al. 2007) also towards gliomas. Diamond nanoparticles reduced tumor mass and volume and inhibited new blood vessel development in GBM tumors cultured *in ovo*. It was observed that DN significantly decreased expression of angiogenic factors: FGF-2 and VEGF (Grodzik et al. 2011). The effect of diamond nanoparticles on morphology of glioblastoma multiforme cells in tumor is still unclear. The objective of the investigation was to evaluate changes in glioma tumor cells ultrastructure after DN treatment being the effect of metabolic changes in the cell.

MATERIALS AND METHODS

Diamond nanoparticles

Diamond nanoparticles (DN) were obtained from SkySpring Nanomaterials (Houston, TX, USA). In the experiment, concentration 500 μg per 1 ml was used. Physicochemical characteristics of DN were performed. The shape and the size of nanoparticles were determined by JEM-2000EX transmission electron microscope (TEM) at 200 kV (JEOL Ltd., Tokyo, Japan). Figure 1 shows the image of the diamond nanoparticles from TEM. The nanoparticles were 4–5 nm size and rounded shape. Zeta potential of nanodiamond hydrocolloid was examined with Zetasizer Nano-ZS90 (Malvern Instruments Ltd., Malvern, UK) and was measured as –39 mV. To prevent particles aggregation, soniphication was performed before every application.

Cells and chicken embryos

U87MG glioblastoma multiforme (GBM) cells (HTB-14; American Type Culture Collection, Manassas, VA) were maintained in Dulbecco's modified Eagle medium (Sigma-Aldrich Corporation, St Louis, MO) with addition of 10% fetal bovine serum (Sigma-Aldrich) and 1% Antibiotic Antimycotic Solution (Sigma--Aldrich).

The fertilized eggs (*Gallus gallus*) were supplied by a commercial, local hatchery (Marylka, Poland). The strains used depending on availability, included various crosses among Ross, Cobb, and

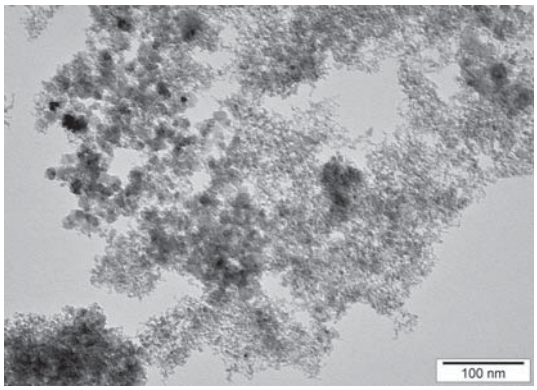

FIGURE 1. TEM image of diamond nanoparticles. Scale bar – 100 nm

Hubbard, kept for 4 days at 12°C. Chicken embryos were incubated at 37°C with 60–70% of relative humidity in incubator (PHU Walenski, Gostyn, Poland). Embryonic day 0 (E0) was designated as the day when the eggs were placed into the incubator.

Glioma tumor culture

The eggs were divided into two groups (2×40 eggs): group I (control) and group II (tumor treatment diamond nanoparticles). The GMB culture suspension (3–4 $\cdot 10^6$ cells in 30 μl cell culture medium) was injected inside the silicon ring placed onto the CAM at day E6. Diamond nanoparticles were injected into the tumor (form II group) after 7 days from start of the culture. The chicken embryos were killed by decapitation at day E18. The GBM tumors that grew inside or near the silicone ring were resectioned and passed for further investigations.

Transmission Electron Microscopy

The tumors were cut into pieces of about 1 mm^3 and fixed in the 2.5% solution of glutaraldehyde (grade I, 25% in H_2O, purified for use as an electron microscopy fixative, Sigma-Aldrich) in 0.1 M phosphate buffer (pH 6.9). Then, the samples were rinsed in the same buffer and transferred to the 1% solution of osmium tetroxide (Sigma-Aldrich) in 0.1 M phosphate buffer (pH 6.9) for 1 h. Subsequently, the samples were rinsed in distilled water, dehydrated in the ethanol gradient and impregnated with epoxy embedding resin (Epoxy – Embedding Kit, Fluka, Sigma-Aldrich). The next day, the samples were embedded in the same resin and baked for 24 h at 36°C. Then, the blocks were transferred to 60°C and baked for another 24 h. The blocks were cut into ultrathin sections (50–80 nm) using an ultramicrotome (LKB Ultratome III, Sweden) and transferred onto copper grids, 200 mesh (Agar Scientific Ltd. GB). Subsequently, the sections were contrasted using uranyl acetate (uranyl acetate dehydrate, puriss. p.a., ACS reagent, ≥98.0% (T) Fluka, Sigma-Aldrich) and lead citrate (lead (II) citrate tribasic trihydrate – purum, for electron microscopy, Sigma-Aldrich). The morphological structure of each GBM was inspected using a JEM-1220 transmission electron microscope (TEM) at 80 keV (JOEL, Japan) coupled with a digital camera (Morada) and Olympus Soft Imaging Solutions software (Olymus, Germany).

