NANOTECHNOLOGY

Proliferation and Survival of Rat C6 Glioma Culture in the Presence of Implants Coated with Modified Carbon-Based Films

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The survival of rat C6 glioma decreased in the presence of implants from VT-16 titanium alloy. Diamond-like carbon coating of VT-16 alloy slightly increased cell death on day 5 of the experiment (39.9±2.1%). The percentage of dead C6 glioma cells inside titanium rings with diamond-like carbon coating, incorporating up to 3.5 atom.% Ag nanoparticles, was 53.7±4.3% on day 5 of culturing, while after doping to 6.7 atom.% Ag cell death reached 66.7±3.2% ($p<0.05$). The maximum toxic effect towards C6 glioma was detected in the specimens coated with diamond-like film with silver nanoparticles.

Key Words: *C6 glioma; proliferation index; titanium implants; diamond-like coating; silver nanoparticles*

The constancy/variability relationship in the process of evolution in nature is the basis for formation of mechanisms of homeostasis control. In the body these mechanisms are stimulated by the entrance of external substances. On the one hand, this promotes the maintenance of the internal medium constancy and on the other, these intrusions are liable to disturb the homeostasis and mobilize the defense reactions of the host. Modern medicine faced these reactions and the biocompatibility problem with development of implantation and transplantation technologies. Along with the positive aspects of implantology and transplantology, promoting partial or complete compensation for lost organs or functions, these technologies are fraught with certain negative consequences, the key of which are rejection reactions, allergic components, autoimmune processes, systemic inflammations, and procarcinogenic reactions. Hence, interactions of the implants widely used in various spheres of modern medicine with the host tissues and systems remain a pressing problem. One of the universally acknowledged biocompatible materials is titanium [5,8]. Its characteristics are improved after coating with diamond-like carbon films 0.1-5-μ thick [5,6]. Diamond-like carbon coating is inert for human tissues and physiological environment. They are hard, chemically inert, abrasion-resistant [5,6,8]. Diamond-like carbon material doped with silver has been obtained by pulsed cathode arch precipitation [8]. How will titanium implants coated

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with diamond-like carbon film behave in biological tissues? The bactericidal effect of silver nanoparticles is well-known [2]. However, discussions on the effects of these articles on the development of tumor tissue, specifically, on the proliferation and survival of C6 glioma never cease.

We compared the development of C6 glioma cells in the presence of titanium implants prepared by different methods, including the implants with Ag nanoparticles incorporated in their surface layers.

MATERIALS AND METHODS

Diamond-like films (80-95 nm) for coating plates from VT-16 titanium alloy were formed (Plasmoteg, Physical Technological Institute of the National Academy of Sciences of Belarus) at the initial voltage of 300 V in the basal discharge condenser battery, ignition voltage of 300 V, discharge pulse frequency of 2 Hz, and sublayer temperatures of 373°K [8]. The structure of carbon diamond-like films containing no other admixtures varied (depending on the sublayer temperature) from quasimorphic at 20°C to polycrystalline finely dispersed with grains of the mean size of 15 nm at 400°C.

Using Ag doping, the films were obtained by co-precipitation of flow of silver from the stationary arch discharge and carbon plasma generated by pulsed arch evaporator [8]. Before spraying the sublayers were cleansed and warmed by the Radical ionic source (Physical Technological Institute, the Russian Academy of Sciences). Silver concentration in the films was measured by regulating the time of its application. The element composition of the coating was controlled by X-ray photoelectron spectroscopy on and ES-2401 device (Nauchpribor Firm), the surface structure and silver concentration were evaluated under a Philips SEN-15 electron microscope (Electroscan Corp.) with a Genesis 2000 headpiece for element composition evaluation (Ellie Mae Inc.). Enriched with 3.5-6.7 atom.% silver (which forms no chemical compounds with carbon), the film surface acquired certain peculiarities. The silver in these films was present in the form of separate evenly distributed nanoparticles of up to 30 nm in size. Few larger particles were also detected, presumably the droplet phase, forming as a result of electric arch spraying of the silver cathode. Silver particles were mainly of regular spherical shape. With increase of silver concentration in the film to 3.5 atom.%, the distance between the particles decreased, and their density increased (Fig. 1, *a*) Further increase of silver concentration to 6.7 atom.% led to appearance of large particles, forming at the expense of coalescence processes (Fig. 1, *b*). The coalescence processes indicated that silver islets were located mainly on the surface of diamond-like film.

