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Formation of multicellular tumor spheroids induced by cyclic RGD-peptides and use for anticancer drug testing *in vitro*

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ABSTRACT

Development of novel anticancer formulations is a priority challenge in biomedicine. However, *in vitro* models based on monolayer cultures (2D) which are currently used for cytotoxicity tests leave much to be desired. More and more attention is focusing on 3D *in vitro* systems which can better mimic solid tumors. The aim of the study was to develop a novel one-step highly reproducible technique for multicellular tumor spheroid (MTS) formation using synthetic cyclic RGD-peptides, and to demonstrate availability of the spheroids as 3D *in vitro* model for antitumor drug testing. Cell self-assembly effect induced by addition of both linear and cyclic RGD-peptides directly to monolayer cultures was studied for 12 cell lines of various origins, including tumor cells (e.i. U-87 MG, MCF-7, M-3, HCT-116) and normal cells, in particular L-929, BNL.CL2, HepG2. Cyclo-RGDfK and its modification with triphenylphosphonium cation (TPP), namely cyclo-RGDfK(TPP) in a range of 10–100 μM were found to induce spheroid formation. The obtained spheroids were unimodal with mean sizes in a range of 60–120 μm depending on cell line and serum content in culture medium. The spheroids were used as 3D *in vitro* model, in order to evaluate cytotoxicity effects of antitumor drugs (doxorubicin, curcumin, temozolomide). The developed technique could be proposed as a promising tool for *in vitro* test of novel antitumor drugs.

1. Introduction

In vitro assessment of novel antitumor drugs and drug delivery systems is rather complicated challenge, since it includes evaluation of biocompatibility, cytotoxicity and immunogenicity of formulations. First of all, cytotoxicity tests should be performed with a large data base previously tested compounds and forms, they allow to evaluate a potential of the proposed drug or formulation. However, a prognostic value of these tests leaves much to be desired. One of the reasons is that routine cytotoxicity tests are performed in monolayer cell cultures. However, this two-dimension (2D) culture can not well mimic solid tumors, in particular the behavior of cancer cells in terms of their interactions with extracellular matrix (ECM) network and cell–to-cell communications. For this reason, a number of three-dimensional (3D) models have been recently proposed. The advantages of 3D tumor models for drug screening have been discussed in numerous papers and reviews (Pampaloni et al., 2007; Ravi et al., 2015; Xu et al., 2014). For example, cells in 2D monolayer culture are exposed to uniform concentrations of oxygen and nutrients, while in solid tumors the cells occur at gradient conditions of vital factors. As a result, it could lead either to enhancement or vice versa inhibitory effects on tumor progression (Mehta et al., 2012). Moreover, chemotherapeutics can be easily delivered to target cells in monolayer cell culture conditions, while *in vivo* drugs have to penetrate through several layers of stromal cells. Finally, cell growth in 3D cultures leads to a divergent gene expression compared to that in a monolayer (Lee et al., 2013; Luca et al., 2013).

Abbreviations: 2D, two-dimensional; 3D, three-dimensional; MTS, multicellular tumor spheroids; RGD, arginine-glycine-aspartic acid; DOX, doxorubicin; CUR, curcumin; TMZ, temozolomide; ULA, ultra-low attachment.

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All the above mentioned issues result in a reduced sensitivity to chemotherapeutic agents in 3D conditions compared to the monolayer culture.

Multicellular tumor spheroids (MTS) are 3D dense spherical aggregates which could mimic solid small size tumors in terms of simulating cell-cell interactions and microenvironment in tumors (LaBarbera et al., 2012). MTS have been proposed as a useful tool in tumor biology, in particular radiation biology, photodynamic and chemotherapies (Benien and Swami, 2014). Several approaches have been proposed to MTS formation, such as spontaneous aggregation, spinner flasks, rotary cell culture systems, hanging drops, liquid overlay, low binding plates or microencapsulation in polyelectrolyte microcapsules (Hickman et al., 2014; Vinci et al., 2012; Zaytseva-Zotova et al., 2011). However, despite the encouraging results there is no "ideal technique" for high-throughput drug screening which could meet all necessary requirements, such as rapidity, reproducibility, simplicity and cost-effectiveness (Ivanov et al., 2014). For example, rotary cell cultivation systems or shakers which are widely used for MTS generation can not provide rather narrow spheroid size distribution, while handing-drop technique is extremely cost- and labor-intensive (Breslin and O'Driscoll, 2013). Nowadays, cell cultivation in ultra-low attachment (ULA) round-bottom plates is one of the most common approaches due to the simple protocol and high reproducibility (Guo et al., 2014; Lee et al., 2014; Mikhail et al., 2013). However, this technique is also limited with a small MTS number (one spheroid per well) and an occasional formation of loose aggregates instead of dense spheroids.

Recently, RGD (Arg-Gly-Asp) motif in fibronectin was found to promote a formation of tight fibroblast spheroids (Salmenperä et al., 2008) or tumor cell spheroids (Serres et al., 2014) through the interactions with $\alpha5\beta1$ integrins. More over, an addition of ECM proteins to cell suspension favors cell self-aggregation with further compact spheroid formation when used in a combination with ultra-low attachment or handing-drop techniques (Benton et al., 2011; Ivascu and Kubbies, 2006). This effect could be also related to RGD-sequence which was found in several ECM proteins, such as laminin, fibronectin, collagen and vitronectin (Hayman et al., 1985; Xu and Mosher, 2011). The RGD-motif is known to be responsible for binding to cell membrane integrins, thus providing cell-to-cell and cell-to-matrix interactions. A contributory role of αv integrins at the initial step of spheroid formation from IGROV1 ovarian cancer cells was demonstrated earlier (Kellouche et al., 2010). We suggested that synthetic RGD-peptides could mimic natural ECM proteins in terms of self-assembly through the mechanism of cell membrane integrins binding. In the current study, we synthesized and evaluated a series of RGD-peptides which could induce cell self-aggregation like natural ECM proteins.