RESULTS AND DISCUSSION

Diamond nanoparticles reduce mass, volume and number of vessels in glioblastoma multiforme tumor cultured on chicken's embryo CAM (Grodzik et al. 2011). In the present experiment we additionally observed, that DN caused changes in the tumor ultrastructure. The electron microscopy images from both groups (control group and group treated with diamond nanoparticles) showed a typical ultrastructure of the glioma tumors with glioblastoma multiforme cells, epithelium cells and erythrocytes (Fig. 2). In the control group (Fig. 2 A, B, C; Fig. 3 A, B, C) glioblastoma multiforme cells with cell structures (nucleus, mitochondria, Golgi apparatus, rough endoplasmic reticulum with ribosomes, transport vesicles) were visible. Cancer cells have characteristic and unique metabolism, that is an adaptation to their microenvironment (low pH,

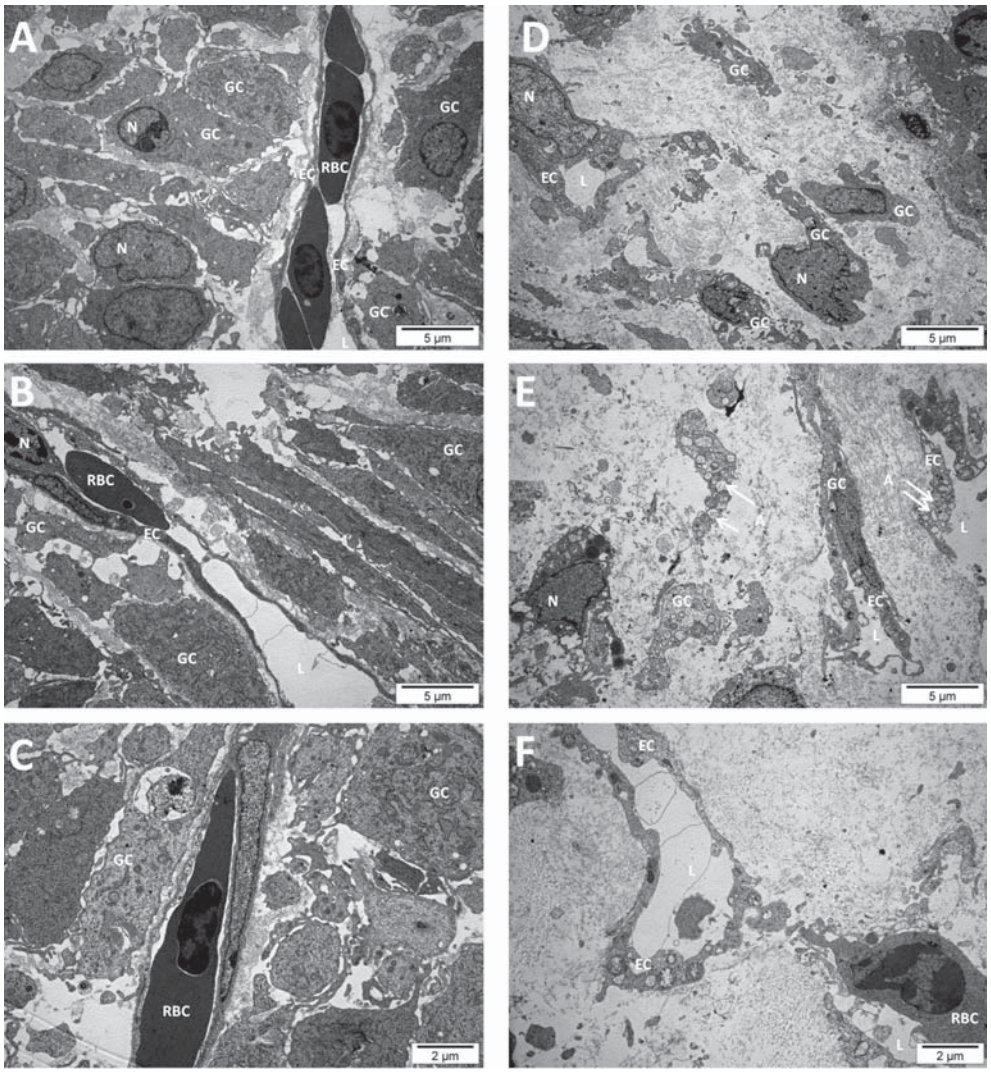

FIGURE 2. Glioblastoma multiforme ultrastructure from control group (A, B, C) and after diamond nanoparticles treatment (D, E, F). Scale bar: A, B, D, E – 5 µm, C, F – 2 µm; GC – glioblastoma multiforme cell, RBC – red blood cells, L – lumen of vessel, EC – endothelial cell, N – nucleus, A – autophagosome

hypoxia, anaerobic glycolysis, intensive cell divisions). Well-developed rough endoplasmic reticulum (ER) and numerous secretory and endocytotic vesicles prove high secretory activity of GBM cells. Endoplasmic reticulum (ER) is the structure of eukaryotic cells, where lipid synthesis, protein folding, and protein maturation take place. ER is the major signal-transducing organelle that senses and responds to changes of homeostasis (Baumann and Walz 2001). In general,