The study was carried out on rat transplanted C6 glioma cells from Russian collection of vertebrate cell cultures (Institute of Cytology, the Russian Academy of Sciences). It is one of the most widely used strains, easily cultured *in vitro*, with large (up to 20-30 μ) structural elements [1]. Tumor gliocytes (10^5 cell/ml) were cultured in Petri dishes (35 mm) in MEM with 10% fetal calf serum and 10^{-4} g/ml gentamicin sulfate. The inoculate was incubated in a CO_2 incubator (Lab. Instruments) with 5% carbon dioxide at 37°C. The cells cultured by this method constituted series 1 (control). In other four series, the gliocytes were cultured inside titanium rings 1 cm in diameter, made by different technologies. Each ring was put into a separate Petri dish. For experimental series II the ring was made from VT-16 titanium alloy; for series III VT-16 titanium alloy was coated with a 90-nm diamond-like carbon film; for series IV VT-16 titanium alloy was coated with diamond-like carbon film and silver-doped to 3.5 atom.%; and in series V VT-16 titanium alloy was coated with diamond-like carbon film and silver-doped to the concentration of 6.7 atom.% (Table 1).

Cell viability and number of dead tumor cells [1] were evaluated by staining with 0.2% trypan blue (Merck) in phosphate buffer saline (pH 7.2). The culture was visualized by computerized device for light microscopy. Mitotic activity (proliferation index) was calculated in a Goryaev chamber as the proportion of grown to inoculated cells [1]. Each studied group was represented by three independent inoculations. Experimental values were presented as the arithmetic mean±standard error in the mean. The level of significance was estimated by Mann—Whitney test for nonparametric samples using StatPlus 2005 software (Statistica Pack). The differences were significant at $p<0.05$.

RESULTS

Significant ($p<0.05$) cell death was observed on days 3 and 5 of C6 glioma culturing inside titanium rings from VT-16 titanium alloy: 9.3±0.1 and 33.2±1.5%, respectively, *vs.* 3.4±0.6 and 5.4±0.3% in series I, respectively. Spraying of VT-16 titanium alloy with diamond-like carbon coating (series III) was associated with an increase in cell death on day 5 of the experiment (39.9±2.1%), the proliferation index increasing ($p<0.05$) to 3.2±0.3 on day 3 in comparison with series I (2.2±0.1). The most significant results were observed in experiments with addition of silver nanoparticles to the diamond-like film. In experimental series IV (diamond-like carbon coating with up to 3.5 atom.% Ag nanoparticles), the death of C6 glioma cells on