The aim of the study was to develop a novel one-step highly reproducible technique for MTS formation using these synthetic RGD-peptides, and to demonstrate the availability of these spheroids as 3D *in vitro* model for anti-tumor drug testing.

2. Materials and methods

2.1. Chemicals

MTT (Thiazolyl Blue Tetrazolium Bromide, 98%), Hoechst 33342, Calcein AM, PI (Propidium iodide), DOX (doxorubicin hydrochloride, ≥98%), CUR (curcumin, ≥65%), TMZ (temozolomid, ≥98%), and fluorophore protector CC/Mount were from Sigma-Aldrich. DMSO (>99.5%), phosphate buffered saline (PBS, pH 7.4), Dulbecco's modified Eagle's medium (DMEM) were from PanEko (Russia). Fetal bovine serum (FBS) was from PAA (Austria). All reagents for RGD-peptide synthesis were purchased from Sigma Chemical Co., Fisher Scientific, Bachem, Reanal and Iris Biotech GMBH. Solvents were purified according standard protocols. The ligand K34c (2-(*S*)-2,6-dimethylbenzamido)-3-[4-(3-pyridin-2-ylamino-prooxy)-phenyl]propionic acid), a RGD-like small molecule, was synthesized as it was described earlier (Heckmann et al., 2008).

2.2. Synthesis of RGD-peptides

All RGD-peptides (cyclo-RGDfK, Mw 603; cyclo-RGDfK(TPP), Mw 1029; RGDF, Mw 494; TPP-RGDF, Mw 919; $(TPP)_2$-KRGDF, Mw 1472) were prepared using standard protocols of solid phase peptide synthesis. Briefly, linear peptides on Wang resin were prepared using semi-automatic NPS 4000 peptide synthesizer (Neosystem Laboratoires, France) according to a standard Fmoc/Bu^t protocol. A piperidine solution (20 vol% in DMF) was used for deprotection (1×2 min; 1×8 min); Fmoc-protected amino acids (3 equiv) were activated in the DMF solution by a mixture of DIC (3 equiv) and Cl-HOBt (3 equiv). A cleavage was carried out in a TFA/H_2O/TIS cleavage cocktail (95:2.5:2.5 v/v) at ambient temperature (2 h). Volatile products were removed under reduced pressure. Residues were triturated with MTBE, filtered, dried *in vacuo*, purified by RP-HPLC and then lyophilized. In case of cyclic peptides, their linear precursors were synthesized on a Cl-Trt resin, then a cyclization process was performed using HCTU (2-(6-chloro-1H-benzotriazole-1-yl)-1,1,3,3-tetramethylaminium hexafluorophosphate). Coupling of 4-carboxybutyltriphenylphosphonium bromide (TPP-acid) was performed in the same way as described above for the Fmoc-protected amino acids. A coupling completeness was monitored by Kaiser test. The peptide purity was confirmed by analytical HPLC and ESI–MS which were recorded on a Bruker micrOTOF SD mass spectrometer.

2.3. Cell culture

Nine human cell lines, MCF-7 (breast adenocarcinoma), MCF-7/ADR (doxorubicin-resistant breast adenocarcinoma), A-375 (melanoma), HaCaT (keratinocytes), HCT-116 (colon colorectal carcinoma), U-87 MG and A-172 (both glioblastoma), HOS (osteosarcoma), HepG2 (hepatocytes) as well as three murine cell lines, namely M-3 (melanoma), L-929 (fibroblasts) and BNL.CL2 (embryotic hepatocytes) were used in this study. MCF-7 and U-87 MG cells were purchased from ATCC collection. MCF-7/ADR cells were a generous gift of Dr. Akatov (Institute of Theoretical and Experimental Biophysics RAS, Pushchino, Moscow region, Russia). The other cells were kindly provided by Drs. Svirchevskaya and E. Kovalenko (Shemyakin-Ovchinnikov Institute of Bioorganic Chemistry RAS, Moscow, Russia). The cells were cultured in DMEM supplemented with 10% FBS at 37 °C in a 5% CO_2 humidified atmosphere. The medium was replaced every 3–4 days.

2.4. Formation of multicellular tumor spheroids

The cells were seeded in DMEM supplemented with 10% FBS in 96-well plate (10^4 cells/well). After cell attachment, the medium was replaced in each well with 100 μl of DMEM supplemented with FBS (2.5; 5; 10 vol%) or with serum free DMEM which contained RGD-peptides. Peptide concentration in the medium was in a range of 1–100 μM. The animated scheme of the multicellular tumor spheroids formation is available in the Supplementary data 1. The concentration of the K34c ligand which was used in some experiments instead of the RGD-peptide, was varied within a range of 10–500 μM. Cell aggregation with subsequent spheroid formation was observed using light microscope (Reichert Microstar 1820E, Germany) equipped with the MDS-320 camera after 24, 48 and 72 h of incubation. Spheroid diameters were measured using ImageJ software and mean values were calculated (at least 200 measurements for each sample). To