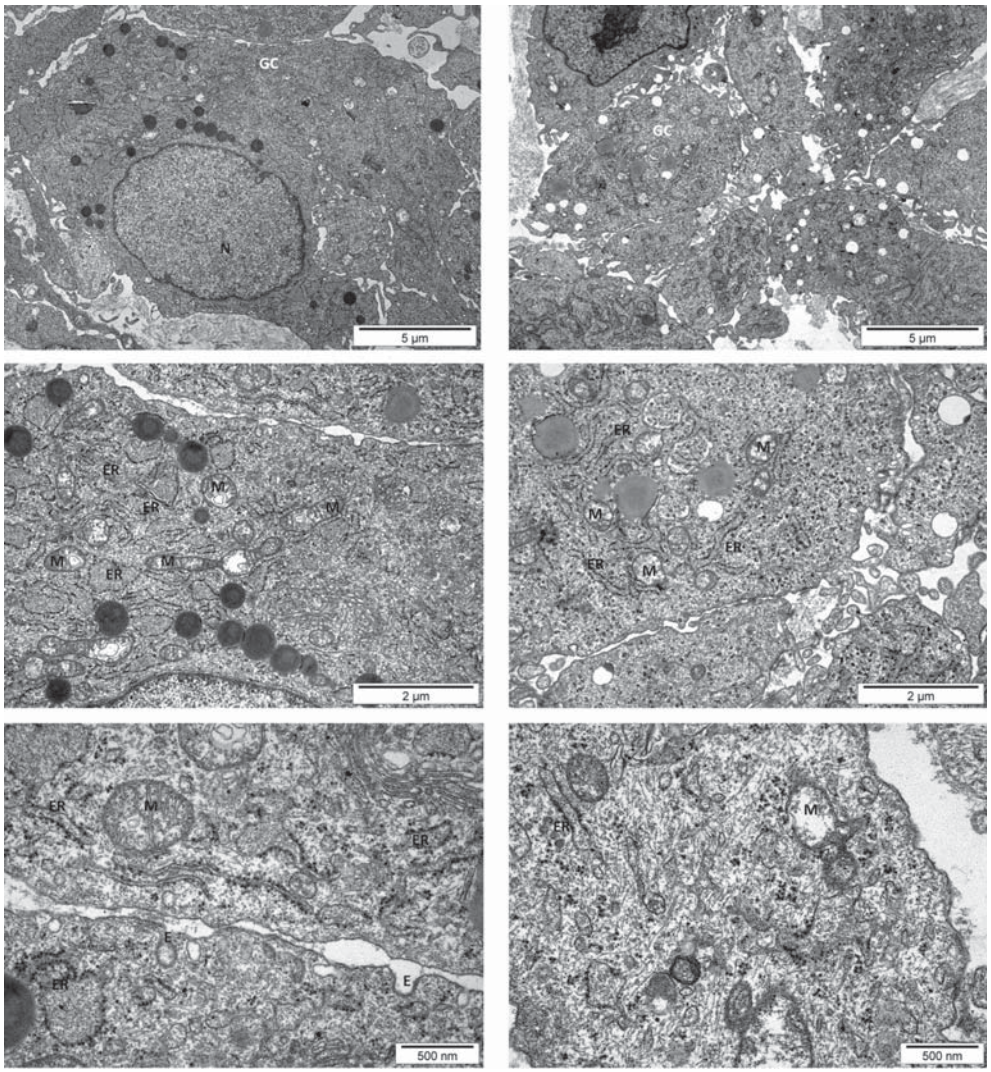

FIGURE 3. Glioblastoma multiforme ultrastructure from control group (A, B, C) and after diamond nanoparticles treatment (D, E, F). Scale bar: A, D – 5 µm, B, E – 2 µm, C, F – 500 nm; GC – glioblastoma multiforme cell, RBC – red blood cells, L – lumen of vessel, EC – endothelial cell, N – nucleus, M – mitochondrion, ER – rough endoplasmic reticulum, E – endocytosis

intense or persistent ER stress induces apoptosis or autophagy, resulting in cell death. Also the structure of glioma cells proves intensive cellular metabolism. Structures characteristic for endocytosis can be observed (membrane cavities of varying width and depth, transport vesicles). Endocytosis is essential for all the cells to internalize nutrients, antigens, pathogens and cell surface receptors, from the plasma membrane into membrane-bounded, endocytic vesicles, to

regulate cell homeostasis, cell signaling and development. There are multiple pathways for endocytic uptake into cell, depending on the size and kind of the transported compound or substance, they can be divided into 4 classes: clathrin--mediated endocytosis, caveolae, macropinocytosis, and phagocytosis (Doherty and McMahon 2009). Very important and well visible cell structures are the mitochondria, which key role is the energy metabolism and regulation of cell death (Wen et al. 2013). The mitochondria of glioblastoma mutliforme cells in control group have clearly defined folds in the inner membrane, named cristae.

In the group treated with diamond nanoparticles, despite the cells that can be identified are the same as in the control group, the morphology of these cells is different (Fig. 2 D, E, F; Fig. 3 D, E, F). First of all, the large spaces between the few deformed cells of glioblastoma multiforme are visible. Primary tumor cells that previously existed in the extracellular matrix probably influenced by ND underwent cell death. Glioma cells have irregular shapes; look like they lost their elasticity and rigidity. Inside the cell, cell