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Microencapsulated multicellular tumour spheroids as a novel *in vitro* model to study liposomal formulations

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INTRODUCTION AND OBJECTIVES

A variety of models are currently used in experimental oncology, including spontaneous, transplantable, and inducible animal tumors, human tumors transplanted to animals, various cultures of human and animal tumor cells, and some molecular-genetic models. Increased requirements to animal experiments stimulated development of *in vitro* models based on cell cultures, including multicellular tumor spheroids (MTS). MTS are artificial small solid tumors which represent a 3D model based on tumor cells. Earlier a technique for generation of MTS within polymer biocompatible alginate-chitosan microcapsules was proposed by the authors (Markvicheva et al., 2003). In recent time, anticancer drug entrapment into nanocarriers including liposomes is widely used in biomedicine. It allows to decrease systemic toxicity of drugs through 1) lower concentration of the intact drug in circulation and 2) passive targeting by means of enhanced permeability and retention effect in a tumor tissue. The aim of the present study was to prepare MTS by cultivation of human breast adenocarcinoma MCF-7 cell line in microcapsules and to study them as an in vitro model for testing anticancer liposomal formulation of a lipophilic methotrexate (MTX) derivative.

MATERIALS AND METHODS

Chemicals

Sodium alginate (medium viscosity), NaCl, $CaCl_2 \times 2H_2O$, EDTA were from Sigma, US. Cultivation medium RPMI-1640 and PBS (PanEco, Russia); foetal bovine serum FBS (HyClone, US); trypan blue dye (Laboratories, Inc., US) were used in the current research. Oligochitosan (Mw 4200 Da, deacetilation degree 87%) was kindly provided by Prof. A. Bartkowiak (Poland). Diglyceride conjugate of MTX (MTX-DG) was synthesized as previously reported (Vodovozova et al., 2004). Phosphatidylcholine (PC) from egg yolk and phosphatidylinositol (PI) from *S. cerevisiae* were purchased from Reakhim (Russia).

Cell cultivation

In the study human breast adenocarcinoma cells MCF-7 were used. Cells were cultured in RPMI-1640 medium supplemented with 10% FBS in 25 cm^2 flasks (Corning Inc.) at 37°C in a 5% CO_2 humidified atmosphere. The medium was replaced every 2-4 days.

Bioencapsulation of cells

Optimal conditions for microencapsulation were adopted earlier (Tsoy et al., 2010). Briefly, a mixture of sodium alginate solution and cell precipitate was extruded by peristaltic pump to $CaCl_2$ solution electrostatic droplet generator to obtain Ca-alginate microbeads. The obtained hydrogel microbeads were washed with physiological saline and then were incubated with oligochitosan solution in order to form an alginate-chitosan membrane on the microbead surface. Then microbeads were washed with physiological saline and were incubated in EDTA solution, in order to dissolve the microbead core and to obtain alginate-chitosan microcapsules. Then microcapsules were again washed with physiological saline and with culture medium.

Cultivation of bioencapsulated cells

Encapsulated cells were cultured in RPMI-1640 medium supplemented with 10% FBS in 25 cm^2 flasks (Corning Inc.) at 37°C in a 5% CO_2 humidified atmosphere. To generate MTS with desired size, the cells were cultivated for 2-3 weeks. The microcapsules size and the membrane thickness were determined by light microscopy.

Preparation of MTX liposomal formulation

Liposomes of the final composition of PC : PI : MTX-DG, 8 : 1 : 1 (mol), were prepared according to a standard technique (Kuznetsova et al., 2009) using membrane filters with a mean pore size of 100 nm (Nucleopore, US) and an Avanti Mini-Extruder (Avanti Polar Lipids, US) under sterile conditions.

Preparation of placebo liposomal formulation

Liposomes based on PC : PI, 9 : 1, mixture containing 0.3 mol % 1-acyl-2-[ω-(4,4-difluoro-1,3,5,7-tetramethyl-4-bora-3a,4a-diaza-*s*-indacene-8-yl)heptanoyl]-*sn*-glycero-3-phosphocholine (B7-PC) (Boldyrev and Molotkovsky, 2006) were prepared according to the same procedure to yield final concentration of 3.0 μM B7-PC.

Liposomal formulation penetration study

In order to study the ability of the MTX-DG liposomal formulation to penetrate both microcapsule membrane and MTS, aliquots of fluorescently labeled placebo liposomes were incubated with MTS 1/10 (v/v). The spheroid aliquots were analyzed by Confocal Laser Scanning Microscopy (CLSM, Multi-mode imaging inverted microscope Nikon TE2000, Japan). To visualize cell nuclei, Dapi dye (Invitrogen, Carlsbad, CA) was used.