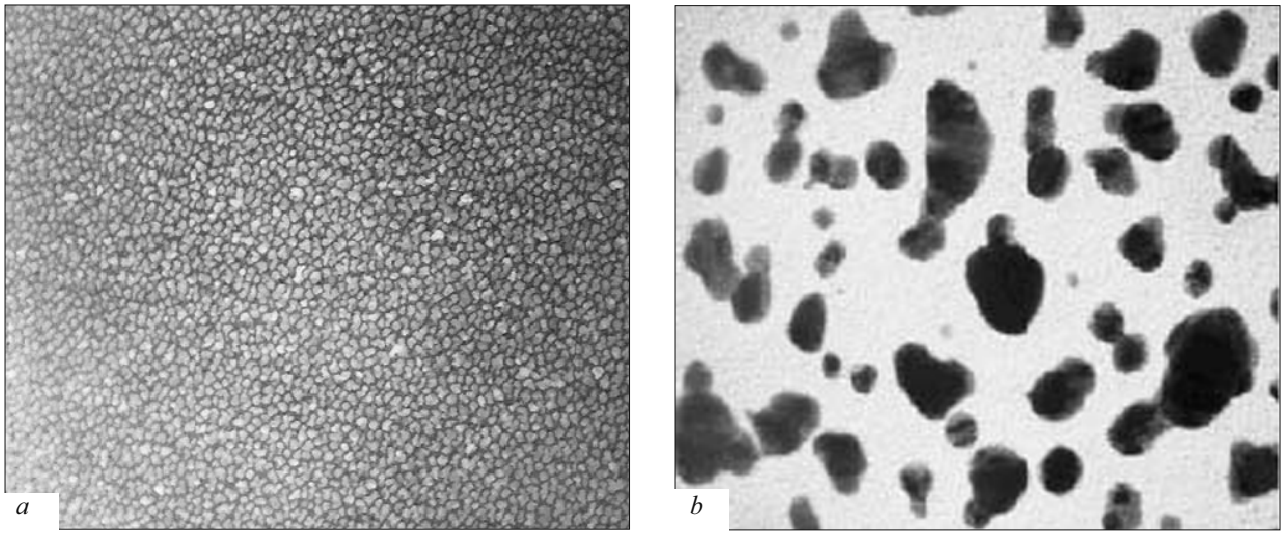

Fig. 1. Structure of carbon-silver films, ×100,000. *a*) silver concentration 3.5 atom.%; *b*) silver concentration 6.7 atom.%.

TABLE 1. Morphological and Functional Changes in C6 Glioma Cells on Days 3 and 5 of Culturing with and without Implants (*M*±*m*)

Series	Day 3		Day 5	
	proliferation index	cell death, %	proliferation index	cell death, %
Series I (control)	2.2±0.1	3.4±0.6	2.4±0.2	5.4±0.3
Series II	2.2±0.2	9.3±0.1*	3.4±0.2*	33.2±1.5*
Series III	3.2±0.3*	6.20±0.67*	3.5±0.4*	39.9±2.1*
Series IV	1.7±0.1*	11.8±3.0*	1.2±0.1*	53.7±4.3*
Series V	2.4±0.2	13.8±2.9*	0.4±0.1*	66.7±3.2*

Note. *$p<0.05$ compared to series I (control).

day 5 was 53.7±4.3% ($p<0.05$ *vs.* series I), while in series V (doping to 6.7 atom.% silver) the cell death reached 66.7±3.2% ($p<0.05$ *vs.* series I). The minimum proliferation index ($p<0.05$) indicating mitotic activity was recorded on day 5 in series IV and V (1.2±0.1 and 0.4±0.1, respectively, compared to 2.4±0.2 in series I).

The state of glioma C6 cells on day 5 in experimental series II (Fig. 1, *a*), III (Fig. 2, *b*), IV (Fig. 2, *c*), and V (Fig. 2, *d*) are shown.

Hence, reduced survival of C6 glioma cells in the presence of implants from VT-16 titanium alloy and from this alloy coated with silver-doped diamond-like carbon film was demonstrated *in vitro*. Other authors demonstrated low proliferation and death of fibroblasts in experiments with silver nitrate in concentrations of 4.1-82.4 μM [4]. These data are comparable with our results obtained on C6 glioma cells. At the same time, activation of neuronal growth after exposure of neuroblastoma cells with diamond-like samples was observed [6]. This is in line with the results of our studies, in which the proliferation index increased on day 5 in series II (VT-16 titanium alloy) and on days 3 and 5 in experimental series III (diamond-like coating of VT-16 alloy, Table 1), but contradicts the results obtained on day 5 in experimental series IV and V, when a significant reduction of proliferation index was observed (Table 1). Since the difference from the previous result [6] in the direction of proliferation was seen on day 5 of the experiment in the series with silver-containing diamond-like coating (and in which the maximum death of C6 glioma cells was observed), comparison with the previous data [2,4,6] suggested that addition of silver nanoparticles to the diamond-like carbon films is a sort of a triggering factor for manifestation of the toxic effects of implants with this coating towards C6 glioma.