structures have also a different morphology comparing to the control group. The number of organelles essential for proper metabolism is decreased. Additionally, in the mitochondria crests were degenerated, endoplasmic reticulum is less visible (smaller network of cisterns and ribosomes), endocytosis is practically stopped. In the ultrastructural image of these cells appeared vesicles characteristic for autophagy – highly conserved cellular homeostatic process. Changes can be observed in the structure of blood vessels as well. Their lumen is irregular and collapsed, vascular endothelial cells have lost their shape and elasticity, inside degenerated mitochondria, deprived of cristae, and numerous autophagocytic vesicles (autophagosomes) can be seen.

Changes visualized in glioblastoma multiforme ultrastructure testify to the ongoing process of cell death, probably carried out by autophagy. Autophagy, also known as programmed cell death type II, can be induced by various cellular stress mediated signaling pathways involved in nutrient signaling, growth factor status, energy sensing, hypoxia, oxidative and ER stress. The role of autophagy is complicated and may have diametrically opposite consequences for the tumor cell, that is important for the regulation of cancer development and maintain, as well as for the response of tumor cells to anticancer therapy (Liu et al. 2013). Reduction of the number of GBM cells, and a strong degeneration of those which remained alive suggest that autophagy activated in these cells leads to their death.

CONCLUSIONS

Diamond nanoparticles administration to glioblastoma multiforme tumor grown on the CAM, not only inhibit the development of the blood vessels but also affect the metabolism of cancer cells. Ultrastructure of the glioma cells after treatment with ND clearly suggests autophagy leading to the cell death. However, the mechanism of initiation of this process is still unclear. The results obtained encourage further research aimed at the anti-cancer application of diamond nanoparticles.

