3-D *in vitro* model based on microencapsulated multicellular tumor spheroids for anticancer therapy

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INTRODUCTION

Presently multicellular tumor spheroids (MTS) are being widely used in various aspects of tumor biology, including studies in the field of radiation biology, photodynamic therapy (PDT) as well as for testing effects of various chemotherapeutics. As well known, cells cultured in monolayer (2-D growth) or suspension can not mimic cells *in vivo* properly. Cellular arrangement of MTS allows to mimic *in vivo* small size tumors much better than 2D *in vitro* models (Hamilton G et al, 1998). Since Sutherland developed the multicellular tumor spheroid (MTS) model to mimic the 3D-structure of small size solid tumors (R. Sutherland et al., 1970). MTS have been found to be useful in several aspects of tumor biology. MTS were demonstrated to represent quite realistically the 3D growth and organization of small size solid tumors, and consequently to simulate well the cell-cell interactions and microenvironmental conditions found in tumor tissue. This similarities allow to consider MTS as a more rapid and valid *in vitro* model for anticancer drug screening compared to a monolayer culture (2D in vitro model). MTS could be formed from monolayer tumor cells grown by various *in vitro* classical methods, such as liquid-overlay, spinner flask and gyratory rotation systems. In order to have model tumors available, several teams throughout the world are generating MTS (for example teams of : (i) Santini M.T et al, Istituto Superiore di Sanita, Italy; (ii) L. Bezdetnaya, F. Guillemin et al, Alexis Vautrin Centre-France; (iii) Funke I., Mayer B, et al Univ Munchen, Germany; (iv) McCready R et al, Inst Cancer research and Royal Marsden NHS Trust, UK; (v) Kerbel R.S., et al. The Rockfeller Univ., USA). However, these classical methods are time consuming and cannot provide the production of MTS with narrow spheroid size distribution within a range of 200 – 900 μm. Moreover, some tumor cells cannot form spheroids in suspension. Therefore a technology providing spheroids in a simpler manner, with narrow size distribution and with cells not able to form spontaneously spheroids would be of interest. Recently we proposed tumor cell encapsulation method as a novel technique to quickly and easily prepare a large number of tumor spheroids with narrow size distribution within a desired diameter range (Markvicheva E.et al, 2003). The current research is aimed at developing *in vitro* 3-D *in vitro* model based on MTS which can be used to study effects of anticancer drug and photodynamic (PDT) treatments.

MATERIALS AND METHODS

Chemicals. Sodium alginate (medium viscosity), NaCl, $CaCl_2 \times 2H_2O$, EDTA, were from Sigma, USA. Cultivation medium DMEM (PanEco, Russia); foetal bovine serum FBS (HyClone, USA); 3-4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT-assay) (Serva, Germany) were used in the current research. Oligochitosan (Mw 4200 Da, deacetilation degree 87%) was kindly provided by Prof. A. Bartkowiak (Poland). Commercially available cytostatic agent Docetaxel (Beijin Lunarsun Pharmaceutical Co., Ltd.), soybean lecithin Lipoid-S100 (Lipoid), bovine serum albumin BSA (Sigma, USA), Soya oil from Glycine max (Fluka), 5% Dextrose, containing 0.026%

NaCl and HCl (pH 3-4) as well as other reagents of special grade were used for docetaxel-loaded nanoemulsion preparation. Chlorine e6 (Ce6) was from Porphyrin Products (Logan, UT, USA). Ce6 stock solution (2 mM) was prepared in DMSO and stored at -20^{0}C. Before its addition to cells Ce6 was diluted in DMEM. Methotrexate (MTX) was from Sigma.

Cell microencapsulation and generation of MTS in alginate-chitosan microcapsules. In the study human breast adenocarcinoma cells MCF-7 were used. Free and encapsulated cells were cultured in DMEM or RPMI-1640 media supplemented with 10% FBS at 37^{0}C in a 5% CO_2 humidified atmosphere. To encapsulate cells, cell precipitate (6-10×10^6 cells) was mixed with sodium alginate solution (1.3% w/v), and the mixture was dispersed to $CaCl_2$ solution (0.5% w/v) using electrostatic droplet generator. The obtained Ca-alginate microbeads were washed with physiological saline and incubated with oligochitosan solution (0.1-0.3% w/v) at agitation for 5-10 min. Then microbeads were washed 3 times with physiological saline and incubated in 50 mM EDTA solution for 10 min to obtain alginate-chitosan microcapsules which were again washed 0.9% NaCl solution and then with DMEM. To get spheroids of desired size (150-400 μm), encapsulated cells were cultivated for 2-4 weeks.