Cytotoxicity study of MTX liposomal formulation

To study cytotoxicity of the liposomal formulation, the obtained MTS were incubated in culture medium in the presence of MTX-liposomes at MTX-DG final concentrations of 1, 2, 10, 50, 100 and 1000 nM in 24-well plates for 48 h. The monolayer culture was used as a control. The number of viable cells was calculated after Trypan Blue dye (0.4%) staining in a Fuchs-Rosenthal chamber. Cell viability was determined according to the following formula: (number of live cells in experimental group/number of live cells in a control) x 100.

RESULTS AND DISCUSSION

MTS were generated by cultivation of MCF-7 cells in biocompatible alginate-chitosan microcapsules with a mean size of 200 μm produced using the electrostatic droplet generator. In order to test the MTX liposomal formulation using MTS a preliminary study of liposomes penetration through the microcapsule membrane was performed. Even after 15 min of MTS incubation with placebo BODIPY-labeled liposomes, fluorescent bilayer-linked dye was observed inside MTS; it reached the spheroid core after 2 h incubation (Fig.1).

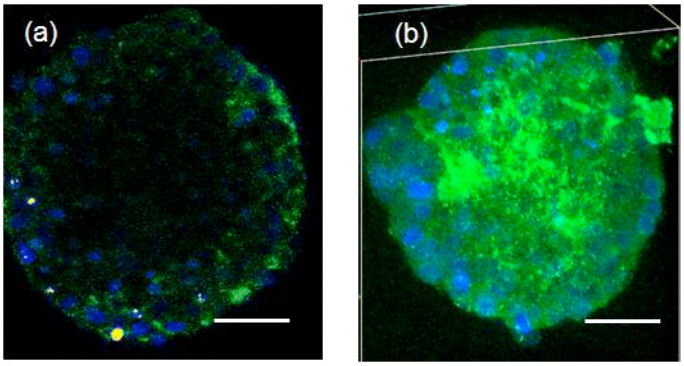

Figure 1 : CLSM images of MTS (nuclei labelled with Dapi, blue) incubated with placebo liposomes (BODIPY-labeled, green) after 15 min (a) and 2 h (b). Scale bar is 50 μm.

MTX cytotoxicity study on MTS showed that spheroids are more resistant to MTX than the monolayer culture (Fig.2 a). Moreover, MTX cytostatic effect decreases with the increase of MTS size (Tsoy et al, 2010).

Liposomes loaded with MTX-DG (90 ± 33 nm) were tested on MTS and monolayer culture (as a control) after 48 h incubation. As can be seen in Fig. 2, cytotoxicity of the liposomal formulation is less than that of the intact MTX due to the changed internalization mechanism and additional stage of prodrug release from liposomes. At 1000 nM MTX-DG in the liposomal form, cell viability in MTS was 1.4-fold higher than that in the monolayer culture.

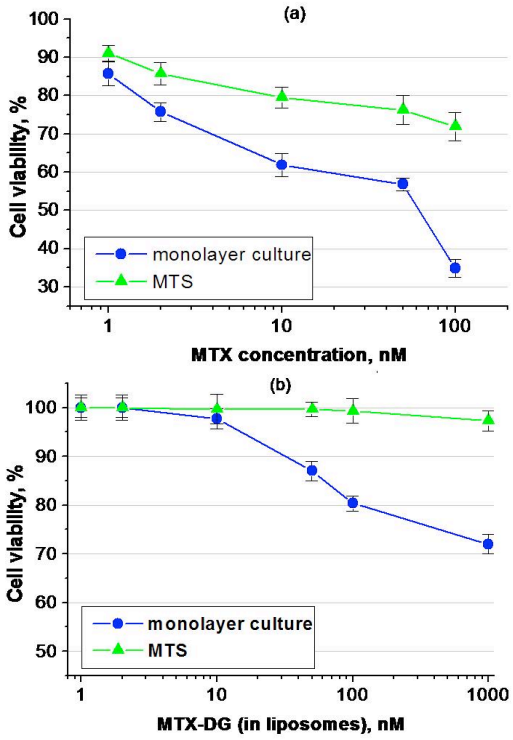

Figure 2 : Viability of MCF-7 cells in MTS and in monolayer culture (a control) after 48 h of incubation with MTX (a) and MTX-DG liposomes (b).

CONCLUSIONS

In vitro comparative study of the effect of MTX liposomal formulation on MTS demonstrated lower cytotoxicity of the formulation compared to MTX. Moreover, MTS were more resistant to both MTX and MTX liposomal formulation than the monolayer culture. Thereby microencapsulated MTS could be proposed as a more adequate *in vitro* model for anticancer drug testing.

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