Acknowledgment

This work was supported by grants from the National Science Centre (NCN NN311540840).

REFERENCES

BAUMANN O.,WALZ B., 2001: Endoplasmic reticulum of animal cells and its organization into structural and functional domains. Int. Rev. Cytol. 205, 149–214.

DOHERTY G.J., McMAHON H.T., 2009: Mechanisms of endocytosis. Annu. Rev. Biochem. 78, 857–902.

GRODZIK M., SAWOSZ E., WIERZBICKI M., ORLOWSKI P., HOTOWY A., NIEMIEC T., SZMIDT M., MITURA K., CHWALIBÓG A., 2011: Nanoparticles of carbon allotropes inhibit glioblastoma multiforme angiogenesis in ovo. Int. J. Nanomedicine. 6, 3041–3048.

HUANG H., PIERSTORFF E., OSAWA E., HO D., 2007: Active nanodiamond hydrogels for chemotherapeutic delivery. Nano. Lett. 7, 3305.

JAIN R.K., 2013: Normalizing tumor microenvironment to treat cancer: bench to bedside to biomarkers. J. Clin. Oncol. 31, 2205–2218.

LIU K.K., CHENG C.L., CHANG C.C., CHAO J.I., 2007: Biocompatible and detectable carboxylated nanodiamond on human cell. Nanotechnology 18, 325102.

LIU W.T., HUANG C.Y., LU I.C., GEAN P.W., 2013: Inhibition of glioma growth by minocycline is mediated through endoplasmic reticulum stress-induced apoptosis and autophagic cell death. Neuro. Oncol. 15, 1127–1141.

ROCO M.C., 2003: Nanotechnology: convergence with modern biology and medicine. Curr. Opin. Biotechnol. 14, 337–346.

SATHORNSUMETEE S., RICH J.N., 2008: Designer therapies for glioblastoma multiforme Ann. NY Acad. Sci. 1142, 108–132.

STROJNIKT.,KAVALARR.,BARONET.A., PLUNKETT R.J., 2010: Experimental model and immunohistochemical comparison of U87 human glioblastoma cell xenografts on the chicken chorioallantoic membrane and in rat brains. Anticancer Res. 30, 4851–860.

WEN S., ZHU D., HUANG P., 2013: Targeting cancer cell mitochondria as a therapeutic approach. Future Med. Chem. 5, 53–67.

ZHANG L., GU F.X., CHAN J.M., WANG A.Z., LANGER R.S., FAROKHZAD O.C., 2008: Nanoparticles in medicine: therapeutic applications and developments. Clin. Pharmacol. Ther. 83, 761–769.

Streszczenie: *Zmiany w ultrastrukturze glioblastoma multiforme po zastosowaniu nanocząstek diamentu. Badania modelowe in ovo.* Glioblastoma multiforme (GBM) jest najczęściej występującym złośliwym nowotworem pierwotnym mózgu o bardzo złych rokowaniach. Pomimo dekad lat badań na tym problemem, niewielki postęp został uczyniony aby wydłużyć życie chorym, nowe strategie terapeutyczne są nadal poszukiwane. Zastosowanie nanotechnologii w leczeniu chorób, diagnostyce, monitornigu, platformach dostarczania substancji aktywnych i kontroli systemów biologicznych daje nadzieję na poprawę aktualnej sytuacji, również w terapii nowotworów. Nanocząstki diamentu (DN) są bioaktywnymi substancjami w stosunku do guza mózgu hodowanego na błonie kosmówkowo-omoczniowej zarodka kury. DN redukuje masę i objętość guza oraz hamuje rozwój nowych naczyń krwionośnych (angiogenezę). W prezentowanym doświadczeniu dodatkowo zaobserwowano zmiany w ultrastrukturze komórek guza pod wpływem działania nanocząstek diamentu, które świadczą o zachodzących procesach śmierci komórkowej, prawdopodobnie na drodze autofagii.

MS. received in November 213

Author's address:
Marta Grodzik
Wydział Nauk o Zwierzętach SGGW
Katedra Żywienia Zwierząt i Gospodarki Paszowej
ul. Ciszewskiego 8, 02-786 Warszawa, Poland
e-mail: marta_grodzik@sggw.pl