Preparation of of docetaxel- loaded nanoemulsion and study of its cytotoxicity. Crude docetaxel-loaded nanoemulsion was obtained by stirring water and oil phases at heating by a high-shear homogenizer (Heidolph, Germany, 10000-13000 rpm.). Further decrease of particle size (50-100 nm) was achieved by high pressure homogenization in APV Homogenizer (Germany) at 1800 bar for 4-5 times. The system was sterilized by filtration (Millipore, d= 0.22 μm) for the further *in vitro* tests. MTS and monolayer culture were incubated in 12 well plates in DMEM medium with previously lyophilized nanoemulsion (20, 300 and 1000 nM/well) was added. Cell viability was determined by MTT assay after incubation for 48 and 72 hs.

Study of cell viability after treatment with methotrexate. Microcapsule aliquots (100 μL) with formed MTS were transferred in 1.5 mL of medium DMEM containing methotrexate (MTX) in 24-well plate (final MTX concentrations were 1, 2, 10, 50 and 100 nM). Then the microcapsules were kept at в CO_2-incubator for 48 hs at 37^{0}C. Cells were counted in hemocytometer using Trypan blue. Monolayer culture (10^5 cells/well) was used as a control. Cell viability was calculated as follows : (live cells in experiment/live cells in control) x 100%.

Study of Ce6 cytotoxicity and phototoxicity : Microcapsulated MTS aliquots (100 μl) were incubated with Ce6 (0-34 nM) in0.5 ml of RPMI medium supplemented with 2 % FBS in 24-well plates for 24h.. Cell viability was measured by MTT-assay. Monolayer MCF-7 culture was used as a control. The cytotoxicity was calculated as described above. To study Ce6 photoxicity, MTS (100 μl) were incubated with Ce6 (8.4 nM) in 24-well plates for 24h, then washed 3 times with PBS, and 0.5 ml of RPMI-1640 was added in each well. The cells were irradiated by 650 nm diode laser (Coherent, France). Light energy densities were 0.5 – 70 J cm^{-2} at power density 30 mW cm^{-2}. The cell viability was measured by MTT-assay in 24 h after irradiation.

RESULTS AND DISCUSSION

To study the comparative effects of anticancer treatments on tumor cells in monolayer culture (2-D model) and in MTS (3-D model), we spheroids both for PDT and chemotherapy treatments. Chemotherapeutics were in free (methotrexate) or in nanoencapsulated form (docetaxel- loaded nanoemulsion). The results of the MTX effect on spheroids as function of their size is shown in Fig.1. As can be seen, monolayer culture was less resistant to MTX than MTS within the range of all MXT concentrations tested (1, 2, 10, 50 and 100 nM). Thus, cell viability in MTS 300 was 2.5-fold higher that that one a for monolayer culture (control). At the same time cell viability was dependent on spheroid size. MTS with mean size of 150 μm (MTS 150) were less resistant than

MTS-300 (300 μm). Thus, the MTS generated in alginate-chitosan microcapsules can be considered as a novel suitable *in vitro* model to test various anticancer drugs before preclinical tests.

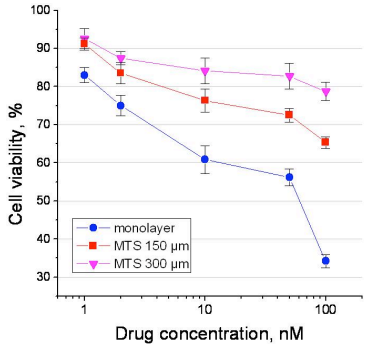

Fig 1. Cytotoxicity of methotrexate for MCF-7 cells in MTS-150 and MTS-300 generated in alginate-chitosan micro-capsules. Control was monolayer culture.