The prospects of using silver-doped diamond-like carbon films can be discussed with consideration for statistical data on mortality of patients with glioblastomas. Despite modern surgical, radio- and chemothera-

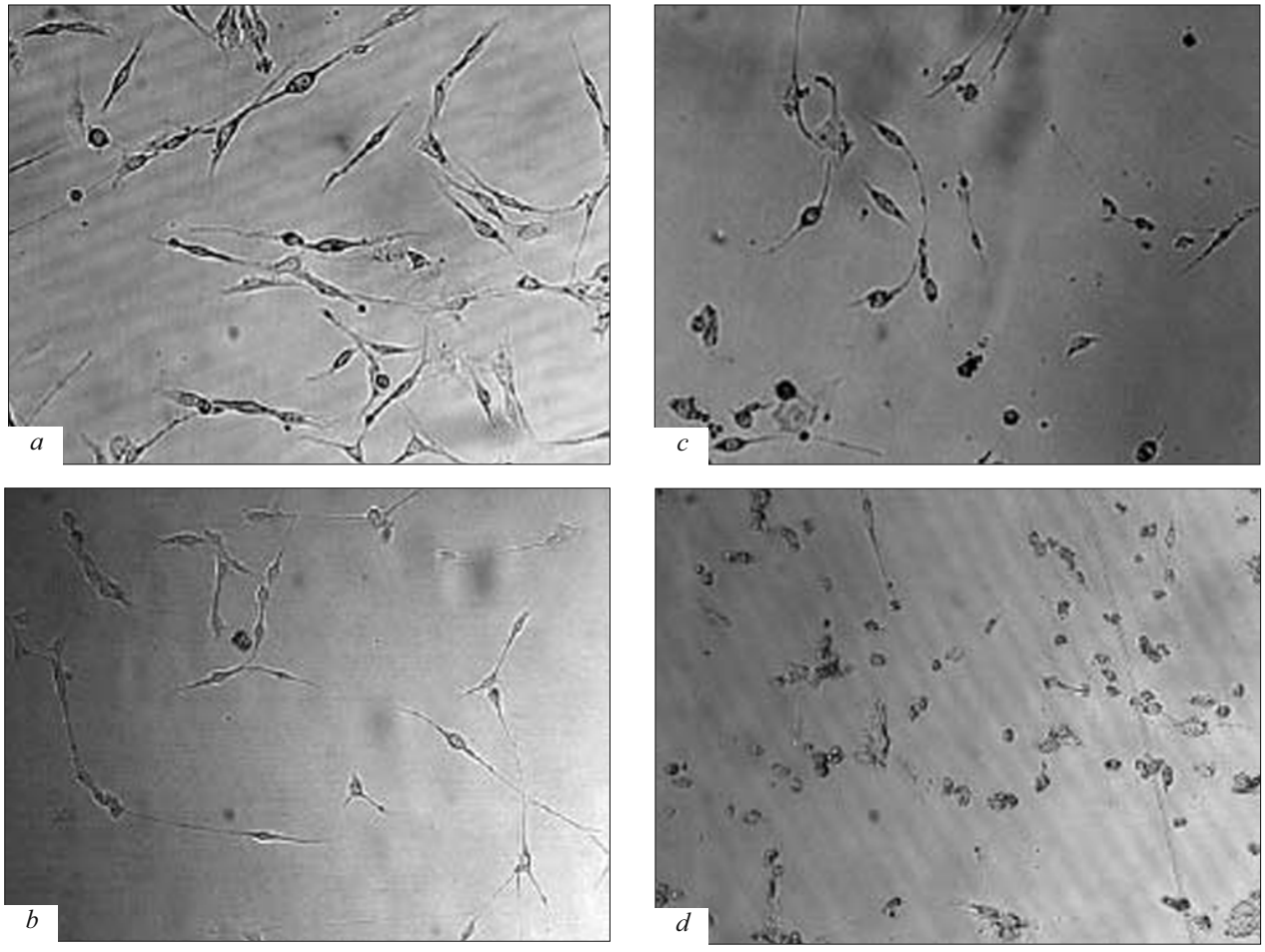

Fig. 2. Glioma C6 cells on day 5 of culturing inside rings made from VT-16 titanium alloy (*a*); VT-16 titanium alloy enveloped in diamond-like film (*b*); VT-16 titanium alloy, coated with diamond-like film doped with silver up to 3.5 atom.% (*c*) and to 6.7 atom.% (*d*), ×312.

peutic methods of treatment, about 15,000 patients in the USA die from this primary tumor every year [3]. This and the fact that glioblastoma ranks third among the causes of oncological mortality of patients aged 15-35 years [7] suggest that the toxic effect of silver-doped diamond-like carbon films deserves further studies in experimental oncology.

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