A similar effect for monolayer culture and MTS was observed when docetaxel-loaded nanoemulsion was used. As can be seen in Fig 2, first of all cytotoxicity values for all 3 tested samples of nanoemultions (concentrations of 20, 300 and 1000 nM) were bigger that that ones for Texotere formulation which is presently used in clinics for cancer treatment.

(a) (b)

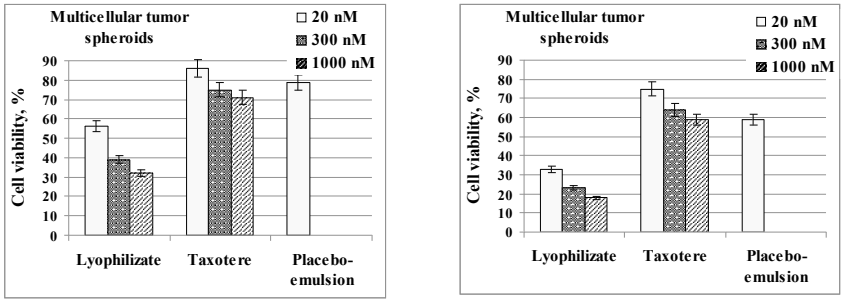

Fig.2. Cell viabilityof MCF-7 cells in MTS after 48 hs (a) and 72hs (b) incubation with docetaxel-loaded nanoemulsions and Taxotere in the culture medium. Placedo emultions are used as a control.

Secondly, the number of alive cells decreased with time (see Fig.2 a and Fig.2 b for 48 and 72 hs of MTS incubation with nanoemulsions,respectively). After 72 hs incubation of MTS and monolayer culture with lyophylized docetaxel-loaded nanoemulsion and Taxotere, for a monolayer culture the numbers of viable cells were 25, 18 and 12% for samples containing lyophilized emulsion at concentrations 20, 300 and 1000 nM, respectively. At the same time for MTS these values were higher, namely 33, 23 и 18%, respectively. Thus, as was expected MTS were more resistant towards

both anticancer drugs (Texotere and docetaxel-loaded nanoemulsions).

Finally, the effect of PDT on MTS and monolayer culture is demonstrated in Fig.3 and Fig.4.

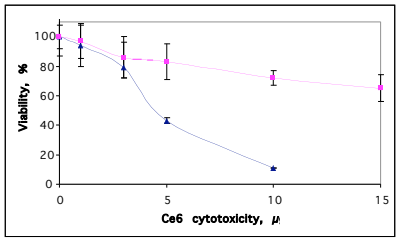

Fig 3. Cell viability in monolayer model (▲) and MTS model (■) after incubation with Ce6 for 24 h.

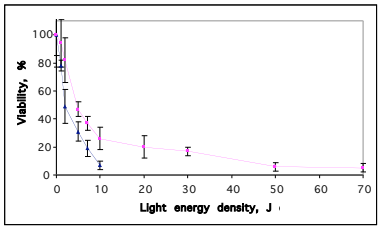

Fig. 4. Cell viability in monolayer model (▲) and MTS model (■) 24h after PDT at light energy densities 1-70 J cm^{-2}

Fig. 3 demonstrates that the difference between monolayer and MTS was remarkable even for these previous experiments. Maximum non-toxic concentrations (8.4 μM and 1.7 μM for MTS and monolayer culture, respectively) were chosen for further experiments. As can be seen in Fig. 4, phototoxicity increased with light energy density enhance both for spheroids and monolayer culture. However, the cell viability of the encapsulated MTS was higher than that one of monolayer culture, in spite of the fifth-fold Ce6 concentration taken for MTS. For instance, a percentage of viable cells in MTS was tree times bigger compared to monolayer culture at light energy density 10 J cm^{-2}.

CONCLUSION

Biocompatible polyelectrolyte alginate-chitosan microcapsules were used to generate encapsulated multicellular tumor spheroids based on human reast adenocarcinoma MCF-7 cells. Our results demonstrated that the proposed 3-D model based on microcapsulated MTS is more resistant to both photodynamic treatment and chemotherapy than a 2-D monolayer model. We concluded that the encapsulated MTS model could mimic small size solid tumors more precisely, than commonly used classical monolayer model and can be considered as a novel promising 3-D in vitro model to be used in tumor biology and experimental oncology.

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